The application of immunological assays for the monitoring and diagnosis of selected infectious diseases, with particular emphasis on neosporosis

by

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Dedicated to Kristin, Robin and Tim: for their patience

“ELISAs for Parasitologists: or Lies, Damned Lies and ELISAs”

(P. Venkatesan and D. Wakelin, Parasitology Today **9**, 228-232)

CERTIFICATE OF AUTHORSHIP & ORIGINALITY

I certify that the work in this thesis has not been previously submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged in the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signed

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AUTHOR'S PUBLICATIONS CITED IN THIS THESIS

ACKNOWLEDGMENTS

Thanks go to a number of collaborators, both in New Zealand and Australia but also further afield, without whom the body of work that is presented in this thesis could not have been accomplished. It is thus totally appropriate that a large number of the papers presented here are collaborative ones, with the important contributors mentioned as co-authors. It has been extremely enjoyable and educational for me to be working with them and to be able to unravel a New Zealand piece of the Neospora story with them.

Thanks are also due to Prof John Ellis who has been a willing collaborator on a number of recent papers and been a constant source of encouragement in this sometimes-wearisome course towards the final thesis. I am particularly grateful to him for maintaining the contact across the globe, via Email and telephone.

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ABSTRACT

The 16 publications presented in this thesis summarise the author's contribution to sero-epidemiological approaches for the diagnosis and monitoring of animal diseases of importance to New Zealand.

The first four publications not only contribute to the above in relation to three important animal pathogens, namely *Brucella ovis, Mycobacterium avium* spp. *paratuberculosis* and Bovine Leukaemia Virus but also give an insight into more general consideration associated with the optimisation and validation of serological assays, namely regarding the definition and choice of gold standard reference sera, the determination of the cut-off threshold and discrimination between negative and positive reference populations.

Two further publications deal with the establishment and validation of serological assays for the diagnosis of *Neospora caninum* infection and abortion in New Zealand. Then, baseline data were obtained for the sero-prevalence of the infection in dog and cattle populations in New Zealand. Three case studies provided initially information about the kinetics of serological responses after a *N caninum* abortion outbreak, and information about the usefulness of herd-based techniques rather than individual cow-based abortion diagnoses. A further study provided some early information about the mode of transmission seemingly predominating in New Zealand, which tends to be mainly via post-natal infection, in contrast to evidence provided by overseas researchers. A final case study, a longitudinal study of serological and other responses over a period of three years also provided data on the production effects of *N caninum*.

The dissertation is completed by a number of reviews on sero-diagnosis of *N caninum* infection, its presence in Australasia and suggests finally control options, based on the present state of knowledge.

1. INTRODUCTION

1.1. General considerations about immunological assays (validation, performance characteristics).

Diagnostic tests are used in veterinary medicine to distinguish between diseased and non-diseased/infected and non-infected individuals. In order for that distinction to be reasonably accurate, information is required about the degree of agreement between the test result and the true status of the animal. The assessment of the accuracy of a test system should be the subject of a detailed study plan, which preferably follows generally accepted guidelines. The Office International des Epizooties (OIE) has published such guidelines (Anonymous. 1996) and they describe in some detail the requirements in regards to a diagnostic assay. Pivotal to the performance of a test system is its accuracy, i.e. the ability to describe the true status of an individual animal and the precision of the assay, i.e. its ability to repeatedly arrive at the same result when testing the same sample.

In reality the latter performance characteristic is linked to adherence to strict quality control measures in the laboratory, the setting up of standard operating procedures and conditions under which the assay is run. The former, accuracy, is described by two independent measures: sensitivity and specificity. Sensitivity describes the (prob)ability of a test system to arrive at a positive test result when the individual is truly infected or diseased, specificity, the prob(ability) to arrive at a negative result when the animal is not infected or diseased. Strictly speaking, measures of either describe the performance characteristics of a test only for the reference population for which they were

determined. The validity of those characteristics depends strongly on the conditions under which they were determined (i.e. the laboratory, the definition of the gold standard population, cut-off determination, etc.).

The definition of the “gold” standard is a very critical determinant and the future characteristics of the assay depend very much on the careful selection of the standard. Often the standard itself, however, might be flawed, or has some serious drawbacks. Sera drawn from reliable not infected animal populations will make the best negative reference population (such as the Johne’s disease negative sera from Western Australia or ram sera from *Brucella ovis* accredited flocks, which will be introduced in the following papers). Often however one will have to rely on test results of another assay, such as microbiological culture, which are in themselves subject to less than perfect performance characteristics and hence the standard is only relative to another.

Positive reference populations may be from infected or diseased animals where their infection status may have been determined by a methodology, which is, at the time, regarded as the standard against which every other assay is compared. This may, in the case of *Brucella ovis* infection be semen culture, in the case of Johne’s disease demonstration of the causal organism in the faeces. These methodologies are usually highly specific and the positive reference population well defined.

In the case of *Neospora caninum* infection the definition of the reference populations is fraught with a great number of problems: while very specific methodologies (such as PCR and Immunohistochemistry) allow the clear identification of infected calves, the pathogenesis of abortion is still not clearly understood (calves with *N caninum* infections acquired *in utero* may well live to term and not be aborted and concurrent

infections with other abortifascients frequently occur) making it difficult to clearly describe a positive aborted calves serum population. Historically the Indirect Fluorescent Antibody Test (IFAT) was developed first for *N caninum* serology and frequently new serological assays are compared to it. As our knowledge of *N caninum* and its epidemiology increases, the IFAT and other serological assays need to be constantly re-evaluated. The same however is probably true for most other diagnostic assays, which require re-evaluation as our knowledge of the specific diseases grows.

Biological factors of the animals are equally important and will determine the performance characteristics of a diagnostic assay. The clinical stage of a disease may determine the sensitivity of an assay, as has been described for cattle varying between preclinical to clinical stages of the disease (Ridge *et al.* 1991, Sockett *et al.* 1992). Generally speaking the time between infection and the first production of antibody (in the case of immunological assays) may vary from weeks to years (Burgess 1982, Chiodini *et al.* 1984). The recent development of molecular techniques often allows to determine the presence of genetic material from a particular pathogen very early on and at very low numbers, but might not be able to distinguish between viable and non-viable organisms, causal or non-causal association with the disease.

The following four papers set out to determine the performance characteristics for serological and molecular techniques for three animal pathogens, namely:

Mycobacterium avium spp *paratuberculosis*, the cause of Johne's disease (a chronic wasting disease) of cattle, sheep and a number of other species. *Brucella ovis*, the cause of chronic epididymitis and infertility of rams and bovine leukaemia virus (BLV), the cause of enzootic bovine leukosis (EBL) in cattle. All three pathogens present(ed)

significant disease problems in New Zealand and serological tests system were in use for their diagnosis. However, the (re-)validation of serological assays at the beginning of an eradication campaign (as in the case of EBL) seemed warranted. Several test systems, mostly serological assays, had been employed overseas, but recently molecular techniques have also been used (Miller *et al.* 1982, Mammerickx 1989, Klintevall *et al.* 1991, Beier and Siakkou 1994, Klintevall *et al.* 1994). When the New Zealand dairy industry elected to embark on an eradication effort in 1996, and as a prerequisite to testing the national herd, a number of serological and molecular assays needed to be evaluated for their respective performance characteristics (sensitivity and specificity) for the accurate detection of the infection status of dairy cows.

In an existing accreditation scheme (in the case of *B ovis*) the larger number of sera tested annually afforded the opportunity to re-validate a serological assay with a very large number of gold standard positive and negative sera. Also, recently, infection with *B ovis* in deer had been recorded in NZ (Bailey 1997) and western blotting and the more traditional test systems were employed to investigate the spread of this infection to a new species.

In the case of Johne's disease, diagnosis is complicated by the long lag between infection and clinical disease, and the late onset of immunological responses to the infection in the host (Hietala 1992). Serological and bacteriological test systems have been plagued by low sensitivity and specificity (Hilbink *et al.* 1994, Jark *et al.* 1997). Here an effort was made to better understand and possibly improve on the notoriously under-performing immunological assay.

These papers also establish the baseline standard for the subsequent larger body of work presented on neosporosis, and some of the techniques mentioned here will also be used in establishing and validating the immunological assays for the diagnosis of *Neospora caninum* infection and abortion.

1.2. Paratuberculosis (Johne's disease)(*Mycobacterium avium* spp. *paratuberculosis*)

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Comparison of serological tests and faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and analysis of the antigens involved

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Abstract

Three hundred and forty-one sera from cattle in Western Australia and 106 sera from *Mycobacterium paratuberculosis* faecal culture positive cattle were used to evaluate the performance of two absorbed enzyme-linked immunosorbent assays (ELISA) (one locally produced, the other a commercial test) and a complement fixation test (CFT) for the detection of Johne's disease in cattle. The diagnostic sensitivity (47.2%) of the local ELISA was significantly higher than that of the commercial ELISA (31.1%), and significantly higher than that for the complement fixation test (17.9%) and immunoblot (20.8%). Diagnostic specificity for the two ELISAs was 99.7% and 97.9% and similar for CFT and immunoblot (97.1% and 97.7%, respectively). The diagnostic sensitivity rose for both ELISAs and the CFT as the number of *M. paratuberculosis* isolated from the faeces increased. The ELISA antigen was characterised by polyacrylamide gel electrophoresis and electrophoretic immunoblotting and was found to consist

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mostly of a carbohydrate-type macromolecule of 32–42 kDa. This macromolecule was identified as lipoarabinomannan (LAM) by using a LAM-specific monoclonal antibody in immunoblots and purified LAM in absorption experiments. By applying more complex antigen preparations in immunoblots, serum antibodies against proteins of 47, 37, 30, 24 and 21 kDa, and against the 32–42 kDa carbohydrate component were frequently found in infected cattle, and of these the 47 kDa protein and the 32–42 kDa antigen were immuno-dominant. Pre-absorption of the sera with *M. phlei* sonicate indicated that the protein antigens contributed markedly to non-specific serological cross-reactions, while the 32–42 kDa non-protein macromolecule appeared to be specific.

Keywords: Cattle-bacteria; Johne's disease; *Mycobacterium paratuberculosis*; *Mycobacterium avium*; Faecal culture; ELISA; Immunoblotting; LAM; Antigens

1. Introduction

Johne's disease is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) (Chiodini et al., 1984). It is of economical importance worldwide and affects cattle, sheep, goats, deer and members of the camelid family. The disease is characterised by intermittent diarrhoea with bacillary excretion. It progresses through several stages and, in the majority of cases, takes several years from infection to manifest itself with clinical signs.

Currently available serological tests for Johne's disease including the complement fixation test (CFT), the gel diffusion test (GDT) and various forms of enzyme-linked immunosorbent assays (ELISA) have significant limitations (Kreeger, 1991). A major reason for this is that seroconversion occurs relatively late during the course of the disease which reduces the sensitivity of such tests. Furthermore, the specificity of serological tests is affected by cross-reactivities with mycobacterial, nocardial and corynebacterial species (Chiodini et al., 1984; De Kesel et al., 1992; Gilot et al., 1992; Hilbink et al., 1994). The specificity of ELISA tests for sheep and cattle was improved by removing cross-reacting antibodies by absorbing sera with *Mycobacterium phlei* (Ridge et al., 1991; Hilbink et al., 1994). This increased the specificity of serological testing to 98.8% (Cox et al., 1991) and 99.8% (Ridge et al., 1991). Depending on the disease status of the animals tested, the sensitivity has been reported as 57% under field conditions in faecal shedders (Milner et al., 1990). The CFT, however, is still the most frequently prescribed test in international trade of cattle (Anon., 1996).

Here we report results from a comparison of the performance (diagnostic sensitivity and specificity) of two absorbed ELISAs with the CFT, using sera from infected (faecal culture-positive cattle) and Johne's disease-free cattle. Changes to the diagnostic sensitivity were assessed in relation to the degree of bacterial shedding. Furthermore, electrophoretic immunoblotting (EIB), a technique which enables the identification of serum antibodies to individual antigenic macromolecules was used for the analysis of the sera used in the comparison of the serological tests. This technique had previously been applied only to small numbers of sera (Bech-Nielsen et al., 1985; Milner et al., 1987; White et al., 1994; El-Zaatari et al., 1997).

2. Materials and methods

2.1. Bacterial culture and antigen preparation

A New Zealand isolate of the *M. avium* complex, not containing IS 900 (WAg 1001), *M. avium* strain 18, formerly identified as *M. paratuberculosis* (Chiodini, 1993) and *M. paratuberculosis* Teps were used for antigen production. The type strain of *M. phlei* (ATCC 11758) was used as immunosorbent. Bacteria for antigen and absorbent were grown as pellicles on modified Watson–Reid medium. Bacteria from the pellicles were washed three times in distilled water by centrifugation for 15 min at 3500 g. Soluble antigen preparations were made by disrupting the bacteria using a VirTis 550 W sonicator (Gardiner, NY, USA). Bacterial cells were chilled during sonication and disrupted for a total of 30 min. Following sonication the suspensions were centrifuged at 15 000 $\times$ g for 60 min. *Lipoarabinomannan* was purified from *M. paratuberculosis* laboratory strain V as described previously (Sugden et al., 1987).

Protein determinations were performed using the Bio-Rad DC protein assay (Bio-Rad Laboratories, CA). Polysaccharide determination was carried out as previously described (Severn et al., 1997).

2.2. Sera and faecal culture

Sera and faecal samples were collected from all the 450 adult animals from three New Zealand dairy cattle herds with a history of Johne's disease and 341 sera were obtained from cattle from Western Australia, a region which is considered to be free of Johne's disease (Cox et al., 1991).

Faecal culturing procedure was a modification of the method described previously (Collins et al., 1993). The modification was that after decontamination in 1% cetylpyridinium chloride for 24 h, the samples were centrifuged for 20 min at 3500 $\times$ g. The supernatants were discarded and the pellets resuspended in 0.4 ml of sterile distilled water. The resuspended pellets were inoculated onto three slopes of Herrold's medium supplemented with mycobactin.

2.3. Serological tests

The CFT and Wallaceville Animal Health Laboratory absorbed ELISA (W-ELISA) were performed as described by Hilbink et al. (1994) with minor modification to the ELISA, using as antigen a sonicate produced from isolate Wag 1001 (prepared as described in 2.1) and as conjugate a horseradish peroxidase conjugated protein G from Zymed, San Francisco, CA.

A reaction of 4 (100% complement fixation) at the 1/4 serum dilution was regarded as positive in the CFT, a reaction of 3 (75% fixation) regarded as suspicious. An absorbed Johne's disease ELISA (C-ELISA) from Commonwealth Serum Laboratories, Australia (Parachek^{TM}), was performed according to the manufacturer's instructions.

2.4. Polyacrylamide gel electrophoresis (PAGE)

Bacterial sonicates were diluted in sample buffer (62 mM Tris-HCl of pH 6.9, 5% SDS, 1% mercaptoethanol, 10% glycerol), boiled for 5 min and then centrifuged for 5 min at 5000 × *g* and the supernatants collected were used for gel electrophoresis. Proteinase K digestion of antigens were performed by adding 1 µl of a 1 mg/ml solution of proteinase K (Boehringer Mannheim, Germany) to an aliquot of 100 µl of antigen, and by incubation at 37°C for 1 h. The reaction was stopped by diluting the antigen in sample buffer and boiling for 5 min. Electrophoresis was performed on homogeneous polyacrylamide gels (12%T, 2.7%C) of 1.5 mm thickness using 5% stacking gels with a discontinuous Tris-glycine buffer system (Laemmli, 1970) in a Mini-Protean II electrophoresis cell (Bio-Rad, USA). Samples of 100 µl of antigens in sample buffer were applied over the whole length of the gel for immunoblotting or 10 µl of sample per well on gels that were used for gel staining. Gels were run for 45 min at 200 V and then either Coomassie-stained or transferred to membranes for EIB.

2.5. Electrophoretic immunoblotting (EIB)

Electrophoretic transfer onto PVDF membranes (Immobilon-P membrane, Millipore, USA) was carried out in a Trans-Blot SD cell (Bio-Rad, USA) at 15 V for 30 min. Membranes were blocked overnight at room temperature in TBS (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, pH 7.5) containing 3% non-fat dry milk powder (Anchor, New Zealand; TBS-MP), then incubated for 2 h at room temperature with bovine serum samples of 50 µl, diluted 1 : 100 in TBS-MP (or absorbed, see below) in a Cassette Miniblot System (Immunetics, Cambridge, USA), followed by three washes with TBS-T. Membranes were subsequently incubated for 1 h at room temperature with alkaline phosphatase (AP)-conjugated recombinant protein G (Zymed, San Francisco, CA), diluted 1 : 5000 in TBS-MP and followed by three washes with TBS-T. Finally, they were incubated for 10 min at room temperature with substrate solution (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.8, 60 mM nitro blue tetrazolium and 30 mM 5-bromo-4-chloro indolyl phosphate) followed by two 5 min washes with distilled water.

Some membranes containing proteinase K-digested antigens were washed three times in water after electrophoretic transfer, then incubated with an aqueous solution of 0.7% periodic acid for 20 min at room temperature, washed again three times in water, and then blocked and further processed as described above.

Absorptions of all sera were performed by diluting the sera 1 : 100 in TBS-MP, containing 30 µg/ml of *M. phlei* sonicate (based on protein content) and incubation for 30 min at room temperature. A few sera were absorbed with double dilution series of sonicates ranging from 2 mg/ml to 2 µg/ml (protein content) of *M. phlei*, *M. avium* S18 and *M. paratuberculosis* Teps from 0.4 mg/ml to 0.4 µg/ml (polysaccharide content) W-ELISA antigen, and from 0.1 mg/ml to 0.02 µg/ml pure LAM, diluted in TBS-MP.

2.6. Immunoblots with a monoclonal antibody (MAB)

The cell culture supernatant of MAB PII/BI (IgM, anti 35–47 kDa carbohydrate) (Mutharia et al., 1997) was used in blots on membranes to which antigen (*M. paratuberculosis*, *M. avium*, W-ELISA antigen) had been transferred. All incubation steps with this MAB were carried out as for cattle sera, except that goat anti-mouse Ig (IgG, IgM, and IgA)-alkaline phosphatase conjugate (Sigma) was used.

2.7. Statistical evaluation

Correlation coefficients (faecal colony counts versus absorbance values for the ELISAs, CFT results and blot staining intensity) were calculated using Microsoft Excel, Version 5. CFT titres were converted on a linear scale as follows: no complement fixation at any serum dilution indicated by 0, a reaction of 1/4 (i.e., 25% fixation at the 1 : 4 dilution) by 1.25, a reaction of 2/4 indicated by 1.5 and so forth adding increments of 0.25. Staining in blots was graded visually by allocating values to the strength of the reaction: negative denoting 0, very weak positive, weak positive, positive to strong positive graded from 1 to 4.

Diagnostic sensitivity and specificity were calculated as previously described (Martin et al., 1987), with the Johne's culture-positive cattle as the positive reference population, the Western Australian cattle as the negative reference population.

The chi-square and McNemar's test were used for statistical significance. Optical density values for the Western Australian cattle sera in the W-ELISA were $\log_{10}$ transformed and the distribution tested for normality. The cut-off value for the W-ELISA was established, based on the mean of the optical density values of the Western Australian cattle sera plus 3.09 standard deviations (99.9% target specificity). The distribution of W-ELISA results for the negative reference population was tested for normality using tests for skewness, kurtosis and the Kolmogorov–Smirnov test (Pearson and Hartley, 1972)

3. Results

3.1. Infection status of cattle from which sera were obtained

M. paratuberculosis was isolated from the faeces of 106 (23%) of the animals indicating a high level of infection in all three herds. Prevalence of infection did not differ significantly ($p = 0.53$) between herds, ranging from 20.6% to 26.3% (Table 1).

Contaminants were observed in the cultures of 26 of the 450 animals. In no case were contaminants observed on all the three slopes inoculated for each animal. In only two cases were there contaminants on two of the three slopes and on one slope only for the remaining 24 cases.

3.2. Establishment of cut-off value in the W-ELISA

For the 341 WA negatives, the $\log_{10}$ transformed W-ELISA values were well fitted by a normal distribution (mean $= -0.9331$, S.D. $= 0.1088$) when the highest value was

Table 1
Number of Johne's disease faecal culture positive cattle on each of three New Zealand dairy farms

Farm	Number cultured	Culture positive	%positive
A	133	35	26.3
B	176	42	23.9
C	141	29	20.6
Total	450	106	23.6

$p = 0.053$

excluded as an outlier. This value was 3.90 S.D. above the mean: the probability of the largest of a normally distributed sample of 341 reaching this level is about 1.6%. The normality of the remaining 340 log transformed results was tested using the third and fourth moments (skewness, $p = 0.23$; kurtosis, $p = 0.99$) and using a Kolmogorov–Smirnov test ($p > 0.15$).

3.3. Comparison of the serological tests

Using faecal culture positivity as the gold standard signifying true infection, the sera of the 106 culture positive animals were used to assess the diagnostic sensitivity of the serological assays. The overall diagnostic sensitivity of the W-ELISA (50/106 = 47.2%) was significantly higher ($p = 0.003$) than that of C-ELISA (33/106 = 31.1%) and significantly higher ($p < 0.001$) than the sensitivity of the CFT (19/106 = 17.9%). Sensitivity increased with increasing faecal culture colony counts and reached 87.5% for W-ELISA, 75% for C-ELISA and 56.3% for the CFT for counts above 100 colonies/culture (Table 2).

Absorbance values in W-ELISA showed the highest correlation ($r = 0.619$) with faecal colony counts, followed by C-ELISA ($r = 0.471$) and CFT ($r = 0.452$). The correlation between the absorbances in the two ELISAs was $r = 0.575$, comparable to the correlation between W-ELISA and the CFT of $r = 0.583$. The correlation between the C-ELISA and the CFT was only with $r = 0.4$ (Table 3).

Testing of the 341 sera from Western Australian cattle showed comparable diagnostic specificity ($p > 0.05$) for the two ELISAs and the CFT, at 99.7% for W-ELISA, 97.9% for

Table 2
Number of serological reactors (% in brackets) in two ELISA and CFT tests (including suspicious results) in relation to faecal culture counts from *M. paratuberculosis* infected cattle

Number of colonies/culture	Number positive	Number of positive sera (sensitivity %)		
		W-ELISA	C-ELISA	CFT
1–10	56 (52.8)	18 (32.1)[a]	5 (8.9)[b]	3 (5.4)[b]
11–100	34 (32.1)	18 (62.9)[a]	16 (47.1)[a]	7 (20.6)[b]
>100	16 (15.1)	14 (87.5)[a]	12 (75.0)[a]	9 (56.3)[a]
Total	106 (100)	50 (47.2)[a]	33 (31.1)[b]	19 (17.9)[c]

Figures in the same row with different superscript differ significantly from each other.

Table 3
Correlation (correlation coefficients) of serological test results for 106 sera from cattle herds with positive faecal culture

	Faecal colonies	W-ELISA	C-ELISA	CFT
W-ELISA	0.619			
C-ELISA	0.471	0.575		
CFT	0.452	0.615	0.400	
EIB Antigen-T, all bands, non-absorbed	0.405	0.731	0.564	0.548
EIB Antigen-T, all bands absorbed	0.459	0.802	0.566	0.522
EIB Antigen-T, 32–42 kDa, non-absorbed	0.459	0.784	0.560	0.593
EIB Antigen-T, 32–42 kDa, absorbed	0.480	0.777	0.608	0.543
EIB Antigen-A , all bands, non-absorbed	0.376	0.516	0.377	0.232
EIB Antigen-A, all bands, absorbed	−0.015	0.220	0.170	0.141
EIB Antigen-A, 47 kDa, non-absorbed	0.258	0.359	0.318	0.129
EIB Antigen-A, 47 kDa, absorbed	−0.020	0.169	0.192	0.169

W-ELISA: Wallaceville Animal Health Laboratory's enzyme-linked immunosorbent assay;
C-ELISA: CSL ELISA; CFT: complement fixation test; EIB: electrophoretic immunoblot.

C-ELISA and 97.1% for the CFT. One serum sample was positive in W-ELISA, seven sera were positive in the C-ELISA and ten in the CFT.

3.4. Immunoblot analysis

3.4.1. Antigen characterisation

Three antigen preparations were used for this study of which two (*M. paratuberculosis* Teps (Antigen-T) and *M. avium* Antigen-A) were used for immunoblotting, while the third was the antigen used in the W-ELISA (Antigen-W). Fig. 1 shows the Coomassie-stained SDS-PAGE profiles of the various antigen preparations and the ELISA absorbent, a *M. phlei* sonicate. Antigen-T, Antigen-A and the absorbent exhibited complex protein

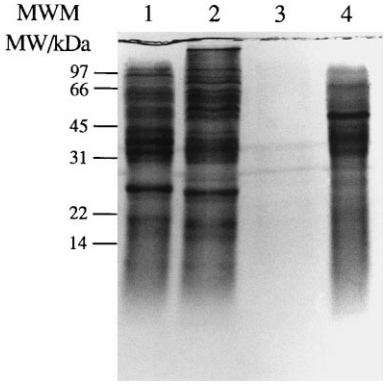

Fig. 1. Polyacrylamide gel electrophoresis analysis of various antigens used in this study in Coomassie- stained gels. (1) Antigen-T, a *M. paratuberculosis* Teps sonicate; (2) Antigen-A, a sonicate of a *M. avium*; (3) Antigen-W, a sonicate used in W-ELISA. (4) *M. phlei* sonicate (used as absorbent in W-ELISA). MWM: molecular weight markers.

staining patterns of protein bands ranging from about 8 to 200 kDa and comprising at least 20 individual bands. The protein concentrations of antigens T, A and the absorbent were 1.9 mg/ml, 10.0 mg/ml and 18.7 mg/ml, respectively. In the gel in Fig. 1, the concentrations are adjusted to 5 mg/ml for all except Antigen-W. Because of their complexity, Antigens T and A presented ideal preparations for immunoblotting in order to identify immuno-dominant antigenic components within these complex macromolecular mixtures.

Antigen-W showed no Coomassie-protein staining in the gel (Fig. 1), which was in accordance with the protein determination. At 0.3 mg/ml this was slightly above the detection limit of 0.2 mg/ml. Polysaccharide determination showed concentrations of 0.14 mg/ml, 0.01 mg/ml and 0.39 mg/ml for Antigens T, A and W, respectively. Therefore, Antigen-W contained predominantly polysaccharide while Antigen-T was composed of proteins and polysaccharides. Antigen-A contained mostly proteins with very little polysaccharide (the value of 0.01 mg/ml was the detection limit).

For immunoblotting Antigen-T and Antigen-A were used at protein concentrations of 1 mg/ml, resulting in 0.07 mg/ml polysaccharide concentration for Antigen-t and a 0.001 mg/ml polysaccharide concentration for Antigen-A.

3.4.2. Immunoblot analysis of sera obtained from M. paratuberculosis faecal culture-positive and non-infected cattle

Immunoblotting results are given in Table 4. The 32–42 kDa antigenic component was the most frequently stained band in blots using Antigen-T. In sera from faecal culture positive animals, 23.6% showed this type of staining which was reduced to 20.8% after pre-absorption of the sera with *M. phlei* sonicate. Bands of 47, 37 and 30 kDa and of variable molecular weight under 21 kDa appeared with low frequency. Highest correlations between the intensity of staining in the immunoblot and other serological tests were found between total bands of Antigen-T or the 32–42 kDa band alone and the W-ELISA. There was little difference between absorbed and non-absorbed sera. All other correlations were poor (Table 3).

Sera from Johne's disease-free cattle showed staining of bands with Antigen-T in 41 sera, which was reduced to nine sera after pre-absorption with *M. phlei* sonicate. The major band recognised was the 32–42 kDa band by 6.7% non-absorbed and 2.1% absorbed sera. The absorbed EIB showed staining of the 32–42 kDa band with five of the C-ELISA positives but not with the one W-ELISA positive serum. One serum, which exhibited the 32-42 kDa staining, was not positive in any of the other serological tests and one was only positive in the CFT. Bands of 47, 37, 30 and 24 kDa and of low molecular weight below 21 kDa stained with low frequency. These bands were mostly absorbed out except for a low frequency of low molecular weight bands staining in 1.2% of absorbed sera.

In faecal culture-positive cattle sera the 47 kDa protein band stained most frequently with Antigen-A, and was observed in 28.3% of non-absorbed and 8.5% of pre-absorbed sera. Other antigenic components (37, 32–42, 30, 24 and 21 kDa and lower) appeared with much lower frequency. Staining at 37 and 21 kDa and below was completely absorbed out, and the frequency of staining reduced for the other bands. Fig. 2 shows a

Table 4
Electrophoretic immunoblotting results (% positives) for sera from Johne's disease faecal culture positive cattle (positive) and non-infected cattle from Western Australia (negative), non-absorbed and absorbed with *M. phlei* sonicate

Antigen used	No. of sera	EIB (all bands)		Individual bands													
				47 kDa		37 kDa		32–42 kDa		30 kDa		24 kDa		21 kDa		<21 kDa	
		non	abs	non	abs	non	abs	non	abs	non	abs	non	abs	non	abs	non	abs
Antigen-T																	
Positive[a]	106	27.4	24.5	0.9	0.0	2.8	0.0	23.6	20.8	0.0	0.9	0.0	0.0	0.0	0.0	2.8	0.9
Negative[a]	341	12.0	2.3	2.6	0.0	5.0	0.0	6.7	2.1	1.2	0.0	0.3	0.0	0.0	0.0	3.2	1.2
Antigen-A																	
Positive[a]	106	31.1	9.4	28.3	8.5	4.7	0.0	5.7	0.9	0.9	0.9	1.9	0.9	0.9	0.0	5.7	0.0
Negative[a]	341	14.1	4.1	9.4	2.3	1.2	0.0	0.9	0.0	3.2	0.9	1.8	0.3	1.5	0.0	6.2	2.9

[a]Indicates infection status.
EIB: electrophoretic immunoblot; non: non-absorbed; abs: absorbed.

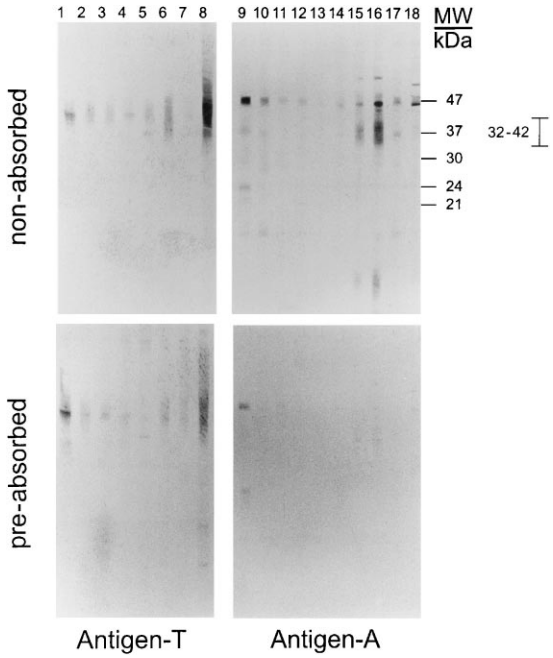

Fig. 2. Staining in blots of a random selection of sera (1–18) from *M. paratuberculosis* faecal culture-positive cattle. Sera were either non-absorbed or pre-absorbed with *M. phlei* sonicate. Antigen-T: a *M. paratuberculosis* Teps sonicate, Antigen-A: a sonicate of a *M. avium* isolate. Molecular weights (MW) indicate antigenic components identified in blots.

selection of these sera and the effects of absorption. Correlation between staining in immunoblots and other serological tests was poor for Antigen-A.

Sera from Johne's disease-free cattle immunostained mostly a 47 kDa protein band with Antigen-A (9.4% non-absorbed, 2.3% absorbed). Three of those sera were identical to those exhibiting the dominant 32–42 kDa staining with Antigen-T. One of those sera was the one positive in the W-ELISA, and three were positive in the C-ELISA. Other bands (37, 32–42, 24, 21 kDa and molecular weight below 12 kDa) stained with low frequency and were generally totally absorbed out.

3.4.3. Characterisation of the 32–42 kDa antigenic component

The finding that the ELISA antigen (Antigen-W) contained mostly polysaccharides, the relatively good correlation between W-ELISA and the immuno-dominant 32–42 kDa component in the *M. paratuberculosis* sonicate (Antigen-T) and the low appearance of this staining in Antigen-A which contained little polysaccharide, suggested that the 32–42 kDa component may represent a polysaccharide. A selection of 12 sera from cattle herds with Johne's disease was used to further analyse the various antigens by immunoblot after proteinase K and periodic acid treatment (Fig. 3).

With Antigen-T the bands of 47, 37 and 30 kDa disappeared after proteinase K digestion, while staining of the 32–42 kDa band remained at equal strength, indicating

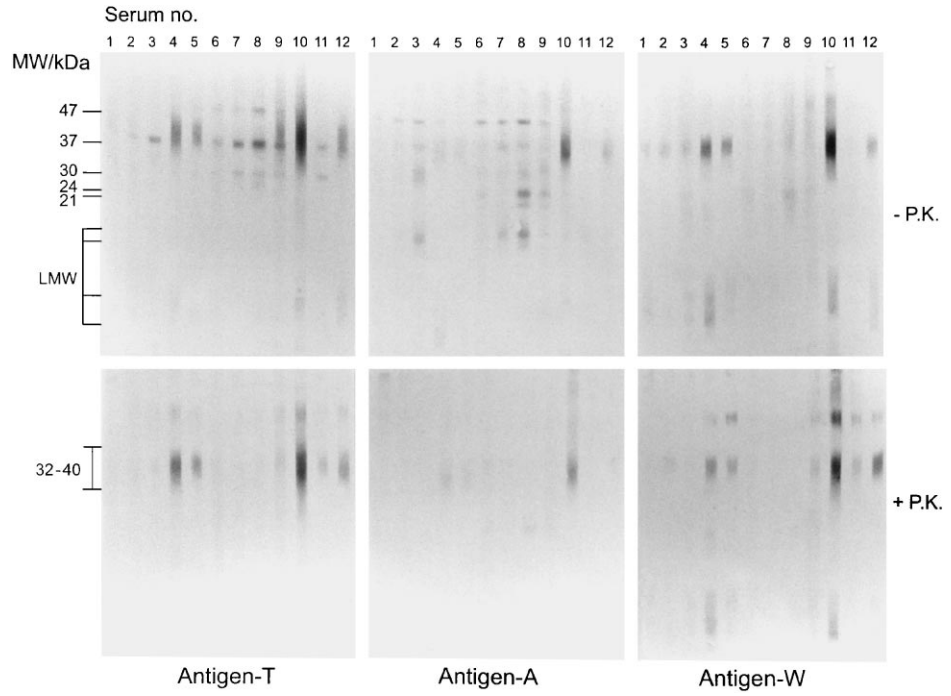

Fig. 3. Immunoblot analysis of antigens by using a panel of twelve sera (lanes 1–12) from cattle herds with Johne's disease. Antigen-T: a *M. paratuberculosis* Teps sonicate; Antigen-A: a sonicate of a local *M. avium* strain; Antigen-W: current W-ELISA antigen. Molecular weights indicate antigenic components identified in blots. LMW–low molecular weight components, ranging from 8–20 kDa. Upper panels show results with antigens not treated with proteinase K (−P.K); lower panels show results with proteinase K-treated antigens (+P.K).

that this band represented a non-protein macromolecule. Pre-treatment of the membrane with periodic acid resulted in the disappearance of the 32–42 kDa band, confirming its polysaccharide character. When using the W-ELISA antigen in blot with or without proteolytic degradation, mainly the 32-42 kDa band was immuno-stained. This staining disappeared completely after periodic acid treatment or was strongly reduced depending on the staining intensity of the serum used. With Antigen-A bands of 47, 37, 30, 24 and 21 kDa were visible and only little 32-42 kDa staining. The latter appeared only with sera that stained this component strongly in Antigens T and W. Except of the 32–42 kDa staining, all other bands disappeared after proteolytic digestion. After periodate treatment the 32–42 kDa staining disappeared.

The staining of the 32–42 kDa component in blots by cattle sera was also compared to the immuno-staining of Mab PII/BI, which is specific for a LAM-like molecule from *M. paratuberculosis* (Fig. 4). Staining with this MAB was similar in appearance and size to the staining of the cattle sera, showing a diffuse band of 32–42 kDa. Only Antigens T and W exhibited staining with this MAB.

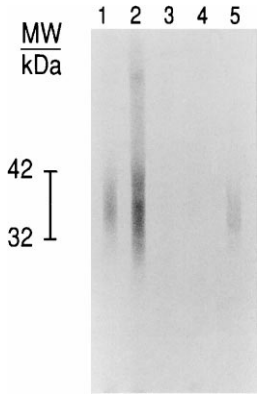

Fig. 4. Comparison of staining in blots of sera from two faecal-culture positive cattle with medium and strong staining of the 32–42 kDa antigenic component of *M. paratuberculosis* (lanes 1 and 2) and two negative cattle sera (lanes 3 and 4), with the staining of the anti-polysaccharide monoclonal antibody PII/BI (lane 5). Antigen was a *M. paratuberculosis* Teps sonicate (Antigen-T).

3.4.4. Absorption studies

Absorption studies were performed with four sera from *M. paratuberculosis* faecal culture positive cattle in order to further characterise the 32–42 kDa component and to identify the presence of *M. paratuberculosis*-specific epitopes in this polysaccharide. The sera used showed medium or strong staining of the 32–42 kDa band in blots absorbed and non-absorbed in the initial screening. Sera were pre-absorbed with increasing

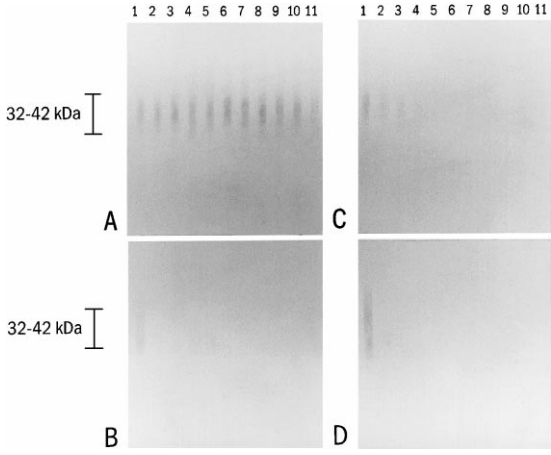

Fig. 5. Immunoblot staining of a serum from a faecal culture positive cow, non-absorbed (lane 1) or pre-absorbed with increasing concentration of mycobacterial sonicates (double dilution series, lanes 2–11, 1/2 to 1/1024). Antigen in blots was *M. paratuberculosis* Teps sonicate (Antigen-T). A: absorption with *M. phlei* sonicate, which had no effect on the staining of the 32–42 kDa band. B, C and D: effect of absorption with *M. avium* S18, *M. paratuberculosis* Teps sonicate (Antigen-T) and the W-ELISA antigen (Antigen-W). Similar results to B, C and D were found with pure LAM as absorbent (not shown).

concentrations of LAM and sonicates from *M. phlei*, the W-ELISA antigen (Antigen-W), *M. avium* S18 and *M. paratuberculosis* Teps (Antigen-T). They were run in blots with *M. paratuberculosis* Teps sonicate (Antigen-T) as antigen. All sera exhibited similar behaviour to absorption (Fig. 5). While absorption of the sera with *M. phlei* had no or only a minor effect on the intensity of staining of the 32–42 kDa band in blots, pre-incubation of sera with pure LAM, Antigen-T, the *M. avium* S18 sonicate and Antigen-W, however, completely removed this staining.

4. Discussion

The sensitivity of serological tests for Johne's disease is limited by the nature of *M. paratuberculosis* infections, with seroconversion occurring relatively late during the course of the disease (Kreeger, 1991). Another limitation is the potential reduction in specificity through cross-reactions with environmental bacterial species (Chiodini et al., 1984; De Kesel et al., 1992; Gilot et al., 1992; Gilot and Misonne, 1994). The specificity of serological testing was improved by the introduction of absorbed ELISAs, which reduced serological cross-reactivities (Yokomizo, 1986; Ridge et al., 1991). In this study the diagnostic specificity of the serological tests was high and comparable to those found in previous reports (Milner et al., 1990; Sherman et al., 1990; Cox et al., 1991; Ridge et al., 1991; Sockett et al., 1992). The W-ELISA compared well with the commercial absorbed C-ELISA and with the other serological tests used. While the C-ELISA is reported to be up to 57% sensitive under field conditions (Milner et al., 1990), none of the serological tests were able to attain such sensitivity in the present study. The diagnostic sensitivity, however, is dependent as demonstrated in the present study on the degree of bacterial shedding. The relative sensitivity of different serological tests can only be determined by examining the same groups of sera. The high proportion of cattle shedding low numbers of mycobacteria in the faeces in this study (43.4% $\leq$ 5 colonies, 52.8% $\leq$ 10 colonies) explains the low level of serological reactions observed. The complement fixation test is still a prescribed test in international trade (Anon., 1996), but its ability to detect culture-positive cattle is less than half of that of the W-ELISA at comparable specificity. Even in high bacterial shedders (>100 colonies/2 g) the sensitivity of the CFT is more than 30% lower than that of W-ELISA, suggesting that the CFT should be replaced by the ELISA in import/export testing, as well as in diagnostic testing.

The total number of infected animals in the three study herds is likely to be considerably greater because many minimally infected animals will not be detected by a single faecal culture (Chiodini et al., 1984).

Analysis of the antigen used in the W-ELISA, by gel electrophoresis and immunoblotting revealed that this antigen preparation consisted mostly of a 32–42 kDa polysaccharide bacterial component, suggesting that this polysaccharide may be immuno-dominant during infections. Polysaccharide antigens such as lipoarabinomannan were used by others in ELISA with sheep sera, attaining 70.6% sensitivity and 95.2% specificity (Sugden et al., 1989). Subsequently, all sera used in the ELISA comparison in the present study were screened in immunoblots, absorbed and non-absorbed with *M. phlei* sonicate against two antigen preparations in order to identify specific and dominant

antigenic components. The 32–42 kDa antigenic component was confirmed as the immuno-dominant component and, because serum antibodies against it could be absorbed out by pure LAM, it represents LAM or a LAM-like molecule. It is most likely identical to the previously reported carbohydrate of 31–43 kDa of similar appearance in blots (Valentin-Weigand and Moriarty, 1992) and it may also be identical to a cluster of diffusely-appearing bands in blots of a 34–38 kDa band, then considered a polypeptide (Bech-Nielsen et al., 1985). The relatively low diagnostic sensitivity of 20.8% for the 32–42 kDa antigen in EIB compared to 47.2% for W-ELISA in this study may be partially due to the loss of carbohydrate epitopes during sample preparation for EIB, which included boiling in denaturing reagents. This would make an immunoblot unsuitable in routine serological diagnosis of Johne's disease in cattle, but the sensitivity is comparable to the one shown by the CFT in this study.

Protein antigens have been reported which are supposed to be specific for *M. paratuberculosis*. These include a 34 kDa protein, a component of the so-called A36 complex, which has been shown to be immunodominant and to contain *M. paratuberculosis*-specific epitopes (Vannuffel et al., 1994). Furthermore, proteins of 43 kDa (Tizard et al., 1992), 42 kDa (White et al., 1994), 34.5 kDa, containing a specific epitope (Mutharia et al., 1997) and 27 kDa (Valentin-Weigand et al., 1991) have been described. In the immunoblotting study reported here, serum antibodies against proteins of 47, 37, 30, 24 and 21 kDa were frequently found, of which the 47 kDa protein was immuno-dominant. It is likely that some of these proteins are identical to the previously reported antigens. In addition, our results show that protein antigens also contribute to non-specific cross-reactivities. This became obvious from the EIB results of sera from Johne's disease free cattle herds directed against protein components of the antigens and the ability to reduce most of these reactivities by pre-absorbing the cattle sera with a *M. phlei* sonicate. There was also a poor correlation between the faecal colony count and the 47 kDa protein band in blots. Others have shown with a limited number of sera in blotting/ absorption studies that certain bands, presumably protein bands, were absorbed out by *M. phlei* sonicate, while staining in the 40–43 kDa region remained unaffected (Milner et al., 1987). These results are identical in principle to our findings, assuming that the 40–43 kDa antigen is identical to the 32–42 kDa polysaccharide reported here.

Attempts were made to find *M. paratuberculosis*-specific epitopes on the 32–42 kDa macromolecule. Absorption of sera with *M. phlei* sonicate even at increased concentrations revealed that epitopes exist in the 32–42 kDa molecule which are not present in *M. phlei* (and thus could not be absorbed out). Absorption experiments with *M. avium* S18 and with Antigen-W, the latter mainly containing the 32-42 kDa polysaccharide of a *M. avium* strain, showed that these epitopes are not unique to *M. paratuberculosis*. Sonicates of both mycobacterial species completely absorbed out immunostaining of the 32–42 kDa component in blots. *M. avium* S18, which was used in the past as a *M. paratuberculosis* reference strain, is now considered another species of slow-growing mycobacteria (Collins and de Lisle, 1986; Chiodini, 1993). These results strongly suggest that *M. paratuberculosis* specific antigens or epitopes, which are also immuno-dominant in infected animals, do not exist and that the 32–42 kDa polysaccharide appears to be specific to the genus *M. avium* only.

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References

Anonymous, 1996. In: Manual of Standards for Diagnostic Tests and Vaccines, Office International des Epizooties, Paris, 218–228.

Bech-Nielsen, S., Burianek, L.L., Spangler, E., Heider, L.E., Hoffsis, G.F., Dorn, C.R., 1985. Characterization of *mycobacterium paratuberculosis* antigenic proteins. Am. J. Vet. Res. 46, 2418–2420.

Chiodini, R.J., 1993. Abolish *mycobacterium paratuberculosis* strain 18. J. Clin. Microbiol. 31, 1956–1958.

Chiodini, R.J., Van Kruiningen, H.J., Merkal, R.S., 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. 74, 218–262.

Collins, D.M., de Lisle, G.W., 1986. Restriction endonuclease analysis of various strains of *Mycobacterium paratuberculosis* isolated from cattle. Am. J. Vet. Res. 47, 2226–2229.

Collins, D.M., Stephens, D.M., de Lisle, G.W., 1993. Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. Vet. Microbiol. 36, 289–299.

Cox, J.C., Drane, D.P., Jones, S.L., Ridge, S., Milner, A.R., 1991. Development and evaluation of a rapid absorbed enzyme immunoassay test for the diagnosis of Johne's disease in cattle. Aust. Vet. J. 68, 157–160.

De Kesel, M., Gilot, P., Coene, M., Cocito, C., 1992. Composition and immunological properties of the protein fraction of A36, a major antigen complex of *Mycobacterium paratuberculosis*. Scand. J. Immunol. 36, 201–212.

El-Zaatari, F.A.K., Naser, S.A., Graham, D.Y., 1997. Characterization of a specific *Mycobacterium paratuberculosis* recombinant clone expressing 35 000 molecular-weight antigen and reactivity with sera from animals with clinical and subclinical Johne's disease. J. Clin. Microbiol. 35, 1794–1799.

Gilot, P., De Kesel, M., Coene, M., Cocito, C., 1992. Induction of cellular immune reactions by A36, an antigen complex of *Mycobacterium paratuberculosis*: comparison of A36 and Johne's components. Scand. J. Immunol. 36, 811–821.

Gilot, P., Misonne, M.C., 1994. *Mycobacterium paratuberculosis* and *Escherichia coli* share common antigenic determinants. Vet. Microbiol. 39, 353–360.

Hilbink, F., West, D.M., de Lisle, G.W., Kittelberger, R., Hosie, B.D., Hutton, J., Cooke, M.M., Penrose, M., 1994. Comparison of a complement fixation test, a gel diffusion test and two absorbed and unabsorbed ELISAs for the diagnosis of paratuberculosis in sheep. Vet. Microbiol. 41, 107–116.

Kreeger, J.M., 1991. Ruminant paratuberculosis – A century of progress and frustration. J. Vet. Diagn. Invest. 3, 373–383.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Martin, S.W., Meek, A.H., Willeberg, P., 1987. In: Veterinary Epidemiology: Principles and Methods. Iowa State University Press, Ames, 48–76.

Milner, A.R., Lepper, A.W.D., Symonds, W.N., Gruner, E., 1987. Analysis by ELISA and western blotting of antibody reactivities in cattle with *Mycobacterium paratuberculosis* after absorption of serum with *M. phlei*. Res. Vet. Sci. 42, 140–144.

Milner, A.R., Mack, W.N., Coates, K.J., Hill, J., Gill, I., Sheldrick, P., 1990. The sensitivity and specificity of a modified ELISA for the diagnosis of Johne's disease from a field trial in cattle. Vet. Microbiol. 25, 193–198.

Mutharia, L.M., Moreno, W., Raymond, M., 1997. Analysis of culture filtrate and cell wall-associated antigens of *Mycobacterium paratuberculosis* with monoclonal antibodies. Infect. Immun. 65, 387–394.

Pearson, E.S., Hartley, H.O., 1972. Biometrika Tables for Statisticians. Cambridge University Press, pp. 118.

Ridge, S.E., Morgan, I.R., Sockett, D.C., Collins, M.T., Condron, R.J., Skilbeck, N.W., Webber, J.J., 1991. Comparison of the Johne's absorbed EIA and the complement-fixation test for the diagnosis of Johne's disease in cattle. Am. Vet. J. 68, 253–257.

Severn, W.B., Jones, A.M., Kittelberger, R., de Lisle, G.W., Atkinson, P.H., 1997. Improved procedure for the isolation and purification of lipoarabinomannan from *Mycobacterium bovis* strain AN5. J. Microbiol. Methods 28, 123–130.

Sherman, D.M., Gay, J.M., Bouley, D.S., Nelson, G.H., 1990. Comparison of the complement fixation test and agar gel immunodiffusion test for the diagnosis of subclinical bovine paratuberculosis. Am. J. Vet. Res. 51, 461–465.

Sockett, D.C., Conrad, T.A., Thomas, C.B., Collins, M.T., 1992. Evaluation of four serological tests for bovine paratuberculosis. J. Clin. Microbiol. 30, 1134–1139.

Sugden, E.A., Corner, A.H., Samagh, B.S., Brooks, B.W., Turcotte, C., Nielsen, K.H., Stewart, R.B., Duncan, J.R., 1989. Serodiagnosis of ovine paratuberculosis, using lipoarabinnomannan in an enzyme-linked immunosorbent assay. Am. J. Vet. Res. 50, 850–854.

Sugden, E.A., Samagh, B.S., Bundle, D.R., Duncan, J.R., 1987. Lipoarabinomannan and lipid-free arabinomannan antigens of *Mycobacterium paratuberculosis*. Infect. Immun. 55, 762–770.

Tizard, M.L.V., Moss, M.T., Sanderson, J.D., Austen, B.M., Hermon-Taylor, J., 1992. p43, the protein product of the atypical insertion sequence IS900, is expressed in *Mycobacterium paratuberculosis*. J. Gen. Microbiol. 138, 1729–1736.

Valentin-Weigand, P., Murray, C., Moriarty, K.M., 1991. Antibody reactivities of *Mycobacterium paratuberculosis* infected sheep as analyzed by enzyme-linked immunosorbent assay and Western blotting. FEMS Microbiol. Lett. 78, 145–148.

Valentin-Weigand, P., Moriarty, K.M., 1992. Protein antigens secreted by *Mycobacterium paratuberculosis*. J. Vet. Med. B39, 762–766.

Vannuffel, P., Gilot, P., Limbourg, B., Naerhuyzen, B., Dietrich, C., Coene, M., Machtelinckx, L., Cocito, C., 1994. Development of species-specific enzyme-linked immunosorbent assay for diagnosis of Johne's disease in cattle. J. Clin. Microbiol. 32, 1211–1216.

White, W.B., Whipple, D.L., Stabel, J.R., Bolin, C.A., 1994. Comparison of cellular and extracellular proteins expressed by various isolates of *Mycobacterium paratuberculosis* and other mycobacterial species. Am. J. Vet. Res. 55, 1399–1405.

Yokomizo, Y., 1986. Evaluation of an enzyme-linked immunosorbent assay (ELISA) using *Mycobacterium phlei*-absorbed serum for the diagnosis of bovine paratuberculosis in a field study. Jpn. Agric. Res. Q. 20, 60–67.

1.3. Brucellosis (*Brucella ovis*)

Reichel, MP; Ross, G; Drake J and Jowett, JH (1999)

Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams.

New Zealand Veterinary Journal **47**, 43-46

Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams

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Abstract

Aim. To describe the performance characteristics (sensitivity and specificity) of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of *Brucella ovis* infection in rams.

Methods. Sera from a negative (n = 2535) and a positive (n = 589) reference population were tested in an ELISA for anti-*B. ovis* antibodies and cut-off values calculated from the raw, $\log_{10}$-transformed and fitted data. Statistical methods were used to fit curves to the frequency distribution of the data and receiver-operated characteristics analysis used to optimise the cut-off values.

Results. Analysis of the frequency distribution of the positive ELISA values suggested a normal distribution of the data, whereas, in the case of the negative population, a Pearson type IV curve appeared to best fit the data. The cut-off values calculated as the mean plus 1.96 standard deviations (s.d.) from the raw, log-transformed and fitted ELISA data did not differ markedly. The differences were much greater at the mean plus 3.09 s.d. cut-off, with the cut-off value calculated from the log-transformed data giving a much better estimate of specificity. Optimisation (minimisation of classification error) of the cut-off calculated from the fitted curves suggested varying cut-off values, depending on the prevalence of *B. ovis* infection.

Discussion. Calculation of cut-off values from curves that were fitted from the observed data give more accurate estimations of the performance characteristics of an assay than traditional calculations from observed values. They also allow the calculation of optimal cut-off values taking into account the prevalence of *B. ovis* infection and give additional information about the performance of the assay at cut-off values varied according to the epidemiological situation.

Key words. *Brucella ovis*, rams, ELISA, sensitivity, specificity, ROC-analysis.

(New Zealand Veterinary Journal 47, 71-74, 1999.)

Introduction

Brucella ovis infection in rams causes epididymitis and may lead to infertility (Burgess, 1982). Since the infection was first described in the early 1950s (Buddle and Boyes, 1953; Simons and Hall, 1953) it has been reported in most sheep-rearing countries around the world (Burgess, 1982). Notable exceptions are the United Kingdom, where it has never occurred, and the Falkland Islands, where it has recently been eradicated (Reichel *et al.*, 1994). Diagnosis of infection is usually achieved by serological testing, and in New Zealand the official test of the voluntary *B. ovis* accreditation scheme is the complement fixation test (CFT) (Reichel and West, 1997). Occasionally, however, anti-complementary reactions occur with a small number of sera and no result can be given. Also, chronically infected rams may appear consistently negative in CFT testing (Worthington *et al.*, 1985) and this may hinder eradication attempts due to an inability to identify those residual foci of infection. Additional serological tests, such as the enzyme-linked immunosorbent assay (ELISA) are required to detect those CFT-negative yet infected individuals. An ELISA (Worthington *et al.*, 1984) has been described in New Zealand for such situations, but some of the critical parameters (incubation temperature and time), reagents (conjugate, control sera) and cut-off levels of the assay have been changed. It was therefore necessary to revalidate this ELISA for use in the accreditation programme.

Materials and Methods

Sera

Negative sera

Two thousand five hundred and thirty-five ram serum samples were obtained from routine submissions for re-accreditation in the New Zealand *B. ovis* voluntary accreditation scheme from sheep flocks which had been accredited free from *B. ovis* infection for several years.

Positive sera

Five hundred serum samples were obtained from rams in infected flocks which had reacted in the agar gel immunodiffusion test (AGID) and 89 serum samples from *B. ovis* semen culture-positive rams.

Serological testing

The AGID test was performed as previously described (Worthington, 1983). The ELISA was carried out as previously described (Worthington *et al.*, 1984), except that a protein G HRPO-conjugate (Zymed, San Francisco, USA) and a different positive control was used in the assay. The incubation temperature and time for the assay was also changed. The antigen concentration was optimised for each batch by checkerboard titration, as was the dilution of the conjugate.

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Table I. Cut-off values (and sensitivity and specificity) for the *B. ovis* ELISA from results of 2535 negative sera calculated from raw ELISA units, after logarithmic (log_{10}) transformation, and from fitted distributions

Data type	Cut-off	Specificity (%)		Sensitivity (%)	
		Observed	Fitted	Observed	Fitted
Mean + 1.96 s.d., target specificity 97.5%					
Raw ELISA units	100	97.5	97.2	97.8	98.1
Log_{10} ELISA units	96	97.1	96.9	98.0	98.4
Fitted curve	105	97.8	97.5	97.4	97.8
Mean + 3.09 s.d., target specificity 99.9%					
Raw ELISA units	139	98.8	98.7	94.9	95.1
Log_{10} ELISA units	219	99.4	99.6	86.0	85.7
Fitted curve	408	99.9	99.9	60.8	60.3

Appropriately diluted serum samples and controls were incubated with the antigen overnight at +4 °C, the plates emptied, tapped dry and washed six times. Conjugate was added to all wells and the plates incubated for 37 °C for 2 hours. After six further washes, the substrate (OPD) was added and the colour developed for 7 minutes before the reaction was stopped with 6% sulphuric acid. Absorbance values were read on a Titertek Multiskan MC reader (Flow Laboratories) at 490 nm and 630 nm. ELISA unit (EU) values were calculated from a standard curve derived from four dilutions of the positive control serum, given an arbitrary value of 1000 ELISA units/ml.

Results

Untransformed ELISA unit values for the 500 AGID and 89 semen culture positive sera were markedly non-normal and skewed. After log-transformation and exclusion of five outliers > 4.6 s.d., the data appeared approximately normally distributed. The means for the two positive groups were not dissimilar at 2.688 (s.d. = 0.303) and 2.699 (s.d. = 0.342). The difference between means and s.d. did not approach statistical significance (p = 0.25 and 0.12 respectively). The combined data (mean = 2.698; s.d. = 0.336) were normally distributed, supported by the coefficient of skewness and kurtosis (p = 0.20 and p = 0.099) and a Kolmogorov-Smirnov test (Pearson and Hartley, 1972) of goodness of fit (p > 0.10).

For the negative sera (mean = 1.353; s.d. = 0.320), a normal distribution did not fit the log-transformed data well (skewness and kurtosis both p < 0.001). However, a type IV distribution from the Pearson system (Kendall and Stuart, 1958) did fit the data, both over the bulk of the data and in the tails, without any need for discarding observations as outliers. The fit was confirmed by a chi-squared test of goodness of fit (p = 0.70). Fitted curves and the relative frequency distribution for the ELISA unit (EU) values of the negative and positive reference populations are given in Figure 1.

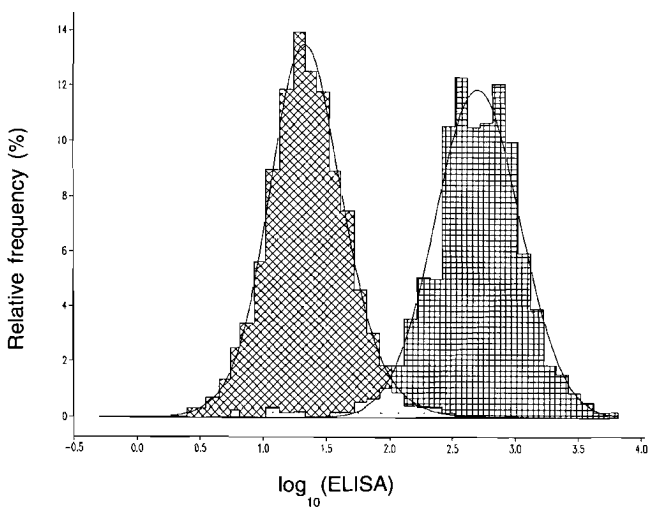

Figure 1. Frequency distributions for *B. ovis* ELISA values from negative (n = 2535) and positive (n = 589) sera with fitted distribution curves.

For the untransformed EU data, the cut-off values (established as the mean plus 1.96 and 3.09 s.d.) resulted in 100 EU and 139 EU, respectively while for the log-transformed data, mean EU values plus 1.96 and 3.09 s.d. resulted in cut-off values of 96 EU and 219 EU, respectively. Cut-offs obtained from the fitted curve (to give fitted specificity of 97.5% and 99.9%) were 105 and 408, respectively. The observed specificities (i.e. percentage of the negative sample at or above the cut-off) and resulting sensitivities for these cut-offs are shown in Table I.

A two-graph receiver operating characteristics (ROC)-type analysis (Greiner *et al.*, 1995) of the fitted negative and positive distributions allows the operator to select cut-off values, according to circumstances, from a range of specificities and sensitivities (Figure 2). The likelihood ratio (Smith, 1995), calculated from the fitted curves, is graphed in Figure 3. Varying cut-off values, which minimise the total number of classification errors as a function of prevalence, are shown in Figure 4.

Discussion

In New Zealand the CFT is the official test in the *Brucella ovis* accreditation scheme (Reichel and West, 1997). Occasionally, however, serum samples react in an anti-complementary manner and a result cannot be given. Chronically infected rams may also consistently not react in the CFT (Worthington *et al.*, 1985). To overcome these situations, an enzyme-linked immunosorbent assay has been used to be able to give serological results for anti-complementary samples, or to successfully detect chronically infected rams.

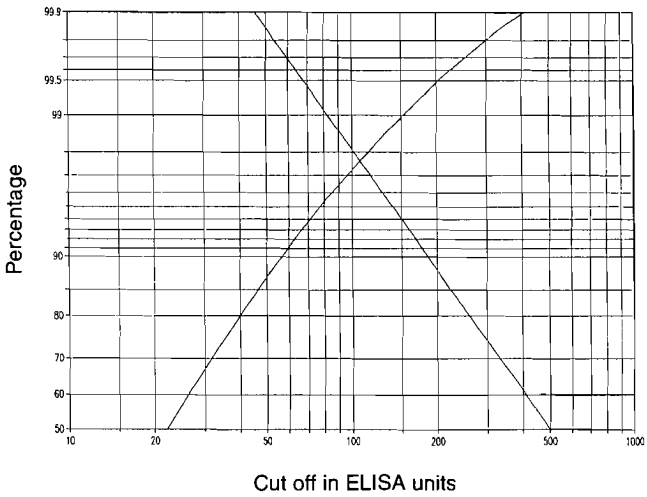

Figure 2. Sensitivity and specificity of the *B. ovis* ELISA calculated from the distribution curves fitted to the negative and positive reference populations.

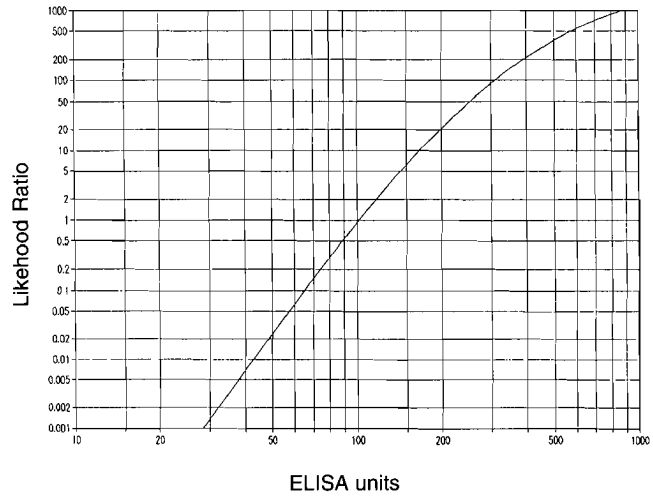

Figure 3. Likelihood ratio for *B. ovis* ELISA results.

The determination of appropriate cut-off criteria in serological assays relies in the first instance on the definition of reliably negative and positive reference populations and secondly on the similarity of those reference groups with the target population. In the case of *B. ovis*-infected animals, semen culture isolation of the organism constitutes the "gold standard" for positivity. Previous publications have also demonstrated that the AGID test possesses 100% specificity (Worthington *et al.*, 1984) and thus the 500 AGID-positive sera appeared to be a reasonable choice as a positive reference population. The comparisons of the $\log_{10}$-transformed ELISA values (frequency distribution, mean, s.d. and kurtosis, skewness and KS-test) suggested no significant difference between the two populations and the combined data set of the two groups appeared to be normally distributed. However, some outliers with very low EU values were ignored in the calculation. It was assumed that these values were aberrations in the performance of the assay, but their existence should be noted, as they may conceivably represent some small subpopulation of rams in which *B. ovis* is essentially undetectable using the ELISA tests with any reasonable cut-off.

Traditional cut-off calculations are based on establishing the threshold between negative and positive populations as the mean plus 2 or 3 standard deviations of the negative population (1.96 s.d. and 3.09 s.d. have been used to achieve a specificity of 97.5% and 99.9% more precisely). Implicit in this method of using standard deviations from the mean of negatives is the assumption that the data from the negative reference population is normally distributed. As there was evidence of non-normality for the ELISA values of the negative reference population, even after log-transformation, a more valid approach seemed to be to determine cut-offs from the fitted Pearson curve to achieve the relevant target specificities. Values for specificity, sensitivity and related parameters can then be estimated with significantly better precision from the fitted data. Cut-off calculations using the two and three s.d. method from the ELISA-values result in a cut-off at 2 s.d., which was very similar for all three methods. However, the 3 s.d. cut-off for un-transformed and log-transformed EUs differed significantly, both from each other and from the one derived from the fitted curve.

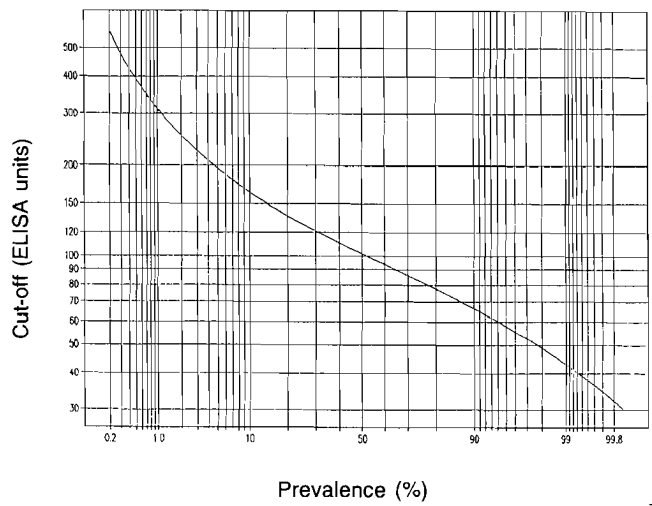

Figure 4. Optimal cut-off values (minimising error rates) in the *B. ovis* ELISA depending on the prevalence of *B. ovis* infection.

Derivation of the cut-off using this method, however, does not take into account effects on the sensitivity. ROC-analysis gives a measure of the impact of changes of the cut-off to both sensitivity and specificity, and allows the operator to pre-select from the available combinations of the two characteristics to suit the circumstances.

The point at which the specificity and sensitivity curves intersect (Figure 2) has some merit if a single cut-off value is to be chosen. The ELISA value of 107.1 results in sensitivity and specificity both being equal at 97.7%. This cut-off is optimal in the sense that it minimises the total error rate (false positives + false negatives) as for any other cut-off there will be prevalences at which this error rate would be exceeded. The total error rate for this cut-off is fixed at 2.3% for all prevalences. This error rate of 2.3% is in fact the minimum obtainable for a prevalence of 45%, and does not change significantly within the range 20-70%, the minimum possible error rate for prevalences within this range all being above 2%. However, if reasonable limits can be placed

on prevalence, better error rates can be achieved. If we could be confident of a prevalence of *B. ovis* infection of 10% or below, a cut-off of 170 ELISA units could be used, with a maximum error rate of 1.5% (Figure 4). In accredited flocks of long standing an assumption of prevalence close to 0% is not unreasonable. The optimum cut-off, as suggested by Figure 4, would thus be well in excess of 400 EU. Test sensitivity at 400 EU is still reasonable at about 60%.

Ideally, one would like to determine, for a given ELISA result, what the relative probabilities of the animal being positive or negative are. Likelihood ratios (Smith, 1995) give these relative probabilities (or "odds") for a prevalence of 50% when the ELISA value is known only to the extent that it falls below (likelihood ratio negative) or above (likelihood ratio positive) a specified cut-off. In common use among statisticians is an alternative definition of likelihood ratio, as the ratio of the relative frequency curves. This would be more appropriate where an actual numerical value is available. This is graphed in Figure 3, calculated from the fitted curves. It gives, for any ELISA result, the factor by which the pre-test odds of an animal being positive should be multiplied to give post-test odds. For example, if the pre-test probability of an animal having *B. ovis* was 10% (i.e. the prevalence of *B. ovis* infection is 10%), and that animal gave an ELISA result of 150, then the pre-test odds of 10% : 90% or 1/9 would be multiplied by about 7 (from the graph in Figure 3) to give post-test odds of 7/9 and a post-test probability of 7/(7+9) or about 44%. Graphs, tables or computer programmes can be constructed reasonably easily to convert a prevalence (or prior probability) and ELISA result into an estimate of the probability of an animal having *B. ovis*, using the likelihood ratio.

An ELISA result of about 100 units is essentially uninformative, as this result is equally likely, whether an animal is infected or not. This value is in the vicinity of the 2 s.d. cut-off. The 2 s.d. cut-off is designed to classify as many infected animals as positive as possible subject to it correctly classifying 97.5% of negatives. The 97.5% have already been obtained by the time we reach 100 ELISA units, and thereafter we count everything as positive. If the distributions of positives and negatives were further apart, a 2 s.d. cut-off could even classify as positive those animals for which the likelihood ratio strongly suggested negativity. Fortunately such animals would be relatively rare, but the situation highlights the difficulties involved in basing a cut-off on specificity alone.

An alternative approach is to try choose a cut-off that optimises some measure of long term test performance, such as the percentage of incorrect decisions, or an appropriately cost weighted combination of the percentages of false positives and false negatives. Such a cut-off will again vary with prevalence, low prevalence suggesting high cut-offs. In Figure 4 the performance criterion being minimised is the percentage of incorrect decisions, but simple calculations would enable the graph to be used in the cost-weighted situation.

Greiner *et al.* (1995) suggested that an intermediate range be set between 95% sensitivity and specificity. In the present example this would suggest a "suspicious" range from 78 to 139 ELISA units.

However, an accreditation scheme such as the one for *B. ovis*, which gives a fairly reliable estimate of the pre-test probability (prevalence) based on the testing history of a flock, affords the opportunity to operate with two thresholds — one maximising sensitivity (in infected flocks), the other specificity (in accredited flocks). From Figure 2, maximum sensitivity and specificity (at 99.9%) arrives at cut-off values of 47 and 408 EU, respectively. The cut-off value of 408 EU could be used in sera requiring ELISA testing in accredited flocks (minimising the probability of a false-positive result) and a cut-off value of 47 EU in flocks where *B. ovis* infection is likely (i.e. other evidence corroborates the infection). For a non-accredited flock (where infection is not suspected, or of low prevalence), a cut-off of 170 EU would minimise the mis-classification rate.

The sensitivity and specificity for the ELISA described here (at a cut-off of 114 and 133 ELISA units) are not dissimilar to those presented by Worthington *et al.* (1984), which were 97.2% (sensitivity) and 98.6% (specificity), respectively, at a cut-off of 400 and 500 ELISA units. However, the Worthington *et al.* (1984) ELISA gave performance characteristics for essentially two cut-off points only, and the evaluation was limited to a smaller number of sera. The statistical evaluation of the test results and the fitting of the curves in the present study provides additional information regarding the performance characteristics of the assay at varying cut-off values, and gives flexibility in choosing a cut-off best fitting the epidemiological information regarding the flock or ram being tested. This will optimise the test performance and minimises misclassifications.

Guidelines issued by the *Office International des Epizooties* (OIE) (Anonymous, 1996), recommend the testing of 1000 positive and 5000 negative sera for the full assessment of the performance characteristics of a serological assay. Further work is in progress and it remains to be seen whether additional testing (essentially doubling the current dataset) will provide even more accurate fits of the curves and better estimations of the assay's performance.

References

Anonymous. Principles of validation of diagnostic assays for infectious diseases. In: Manual of Standards for Diagnostic Tests and Vaccines. Vol. 3. Pp 8-15. Office International des Epizooties, Paris, 1996.

Buddle MB, Boyes BW. A *Brucella* mutant causing genital disease of sheep in New Zealand. Australian Veterinary Journal 29, 145-53, 1953.

Burgess GW. Ovine contagious epididymitis: A review. Veterinary Microbiology 7, 551-75, 1982.

Greiner M, Sohr D, Göbel P. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. Journal of Immunological Methods 185, 123-32, 1995.

Kendall MG, Stuart A. Standard distributions — (2). In: The Advanced Theory of Statistics. Griffin & Co., London, Pp. 148-54, 1958.

Pearson ES and Hartley HO. Biometrika Tables for Statisticians. P 118. Cambridge University Press, Cambridge, 1972.

Reichel MP, Baber DJ, Armitage PW, Lampart D, Whitley RS, Hilbink F. Eradication of *Brucella ovis* from the Falkland Islands 1977-1993. Veterinary Record 134, 595-7, 1994.

Reichel MP, West DM. *Brucella ovis* accreditation in New Zealand. Surveillance 24, 19-20, 1997.

Simons GC, Hall WTK. Epididymitis of rams: Preliminary studies on the occurrence and pathogenicity of a *Brucella*-like organism. Australian Veterinary Journal 29, 33-40, 1953.

Smith RD. Evaluation of diagnostic tests. In: Veterinary Clinical Epidemiology. Pp. 31-52. CRC Press, Inc., Boca Raton, 1995.

Worthington RW. The complement fixation test for the diagnosis of *Brucella ovis* infection in rams. New Zealand Veterinary Journal 31, 157-60, 1983.

Worthington RW, Stevenson BJ, de Lisle GW. Serology and semen culture for the diagnosis of *Brucella ovis* infection in chronically infected rams. New Zealand Veterinary Journal 33, 84-6, 1985.

Worthington RW, Weddell W, Penrose ME. A comparison of three serological tests for the diagnosis of *B. ovis* infection in rams. New Zealand Veterinary Journal 32, 58-60, 1984.

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Kittelberger, R and **Reichel, MP** (1998)

Evaluation of electrophoretic immunoblotting for *Brucella ovis* infection in deer using ram and deer serum.

New Zealand Veterinary Journal **46**, 32-34

Short Communications

Evaluation of electrophoretic immunoblotting for *Brucella ovis* infection in deer using ram and deer serum

R. Kittelberger[†*] and M.P. Reichel[*]

Abstract

Aims. Recently the first case of natural infection of deer with *Brucella ovis* was discovered. The aim of this study was to develop and evaluate an electrophoretic immunoblotting method for testing deer serum for specific *B. ovis* antibodies.

Methods. An existing immunoblotting method for sheep serum was altered by using a recombinant protein G-alkaline phosphatase conjugate and Tris-buffered saline containing 3% non-fat dry milk powder for the blocking step and the serum and conjugate dilutions. The method was evaluated using 106 sheep sera from *B. ovis*-negative, accredited flocks, 69 sera from chronically infected rams shedding *B. ovis* in their semen, 110 sera from a *B. ovis*-infected flock, 18 sera from stags from which *B. ovis* was isolated, and 48 sera from deer flocks free from *B. ovis* infections. The immunoblotting method was applied to another 85 deer sera.

Results. The sensitivity of the new immunoblotting method was 98.6% for sheep and 94.4% for deer, and the specificity 99.1% for sheep and 100% for deer. Sixty-nine out of 97 deer sera, originating from the property from which the first *B. ovis* deer case had been reported, tested positive or suspicious in the complement fixation test. Of these, 53 sera exhibited staining patterns in blots typical for *B. ovis* infections and also one serum which was negative in the CFT. Only six out of 1498 deer sera from throughout New Zealand had positive or suspicious reactions in the *B. ovis* complement fixation test. Of these, one exhibited a staining pattern in the blot suggestive of a *B. ovis* infection, while four showed patterns of suspicious reactions.

Conclusion. The new immunoblotting technique is useful as a confirmatory serological test method for *B. ovis* infections in deer.

Key words: *Brucella ovis*, deer, immunoblot. (New Zealand Veterinary Journal 46, 32–34, 1998.)

Introduction

Until recently, *Brucella ovis* infection in deer has only been reported from experimentally infected animals[1]. The first case of naturally acquired *B. ovis* infection in deer was reported in New Zealand from a commercial deer herd comprising 1375 stags, 385 hinds and 420 weaners[2][3]. *Brucella ovis* infection was diagnosed in one stag (Stag A) by the complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and gel diffusion test (GDT), and confirmed by isolation of *B. ovis* from the semen. One in-contact hind (Hind B) was also positive in the CFT and suspicious in the ELISA and GDT, while eight in-contact deer were negative in all three serological tests. At the time of testing, electrophoretic immunoblotting (EIB), which is in routine use as a confirmatory serological method for *B. ovis* infection in sheep in our laboratory[4][5] (sheep EIB), could not be applied to the deer samples because of the poor reactivity of the monoclonal anti-bovine/ovine-alkaline phosphatase conjugate with deer immunoglobulins (IgGs).

An alternative EIB method (sheep-deer EIB) was developed and applied to the 10 sera mentioned above, to a further 87 sera from the property from which Stag A and Hind B had originated, and to six CFT-positive and 48 CFT-negative sera from 1498 routinely submitted deer sera from throughout New Zealand which had been tested in the *B. ovis* CFT.

Methods

The newly developed sheep-deer EIB used recombinant protein G-alkaline phosphatase conjugate (Zymed, San Francisco, CA), and Tris-buffered saline, containing 3% non-fat milk powder for the blocking step and for the serum and conjugate dilutions. Other conditions remained the same as in the sheep EIB test[5]. Equally strong binding of protein G-alkaline phosphatase conjugate to both sheep and deer IgG was confirmed in an indirect ELISA, using pure sheep and deer IgG. The sheep-deer EIB procedure was evaluated using 106 sheep sera from *B. ovis*-negative, accredited flocks, 69 sera from chronically infected rams, 18 sera from stags from which *B. ovis* had been isolated, 48 sera from deer herds presumably free from *B. ovis* infections, and against 110 sera from a *B. ovis* naturally infected flock, comprising 45 serologically positive and 65 negative animals. Data were processed as reported elsewhere[5][6].

Results and Discussion

Test evaluation data for the sheep-deer EIB test are given in Table I. The performance of the new sheep-deer EIB method was comparable to the traditional sheep EIB. In an uninfected sheep flock its specificity was marginally lower, which was due to the very weak staining of a 19 kDa band in one serum. None of the negative sera exhibited staining of non-specific

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bands with the new sheep-deer EIB method, while such staining was found at a 4.5% frequency in the traditional sheep EIB test[5]. As *B. ovis* infection in deer has only been diagnosed recently, one can assume that deer herds testing negative in the CFT are free from the disease. By using deer sera from such a herd, the specificity of the new sheep-deer EIB for deer was 100%. As was the case with the negative sheep sera, none of these deer sera exhibited non-specific staining. The sensitivity was 98.6% in 69 sera from infected rams shedding *B. ovis* in their semen and 94.4% in 18 sera from infected stags, from which *B. ovis* was isolated. A direct comparison of both EIB tests by using sera from a naturally infected sheep flock resulted in a kappa-value[6] of 0.924, indicating very good agreement between the two blotting methods (Table II). Both the traditional EIB and the new sheep-deer EIB were in close agreement with the other serological test results ($\kappa = 0.989$ and 0.968), when the combined results of CFT, ELISA and GDT were used (a serum was considered positive when it showed a positive or suspicious result in at least two tests).

Sera from Stag A and Hind B, which were either positive or suspicious in the CFT, showed staining patterns in the blot which are typical for *B. ovis* infections. Sera from eight in-contact deer were negative in the CFT and in the EIB test. In a further 87 sera from the property from which Stag A and Hind B had originated, 67 samples were CFT-positive or suspicious (titres ranged from 3/8 to 4/128) and *B. ovis* was isolated from 17 stags (from 23 animals tested). Forty-three of these sera, the serum from Stag A and one serum which tested negative in the CFT exhibited staining patterns in blots typical for sera from *B. ovis*-infected sheep (Figure 1), showing immuno-dominant bands of 19 and/or 29 kDa, representing outer membrane proteins, and frequently a broad band of 8–12 kDa, typical for rough lipopolysaccharide (RLPS)[7], which represents another immuno-dominant antigen. Eight of these 87 sera and the serum from Hind B showed weak to strong staining of RLPS without the appearance of other bands, a feature found in about 5% of the sera from the naturally infected sheep flock.

Six CFT-positive deer sera (titres ranged from 3/8 to 2/128) were identified in a further 1498 deer sera from 127 submissions to this laboratory. These six sera were tested in the sheep-deer EIB test. Four had weak RLPS staining but no other bands, indicating possible *B. ovis* infections. Only one serum was definitely positive in the EIB test, showing bands of 19 and 29 kDa. One of the sera with a high CFT titre (2/128) tested negative in the EIB, ELISA and GDT. The appearance of weak RLPS staining was reported in about half of the cases of false positive sera in sheep[8]. The four deer sera here which exhibited this type of staining may well be from animals not infected with *B. ovis*, which is further supported by the absence of other reactors in these submissions.

Table I. Serological test results for the sheep and the sheep-deer EIB

	Number of sera tested	Sheep EIB	Sheep-deer EIB
Sensitivity in sheep (%)[a]	69	98.6	98.6
Sensitivity in deer (%)[b]	18	n.a.[c]	94.4
Specificity in sheep (%)[d]	88/106[d]	100	99.1[e]
Specificity in deer (%)[f]	48	n.a.	100
Agreement[g]	110	0.968	0.989

a All sera were from rams which were shedding *B. ovis* in their semen. One serum was negative in all tests.
b Sera from stags from which *B. ovis* was isolated.
c n.a. = not applicable.
d Sera were from accredited ram flocks which were free from *B. ovis* infections for more than 4 years. All sera were negative in the CFT. Negative sera used for the sheep EIB (88) and the sheep-deer EIB (106) were not identical. This was due to the unavailability of the sera previously used in the sheep EIB, and the unavailability of the commercial Mab anti-bovine/ovine IgG-alkaline phosphatase conjugate.
e One serum showed very weak staining of a 19 kDa band.
f Deer sera from a herd where all animals tested negative in the CFT.
g The level of agreement (κ) between combined serological results and the two blots. Sera were from a *B. ovis*-infected flock, comprising 45 serologically positive and 65 negative animals. A serum was considered positive when it showed a positive or suspicious result in at least two tests of the CFT, ELISA and GDT.

Table II. Level of agreement ($\kappa = 0.924$) of the sheep and the sheep-deer *B. ovis* EIB methods in 110 sera from a naturally *B. ovis*-infected sheep flock

		Sheep EIB		
		Positive	Negative	Totals
Sheep-deer EIB	Positive	41	3	44
	Negative	1	65	66
Totals		42	68	110

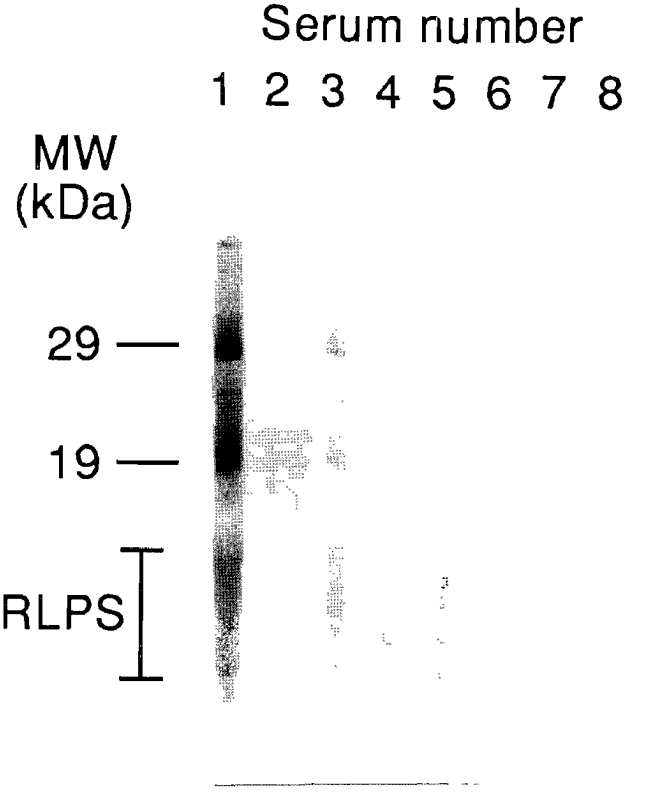

Figure 1. Electrophoretic immunoblot (sheep-deer EIB) of various sera. Lane 1: positive sheep serum; lane 2: negative sheep serum; lanes 3–8: positive deer sera. Numbers on the left show the molecular weights of immuno-dominant antigens of 19 and 29 kDa. RLPS = rough lipopolysaccaride. Note: Negative deer sera had the same appearance as the negative sheep serum in lane 2.

If these reactions are in fact with sera from uninfected animals, then they are false positive reactions, so the specificity of the sheep-deer EIB test may be less than 100% with certain batches of deer sera.

After the first case of *B. ovis*-infection in deer was discovered more than a year ago, which triggered the development of this sheep-deer EIB test, another two submissions from the same property were derived and included in the evaluation of this method. The relatively large number of *B. ovis* serologically positive deer on this property alone suggests that infection may spread easily in this species. While the serological test results in this study from deer sera from throughout New Zealand indicate that *B. ovis* infections in deer are generally uncommon, further work is needed to ascertain the route of spread from sheep to deer and into whether further foci of infection may be present on isolated deer properties. The sheep-deer EIB results here show that this blotting method is well suited as a confirmatory test for such studies and for routine serological testing.

Acknowledgments

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References

(1) Barron SJ, Kocan AA, Morton RJ, Thedford TR, McCain CS. Susceptibility of male white-tailed deer (*Odocoileus virginianus*) to *Brucella ovis* infection. American Journal of Veterinary Research 46, 1762–4, 1985.

(2) O'Neil B. *Brucella ovis* in a stag (*Cervus elaphus*) in New Zealand. Bulletin Office International des Epizooties 8, 613–5, 1996.

(3) Bailey, K. Naturally acquired *Brucella ovis* infection in a deer. Surveillance 24, 10–11, 1997.

(4) Kittelberger R, Hansen M, Ross GP, Hilbink F. A sensitive immunoblotting technique for the serodiagnosis of *Brucella ovis* infections. Journal of Veterinary Diagnostic Investigation 6, 188–94, 1994.

(5) Kittelberger R, Diack DS, Ross GP, Reichel MP. An improved immunoblotting technique for the serodiagnosis of *Brucella ovis* infections. New Zealand Veterinary Journal 45, 75–7, 1997.

(6) Martin SW, Meek AH, Willeberg P. Measuring agreement. In: Veterinary Epidemiology — Principles and Methods. Pp 73–76. Iowa State Press, Ames, 1987.

(7) Kittelberger R, Hilbink F, Hansen MF, Ross GP, De Lisle GW, Cloeckaert A, De Bruyn J. Identification and characterization of immunodominant antigens during the course of infection with *Brucella ovis*. Journal of Veterinary Diagnostic Investigation 7, 210–8, 1995.

(8) Kittelberger R, Laybourn BJ, Reichel MP, Ross GP, deLisle GW, Joyce MA. Attempted definition by immunoblotting of the causes of reactivity in suspected false-positive sera in the *Brucella ovis* complement fixation test. New ZealandVeterinary Journal 44, 203–207, 1996.

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1.4. Enzootic Bovine Leukosis (Bovine leukaemia virus)

Reichel, MP; Tham, KM; Barnes, S and Kittelberger, R (1998)

Evaluation of alternative methods for the detection of Bovine Leukaemia virus in cattle.

New Zealand Veterinary Journal **46**, 140-146

Evaluation of alternative methods for the detection of bovine leukaemia virus in cattle

M.P. Reichel *†, K.M. Tham*, S. Barnes* and R. Kittelberger*

Abstract

Aim. To evaluate commercially available enzyme-linked immunosorbent assays (ELISAs) and the polymerase chain reaction (PCR) for their ability to detect antibodies against or nucleic acid of the bovine leukaemia virus (BLV), the causal agent of enzootic bovine leukosis (EBL), and to assess their usefulness in a national eradication programme.
Methods. Eighty-two well-defined sera (including 18 from an OIE reference laboratory) and 399 field sera from New Zealand cattle were tested in five ELISAs and the results compared with the agar gel immunodiffusion (AGID) test and electrophoretic immunoblotting (EIB) results. A polymerase chain reaction-based technique, which could detect BLV-RNA and proviral-DNA, was also evaluated on a subsample of the field cases.
Results. Two commercial ELISAs classified 99% of the defined sera correctly, with the other three ranging in their correct classification between 88% and 95%. The ELISAs agreed in their general classification on the majority of the 399 blood samples (91.7%), and with the AGID for more than 95 % of the sera. In a dilution series of the international reference serum E4, the highest dilution with a positive (or suspicious) result ranged from 1: 80 to 1:5120. A dilution series of 202 field positive samples tested in the preferred ELISA detected 98% of positive sera at a 1:5 and 1:10 dilution, reducing to 78% at a 1:80 dilution of the sera. Agreement between serological tests and PCR was poor, mainly due to failure of the PCR to detect a number of serologically positive animals.
Conclusion. ELISA tests detected about 10% more reactors than the AGID and the EIB combined. Some ELISA-positive animals were not detected by PCR, raising doubts about the usefulness of PCR-based technology in EBL eradication programmes.
Key words. Enzootic bovine leukosis, ELISA, AGID, PCR, electrophoretic immunoblotting, assay evaluation, disease eradication.

(New Zealand Veterinary Journal 46, 140-146, 1998.)

Introduction

Bovine leukaemia virus (BLV), the causal agent of enzootic bovine leukosis (EBL), is a retrovirus which naturally infects cattle and sheep, causing clinical disease associated with lymphosarcoma in a small percentage of infected, mainly mature, cattle (Ferrer, 1980). Clinical cases of lymphosarcoma are occasionally reported in New Zealand (Thompson *et al.*, 1993). Once infected, cattle remain virus carriers for life and start to show a serological reaction within a few weeks of infection (Klintevall *et al.*, 1994). Serological tests have been used for a number of years to identify infected cattle and traditionally the agar gel immunodiffusion (AGID) test (Miller and van der Maaten, 1976) has been used. In more recent years, the enzyme-linked immunosorbent assay (ELISA) (Portetelle *et al.*, 1983) has replaced the AGID in eradication programmes and there are several commercial ELISA kits available to detect antibodies mainly against the glycoprotein gp51, which occur earlier in the course of an immune response than antibodies against the core protein p24 (Mammerickx *et al.*, 1980; Klintevall *et al.*, 1994).

In New Zealand, it is estimated that about 6.5% of the 15 000 dairy herds have infected cattle, with an estimated within herd prevalence of 3.7% (Burton *et al.*, 1997). In previous surveys on milk in New Zealand, a commercial ELISA kit had been used to estimate herd prevalence on the basis of bulk milk samples, while in routine diagnostic testing of blood samples the AGID has been the serological test of choice, based on its high sensitivity and specificity, estimated to be 98.5 and 99.8%, respectively (Monke *et al.*, 1992).

Before the start of an eradication campaign for BLV infections in dairy cows in New Zealand, a number of commercially available BLV ELISA kits were evaluated and compared with the AGID for their usefulness in the eradication campaign. Initial screening of dairy herds is being carried out on milk samples obtained from the dairy industry's national milk analysis centre (Hayes *et al.*, 1997) and blood samples of milk-positive animals are to be tested in a confirmatory second test. Because of the low initial herd and within-herd prevalence, in the eradication campaign the test to be used must have a high level of specificity, to ensure the highest possible positive predictive value for the test (which was estimated to be as low as 37.9% for milk ELISA testing alone (Hayes *et al.*, 1997). This will minimise the number of test- (false-) positive reactions, thus preventing disease-negative reactors being slaughtered unnecessarily. However, the overall sensitivity of the combined (serial) testing strategy is another consideration, as false-negative test results may unnecessarily prolong the eradication effort if truly infected animals are not detected.

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Materials and Methods

Eighty-two defined sera (45 positive, 37 negative) of which eighteen (eleven positive, seven negative) were obtained from the OIE reference laboratory, Tübingen, Germany, the others from infected or all herd-negative New Zealand herds and tested in the AGID test (Miller and van der Maaten, 1976), were tested in each of five commercially available BLV ELISA diagnostic kits (Institut Pourquier, Montpellier, France; Svanovir, Svanova Biotech, Uppsala, Sweden; Serelisa Monoblocking, Rhône-Mérieux, Lyon, France; Chekit Leucotest Dr. Bommeli AG, Bern, Switzerland; and Dako A/S, Copenhagen, Denmark) for the presence of specific anti-BLV antibodies. Assays were carried out according to the test protocols provided by the manufacturers. In addition, all five ELISAs were used to test a dilution series of the EBL reference serum E4.

Three hundred and ninety-nine bovine sera originating from 19 New Zealand dairy herds which contained reactors were tested using the AGID and the five commercial ELISAs. All sera were also tested by electrophoretic immunoblotting (EIB), as previously described (Kittelberger *et al.*, 1996).

Two hundred and two BLV-antibody positive New Zealand field sera were tested by the preferred ELISA assay in a dilution series of five doubling dilutions, starting with an initial serum dilution of 1:5.

One hundred and sixty-eight EDTA blood samples obtained from a sub-sample of the above animals (137 AGID-positive and 31 AGID-negative) were tested by PCR for both viral RNA and proviral DNA (the application of RT-PCR in this study was to determine whether gene amplification assay could be used to identify animals with active infections, as has been demonstrated by others previously (Murtaugh *et al.*, 1993; Poon *et al.*, 1993)) of the BLV-virus as follows.

Nucleic acid extraction

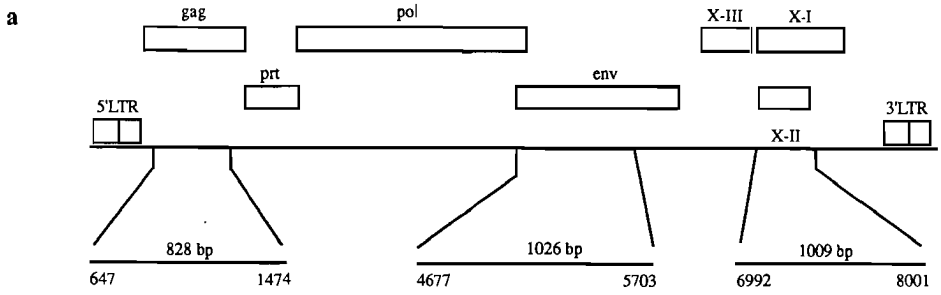
EDTA blood was centrifuged at 3000 rpm for 20 min and the buffy coat was collected and treated with 10 ml of ice cold 0.17 M ammonium chloride to lyse any residual red blood cells. The cell suspension was then centrifuged and the cell pellet was washed once with sterile phosphate-buffered saline, pH 7.4. The buffy coat cells were resuspended in sterile PBS and aliquoted into sterile tubes for RNA and DNA extraction.

RNA was extracted from the buffy coat cells using the commercial Tri-zol LS (Life Technologies, Gaithersburg, MD, USA) protocol according to the recommendation of the manufacturer. Briefly, 750 µl of Tri-zol LS was added to 250 µl of buffy coat cells and the mixture was left at room temperature for 5 min. A 200 µl volume of chloroform was added to the mixture which was vortexed vigorously for 15 s and then incubated at room temperature for 15 min. After centrifugation to remove cellular debris, the aqueous phase was transferred to a clean Eppendorf tube, 500 µl of isopropylalcohol added and the mixture was incubated at room temperature for 10 min. The mixture was centrifuged and the supernatant was transferred to a clean tube containing 1 ml of 75 % absolute ethanol. The mixture was vortexed, centrifuged and the supernatant discarded. The pelleted RNA was air-dried and resuspended in 50 µl of water pretreated with diethyl pyrocarbonate.

Enzootic bovine leukosis provirus DNA was extracted from the buffy coat cells using a commercial high pure PCR template preparation kit (Boehringer Mannheim, Heidelberg, Germany).

Primer and probe oligonucleotides

Three sets of primers (B5.1/B5.2, B5.3/B5.4 and B5.5/B5.6) and their corresponding internal probes (B5.a, B5.b and B5.c) (Figure 1) were constructed based on the



c

Primers and Probes (Orientation)	5'-sequence-3'	Location in genome (bp)
B5.1 (sense)	TGGCCACCCTAAACGAAGTA	647-666
B5.2 (antisense)	TGAGGCATCGATAGCATGGT	1474-1455
B5.3 (sense)	TCTTACTCTCCTCGCTCTCT	4677-4696
B5.4 (antisense)	CACAGAGGCCACATTAAGCA	5703-5684
B5.5 (sense)	CTTCGGGATCCATTACCTGA	6992-7011
B5.6 (antisense)	GGCCTGCATGATCTTTCATA	8001-7982
B5.a (antisense)	AATGTGCACAAGCCTGCAGT	1357-1338
B5.b (antisense)	GGAGGAAACCGTAGAGAGAT	5493-5474
B5.c (antisense)	GTCATGGAGGAAGCGTTGTA	7862-7843

Figure 1: a. Schematic representation of the genomic organisation of EBL provirus T15(11) showing the enlargement of the amplified fragments of the gag, pol and X-II genes. b. Locations of the primers and probes used in the EBL PCR assays. c. Nucleotide sequences of the primers and probes.

Table I. Dilution series of the international reference serum (E4) for EBL and correct classification (CC), sensitivity (se) and specificity (Sp) by five commercial ELISA kits of 82 defined sera

ELISA	CC	Se	Sp	E4 detection limit
Bommeli	98.8	97.1	100.0	40[a] (80 suspicious)
Dako	90.8	100.0	86.0	5120
Pourquier	98.8	97.1	100.0	160 (320 suspicious)
Rhône-Mérieux	95.1	97.1	93.6	320 (2560 suspicious)
Svanovir	87.8	100.0	78.7	640

a Reciprocal of highest E4 dilution positive or suspicious.

Table II. Level of agreement (measured as correct classification (CC) and kappa-value[a]) between agar gel immunodiffusion test results (AGID) and electrophoretic immunoblotting (EIB) for 399 bovine sera

	EIB		
AGID	Positive	Negative	Total
Positive or suspicious	162	10	172
Negative	7	220	227
Total	169	230	399

a Kappa= 0.913 (CI 95%: 0.872, 0.953); CC = 95.7%.

published nucleotide sequences of the gag, env and XII genes of the BLV provirus T15 DNA (Weiss *et al.*, 1985).

PCR assays

For the RT-PCR, pilot experiments were performed to optimise the reaction conditions for cDNA synthesis and for gene amplification by PCR using the procedures described previously for another RNA virus (Tham *et al.*, 1995). Briefly, the optimised standard RT-PCR consisted of the synthesis of first strand cDNA at 37 °C for 1 h with 250 ng per reaction of antisense primers B5.2, B5.4 or B5.6 for the gag, env or XII genes respectively. A 10 µl aliquot of the cDNA template was used for PCR as follows. A 5 µl volume of 10X buffer (500 mM Tris-HCl, pH 9.0, 200 mM NH_2SO_4, 15 mM $MgCl_2$), 2 µl of an equimolar mixture of 2 mM dNTP, 500 ng of each primer B5.1 and B5.2, B5.3 and B5.4 or B5.5 and B5.6 (Figure 1) per reaction and 5 U of Tub polymerase were added in a final volume of 50 µl. With a programmable thermocycler (Hybaid Omnigene, Hybaid Ltd, Teddington, Middlesex, UK), the mixtures were amplified by 30 repetitive cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 74 °C for 2 min, with a final extension of 10 min for the completion of the reaction. Positive (foetal lamb kidney cell line, persistently infected with BLV (FLK-BLV) and negative (a primary bovine foetal lung cell culture) controls were included in all experiments.

For EBL provirus PCR, the cDNA amplification protocol described above was used.

Analysis of PCR products

The amplified products were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining and UV fluorescence as described previously (Tham and Stanisławek, 1992). The specificity of the PCR products was confirmed by the appearance of the fragments of 828 bp, 1026 bp or 1009 bp for the gag, env or XII genes respectively and chemiluminescence Southern blot hybridisation with digoxigenin (DIG)-labelled probes B5.a, B5.b and B5.c for the 828 bp, 1026 bp, and 1009 bp fragments respectively (Tham and Stanisławek, 1992; Tham *et al.*, 1995).

Thirty-three buffy coat preparations were also sent to the OIE reference laboratory for EBL in Wusterhausen, Germany, where they were tested by the PCR (Proviral, env-nested) in use at that laboratory.

Analyses of tests

Kappa-values were calculated using Winepiscope 1.0 (EPIDECON, Veterinary Faculty Zaragoza, Spain; Agricultural University Wageningen, The Netherlands). Discriminatory values for the separation of negative and positive populations were calculated as previously described (Lusted, 1971).

Results

Testing of 82 defined sera resulted in between 87.8% and 98.9% of samples being correctly classified by the commercial ELISA kits (Table I). The sensitivity was very similar at between 97.1% and 100% for all the ELISAs, the specificity ranged from 78.7% to 100%. In a dilution series of the international EBL reference standard E4, the highest dilution of E4 still classified by the ELISAs as either positive or suspicious ranged from 1:80 to 1:5120 (Table I).

Table III. Performance characteristics (relative sensitivity (rSe), specificity (rSp), correct classification (CC) and kappa-value (95% confidence interval)) of five commercial enzyme-linked immunosorbent assays relative to the agar gel immunodiffusion test (AGID) for 399 bovine sera from infected herds

ELISA	rSe	rSp	CC	Kappa	95% CI
Bommeli	99.4	97.8	98.5	0.969	(0.945; 0.994)
Dako	100.0	90.3	94.5	0.889	(0.845; 0.934)
Pourquier	100.0	94.3	96.7	0.934	(0.899; 0.969)
Rhône-M	93.0	96.5	95.0	0.898	(0.854; 0.941)
Svanovir	100.0	93.4	96.2	0.924	(0.887; 0.962)

Table IV. Comparison (correct classification (CC) and kappa-values) of agar gel immunodiffusion (AGID), electrophoretic immunoblotting (EIB), enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) results for 168 cattle from BLV-infected dairy herds

		Correct classification		
Kappa	AGID	EIB	ELISA	PCR
AGID		0.869	0.928	0.833
EIB	0.800			
ELISA	0.780	0.722		
PCR	0.578	0.668	0.565	

Table V. Dilution series of 202 New Zealand BLV antibody-positive field samples tested in the Pourquier ELISA and percentage with positive and negative results

	Dilution[a]					
	1	5	10	20	40	80
% + ve	100	98	98	96	92	78
% - ve	0	2	2	4	8	22

a Reciprocal of serum dilution.

Of the 399 sera from reactor herds characterised, 168 were positive by the AGID, four regarded as suspicious and 227 were negative. Electrophoretic immunoblotting was in very good agreement with those results, with 382 (95.7%) of sera having the same classification. Immunoblotting identified a further seven reactors. The resulting kappa (K) value (as a measure of agreement beyond chance) was 0.913 (Table II).

The number of sera identified by the various commercial ELISAs as either positive or negative varied, but agreed for a core of 91.7% of samples. Compared to the AGID, the percentage of correctly classified sera ranged from 98.5% (relative specificity (rSp) 96.9%, relative sensitivity (rSe) 94.2%) for the Bommeli-ELISA, to 94.5% (rSp 90.3%; rSe 100%) for the Dako-ELISA. Kappa values varied accordingly, ranging from 0.969 (Bommeli) to 0.889 (Dako) (Table III).

Three ELISAs identified all AGID positive samples correctly (100% relative sensitivity). One ELISA (Rhône-Mérieux) failed to identify twelve AGID (and EIB) positive serum samples, and the Bommeli-ELISA failed to identify just one AGID positive serum sample. Overall, ELISAs identified an additional 20 sera (11.6%), which had not been identified in either the AGID or EIB as positive, increasing the total number of samples with serologically positive or suspicious results to 199. Agreement between the five ELISA tests over the 20 additional positive samples was poor, however, with only five of the 20 samples being identified by four or five of the ELISAs.

Of the two ELISA assays with the highest correct classification score (Bommeli and Pourquier), the Pourquier-ELISA discriminated better between AGID-positive and negative serum samples. The discriminatory value was 7.2 for the Pourquier-ELISA, 4.5 for the Bommeli-ELISA. Frequency distributions for the two assays are shown in Figure 2a and 2b. Frequency distributions of AGID positive and negative sera for the other ELISAs are given in Figures 2c - 2e.

The proviral DNA PCR and RT-PCR assays combined, using three sets of primer (Figure 1) correctly identified 83.8% of blood samples compared with the AGID results, but failed to identify 20/129 (15.5%) of AGID and 17/129 (13.2%) of EIB-positive animals. Thus the PCR results had only moderate agreement with the AGID results (K = 0.578), and slightly better agreement (0.668) with the EIB results. Compared to the results of the preferred ELISA (Pourquier), the PCR correctly classified 84.4% of samples characterised by ELISA, and the agreement was moderate (K = 0.575), due to the failure to identify 25/140 (17.9%) of ELISA-positive animals in the PCR (Table IV). The PCR detected an additional two (0.5%) animals, which had not been identified by the ELISA, as BLV-infected.

Of the 20 animals which had been identified by the five ELISA assays as additional positives (over and above the number of AGID positive reactors) only two were also positive in the PCR. The Pourquier ELISA was the only serological test which detected both of those two animals.

The comparison of PCR results with the results of the reference laboratory showed agreement for 28 (84.8%) of 33 of the samples, resulting in a high kappa-value of 0.693. Comparison of the results of the two PCRs with the preferred ELISA showed moderate agreement at kappa-values of 0.433 and 0.476, respectively.

A dilution series of 202 New Zealand BLV-antibody positive field samples resulted in the mis-classification as negative of 2% of sera at the 1:5 and 1:10 dilution, rising to 4% at 1:20, to 8% (1:40) and 22% (1:80), respectively (Table V).

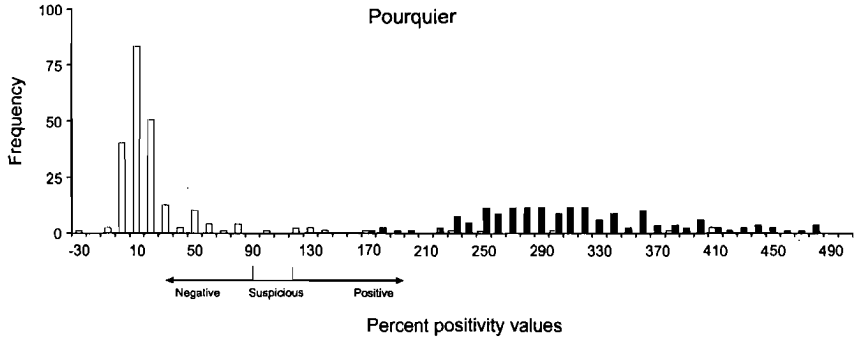
Discussion

The AGID test has been the prescribed test for international trade for a number of years (Anon., 1996). It is characterised by a high level of sensitivity (98.5%) and specificity (99.8%) (Monke *et al.*, 1992), but doesn't lend itself to large scale testing regimes and cannot be used on milk (Mammerickx *et al.*, 1985). In more recent years, ELISA-based testing has replaced the AGID in eradication campaigns in several countries (Mammerickx and Strobbe, 1986; Klintevall *et al.*, 1991; Straub and Bruchhof, 1994; Gibson, 1995) and is used for testing of milks in the New Zealand Dairy Industry eradication campaign (Burton *et al.*, 1997).

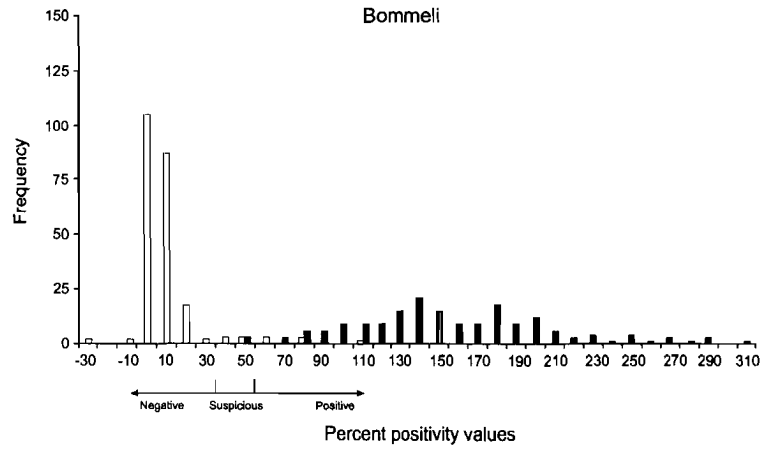
An ELISA test for BLV-antibodies in the New Zealand EBL eradication programme should possess a high degree of specificity, relative to the AGID, as blood testing is used as a confirmatory test to complement the initial screening on milk. The milk ELISA testing has demonstrated a low positive predictive value of only 0.37 (Hayes *et al.*, 1997). The specificity of two ELISA assays (Bommeli and Pourquier) was 100% over the defined sera, and not too dissimilar compared over the 399 field sera. The Pourquier-ELISA showed good (100%) sensitivity, relative to the AGID, and overall good agreement with the AGID with a kappa value of 0.934 and correctly classified (CC) 96.7% of the 399 field sera. The Bommeli-ELISA attained higher values for relative specificity, kappa and CC, but the discrimination between negative and positive population was not as good as for the Pourquier-ELISA. In routine applications of the ELISA test, the ease with which positive and negative samples can be distinguished becomes important; good discrimination between positive and negative test results obviates any unnecessary expenditure on further (and costly) re-testing or confirmatory testing with alternative test systems.

Also, the five ELISA assays combined detected an additional 20 sera as positive, which had not been detected by the AGID (or EIB), but only two of them could be confirmed as positive in the PCR. While all ELISAs detected one of the PCR-positive animals, only the Pourquier-ELISA detected both of them. The AGID-status was used in this study as the "gold" standard, but all the AGID-negative sera were drawn from infected herds, and it is reasonable to assume that a number of additional animals were EBL-infected — this would include the seven additional reactors in the immunoblot, the two PCR-positive animals and a number of the ELISA-positive animals.

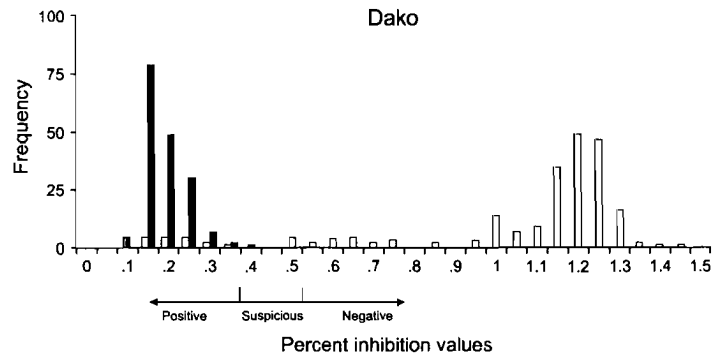
a. Frequency distribution of percentage positivity values for AGID-positive (■) and AGID-negative (□) values in the Pourquier ELISA.



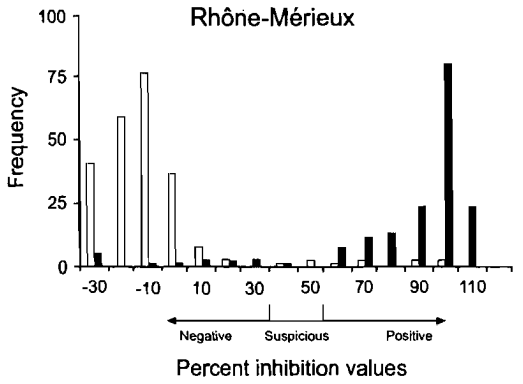
b. Frequency distribution of percentage positivity values for AGID-positive (■) and AGID-negative (□) values in the Bommeli ELISA.



c. Frequency distribution of percentage inhibition values for AGID-positive (■) and AGID-negative (□) values in the Dako ELISA.



d. Frequency distribution of percentage inhibition values for AGID-positive (■) and AGID-negative (□) values in the Rhône-Mérieux ELISA.



e. Frequency distribution of corrected optical density (OD) values for AGID-positive (■) and AGID-negative (□) values in the Svanovir ELISA.

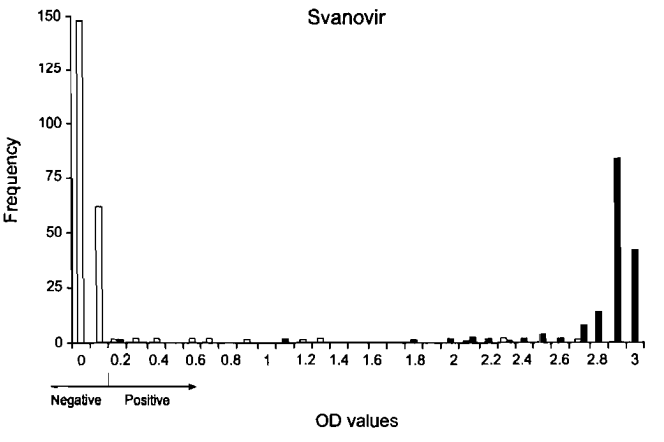


Figure 2. Frequency distribution of test results from five commercial BLV-ELISAs for the testing of 399 bovine sera for BLV-antibodies

Further work is needed when more field sera can be obtained from reliably BLV-free dairy herds as the eradication campaign pro-gresses.

The specificity of the confirmatory assay will become even more important as the eradication campaign progresses in future years, with declining numbers of truly infected cattle and the choice of assay (and/or cutoff) will need to be re-addressed.

In eradication campaigns it may be economical to test pooled blood and it is generally accepted that the reciprocal value of the highest dilution of E4 detected as positive should be 10 times greater than the number of animals used to make up a pooled blood sample (Hoff Jørgensen, 1989). The Bommeli-ELISA showed positive (or suspicious) results for a dilution series of E4 only to a 1:40 (1:80) dilution, while the Pourquier-ELISA detected a 1:160 dilution of E4 as positive and 1:320 as suspicious. Thus the Bommeli-ELISA would allow pooling of sera of up to four animals only, while the Pourquier-ELISA would allow the pooling of the blood samples of up to 16 animals. However, the testing of dilution series of 202 New Zealand BLV-positive field samples in the Pourquier-ELISA, suggested that pooling (even of only up to five sera) may result in a slight loss of sensitivity (2%) compared to individual test results. This suggests that the E4 dilution series should only be seen as a theoretical guideline and that criteria for the number of sera making up a test pool should be ultimately be derived from field samples. The loss of sensitivity relative to individual test results became clearly unacceptable for dilutions equal or greater than 1:40, with only 92% relative sensitivity.

The highest technical sensitivity (i.e. highest dilution positive of E4) was achieved by the DAKO-ELISA, theoretically allowing up to 500 blood samples to be pooled. Generally, however, the performance of the DAKO-kit was not sufficiently satisfactory for it to be used as a confirmatory blood test.

The failure of the PCR-based test to identify a large number of the animals which were positive by the indirect methods (AGID, EIB, ELISA) is disappointing, as, at least theoretically, the PCR should have the potential to be a very sensitive and specific test, being able to identify a very small number of BLV genome copies against a background of millions of negative lymphocytes (Ballagi-Pordány *et al.*, 1992). In this comparison with antibody-based tests, the PCR showed the least agreement of any test with the AGID, and failed to detect a large number of AGID-, EIB- and ELISA-positive animals. The failure of PCR assays to detect BLV gene sequences in some BLV seropositive animals has been reported previously (Eaves *et al.*, 1994; Murtaugh *et al.*, 1991). A plausible explanation for the failure of the PCR assays to detect some seropositive animals is that a low proportion of circulating lymphocytes was infected with BLV at the time of sampling of these animals. A similar observation has been noted with human immunodeficiency virus (HIV) infection in which it was difficult to find the infectious virus, even in people suffering from the acquired immune deficiency syndrome. This was shown to be a result of virus sequestration into the lymphoid organs, in which large amounts of virus were present even during the asymptomatic stage of the disease, although it was not always in the form that might have been expected (Embretson *et al.*, 1993; Luque *et al.*, 1993; Pantaleo *et al.*, 1993). Indeed, re-examinations of some BLV seropositive blood samples which initially tested PCR-negative showed that following lysis of the blood clots in the EDTA samples, BLV gene sequences could be detected by PCR assays, suggesting that the number of virus-infected lymphocytes in the blood samples is an important factor that affects the sensitivity of the PCR assays. Other authors report BLV genome copies detected by PCR in spleen extracts, but not in PBL (Klintevall *et al.*, 1994). In our study it was not possible to obtain tissue samples for PCR from any of the animals, and thus the question of whether or not tissues may have yielded PCR-positive results remains unanswered. Jacobs *et al.* (1992) reported a level of agreement between ELISA and PCR of 83.6%, not dissimilar from the 84.4% in the present study. In the present study, however, the numbers of serologically positive yet PCR-negative animals was disproportionally greater than the number of serologically negative, PCR-positive ones. Recent PCR results for a related virus (bovine immunodeficiency virus (BIV)) point to similar problems in detecting virus in field samples, as compared to cell culture or experimentally infected animals (Suarez and Whetstone, 1997). The unsatisfactory performance of the PCR compared to the serological tests suggests, however, that using blood samples for PCR assays is not appropriate for large scale application in an eradication campaign, but may in selected cases be of use as a confirmatory test where the AGID fails to give a positive result.

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References

Anonymous. Enzootic bovine leukosis. In: Manual of Standards for Diagnostic Tests and Vaccines. Pp 276-80. Office International des Epizooties, Paris, 1996.

Ballagi-Pordány A, Klintevall K, Merza M, Klingeborn B, Belak S. Direct detection of bovine leukemia virus infection: Practical applicability of a double polymerase chain reaction. Journal of Veterinary Medicine Series B 39 69-77, 1992.

Burton L, Allen G, Hayes D, Pfeiffer D, Morris R. A novel approach to disease control. An industry operated programme for bovine leukaemia virus in New Zealand. In: 8th Symposium of the International Society for Veterinary Epidemiology and Economics. Special issue of Epidemiologie et Santé Animale. Vol. 31-32. Pp 08.08.1-3. International Society for Veterinary Epidemiology and Economics, Paris, 1997.

Eaves FW, Molly JB, Dimmock CK, Eaves LE. A field evaluation of the polymerase chain reaction procedure for the detection of bovine leukaemia virus proviral DNA in cattle. Veterinary Microbiology 39, 313-21, 1994.

Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tenner-Racz K, Haase AT. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 362, 359-62, 1993.

Ferrer JF. Bovine lymphosarcoma. Advances in Veterinary Science and Comparative Medicine 24, 1-68, 1980.

Gibson LAS. Testing for enzootic bovine leukosis. Veterinary Record 136, 156, 1995.

Hayes D, Hoggard G, Reichel M, Burton L, Pfeiffer D, Morris R. The suitability of milk samples from a national milk analysis centre for bovine leukaemia virus surveillance, In: 8th Symposium of the International Society for Veterinary Epidemiology and Economics. Special issue of Epidemiologie et Santé Animale. Vol 31-32. Pp 11.C.14. International Society for Veterinary Epidemiology and Economics, Paris, 1997.

Hoff-Jørgensen R. An international comparison of different laboratory tests for the diagnosis of bovine leukosis: Suggestions for international standardization. Veterinary Immunology and Immunopathology 22, 293-7, 1989.

Jacobs RM, Song Z, Poon H, Heeney JL, Taylor JA, Jefferson B, Vernau W, Valli VEO. Proviral detection and serology in bovine leukemia virus-exposed normal cattle and cattle with lymphoma. Canadian Journal of Veterinary Research 56, 339-48, 1992.

Kittelberger R, Laybourn BJ, Diack DS, Penrose ME, Reichel MP, Motha J, Molloy BJ, Merza M. Evaluation of electrophoretic immunoblotting for the detection of antibodies against the bovine leukosis virus in cattle. Journal of Virological Methods 61, 7-22, 1996.

Klintevall K, Ballagi Pordany A, Naslund K, Belak S. Bovine leukaemia virus: Rapid detection of proviral DNA by nested PCR in blood and organs of experimentally infected calves. Veterinary Microbiology 42, 191-204, 1994.

Klintevall K, Naslund K, Svedlund G, Hajdu L, Linde N, Klingeborn B. Evaluation of an indirect ELISA for the detection of antibodies to bovine leukaemia virus in milk and serum. Journal of Virological Methods 33, 319-33, 1991.

Luque F, Leal M, Pineda JA, Torres Y, Aguado I, Olivera M, Hernandez-Qucro J, Sanchez-Quijano A, Rey C, Lissen E. Failure to detect silent HIV infecxtion by polymerase chain reaction in subjects at risk for heterosexually transmitted HIV type 1 infection. European Journal of Clinical Microbiology and Infectious Diseases 12, 663-7, 1993.

Lusted LB. Decision-making studies in patient management. The New England Journal of Medicine 284, 416-24, 1971.

Mammerickx M, Portetelle D, Burny A. Application of an enzyme-linked immunosorbent assay (ELISA) involving monoclonal antibody for detection of BLV antibodies in individual or pooled bovine milk samples. Zentralblatt für Veterinärmedizin B 32, 526-33, 1985.

Mammerickx M, Portetelle D, Burny A, Leunen J. Detection by immunodiffusion- and radioimmunoassay tests of antibodies to bovine leukaemia virus antigens in sera of experimentally infected sheep and cattle. Zentralblatt für Veterinärmedizin B 27, 291-303, 1980.

Mammerickx M, Strobbe R. L'épizootologie de la leucose bovine enzootique en fonction de la taille des troupeaux. Annales Médicine Véterinaire 130, 53-9, 1986.

Miller JM, van der Maaten MJ. Serologic detection of bovine leukaemia virus infection. Veterinary Microbiology 1, 195-202, 1976.

Monke DR, Rohde RF, Heston WD, Milburn RJ. Estimation of the sensitivity and specificity of the agar gel immunodiffusion test for bovine leukaemia virus: 1,296 cases (1982-1989). Journal of the American Veterinary Medical Association 200, 2001-4, 1992.

Murtaugh MP, Lin GF, Haggard DL, Weber AF, Meiske JC. Detection of bovine leukemia virus in cattle by the polymerase chain reaction. Journal of Virological Methods 33, 73-85, 1991.

Murtaugh MP, Lin GF, Haggard DL, Weber AF, Meiske JC. Detection of bovine leukemia virus in cattle by the polymerase chain reaction. Journal of Virological Methods 33, 73- 85, 1993.

Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, Orenstein JM, Kotler DP, Fauci AS. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362, 355-8, 1993.

Poon H, Jimenez E, Jacobs RM, Song Z, Jefferson B. Detection of bovine leukemia virus RNA in serum using the polymerase chain reaction. Journal of Virological Methods 41, 101-12, 1993.

Portetelle D, Bruck C, Mammerickx M, Burny A. Use of monoclonal antibody in an ELISA test for the detection of antibodies to bovine leukaemia virus. Journal of Virological Methods 6, 19-29, 1983.

Straub OC, Bruchhof BR. A comparison of examinations of bovine sera by ELISA kits for determination of antibodies against bovine leukosis virus. Tierärztliche Umschau 49, 628-30, 635, 1994.

Suarez DL, Whetstone CA. Comparison of different PCR tests to detect bovine lentivirus in cell culture and experimentally and naturally infected cattle. Journal of Veterinary Diagnostic Investigation 9, 421-4, 1997.

Tham KM, Stanisławek WL. Detection of chicken anaemia agent DNA sequences by the polymerase chain reaction. Archives of Virology 127, 245-55, 1992.

Tham KM, Young LW, Moon CD. Detection of infectious bursal disease virus by reverse transcription-polymerase chain reaction amplification of the virus segment A gene. Journal of Virological Methods 53, 201-12, 1995.

Thompson KG, Johnstone AC, Hilbink F. Enzootic bovine leukosis in New Zealand — A case report and update. New Zealand Veterinary Journal 41, 190-4, 1993.

Weiss R, Teich N, Varmus H, Coffin J (eds). In: RNA Tumour Viruses. Vol. 2. Pp. 1086-101. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1985.

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1.5. Discussion and Conclusions

Diagnostic assays were established for three animal pathogens of significant proportion in New Zealand, and the general principles for the validation of those assays followed.

Serological assays had previously been used in New Zealand for paratuberculosis (Hilbink *et al.* 1994), the sera used however had not been as well defined as in the present study. The quantitative analysis of faecal culture results, in particular, allowed to establish a clear correlation between an increase in the extent of faecal shedding and increase in the serological responses in ELISA. The serological response in high faecal shedders was equal to or exceeded that reported in other studies (Milner *et al.* 1990).

Brucella ovis infection of rams is thought to be an economically important disease in New Zealand and an accreditation scheme has been in place for a number of years (Reichel and West 1997). The complement fixation test is the standard test in use, but additional serological tests for the diagnosis of the infection had previously been evaluated, however only with a small number of sera (Worthington *et al.* 1984). The ELISA in use had also been extensively modified and was in need for re-validation. The present study was hence the first major validation of the serological tests in use in NZ for 15 years and used in excess of 500 positive and 2500 negative “gold-standard” sera. It established reliable performance criteria for the ELISA, and with ROC-analyses (Greiner *et al.* 1995) tools that allow the operator to select cut-off thresholds and with it a desired sensitivity and specificity, in line with the epidemiological situation. Likelihood ratio analysis was only gaining respect as an appropriate use of non-dichotomised data in 1999-2000 and its use is a significant enhancement of the interpretation of the results.

Naturally occurring *B ovis* infection had previously only once been described in deer in the US. When it emerged in NZ farmed deer (Bailey 1997), another livestock industry of increasing importance, there was an urgent need to re-validate all serological assays presently used in rams, for their utility in deer. Electrophoretic immunoblotting and ELISA had not been previously used in deer, and hence their validation for use in deer were novel.

Enzootic bovine leukosis was thought to be infecting a small number of herds only and within those herds a small percentage of animals (Burton *et al.* 1997). Tests of high sensitivity and specificity were required to identify the infected individuals within those herds. Previously (Hilbink and Penrose 1990), two ELISA assays had been evaluated, but the eradication campaign called for a two-stage sampling plan: the first test being based on milk sampling, the second on blood sampling. The milk sampling was highly sensitive, but suffered from deficiencies in its specificity (Hayes *et al.* 1997). Hence the blood testing had to be both, highly sensitive and specific (with particular emphasis on the latter). Previously, overseas, ELISA assays had been established and performed with high characteristics (Mammerickx *et al.* 1985, Klintevall *et al.* 1991), however in the present study, clear differences emerged between the five commercial assays compared, confirming the need for re-validation.

In addition, confirmatory tests in the form of electrophoretic immunoblotting and PCR were established and validated. Surprisingly, PCR failed to establish presence of pro-viral DNA in about 15% of ELISA-positive blood samples. This is partially in agreement with other studies, which report similar levels of agreement between ELISA and PCR for the diagnosis of BLV infection in cattle (Jacobs *et al.* 1992). In the present

study, however, there was a clear bias towards failure of the PCR to detect ELISA-positive cattle, while the former also detected pro-viral DNA in ELISA-negative cattle in an equal number of cases.

1.6. Neosporosis (*Neospora caninum*)

Another animal pathogen of major importance in New Zealand appeared to emerge towards the end of the 1980's in the form of *N caninum*, which was soon recognised as a major cause of epidemic abortions in dairy cattle and, to a lesser degree, as a disease of dogs. Epidemic abortions were still being reported after the conclusion of the successful eradication of the abortifascient *Brucella abortus* from New Zealand (MacDiarmid 1994) and the CNS of aborted bovine foetuses was examined more closely for the characteristic lesions simultaneously reported from overseas.

Neosporosis was first described from dogs in Norway in 1984 (Bjerkås *et al.* 1984) and the causal agent (*Neospora caninum*) was described in detail by (Dubey *et al.* 1988). Since then neosporosis has also been recognised as a an important cause of abortions in cattle world-wide (Dubey 1999). Some species have been known to be (rarely) naturally infected, such as white-tailed deer (Woods *et al.* 1994) and horses (Lindsay 2001). Other species, such as mice, gerbils, sheep and others can be experimentally infected (Dubey *et al.* 1990b, Dubey and Lindsay 1990, Cole *et al.* 1995, Dubey and Lindsay 2000).

While the recognition of this disease was only relatively recent, infection had previously, most likely, been misdiagnosed and retrospectively was now reported from stored tissues of dogs and cattle, dating back to 1957 (Dubey 1992) and 1974 (Dubey *et al.* 1990a), respectively. In New Zealand, *N caninum* infection was diagnosed retrospectively in tissues of dogs dating from 1972 (Patitucci *et al.* 1997).

1.6.1. Identification

N caninum is a protozoan parasite with a close relationship to particularly *Toxoplasma gondii* and *Hammondia heydorni*. In fact, discussions are still quite heated, as to whether *N caninum* is not in fact *H heydorni*, and this issue may yet need to be resolved (Ellis *et al.* 1999, McAllister 2000, Mehlhorn and Heydorn 2000, Hill *et al.* 2001, Heydorn and Mehlhorn 2002). Very recently, however, a re-description of *N caninum* by a great number of eminent workers in that field has shown its relationship to, and distinctiveness from, closely related protozoan (Dubey *et al.* 2002).

A closely related species of Neospora, with only subtle differences in the ITS-1 region, occurs in horses (Dubey *et al.* 2001).

1.6.2. Presence and disease in dogs

Since its first description in dogs in 1984 (Bjerkås *et al.* 1984), there have been a great number of reports of neosporosis in dogs around the world (Dubey and Lindsay 1996). The clinical aspects of the disease in dogs have been extensively reviewed by Ruehlmann *et al.* (1995) and Barber and Trees (1996).

Disease in dogs

In the majority of clinical cases in dogs, signs are those of an ascending paresis or paralysis of the hind limbs (Ruehlmann *et al.* 1995) with dogs under 6 months old more often affected. Clinical signs may include difficulty in swallowing (Hay *et al.* 1990), myocarditis (Odin and Dubey 1993), dermatitis (Dubey *et al.* 1995) and pneumonia (Greig *et al.* 1995). Disseminated infection is more common in older dogs (Dubey *et al.* 1988, Hoskins *et al.* 1991, Barber and Trees 1996).

An early sero-epidemiological, cross-sectional study was initiated to establish the sero-prevalence of *N caninum* infection in various canid populations around the world (Barber *et al.* 1997a). This was thought to establish data regarding the prevalence of infection, versus the level of clinical disease alone, which had hitherto been the focus of scientific attention. The study would also provide a more complete picture of the level of infection in the canid populations. It would also provide better information for the understanding of the epidemiology and possibly the life cycle of the parasite, by establishing the presence or absence of the parasite in different continents and different ecological niches.

An additional paper discussed the utility of diagnostic test systems (serological, histological and molecular) for confirmation of clinical neosporosis in a dog (Reichel *et al.* 1998b)

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PREVALENCE OF ANTIBODIES TO *NEOSPORA CANINUM* IN DIFFERENT CANID POPULATIONS

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ABSTRACT: A total of 1,554 dogs from 5 countries on 3 continents were tested for antibodies to *Neospora caninum* using an indirect fluorescent antibody test. In Australia, overall, 42/451 (9%, 95% confidence interval [CI] 6–12%) dogs were seropositive (Melbourne 11/207 [5%, 95% CI 2–9%]; Sydney 18/150 [12%, 95% CI 7–18%]; Perth 13/94 [14%, 95% CI 8–22%]). Antibodies to *N. caninum* were also detected in dogs in South America (Uruguay [20%, 95% CI 16–24%, n = 414]) and sub-Saharan Africa (Tanzania [22%, 95% CI 12–36%, n = 49]). In contrast, only 1 of 500 dogs tested from the Falkland Islands and none of 140 dogs from Kenya was seropositive. Of wild canids, 1/54 (2%, 95% CI 0–10%) British foxes and 15/169 (9%, 95% CI 5–14%) Australian dingoes had antibodies to *N. caninum*.

Neospora caninum is a recently recognized sporozoan parasite, closely related to *Toxoplasma gondii* but from which it is ultrastructurally and antigenically distinct. Since it was described by Dubey, Carpenter et al. (1988), *N. caninum* has emerged as a significant pathogen, causing predominately neuromuscular signs in dogs. What is probably the same parasite also causes abortion in cattle, and naturally acquired infections have been found in other farm animal species and in a wild California black-tailed deer (Woods et al., 1994). Cases of neosporosis in dogs have been reported from most of the developed countries of the world, including North America, Europe, South Africa, Japan, Australia, and Costa Rica (Dubey and Lindsay, 1993; Morales et al., 1995).

The life cycle and modes of transmission of the parasite remain unclear, and no definitive host (in which sexual reproduction of the parasite occurs) has yet been identified. Vertical transmission, from a subclinically infected dam to her offspring, is the only proven natural route of infection, although epidemiological evidence suggests that postnatal infection can occur in dogs (Barber et al., 1997). Although many cases of canine neosporosis have been reported, the prevalence of disease is difficult to estimate. Many cases will not be confirmed because the clinical signs are not specific for neosporosis, and it is often not possible to conduct immunohistochemistry to make a definitive diagnosis. Hence, surveys to estimate the prevalence of serum antibodies to *N. caninum* can help to indicate the number of dogs exposed to the parasite in a given geographic area and thus the proportion of the population that could be considered to be, or have been, "at risk" of developing the disease.

Seroprevalence studies carried out in different groups of dogs may help to elucidate the epidemiology of the parasite, particularly with regard to transmission. Studies have been reported from Kansas (Lindsay et al., 1990), where 2% of 229 dogs were seropositive; Liverpool (Trees et al., 1993), where 17% of 163 dogs had titers of ≥1:50 in an indirect fluorescent antibody test (IFAT), Denmark (Rasmussen and Jensen, 1996), where 15.3% of 98 dogs were seropositive; and Sweden (Björkman et al., 1994), where only 0.05% of 398 dogs were seropositive in an iscom–enzyme-linked immunosorbent assay. Recent age prevalence studies have shown an overall prevalence of around 8% (but with evidence that this is declining) for U.K. dogs (J. S. Barber, pers. comm.) and 11% in 300 Belgian dogs (Barber et al., 1997). This study examined seroprevalence of antibodies to *N. caninum* in dogs and other canids from several parts of the world.

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MATERIALS AND METHODS

Sera were obtained from different canid populations from Australia, the United Kingdom, Africa, and South America (Table I). Blood samples were obtained between 1991 and 1995 from dogs of both sexes and of various ages (from 3 mo to 8 yr). Blood was collected by jugular venipuncture or by cardiac puncture from dead animals. Blood was allowed to clot for 8–12 hr at 22–37 C, and the serum was collected and stored at −20 C. Individual sera were tested for the presence of IgG antibodies to *N. caninum* by the IFAT as described by Trees et al. (1993) using cell culture-derived tachyzoites of either NC-1 (Dubey, Hattel et al., 1988) or NC-Liv (Barber et al., 1995) isolates as antigen. We have no evidence from IFATs conducted in this laboratory that the choice of isolate for antigen affects the IFAT result. All sera were tested at a dilution of 1:50 in phosphate-buffered saline (PBS, pH 7.2), allowing 30 min incubation at 20 C, prior to rinsing in PBS and 30 min incubation with an anti-dog IgG fluorescein isothiocyanate conjugate at 20 C. Those sera giving uniform fluorescence of tachyzoites were regarded as positive and were then titrated in quadrupling dilutions until fluorescence was lost.

RESULTS

Seropositive dogs were identified in Australia, South America, and Africa (Table II). In Australia, the overall prevalence was 9% (95% CI 6–12%). The prevalences between cities were similar, although in Melbourne the prevalence was significantly lower than in Sydney and Perth. However, these were not matched, equivalent population samples. Seroprevalence varied from 0 in 140 Kenyan dogs and 0.2% (95% CI 0–1%) in 500 Falkland Island dogs to 20% (95% CI 16–24%) in Uruguayan dogs and 22% (95% CI 12–36%) in Tanzanian dogs. The titers in all cases were low; none exceeded 1:800, and 92% (125/136) were ≤1:200. One (urban) of 54 foxes (2%; 95% CI 0–10%) was positive at a dilution of 1:200. One of 117 (0.9%, 95% CI 0–5%) New South Wales and 14/52 (27%, 95% CI 16–41%) Queensland dingoes were seropositive, with titers of 1:50 (n = 4), 1:200 (n = 4), 1:800 (n = 3), and 1:3200 (n = 4).

TABLE I. Serum groups from canid populations tested for the presence of antibodies to *Neospora caninum*.

Geographic origin	Number tested	Species of canid	Description
Australia			
Melbourne	207	*Canis familiaris*	Domestic pets; urban, semiurban, rural; killed at lost dogs home
Sydney	150	*C. familiaris*	Domestic pets; killed at lost dogs home*
Perth	94	*C. familiaris*	Domestic pets and farm dogs; killed at lost dogs home
Queensland	52	*C. familiaris dingo*	Feral; mainly rural; trapped and shot
New South Wales	117	*C. familiaris dingo*	Feral; mainly rural; trapped and shot
United Kingdom			
United Kingdom	54	*Vulpes vulpes*	Feral; urban, semiurban, rural; 35 trapped and bled, 15 shot and bled, 4 RTAs†
South America			
Uruguay	414	*C. familiaris*	Domestic farm dogs; bled
Falkland Islands	500	*C. familiaris*	Domestic farm dogs and pets; bled
Africa			
Kenya	140	*C. familiaris*	Feral dogs; rural; shot and bled
Tanzania	49	*C. familiaris*	Domestic dogs; rural; bled for various serological tests

* Bidgood and Collins (1996).
† Road traffic accidents.

DISCUSSION

Assessing seroprevalence, and hence the exposure of dog populations to *N. caninum*, is an important part of investigating possible transmission routes of the parasite as well as identifying populations in which neosporosis may occur. The IFAT is a well established test for detecting anti-*N. caninum* antibodies in dogs. An IgG response occurs within 3 wk of infection (Cole et al., 1995) and persists for years (J. S. Barber, pers. comm.). Thus, positive reactivity in the IFAT is an indicator of past exposure as well as recent infection. Antibodies to *T. gondii, Sarcocystis* spp., and *Babesia canis* do not cross-react with *N. caninum* tachyzoites in the IFAT at dilutions of 1:50 or more (Dubey and Lindsay, 1993; J. S. Barber, pers. comm.). Yamane et al. (1993) showed that antibodies produced by 2 dogs experimentally infected with *Babesia gibsoni* reacted with *N. caninum* antigen in an IFAT. However, 1 of the dogs was seropositive in a *N. caninum* IFAT at the time of experimental infection (day 0), and the *N. caninum* antibody status of the donor dogs providing the *B. gibsoni* infected erythrocytes was not shown. *Babesia gibsoni* infections occur primarily in Asia and Egypt and, more recently, in the U.S.A. but have not been recognized in Australia, sub-Saharan Africa, or South America. It is not certain that at low dilutions antisera to other protozoa, especially those infecting canids in tropical areas, would not cross-react with *N. caninum* tachyzoites in an IFAT, but in immunohistochemical studies, cross-reactions with a variety of protozoa, including *Sarcocystis* sp., *Besnoitia jellisoni, Caryospora* sp., and *Hepatozoon canis,* did not occur (Lindsay and Dubey, 1989).

TABLE II. *Neospora caninum* IFAT titers in domestic dogs from 5 countries.

Origin of samples	Number tested	Number positive (≥1:50)	Seroprevalence*	Titers 1:50	1:200	1:800
Australia						
Melbourne	207	11	5%	8	2	1
Sydney	150	18	12%[a]	12	4	2
Perth	94	13	14%[ab]	9	2	2
Total Australia	451	42	9%	29	8	5
South America						
Uruguay	414	82	20%[b]	45	32	5
Falkland Islands	500	1	0.2%[c]	1		
Africa						
Kenya	140	0	0%[c]			
Tanzania	49	11	22%[ab]	5	5	1
Total all countries	1,554	136	9%	80	45	11

* Prevalences with common superscripts are not significantly different ($P > 0.05$, Fisher's exact test).

Although cases of canine neosporosis have been described from Australia (Munday et al., 1990; Gasser et al., 1993), these data give the first indication of the prevalence of infection in the Australian dog population, which is similar to that observed in Europe. No case of neosporosis has been described from South America, but the data from Uruguay suggest infection is common there, in marked contrast to the insular population of the Falkland Islands. Epidemiological studies in the U.K. carried out by the authors have indicated that dogs may be postnatally infected from meat of bovine origin, and it is tempting to speculate that the disparate prevalences in Uruguay and the Falkland Islands may be connected to the difference in the sizes of the cattle populations in these 2 countries. If, additionally, the Falkland Islands canine population has been derived from a restricted number of noninfected breeding bitches, this would account for the almost complete absence of infection. The results from the Falkland Islands are interesting because a large proportion of the entire dog population in 1991 has been included (Reichel et al., 1996). Hence, the results genuinely reflect an almost complete absence of neosporosis in that dog population. In Africa, there is no report of neosporosis in dogs or cattle north of Zimbabwe. The results from Tanzania suggest infection may be common, although they are in marked contrast to results from Kenya. Both samples are small, and these data may not reflect true differences in prevalence.

Antibody titers were generally low in the dogs tested in this survey. These dogs were not known to be clinically affected,

and these results are consistent with other seroepidemiological studies (J. S. Barber, pers. comm.) that have indicated that IFAT titers are low in subclinically infected dogs compared with clinically ill dogs, which latter may have titers of up to 1:51,200.

The serological results suggest that foxes in the UK and dingoes in Australia can become infected, but the specificity of the IFAT in these species has not been established, so interpretation of the significance of these titers must be made with caution. It is likely that vertical transmission could occur in these animals, but epidemiological studies in Britain (J. S. Barber, pers. comm.) suggest that an oral route of postnatal infection may occur in dogs, and this could be an important route of infection for scavenging foxes and dingoes. Because of the gaps in our knowledge, a wider ranging investigation of *N. caninum* infection in both domesticated and wild animals might help to elucidate the life cycle and transmission patterns of this parasite.

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LITERATURE CITED

BARBER, J. S., O. J. M. HOLMDAHL, M. R. OWEN, F. GUY, A. UGGLA, AND A. J. TREES. 1995. Characterization of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper and Uggla). Parasitology **111:** 563–568.

BARBER, J. S., L. VAN HAM, I. POLIS, AND A. J. TREES. 1997. Seroprevalence of antibodies to *Neospora caninum* in Belgian dogs. Journal of Small Animal Practice **38:** 15–16.

BIDGOOD, A. J., AND G. H. COLLINS. 1996. The prevalence of *Dirofilaria immitis* in dogs in Sydney. Australian Veterinary Journal **73:** 103–104.

BJÖRKMAN, C., A. LUNDÉN, AND A. UGGLA. 1994. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in Swedish dogs. Acta Veterinaria Scandinavia **35:** 445–447.

COLE, R. A., D. S. LINDSAY, B. L. BLAGBURN, D. C. SORJONEN, AND J. P. DUBEY. 1995. Vertical transmission of *Neospora caninum* in dogs. Journal of Parasitology **81:** 208–211.

DUBEY, J. P., J. L. CARPENTER, C. A. SPEER, M. J. TOPPER, AND A. UGGLA. 1988. Newly recognised fatal protozoan disease of dogs. Journal of the American Veterinary Medical Association **192:** 1269–1285.

———, A. L. HATTEL, D. S. LINDSAY, AND M. J. TOPPER. 1988. Neonatal *Neospora caninum* infection in dogs: Isolation of the causative agent and experimental transmission. Journal of the American Veterinary Medical Association **193:** 1259–1263.

———, AND D. S. LINDSAY. 1993. Neosporosis. Parasitology Today **9:** 452–458.

GASSER, R. B., G. EDWARDS, AND R. A. COLE. 1993. Neosporosis in a dog. Australian Veterinary Practitioner **23:** 190–193.

LINDSAY, D. S., AND J. P. DUBEY. 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. American Journal of Veterinary Research **50:** 1981–1983.

———, S. J. UPTON, AND R. K. RIDLEY. 1990. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. Journal of the Helminthological Society of Washington **57:** 86–88.

MORALES, J. A., J. P. DUBEY, F. RODRIGUEZ, R. L. ESQUIVEL, AND D. FRITZ. 1995. Neosporosis and toxoplasmosis-associated paralysis in dogs in Costa Rica. Applied Parasitology **36:** 179–184.

MUNDAY, B. L., J. P. DUBEY, AND R. W. MASON. 1990. *Neospora caninum* infection in dogs. Australian Veterinary Journal **67:** 76.

RASMUSSEN, K., AND A. L. JENSEN. 1996. Some epidemiological features of canine neosporosis in Denmark. Veterinary Parasitology **62:** 345–349.

REICHEL, M. P., D. J. BABER, P. S. CRAIG, AND R. B. GASSER. 1996. Cystic echinococcosis in the Falkland Islands. Preventive Veterinary Medicine **27:** 115–123.

TREES, A. J., F. GUY, B. J. TENNANT, A. H. BALFOUR, AND J. P. DUBEY. 1993. Prevalence of antibodies to *Neospora caninum* in an English urban dog population. Veterinary Record **132:** 125–126.

WOODS, L. W., M. L. ANDERSON, P. K. SWIFT, AND K. W. SVERLOW. 1994. Systemic neosporosis in a California black-tailed deer (*Odocoileus hemionus columbianus*). Journal of Veterinary Diagnostic Investigation **6:** 508–510.

YAMANE, I., J. W. THOMFORD, I. A. GARDNER, J. P. DUBEY, M. LEVY, AND P. A. CONRAD. 1993. Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. American Journal of Veterinary Research **54:** 1579–1584.

Reichel, MP; Thornton, RN; Morgan, PL; Mills, RJM and Schares G (1998)

Neosporosis in a pup

New Zealand Veterinary Journal **46**, 106-110

Clinical Communication

Neosporosis in a pup

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Abstract

Case. A 13-week-old female boxer pup was found to be suffering from rigidity of the left hindleg. Antibiotic and anti-inflammatory treatment over a 3-week period failed to improve the condition and the pup was humanely killed.
Methods. Serological examination for *Neospora* antibodies was carried out by the indirect fluorescent antibody test and for *Toxoplasma gondii* antibodies with a latex agglutination test. A variety of tissues were examined histologically, and the central nervous system by immunohistochemistry and the polymerase chain reaction.
Results. The IFAT for anti-*Neospora* antibodies showed a titre of 1:51 200 in the clinically affected pup while the latex agglutination test for *Toxoplasma* antibodies was negative. The dam and one of two tested litter mates had anti-*Neospora* IFAT titres of 1:1600, the other litter mate was negative. All three were not clinically affected. Histological, immunohistochemical and polymerase chain reaction examinations of the affected pup confirmed the diagnosis of *Neospora* infection.
Conclusion. In the live animal, serological examination is thought to be the most useful specific test. Post-mortem examination by traditional histology, immunohistochemistry and the polymerase chain reaction confirmed the diagnosis. The case is discussed in the context of present knowledge about *Neospora* infection in New Zealand.
(New Zealand Veterinary Journal 46, 106-110, 1998.)

Introduction

Neospora caninum infects a variety of hosts, but mainly dogs and cattle (Dubey and Lindsay, 1996). *Neospora caninum* infection was recognised as a new disease entity in 1984, when it was reported for the first time in Norway (Bjerkås *et al.*, 1984). Retrospective analysis, however, suggests that the organism has been infecting dogs for a much longer period of time (Dubey, 1992). Since 1984, it has been reported from many countries around the world (Jardine and Dubey, 1992; Gasser *et al.*, 1993; Barber *et al.*, 1993; Burkhardt *et al.*, 1992; Dubey *et al.*, 1988a; Flagstad *et al.*, 1995; VanHam *et al.*, 1996; Weissenböck *et al.*, 1997; Uggla *et al.*, 1989). In dogs, it affects mainly animals under 6 months of age with an ascending paralysis of the hindlegs and shows no predilection for sex or breed (Ruehlmann *et al.*, 1995). In New Zealand, clinical cases of neosporosis in dogs have not previously been reported, although it has been diagnosed, retrospectively by immunohistochemistry in tissues held at Massey University (Patitucci *et al.*, 1997). Infection with *N. caninum*, however, appears to be relatively common in dogs, as evidenced by the relatively high number of dogs in New Zealand with anti-*Neospora* antibodies (Reichel, 1998).

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Clinical History and Diagnosis

A 13-week-old female boxer pup, one of a litter of five, was presented with a fixated left stifle (Figure 1). Two other litter mates had died. One had "faded" when only a few days old, and the other had died after an episode of head pressing when 3 weeks old. Neither had been examined.

The serum creatine kinase concentration of the affected pup was slightly elevated at 889 IU/l (normal 53-821 IU/l) and the aspartate transaminase concentration was normal at 54 IU/l (normal 2-79 IU/l).

Serology

An indirect fluorescent antibody test (IFAT) of the affected pup for *Neospora* (Veterinary Medical Research and Development, Pullman, USA) was positive, up to a serum dilution of 1:51 200. A latex agglutination test (Toxoreagent, Eiken Chemical Co., Tokyo, Japan) for *Toxoplasma* antibodies was negative.

Sera from the dam and two clinically normal male litter mates of the affected pup were tested using the IFAT for anti-*Neospora* antibodies. The dam and one pup tested positive at a serum dilution of 1:1600, while the second pup tested negative. The positive pup was re-bled and tested using the IFAT 3 months later, with a positive result at the 1:800 dilution. The dam and the positive pup were re-tested a further 9 months later, and were positive with an IFAT titre of 1:3200 and 1:800, respectively.

Treatment

Treatment of the pup with the fixated leg was initiated with prednisolone (5 mg daily) for a fortnight, with the addition of trimethoprim (120 mg twice daily) and sulphamethoxazole (600 mg twice daily) for a further week. There was no clinical improvement and the pup was humanely killed.

Laboratory Investigations

Pathology

The left stifle was fixated in extension due to atrophy and fibrosis of the quadriceps muscles (Figure 1). No other gross abnormalities were visible.

Histology

Brain and sections of cervical, thoracic and lumbar spinal cord, peripheral nerve, heart, lungs, liver, kidney, adrenal, bladder and hind limb muscles were fixed in formalin, paraffin embedded and stained with haematoxylin and eosin (H&E) for histopathological examination.

Numerous foci of gliosis were visible throughout the grey matter of the brain, including the cerebellum. Occasional foci of leucomalacia and mild neutrophil infiltration were also present. Moderately severe mononuclear vasculitis was a common finding, particularly in medium sized blood vessels close to the foci of gliosis. Both intact cysts and free tachyzoites were occasionally visible near foci of gliosis.

Areas within the left quadriceps muscle showed generalised fibre atrophy and mild, diffuse interstitial fibrosis. Also present were occasional small foci of acute myonecrosis, with small numbers of infiltrating neutrophils and mononuclear leucocytes. More frequent were larger foci of lymphocytes and plasma cells that appeared to have replaced muscle fibres. Individual regenerative fibres were identifiable by their basophilic cytoplasm and large, central, nuclei. Very occasional muscle fibres contained aggregated tachyzoites.

No histological abnormalities were detected in the right quadriceps muscle or in the other tissues examined.

No abnormalities were detected in any other tissues, including the heart and sections of cervical, thoracic and lumbar spinal cord.

Immunohistochemistry

Sections of phosphate-buffered formalin-fixed brain were tested by immunohistochemistry using using a peroxidase-anti-peroxidase (PAP) reaction described previously (Schares *et al.*, 1997).

Immunohistochemistry of tissue sections of the CNS, using rabbit serum developed against *N. caninum* tachyzoites, revealed numerous small groups of protozoan stages reacting strongly with the antiserum (Figures 2 and 3). In addition, large groups of protozoan stages were also observed reacting with the antiserum (Figure 4). The stages observed in large groups seemed to be smaller in size than those observed in small groups. In some cases a thin cyst-wall like structure was visible surrounding the larger groups of organisms.

Polymerase chain reaction

To confirm the diagnosis of neosporosis, sections of two immunohistologically positive tissue blocks were analysed by the polymerase chain reaction (PCR) for *N. caninum* and *T. gondii* DNA. For the PCR, three 15 µm sections of paraffin-embedded central nervous system (CNS) material were deparaffinised with xylene and the resulting tissue pellet washed three times in ethanol. After evaporation of the ethanol, the tissue pellet was digested with proteinase K and the DNA isolated using a DNA extraction kit (NucleoSpin C+T, Macherey & Nagel, Düren, Germany) following the instructions of the manufacturer. The resulting samples were examined by PCR for *N. caninum* DNA using primers Np6 and Np21 (Yamage *et al.*, 1996). For the detection of *Toxoplasma gondii* DNA, a B1 gene fragment was amplified using oligo 1 and oligo 4 (Burg *et al.*, 1989).

The PCR specific for *N. caninum* amplified in both CNS DNA samples a product with an expected size of 328 bp (Figure 5A, lanes 3 and 4). In addition, a fragment of 150 bp could be observed. The *T. gondii* PCR revealed in both samples no product of the expected size (194 bp; Figure 5B). However, in one of the CNS samples, a fragment of about 310 bp was amplified (Figure 5B, lane 3).

Discussion

This is the first full clinical report of canine neosporosis in New Zealand. Previously, three cases of *N. caninum* infection in New Zealand dogs had been diagnosed retrospectively from tissue sections held at Massey University, one of which dated back to 1972 (Patitucci *et al.*, 1997). Most (over 90%) canine infections with *N. caninum* appear to afflict younger animals, less than 12 months of age (Ruehlmann *et al.*, 1995). As in the case presented here, nearly 90% of these younger dogs show ataxia and a progressive, ascending paralysis of (often) the pelvic limbs, with hyperextension of the pelvic limb being a common finding in pups less than 4 months of age. Other clinical signs may include difficulty in swallowing (Hay *et al.*, 1990), myocarditis associated with sudden death (Odin and Dubey, 1993), dermatitis (Dubey *et al.*, 1995) and pneumonia (Greig *et al.*, 1995). As in the present case, creatinine kinase concentrations are often elevated in clinical cases, and so too are liver enzymes (Ruehlmann *et al.*, 1995).

Clinical signs in older dogs are often not characteristic (although hind leg ataxia leading to paralysis was reported from all three Massey dogs) and may include signs of disseminated infection (Dubey *et al.*, 1988a; Barber and Trees, 1996; Hoskins *et al.*, 1991), including pneumonia (Greig *et al.*, 1995) or dermatitis (Dubey *et al.*, 1995).

Most specific diagnostic tests for neosporosis (such as histology, immunohistochemistry and PCR) are post-mortem tests. The most useful test for neosporosis in live animals appears to be serology. Titres of 1:50 in the IFAT are thought to be specific and indicative of *N. caninum* infection (Dubey *et al.*, 1988b). IFAT titres of 1:800 occasionally occur in clinically normal dogs (Trees *et al.*, 1993) but in dogs presenting with clinical signs titres at this level or greater (as in the present case) should be regarded as diagnostic (Barber and Trees, 1996).

Clinical cases in puppies are thought to be due to congenital infection and this is supported in the present case by the strongly positive titre (1:1600) in the *N. caninum* IFAT, in the dam of the pup. The dam may have been infected with *N. caninum* and passed the infection to her offspring. Her serological response did not appear to change much over time and was, at an IFAT titre of 1:3200 9 months later, quite similar to the first serological result. Two litter mates of the present case were available for serological testing, and one was also positive at 1:1600 initially, reducing to 1:800 at 3 and 9 months later. These reactions were within one serum dilution of the original 1:1600 reaction and are within the accepted limits of variability for laboratory testing and not likely to present real changes in antibody levels. Another litter mate was negative, however, and neither litter mate, even the serologically positive litter mate, showed any clinical signs. These results are in agreement with overseas findings, which have so far demonstrated only transplacental infection in

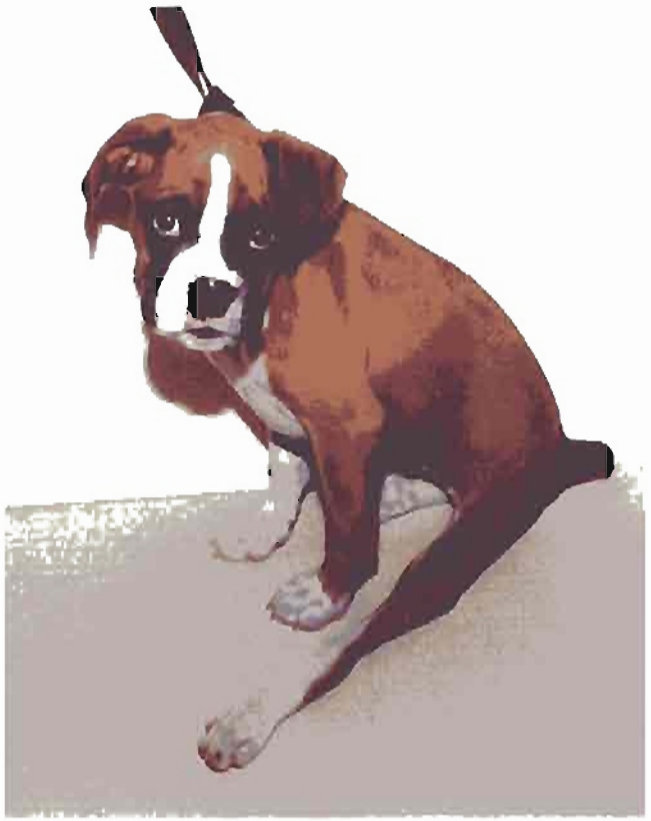

Figure 1. Patient with fixated left hindleg.

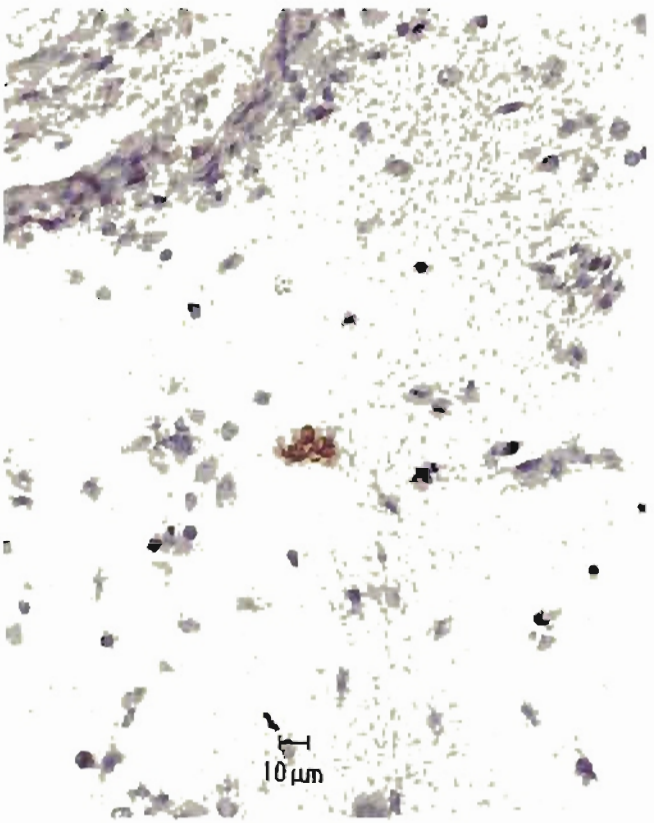

Figure 2. Section of CNS tissue. Small group of protozoan stages reacting with an antiserum against *Neospora caninum* tachyzoites (PAP reaction counterstained with Papanicolaou stain).

Figure 3. Section of CNS tissue. Small group of protozoan stages reacting with an antiserum against *Neospora caninum* tachyzoites (PAP reaction counterstained with Papanicolaou stain).

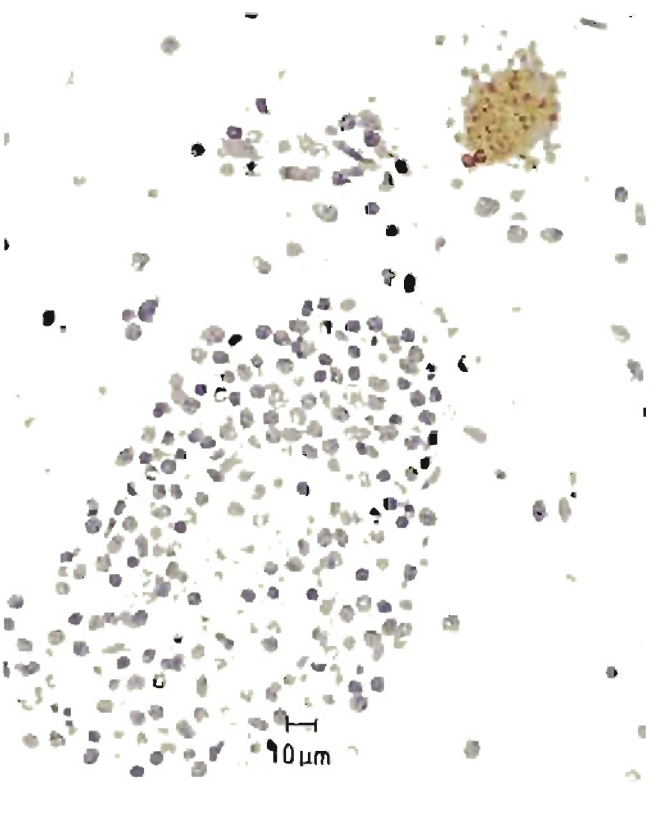

Figure 4. Section of CNS tissue. Large group of protozoan stages reacting with an antiserum against *Neospora caninum* tachyzoites (PAP reaction counterstained with Papanicolaou stain).

dogs, that may also occur repeatedly in subsequent gestations (Bjerkas *et al.*, 1984; Dubey *et al.*, 1988b; Dubey *et al.*, 1990). As in the case presented here, not all individuals of an affected litter show clinical signs (Barber and Trees, 1996; Dubey *et al.*, 1990; Jacobson and Jardine, 1993). It has, however, been suggested that clinical neosporosis in older dogs may be a result of reactivation of a previously acquired infection associated with immunosuppression, sometimes only temporary, such as may be caused by vaccination with an attenuated virus (Patitucci *et al.*, 1997). In this case, the serologically positive, presumably infected pup may be at risk of developing clinical neosporosis at a future date. A large proportion of New Zealand dogs have antibodies to *N. caninum* (Reichel, 1998) and they may therefore be at risk of developing clinical disease if they became immuno-compromised in any way.

The histological findings in the case reported here are in general agreement with the histology reported from overseas cases (Dubey *et al.*, 1988a) and findings from the three cases reported from Massey University which were all older (1-6 years) than the present case.

Lesions were found only in the CNS and skeletal muscle in the present case, and the CNS was also the most rewarding site for examination in previous cases reported from New Zealand (Patitucci *et al.*, 1997). However, Barber *et al.* (1996) have reported lesions from a variety of organs, including heart, lung and liver, and noted that tachyzoites were common in striated muscle, particularly the quadriceps muscle in dogs presenting with hindleg paresis/paralysis and the forelimb muscles in puppies which had developed locomotor problems in those limbs. Barber *et al.* (1996) also suggested that parasitic stages may be found in more tissues during the acute phase of infection with *N. caninum*, while they appear to be confined to the CNS in more chronic cases, at least in younger dogs. It may seem surprising that there were no more general CNS signs in the present case, in view of the extensive histological lesions, but Barber *et al.* (1996) also noted no clear relationship between parasite distribution and severity of lesions and clinical signs.

Immunohistochemistry confirmed the histological diagnosis through the specific staining of *N. caninum* parasitic stages. This study also revealed two different types of parasitic stage reacting with an anti-*N. caninum* serum. It is likely that the larger organisms lying in small groups represent tachyzoites whereas the smaller organisms lying in larger groups may represent bradyzoites. However, the latter were not always surrounded by a cyst wall. Further confirmation was obtained by the amplification of a *N. caninum*-specific DNA fragment by PCR. The additional amplification product of 150 bp may have been caused by an unspecific reaction of primers, e.g. with the host DNA.

Treatment for neosporosis involves a number of drugs, including clindamycin, pyrimethamine and potentiated sulphonamides (Barber and Trees, 1996; Ruehlmann *et al.*, 1995) (see Dubey and Lindsay, 1996, for a review of drugs). While our case did not respond to treatment, prognosis is usually good in cases which are treated early (Jacobson and Jardine, 1993; McGlennon *et al.*, 1990; Mayhew *et al.*, 1991; Barber and Trees, 1996), where clinical signs are initially not severe, progress slowly and do not involve atrophy and fibrosis (Knowler and Wheeler, 1995).

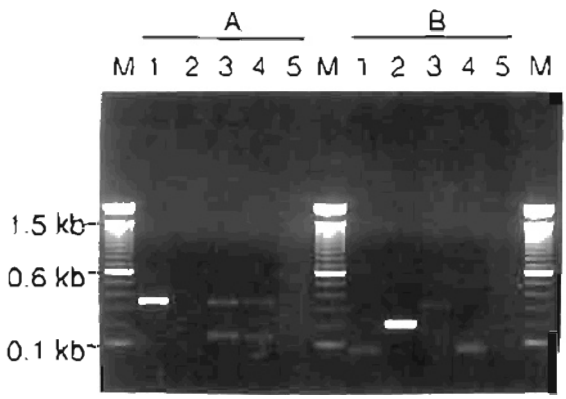

Figure 5. Products of *Neospora caninum*-specific (A) and *Toxoplasma gondii*-specific (B) amplification of nucleic acid in the polymerase chain reaction of: (1) *N. caninum* positive control; (2) *T. gondii* positive control; (3+4) CNS tissue sample DNA, (5) negative control. The 100 bp ladder is the marker (M).

Acknowledgments

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References

Barber JS, Trees AJ. Clinical aspects of 27 cases of neosporosis in dogs. Veterinary Record 139, 439-43, 1996.

Barber J, Trees AJ, Owen M. Isolation of *Neospora caninum* from a British dog. Veterinary Record 133, 531-2, 1993.

Bjerkas I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Zeitschrift fur Parasitenkunde 70, 271-4, 1984.

Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. Journal of Clinical Microbiology 27, 1787-92, 1989.

Burkhardt E, Dubey JP, Korte G, Bauer C. Illness following *Neospora caninum* infection in two pups in Germany. Kleintierpraxis 37, 701-6, 1992.

Dubey JP. Neosporosis — A newly recognized protozoal infection. Comparative Pathology Bulletin 24, 4-6, 1992.

Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A. Newly recognized fatal protozoan disease of dogs. Journal of the American Veterinary Medical Association 192, 1269-85, 1988.

Dubey JP, Hattel AL, Lindsay DS, Topper MJ. Neonatal *Neospora caninum* infection in dogs: Isolation of the causative agent and experimental transmission. Journal of the American Veterinary Medical Association 93, 1259-63, 1988.

Dubey JP, Koestner A, Piper RC. Repeated transplacental transmission of *Neospora caninum* in dogs. Journal of the American Veterinary Medical Association 197, 857-60, 1990.

Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Veterinary Parasitology 67, 1-59, 1996.

Dubey JP, Metzger PL, Jr., Hattel AL, Lindsay DS, Fritz DL. Canine cutaneous neosporosis: Clinical improvement with clindamycin. Veterinary Dermatology 6, 37-44, 1995.

Flagstad A, Jensen HE, Bjerkas I, Rasmussen K. *Neospora caninum* infection in a litter of Labrador Retriever dogs in Denmark. Acta Veterinaria Scandinavica 36, 387-91, 1995.

Gasser RB, Edwards G, Cole RA. Neosporosis in a dog. Australian Veterinary Practitioner 23, 160-3, 1993.

Greig B, Rosow KD, Collins JE, Dubey JP. *Neospora caninum* pneumonia in an adult dog. Journal of the American Veterinary Medical Association 206, 1000-1, 1995.

Hay WH, Snell EG, Lindsay DS, Dubey JP. Diagnosis and treatment of *Neospora caninum* infection in a dog. Journal of the American Veterinary Medical Association 197, 1, 1990.

Hoskins JD, Bunge MM, Dubey JP, Duncan DE. Disseminated infection with *Neospora caninum* in a ten-year-old dog. Cornell Veterinarian 81, 329-34, 1991.

Jacobson LS, Jardine JE. *Neospora caninum* infection in three Labrador littermates. Journal of the South African Veterinary Association 64, 47-51, 1993.

Jardine JE, Dubey JP. Canine neosporosis in South Africa. Veterinary Parasitology 44, 291-4, 1992.

Knowler C, Wheeler SJ. *Neospora caninum* infection in three dogs. Journal of Small Animal Practice 36, 172-7, 1995.

Mayhew IG, Smith KC, Dubey JP, Gatward LK, McGlennon NJ. Treatment of encephalomyelitis due to *Neospora caninum* in a litter of puppies. Journal of Small Animal Practice 32, 609-12, 1991.

McGlennon NJ, Jefferies AR, Casas C. Polyradiculoneuritis and polymyositis due to a toxoplasma-like protozoan: Diagnosis and treatment. Journal of Small Animal Practice 31, 102-4, 1990.

Odin M, Dubey JP. Sudden death associated with *Neospora caninum* myocarditis in a dog. Journal of the American Veterinary Medical Association 203, 831-3, 1993.

Patitucci AN, Alley MR, Jones BR, Charleston WAG. Protozoal encephalomyelitis of dogs involving *Neospora caninum* and *Toxoplasma gondii* in New Zealand. New Zealand Veterinary Journal 45, 231-5, 1997.

Reichel MP. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. New Zealand Veterinary Journal 46, 38, 1998.

Ruehlmann D, Podell M, Oglesbee M, Dubey JP. Canine neosporosis: A case report and literature review. Journal of the American Animal Hospital Association 31, 174-83, 1995.

Schares G, Peters M, Wurm R, Tackmann K, Henning K, Conraths FJ. *Neospora caninum* causes abortions in a cattle herd in North Rhine Westphalia. Deutsche Tierärztliche Wochenschrift 104, 208-12, 1997.

Trees AJ, Guy F, Tennant BJ, Balfour AH, Dubey JP. Prevalence of antibodies to *Neospora caninum* in a populaton of urban dogs in England. Veterinary Record 132, 125-6, 1993.

Uggla A, Dubey JP, Lundmark G, Olson P. Encephalomyelitis and myositis in a Boxer puppy due to a Neospora-like infection. Veterinary Parasitology 32, 255-60, 1989.

VanHam LML, Thoonen H, Barber JS, Trees AJ, Polis I, DeCock H, Hoorens JK. *Neospora caninum* infection in the dog: Typical and atypical cases. Vlaams Diergeneeskundig Tijdschrift 65, 326-35, 1996.

Weissenböck H, Dubey JP, Suchy A, Sturm E. Neosporose als Ursache von Encephalomalazie und Myocarditis bei Hundewelpen. Wiener Tierärztliche Wochenschrift 84, 233-7, 1997.

Yamage M, Flechtner O, Gottstein B. *Neospora caninum*: Specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). Journal of Parasitology 82, 272-9, 1996.

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1.6.3. Discussion and Conclusions

The work described in the former paper established the widespread nature of *N caninum* infection in dog populations around the world, with many shown to be infected with percentages exceeding 10%. The sero-prevalences for canid populations demonstrated in that paper were in-line with the observations of others. These describe the sero-prevalence of infection ranging from single figure percentages to around 30% (Lindsay *et al.* 1990, Trees *et al.* 1993, Björkman *et al.* 1994, Barber *et al.* 1997b, Sawada *et al.* 1998, Gennari *et al.* 2002). Gennari *et al.* (2002) describe a 4-fold higher sero-prevalence in stray dogs over that of domestic dogs. (Sawada *et al.* 1998) also report a 4-fold increase in sero-prevalence in dogs on dairy farms with abortions versus urban dogs. Both studies hence provide valuable epidemiological information about *N caninum*-infection in canid populations. Nothing however was known about the prevalence and distribution of infection in dogs in New Zealand.

The full life cycle of *N caninum* was not completely understood at that time, but this paper demonstrated the widespread nature of infection in canid population on almost all continents (with the exception of Asia, which was not part of the study). This suggested then that the hitherto unknown definitive host must be very common and present in all those countries.

Recent sero-epidemiological work appears to demonstrate some correlation between the sero-prevalence in dog populations in Germany and that in dairy cattle (Schares 2001). A number of further recent publications point to associations between the presence of dogs and abortions on dairy farms (Sawada *et al.* 1998, Wouda *et al.* 1999, Dijkstra *et al.* 2002a, Dijkstra *et al.* 2002b). In hindsight, therefore, it would have therefore been

extremely useful to test cattle populations in the same geographical regions at the same time.

The Reichel *et al*. paper (1998) describes a clinical case of *N caninum* infection in a dog in New Zealand. Prior to this paper, work performed in New Zealand hat described neosporosis retrospectively in specimen of dogs that had previously been diagnosed as suffering from toxoplasmosis (Patitucci *et al.* 1997). Similarly in Australia, diagnostic cases were re-evaluated and reassigned a diagnosis of *N caninum* (Munday et al. 1990) or singular clinical cases described (Gasser *et al.* 1993).

This was the first full description of the clinical features of *N caninum* infection in a dog in New Zealand, with the entire state-of-the-art diagnostic work-up, but into the context of contemporary knowledge. The clinical symptoms were in accordance with overseas observations, i.e. that of an ascending paralysis of the hindlimbs in a dog less than six months of age (Ruehlmann *et al.* 1995, Barber and Trees 1996), which was refractory to treatment (Ruehlmann *et al.* 1995). Some of the littermates had died shortly after birth, an occurrence not uncommonly also reported from overseas (Dubey and Lindsay 1989). Serological testing by IFAT demonstrated high titres in the clinically affected pup, as well as in the dam and in clinically unaffected littermates, suggesting that the infection was acquired from the dam (Dubey and Lindsay 1989, Barber *et al.* 1998) as is observed overseas.

Histological testing demonstrated characteristic lesions of meningoencephalomyelitis in the Central Nervous System (CNS) and myositis (Dubey *et al.* 1988), immunohistochemical and molecular techniques demonstrated *N caninum* in the brain (Ellis 1998).

1.6.4. Presence and disease in cattle

Not long after the first detailed description of the causal agent of neosporosis in dogs by Dubey *et al.* (1988) came the realisation that *N caninum* was also a major cause of infectious abortion in (dairy) cattle (Dubey *et al.* 1989). Subsequent years added a long list of countries with *N caninum*- induced abortions (Thornton *et al.* 1991, Anderson *et al.* 1995, Collery 1995, Jardine and Last 1995, Obendorf *et al.* 1995, Björkman *et al.* 1996, Conraths *et al.* 1996, Klein *et al.* 1997). Several epidemiological studies established that *N caninum* infection increases the risk of abortion in cattle threefold (Moen *et al.* 1998, Wouda *et al.* 1998). In addition, some reports suggest that infection of *N. caninum*-infected cows with bovine pestivirus increases the risk of *Neospora* abortions threefold (Pfeiffer *et al.* 2000). A Swedish study (Björkman *et al.* 2000) also reports a significant association between Bovine Viral Diarrhoea Virus (BVDV)-infection in cattle, *Neospora*, and abortion risk while another failed to demonstrate this association (Gottstein *et al.* 1998, Mainar-Jaime *et al.* 2001).

1.6.5. *N. caninum* in Australia and New Zealand

Not long after the first published descriptions from North America of *N caninum* causing abortions in cattle came its description as an abortifascient in dairy cattle in Australia and New Zealand (Thornton *et al.* 1991, Boulton *et al.* 1995, Obendorf *et al.* 1995). Subsequent years added a small number of further reports, which also started to address epidemiological aspects of the disease (Thornton *et al.* 1994, Patitucci *et al.* 1999).

The history and epidemiology of *N caninum* in Australasia was reviewed in the following paper:

Reichel, MP (2000)

Neospora caninum infections in Australia and New Zealand

Australian Veterinary Journal **78**, 258-261

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'Instructions for authors' were published in the February 1999 issue. They can be found in http://www.farmwide.com.au/nff/vetasscn/aboutava/instruct.htm.

'Statistical guidelines for authors' were published in December 1998. They can be found in http://www.farmwide.com.au/nff/vetasscn/aboutava/statguid.htm.

Invited Review

Neospora caninum infections in Australia and New Zealand

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Objective To review the current state of knowledge of *Neospora caninum* infections with particular reference to Australia and New Zealand.

Procedure Several databases were searched electronically including Medline, Current Contents, Vet CD using several key words (*Neospora caninum*, neosporosis, abortion, cattle, dogs) and authors names. References in original articles were also traced and use made of the author's own original research in the field.

Conclusion *N caninum* is recognised worldwide and is also widespread, in particular in dairy cattle, in Australia and New Zealand. It has been reported in both countries retrospectively (in dogs) from the early 1970s. Abortion storms in dairy herds appear to be the most common feature. Recent reports indicate that the dog is the definitive host, in which the sexual cyle is completed. Further studies are however required to establish important aspects of the epidemiology, such as mode of transmission. In the absence of an effective vaccine, the most effective control strategy is selective culling of infected animals and the prevention of access of dogs to expelled placentas and foetuses and, possibly, to raw beef.
Aust Vet J 2000;78:258-261

Neospora caninum is a protozoan parasite that was first described in a litter of dogs in Norway in 1984.[1] Today it is recognised world-wide as an infection of, predominantly, dogs and cattle.[2] Other species, such as sheep, goats, deer and horses[3-8] have also infrequently been reported as naturally infected. Infection has been reported retrospectively from stored tissues of dogs and cattle, dating back to 1957[9] and 1974,[10] respectively.

Since its recognition, *N caninum* has emerged as a major cause of abortion in cattle. The economic impact of *N caninum* infection in cattle has been estimated at A$85 million per annum for the dairy and $25 million for the beef cattle industry in Australia[11] and NZ$17.8 million for the dairy industry in New Zealand.[12] The true costs are probably higher, since these calculations only take account of abortion outbreaks. It is likely though, that sporadic and low-level endemic abortions also occur, as they do overseas.[13] In addition, a study from the USA reported a decrease in milk yield of approximately 1kg/day[14] in cattle that had serum antibody to *N caninum*. Cattle with serum antibody to *N caninum* are considered to be persistently infected.

Occurrence in Australia and New Zealand

A paper published in 1990 reports that *N caninum* infection in Australia occurred as early as 1971.[15] Infection was retrospectively diagnosed in canine tissues by immunohistochemical techniques; clinical neosporosis was first described in a dog in 1993.[16] In both beef and dairy cattle, infection has been described from the early 1980's onwards.[17,18]

From the end of the 1980's, *N caninum* infection has been described in New Zealand mainly as a cause of abortion in dairy cattle.[19,20] However, infection of dogs has also been described,[21,22] and retrospective immunohistochemical analyses of stored canine tissues have established that *N caninum* infections have been present in that country since at least 1972.[21] Serological surveys indicate that the prevalence of *Neospora* infection in New Zealand dairy cattle is stable[23] and suggest that neosporosis is not a recently introduced disease.

Epidemiology and Life cycle

It has recently been shown that the sexual part of the life cycle of *N caninum* is completed in dogs, where oocysts are released in faeces.[24] Nonetheless, it is still unclear how the disease is transmitted naturally.

There is serological evidence, which indicates that dogs are the reservoir of infection for cattle.[25-27] However, it appears that the number of oocysts shed in dog faeces is low, except when dogs were immunosuppressed,[28] and it is difficult to understand how they could initiate the abortion epidemics that have been observed.

In cattle, evidence suggests that the vertical route represents the major mode of transmission with greater than 80% efficiency.[29,30] Experimentally, colostrum has also been reported as a vehicle of infection for calves.[31] In dogs the efficiency of vertical transmission appears more variable.[32] Many published reports, however, also assert transmission from bitch to pup.[33,34] Modelling suggests that, at least for cattle, vertical transmission alone is not sufficient to sustain infection.[35] There are reports, which suggest that horizontal transmission patterns exist, with point-source exposure accounting for the abortion pattern in cattle.[20,36-38] At this time the relative importance of horizontal compared to vertical transmission remains unclear.

The prevalence of antibodies to *N caninum* in affected dairy herds in Australia and New Zealand is remarkably similar, usually about 30 to 35%,[20,38-40] pointing to a large number of already potentially infected animals. Abortion may be the result of recrudescence of chronic infection triggered by an immunomodulating or suppressive event, such as concurrent infection with bovine viral diarrhoea virus.[39]

Given the relative ease with which they can be infected experimentally[41,42] one might expect *N caninum* abortions to occur naturally in sheep. Recent surveys suggest, however, that neosporosis is not a significant cause of abortion in sheep.[4]

Disease in dogs

In the majority of clinical cases in dogs, signs are those of an ascending paresis or paralysis of the hind limbs,[43] with dogs under 6 months old more often affected. Clinical signs may include difficulty in swallowing,[44] myocarditis,[45] dermatitis[46] and pneumonia.[47] Disseminated infection is more common in older dogs.[48-50] Diagnosis in dogs is usually on the basis of clinical signs, aided by serology (Indirect fluorescent antibody test [IFAT]) and can be confirmed at necropsy by histological, immunohistochemical and molecular techniques.[22] From 5 to 15% of Australian and 22% of New Zealand dogs have specific antibodies to *N caninum*,[23,51] which suggests that a relatively large proportion of them are infected with, or have been exposed to the parasite. This prevalence is similar to that in countries such as Uruguay, Tanzania and the UK, but differs from the situation in Kenya, the Falkland Islands and the USA where the dog population appears to be unaffected or to have a low exposure.[51-53]

Disease in cattle

Abortions are the most dramatic and visible effect of infection in cattle, but congenital infection with no clinical signs appears to be by far the most frequent outcome. Congenitally infected calves may be born underweight, unable to rise with limbs flexed or hyperextended and with neurological signs including ataxia, decreased patellar reflexes and loss of proprioception.[2]

Overseas authors differentiate between sporadic, endemic and epidemic abortion patterns.[13] In Australasia, outbreaks appear to be more often of the epidemic type,[12,17,20,37-40] with the proportion of aborting cattle ranging from 5 to 33%. Repeat abortions appear to be rare, however.[39]

Lesions in aborted foetuses are usually only visible upon histological examination and are located predominantly (> 85% of cases) in the CNS, but heart, skeletal muscle, liver and kidney may be affected.[13,54,55] Characteristic is a multifocal, non-suppurative encephalitis, but lesions are often difficult to detect. Myocardial lesions may be more pronounced and causally associated with foetal death,[56] but these can be masked by autolysis.

Diagnosis

In dogs, clinical signs of paresis or paralysis in an animal that is less than 6 months old are suggestive of *N caninum* infection. Toxoplasmosis may present with similar signs and serological testing should be performed for differentiation. Titres of greater than 800 in the IFAT for *N caninum* are rarely found in clinically normal dogs[49] and can be considered diagnostic. Histology, immunohistochemistry and polymerase chain reaction can be used at necropsy to confirm the clinical diagnosis.

In cattle, diagnosis is somewhat more complicated. Aborted foetuses are frequently not available or are in advanced stages of autolysis, making histological diagnosis difficult. Serological testing of foetal body fluids can yield false-negative results as infected foetuses may lack the immunocompetence to produce significant amounts of anti-*Neospora* antibodies. In one study, only 21% of 3- to 5-month-old *Neospora*-aborted foetuses had specific antibodies compared with 93% of 8- to 9-month-old foetuses.[57] Antibodies in cows decrease in titre within a matter of weeks following an abortion.[39,58] The prevalence of *N caninum* infection in some herds can be high, with a subsequently high level of congenital infection. The presence of congenitally infected calves that have not been aborted as a consequence of *N caninum* infection may falsely suggest that other abortions occurring in the herd are due to neosporosis.[59] In order to determine whether or not there is an association between the abortion and *N caninum* infection, calculation of odds ratios and relative risk have been advocated.[39,60]

Recently, differences in pattern of the serological reactions between endemically and epidemically affected herds have been demonstrated, and these may be useful in epidemiological investigations.[61]

Zoonotic aspects

Nonhuman primates have been shown to be susceptible to *N caninum* infection by intra-uterine inoculation, or by transplacental spread, after intravenous and intramuscular infection of their dams with tachyzoites.[62] Serological evidence of human exposure to *N caninum* has recently been obtained from testing blood samples at a blood bank.[63] Of 1029 sera tested in IFAT, 69 (6.7%) were positive for *N caninum* at a titre of 100, and 50 of those were negative for *T gondii* antibodies. The specificity of this test for *N caninum* antibodies in human serum was confirmed in 12 sera by immunoblotting. The general consensus appears to be however, that *N caninum* does not naturally infect humans[64] and antibodies to *N caninum* have not been detected in sera from women who have experienced abortions.[65]

Conclusions

Neospora caninum has emerged as a major cause of abortion in cattle world-wide and, in the past decade, consistently rated as the single most important infectious cause of abortion in cattle

in Australia and New Zealand. It causes significant losses to cattle producers, estimated to exceed A$100 million per year. While the life cycle of *N caninum* has been fully described, much of the epidemiology remains poorly understood. Vertical transmission appears to be the main route of infection in cattle in the Northern Hemisphere. In Australia and New Zealand, on the other hand, there is evidence for horizontal transmission, which suggests that cattle may be infected by a definitive host, the dog or possibly other canids. Preventive measures to break the protozoan life cycle should at least include the prevention of access of dogs to aborted foetal tissues from cattle. It may be prudent however to exclude also foetal membranes and fluids, as well as raw beef. Modelling suggests that vertical transmission can be effectively controlled by selected culling of antibody-positive cows, provided external sources of infection are controlled.[35] Vaccine development is being pursued[66] but currently there is no effective vaccine available that prevents the shedding of oocysts in dogs or abortions in cattle.[2]

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References

1. Bjerkås I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Z Parasitenkd* 1984;70:271-274.
2. Dubey JP. Recent advances in *Neospora* and neosporosis. *Vet Parasitol* 1999;84:349-367.
3. Dubey JP, Lindsay DS. *Neospora caninum* induced abortion in sheep. *J Vet Diagn Invest* 1990;2:230-233.
4. Otter A, Wilson BW, Scholes SFE et al. Results of a survey to determine whether Neospora is a significant cause of ovine abortion in England and Wales. *Vet Rec* 1997;140:175-177.
5. Barr BC, Anderson ML, Woods LW et al. Neospora-like protozoal infections associated with abortion in goats. *J Vet Diagn Invest* 1992;4:365-367.
6. Woods LW, Anderson ML, Swift PK et al. Systemic neosporosis in a California black-tailed deer (*Odocoileus hemionus columbianus*). *J Vet Diagn Invest* 1994;6:508-510.
7. Dubey JP, Porterfield ML. *Neospora caninum* (Apicomplexa) in an aborted equine fetus. *J Parasitol* 1990;76:732-734.
8. Lindsay DS, Steinberg H, Dubielzig RR et al. Central nervous system neosporosis in a foal. *J Vet Diagn Invest* 1996;8:507-510.
9. Dubey JP. Neosporosis - a newly recognized protozoal infection. *Comp Path Bull* 1992;24:4-6.
10. Dubey JP, Hartley WJ, Lindsay DS. Congenital *Neospora caninum* infection in a calf with spinal cord anomaly. *J Am Vet Med Assoc* 1990;197:1043-1044.
11. Ellis JT. *Neospora caninum*: prospects for diagnosis and control using molecular methods. In: Shirley MW, Tomley FM, Freeman BM, editors. *Control of Coccidiosis into the next millennium*. Compton Newbury: Institute for Animal Health, 1997:80
12. Pfeiffer DU, Wichtel JJ, Reichel MP et al. Investigations into the epidemiology of *Neospora caninum* infection in dairy cattle in New Zealand. Dairy Cattle and Industry Sessions. 75th Jubilee New Zealand Veterinary Association Annual Conference, Rotorua. Continuing Education, Massey University, 1998:279-292.
13. Wouda W, Moen AR, Visser IJR et al. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. *J Vet Diagn Invest* 1997;9:180-185.
14. Thurmond MC, Hietala SK. Effect of *Neospora caninum* infection on milk production in first-lactation dairy cows. J *Am Vet Med Assoc* 1997;210:672.
15. Munday BL, Dubey JP, Mason RW. *Neospora caninum* infection in dogs. *Aust Vet J* 1990;67:76-77.
16. Gasser RB, Edwards G, Cole RA. Neosporosis in a dog. *Aust Vet Practitioner* 1993;23:190-193.
17. Boulton JG, Gill PA, Cook RW et al. Bovine Neospora abortion in north-eastern New South Wales. *Aust Vet J* 1995;72:119-120.
18. Obendorf DL, Murray N, Veldhuis G et al. Abortion caused by neosporosis in cattle. *Aust Vet J* 1995;72:117-118.
19. Thornton RN, Thompson EJ, Dubey JP. Neospora abortion in New Zealand cattle. *NZ Vet J* 1991;39:129-133.
20. Thornton RN, Gajadhar A, Evans J. Neospora abortion epidemic in a dairy herd. *NZ Vet J* 1994;42:190-191.
21. Patitucci AN, Alley MR, Jones BR et al. Protozoal encephalomyelitis of dogs involving *Neospora caninum* and *Toxoplasma gondii* in New Zealand. *NZ Vet J* 1997;45:231-235.
22. Reichel MP, Thornton RN, Morgan PL et al. Neosporosis in a pup. *NZ Vet J* 1998;46:106-110.
23. Reichel MP. Prevalence of Neospora antibodies in New Zealand dairy cattle and dogs. *NZ Vet J* 1998;46:38.
24. McAllister MM, Dubey JP, Lindsay DS et al. Dogs are definitive hosts of *Neospora caninum*. *Int J Parasitol* 1998;28:1473-1478.
25. Paré J, Fecteau G, Fortin M et al. Seroepidemiologic study of *Neospora caninum* in dairy herds. *J Am Vet Med Assoc* 1998;213:1595-1598.
26. Wouda W, Dijkstra T, Kramer AMH et al. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. *Int J Parasitol* 1999;29:1677-1682.
27. Sawada M, Park CH, Kondo H et al. Serological survey of antibody to *Neospora caninum* in Japanese dogs- Note. *J Vet Med Sci* 1998;60:853-854.
28. Lindsay DS, Dubey JP, Duncan RB. Confirmation that the dog is a definitive host for *Neospora caninum. Vet Parasitol* 1999;82:327-333.
29. Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. *Can J Vet Res* 1996;60:133-139.
30. Anderson ML, Reynolds JP, Rowe JD et al. Evidence of vertical transmission of *Neospora* sp infection in dairy cattle. *J Am Vet Med Assoc* 1997;210:1169.
31. Uggla A, Stenlund S, Holmdahl OJM et al. Oral *Neospora caninum* inoculation of neonatal calves. *Int J Parasitol* 1998;28:1467-1472.
32. Barber JS, Trees AJ, Johnson AM. Naturally occurring vertical transmission of *Neospora caninum* in dogs. *10th International Congress of Protozoology* 1998;28:57-64.
33. Dubey JP, Hattel AL, Lindsay DS et al. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *J Am Vet Med Assoc* 1988;93:1259-1263.
34. Dubey JP, Koestner A, Piper RC. Repeated transplacental transmission of *Neospora caninum* in dogs. *J Am Vet Med Assoc* 1990;197:857-860.
35. French NP, Clancy D, Davison HC et al. Mathematical modelling of *Neospora caninum* infection in dairy cattle: transmission and options for control. *Int J Parasitol* 1999;29:1691-1704.
36. McAllister MM, Huffman EM, Hietala SK et al. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. *J Vet Diagn Invest* 1996;8:355-357.
37. Schares G, Conraths FJ, Reichel MP. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. *Int J Parasitol* 1999;29:1669-1676.
38. Atkinson RA, Cook RW, Reddacliff LA et al. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a New South Wales dairy cattle herd. *Aust Vet J* 2000;78:262-266.
39. Cox BT, Reichel MP, Griffiths LM. Serology of a Neospora abortion outbreak on a dairy farm in New Zealand: a case study. *NZ Vet J* 1998;46:28-31.
40. Patitucci AN, Charleston WAG, Alley MR et al. Serological study of a dairy herd with a recent history of Neospora abortion. *NZ Vet J* 1999;47:28-30.
41. McAllister MM, McGuire AM, Jolley WR et al. Experimental neosporosis in pregnant ewes and their offspring. *Vet Pathol* 1996;33:647-655.
42. Buxton D, Maley SW, Wright S et al. The pathogenesis of experimental neosporosis in pregnant sheep. *J Comp Pathol* 1998;118:267-279.
43. Ruehlmann D, Podell M, Oglesbee M al et. Canine neosporosis: a case report and literature review. *J Am Animal Hosp Assoc* 1995;31:174-183.
44. Hay WH, Shell LG, Lindsay DS et al. Diagnosis and treatment of *Neospora caninum* infection in a dog. *J Am Vet Med Assoc* 1990;197:87-89.
45. Odin M, Dubey JP. Sudden death associated with *Neospora caninum* myocarditis in a dog. *J Am Vet Med Assoc* 1993;203:831-833.
46. Dubey JP, Metzger FL Jr., Hattel AL et al. Canine cutaneous neosporosis: clinical improvement with clindamycin. *Vet Dermatol* 1995;6:37-43.
47. Greig B, Rossow KD, Collins JE et al. *Neospora caninum* pneumonia in an adult dog. *J Am Vet Med Assoc* 1995;206:1000-1001.
48. Dubey JP, Carpenter JL, Speer CA et al. Newly recognized fatal protozoan disease of dogs. *J Am Vet Med Assoc* 1988;192:1269-1285.
49. Barber JS, Trees AJ. Clinical aspects of 27 cases of neosporosis in dogs. *Vet Rec* 1996;139:439-443.
50. Hoskins JD, Bunge MM, Dubey JP et al. Disseminated infection with *Neospora caninum* in a ten-year-old dog. *Cornell Vet* 1991;81:329-334.
51. Barber JS, Gasser RB, Ellis J et al. Prevalence of antibodies to *Neospora caninum* in different canid populations. *J Parasitol* 1997;83:1056-1058.
52. Lindsay DS, Dubey JP, Upton SJ et al. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. *J Helminth Soc* 1990;57:86-88.
53. Trees AJ, Guy F, Tennant BJ et al. Prevalence of antibodies to *Neospora caninum* in a populaton of urban dogs in England. *Vet Rec* 1993;132:125-126.
54. Anderson ML, Blanchard PC, Barr BC et al. Neospora-like protozoan infection as a major cause of abortion in California dairy cattle. *J Am Vet Med Assoc* 1991;198:241-244.

55. Barr BC, Anderson ML, Blanchard PC et al. Bovine fetal encephalitis and myocarditis associated with protozoal infections. *Vet Pathol* 1990;27:354-361.
56. Dubey JP, Miller S, Lindsay DS et al. *Neospora caninum*-associated myocarditis and encephalitis in an aborted calf. *J Vet Diagn Invest* 1990;2:66-69.
57. Barr BC, Anderson ML, Sverlow KW et al. Diagnosis of bovine fetal Neospora infection with an indirect fluorescent antibody test. *Vet Rec* 1995;137:611-613.
58. Conrad PA, Sverlow K, Anderson M et al. Detection of serum antibody response in cattle with natural or experimental *Neospora* infections. *J Vet Diagn* Invest 1993;5:572-578.
59. Thurmond MC, Hietala SK, Blanchard PC. Predictive values of fetal histopathology and immunoperoxidase staining in diagnosing bovine abortion caused by *Neospora caninum* in a dairy herd. *J Vet Diagn Invest* 1999;11:90-94.
60. Thurmond M, Hietala S. Strategies to control neospora infection in cattle. *Bovine Pract* 1995;No. 29:60-63.
61. Schares G, Rauser M, Zimmer K et al. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. *J Parasitol* 1999;85:688-694.
62. Barr BC, Conrad PA, Sverlow KW et al. Experimental fetal and transplacental Neospora infection in the nonhuman primate. *Lab Invest* 1994;71:236-242.
63. Tranas J, Heinzen RA, Weiss LM et al. Serological evidence of human infection with the protozoan *Neospora caninum*. *Clin Diagn Lab Immunol* 1999;6:765-767.
64. Barr BC, Bjerkas I, Buxton D et al. Neosporosis - Report of the International Neospora Workshop. *Comp Cont Edu Pract Vet* 1997;19:S120.
65. Petersen E, Lebech M, Jensen L et al. *Neospora caninum* infection and repeated abortions in humans. *Emerg Infect Dis* 1999;5:278-280.
66. Andrianarivo AG, Choromanski L, McDonough SP et al. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *Int J Parasitol* 1999;29:1613-1625.

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Bakker EL, Ball FN, Bell KJ, Belvedere BJ, Brain ME, Buckle WM, Cain DM, Carmichael S, Coles ML, Costa MP, De Gooyer E, Earl IF, Ervin JL, Furlan J, Golden BC, Griffith JE, Grull DA, Hall DA, Hang D, Hennel AF, Hillier N, Hulme KL, Irvin RA, Kawecki ML, Kirkham LE, Kirley AR, Lacorcia L, Le Nepveu J, Luttrell AW, Luxford K, Mason DR, Mason SJ, McCann SA, Mcmeekin B, Mcshane MJ, Morgan MD, Morgan KM, Morrison KE, Mullen TL, O'Dwyer JK, Pankhurst AM, Pereira PC, Picot RJ, Porter AJ, Richards NJ, Rushford ER, Scott RJ, Semp GT, Smith KA, Stanley PE, Stasiuk DJ, Sutcliffe NB, Thai Q, Touzel GR, Turnbull C-L, Underwood PL, Wilcox RS, Willis BR, Wilson DA

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Armit SJ, Astley CE, Azimi SA, Baer MA, Bajwa HK, Baker L, Baker NJ, Boden LA, Breckenridge HJ, Brennan TP, Brooks BK, Bubb LM, Butcher KR, Caldwell KM, Calle GP, Calleja KJ, Cavanagh CA, Cehun ST, Cloet BS, Comyn AR, Conway M, Cossey MG, Crawford TW, Day RJ, De Crewis KL, Eades JM, Eagles DA, Ferguson MC, Flett ARJ, Gray HL, Grehan T, Griffiths KJ, Griffiths MA, Hall KA, Hansell B, Harmsenb CJ, Harper BS, Hendrie FJ, Holt CN, Hulands AN, Johannesen NE, Jones ND, Keune RJ, Langsford NR, Linley VR, Macdonald C, Mansie RIG, Masala M, McFarland PJ, McGregor ML, Morton EJ, Newton PL, O'Halloran TJ, Osman EJ, Parker AC, Paterson S, Paul AEH, Pratap B, Raff SE, Reynolds D, Richards KJ, Robinson P, Rope M, Russell TJ, Schmidt CG, Scott M, Scott P, Sharman BJ, Skinner CH, Spinks NK, Stenberg TF, Sternberg AW, Stewart BP, Tait DJ, Tan BL, Tonion KL, Tracey JJ, Van Eps AW, Vaughan FG, Volny VV, Walker SC, Wearing ZLL, Wilks NN, Williamson CL

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Bhat N, Blick C, Bockmann M, Bridger RL, Bugg RJ, Burgess DN, Burke AM, Caratti RT, Carter CA (was Anderberg), Collins BJ, Constable FA, Dilley SM, Dobson EC, Durey FA, Ford M, Hambling FT, Hartley AJ, Howard BE, Humphrey KL, Humphris MA, James VW, Jay ML, Kelman AN, Mallyon K, Marriott CA, Mincham NJ, Mochankana EM, Moore SE, Nesbit LA, Ng HW, Owen HC, Power JK, Rawlings KL, Reid E, Reynolds AH, Rhodes CV, Robert MF, Roche AL, Schroder D-A, Sherwood MJ, Snader S, Stewart JM, Tan TR, Tarala A-MJ, Tilley LJ, Trotter BJ, Trumper JE, Tucak PB, Walsh TA, Westcott R, Willmer KL, Young LS

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Allison NL, Arslett NH, Ball MC, Balneaves C, Barnett KD, Bilous NS, Booth CJ, Booth MG, Bradbury LA, Cheesman TRA, Chernishev A, Chiam KG, Choi K, Cole AJ, Collis IL, Cooper SA, Crofts LA, Della Torre PK, Drake JC, Fearnside KG, Feary DJ, Feldman RS, Fok KYV, Fortunaso PA, Gavazov I, Graham TB, Hanlon K, Heng LEG, Hick PM, Hopwood J, Howard GJ, Hufschmid J, Hunt TB, Jamieson NV, Kery AR, Klocker J, Kluger EK, Lambeth CR, Lawson SL, Lee P, Lenard ZM, Leung Y, Lockley AE, Lynch GI, Mail KL, Man WYN, Manyweathers JJ, Massy Greene EK, Matthew SRA, McLennan MJ, Mendonca JL, Micklethwaite L, Mok MCY, Nguyen CT, Oakes WP, Payten AD, Pell SM, Perry JR, Pickles KL, Prados Valerio VD, Radford CM, Ricciardo MR, Robinson M, Rose SA, Schniering C, Shah S, Skuja JA, Sweaney R, Tarlinton RE, Tazawa SP, Walker BE, Watts MJ

1.7. Serological diagnosis of neosporosis

1.7.1. Introduction

Serological tests were only just becoming available for routine diagnosis of neosporosis in cattle (Paré *et al.* 1995b, Paré *et al.* 1995a). In addition, a considerable amount of work was still required to determine the magnitude of a specific antibody response indicative of a causal association between *Neospora* infection and abortion in cattle (Trees *et al.* 1994, Barr *et al.* 1995) as well as clinical disease in dogs (Barber and Trees 1996).

Neospora abortions are a major cause of reproductive failure in the New Zealand dairy industry. Little was known about the prevalence and epidemiology (in NZ or globally) in the late 1980s and early 1990s and there was no serological diagnostic testing available for *N caninum* at the time. In New Zealand, *Neospora* abortions were being diagnosed in dairy cattle, by histopathological means, in increasing numbers (Thornton 1992, Thornton 1996) but the lack of any immunological, biochemical or molecular biological test in that country hampered epidemiological investigations. Two papers on *N caninum* abortions had been published in the NZ Veterinary Journal at that time (Thornton *et al.* 1991, Thornton *et al.* 1994). One of those utilised serological data, but the serological testing needed to be performed overseas. Interpretation of the data however was difficult, because there was only limited knowledge and understanding at the time of the kinetics of antibody titres.

The serological diagnosis of *N caninum* infections in cattle was extensively reviewed in the following paper:

Atkinson, R; Harper, PAW; **Reichel, MP** and Ellis, JT (2000)

Progress in the Serodiagnosis of *Neospora caninum* Infections in Cattle

Parasitology Today **16**, 110-114

3 Ormerod, W.E. (1975) Ablastin in *Trypanosoma lewisii* and related phenomena in other species of trypanosomes. *Exp. Parasitol*. 38, 338–341

4 Targett, G.A.T. and Viens, P. (1975) Ablastin: control of *Trypanosoma musculi* in mice. *Exp. Parasitol*. 38, 309–316

5 Targett, G.A.T. and Viens, P. (1979) Immunity to *Trypanosoma* (*Herpetosoma*) infections in rodents, in *Biology of the Kinetoplastida* (Vol. 2) (Lumsden, W.H.R. and Evans, D.A., eds), pp 461–479, Academic Press

6 Viens, P. (1985) Immunology of non-pathogenic trypanosomes of rodents, in *Immunology and Pathogenesis of Trypanosomiasis* (Tizard, I., ed.), pp 201–223, CRC Press

7 Albright, J.W. and Albright, J.H. (1991) Rodent trypanosomes: their conflict with the immune system of the host. *Parasitol. Today* 4, 137–140

8 D'Alessandro, P.A. and Behr, M.A. (1991) in *Parasitic Protozoa* (Vol. I) (Kreier, J.P. and Baker, J.R., eds), pp 225–245, Academic Press

9 Wilson, V.C.L.C. (1971) The morphology of the reproductive stages of *Trypanosoma (Herpetosoma) musculi* in C3H mice. *J. Protozool*. 18, 43

10 Viens, P. *et al.* (1972) The persistence of *Trypanosoma (Herpetosoma) musculi* in the kidneys of immune CBA mice. *Trans. R. Soc. Trop. Med. Hyg*. 66, 669–670

11 Monroy, F.P. and Dusanic, D.G. (1997) Mechanisms of survival of *Trypanosoma musculi* in the kidneys of chronically infected mice: kidney form reproduction and immunological reactions. *J. Parasitol.* 83, 848–851

12 Monroy, F.P. *et al*. (1998) *Trypanosoma musculi* survival in the kidneys of chronically infected mice: kidney form ultrastructure, surface characteristics, serological interactions. *J. Parasitol.* 84, 914–919

13 Oliver, M. and Viens, P. (1985) *Trypanosoma musculi* persisting in the kidneys of mice are responsible for maintaining the host immunity. *Trans. R. Soc. Trop. Med. Hyg*. 79, 516

14 Dusanic, D.G. (1985) Trypanocidal drugs and the role of kidney forms of *Trypanosoma (Herpetosoma) musculi* in immunity of mice against reinfection. *Z. Parasitenkd*. 71,19–31

15 Monroy, F.P. *et al*. Cytokine responses during *Trypanosoma musculi* infection. *Int. J. Parasitol.* (in press)

16 Lu, W.C. *et al*. (1993) Characteristics of *Trypanosoma conorhini* infections in neonatal mice. *Chin. J. Parasitol.* 6, 83–85

17 Steinhausen, M. *et al.* (1983) Hydronephrosis: a new method to visualize vas afferens, efferens, and glomerular network. *Kidney Int*. 23, 794–806

18 Bührle, C.P. *et al.* (1986) The hydronephrotic kidney of the mouse as a tool for intravital microscopy and *in vitro* electrophysiological studies of renin-containing cells. *Lab. Invest*. 54, 462–472

19 Steinhausen, M. *et al.* (1989) Visualization of renal autoregulation in the split hydronephrotic kidney. *Kidney Int*. 35, 1151–1160

Progress in the Serodiagnosis of *Neospora caninum* Infections of Cattle

R. Atkinson, P.A.W. Harper, M.P. Reichel and J.T. Ellis

Neospora caninum *is an apicomplexan protozoan that has become the focus of significant research attention worldwide. This organism infects a range of host species, including dogs, from which it was originally reported in 1984, but it is most important as a major cause of bovine abortion. As a result of the global importance of* N. caninum*, researchers have developed a number of serological tests to investigate the epidemiology of infection and disease. In this article, Robert Atkinson, Peter Harper, Michael Reichel and John Ellis consider progress made in the serodiagnosis of* N. caninum.

Neospora species are cyst-forming coccidia of the family Toxoplasmatinae, which is probably a monophyletic group composed of *Toxoplasma gondii*, *Besnoitia*, *Neospora* and *Hammondia* species[1]. There are believed to be at least two species in the genus *Neospora*: *N. caninum* and *N. hughesi*[2]. *Neospora caninum* is associated with reproductive loss in cows worldwide and is also responsible for clinical disease in dogs[3]. As with all cyst-forming coccidia, there are three major life cycle stages. Tachyzoites are rapidly dividing forms that invade a variety of cell types, often in association with adverse pathology in bovine foetuses, congenitally infected calves and clinically affected pups and dogs. Bradyzoites appear to represent the latent stage of infection and encyst in the tissues of the central nervous system of cows and dogs. Oocysts have been shown to be shed in the faeces of experimentally infected dogs at very low levels, thereby confirming this species as a definitive host for *N. caninum*[4]. The global importance of *N. caninum* has prompted the development of a number of serodiagnostic tests that are currently based on antigenic extracts and antigens of *N. caninum* tachyzoites.

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Antigens of *N. caninum*

A wide range of immunoreactive antigens has been described from *N. caninum*[3]. Immunodominant antigens (IDAs) of *N. caninum* tachyzoites were first identified in 1992 using the western immunoblot technique[5]. In Fig. 1, an immunoblot (IB) is presented that shows the antibody responses of sera from seropositive cows in a *Neospora*-infected herd[6] to non-reduced soluble tachyzoite extract of *N. caninum*. *Neospora caninum* IDAs are detected as a group comprising molecules of molecular mass 37, 29/30, 16/17 and 46 kDa[5,7]. At lower antibody titres, it is clear that some antigens might not be detected at all. The 37 kDa and 29/30 kDa IDAs (p37 and p29/30) are the most commonly detected antigens[6–9] and might be considered as the main target for a diagnostic serological test. These antigens have recently been cloned[9–11] and the gene sequences obtained reveal homologies to those encoding the SRS-2 (surface antigen 1-related sequence 2) and SAG1 (surface antigen 1) antigens of *T. gondii*. No results have yet been presented on the utility of these antigens in serological tests.

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Table 1 lists a number of antigens that have been characterized from *N. caninum*. NCDG1/N57, NCDG2, N54 and p65 have been used in serological tests and shown to be of value[13,14,17]. NCDG1, NCDG2, N54 and NCGRA2 are cloned antigens that were isolated by immunoscreening cDNA expression libraries with serum from infected cows. NCDG1 and NCDG2 appear to be minor tachyzoite antigens associated with dense granules[12,15]. NcGRA1 and -2, by analogy to *T. gondii*, are also likely to be located in the dense granules of the tachyzoite.

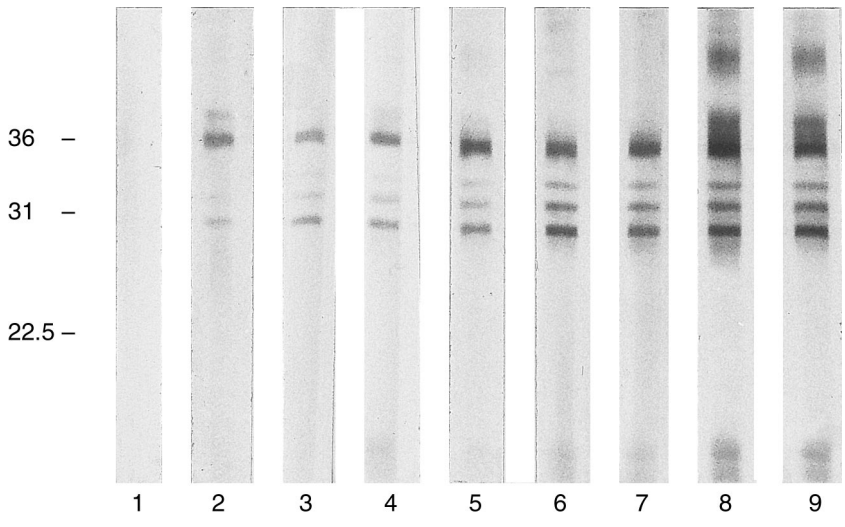

Fig. 1. Immunoblot responses and indirect fluorescent antibody test (IFAT) titres of *Neospora*-infected cows. Lane 1, uninfected cow; lanes 2–9, infected cows with IFAT titres of 160 (lanes 2 and 3), 320 (lanes 4 and 5), 640 (lanes 6 and 7) and >1280 (lanes 8 and 9). Sera were diluted 1:100 for immunoblotting[6]. Molecular mass values (kDa) are shown on the left.

Serodiagnostic tests for neosporosis

Table 2 lists serological tests that have been used successfully in diagnosing exposure of cattle to *N. caninum* infection (for further information see Ref. 28). The indirect fluorescent antibody test (IFAT), which was first applied to neosporosis serology in 1988, has since been used widely for diagnosis of infection in dogs and cows and is a useful standard for comparison with other tests. A positive result is indicated by whole-tachyzoite fluorescence, which occurs when moderate or high-titre sera are tested. When low-titre sera are tested, apical or reduced fluorescence occurs[18,27]. This might also occur as a result of crossreactivity with *T. gondii*, so results should be interpreted with caution. The conoid, a prominent structure in the apical complex, is known to contain epitopes that are crossreactive among *Eimeria* spp, *T. gondii* and *N. caninum*[29]. A cut-off for foetal serology of 1:80 might also be useful[3], whereas for adult cows, titres between 1:25 and 1:640 have been suggested[3,18,30].

The IB is often used as an adjunct to other tests already in use, rather than as a tool for routine screening of herd sera. However, IB is important in identifying IDAs and has been used in limited herd surveys[6,20]. A cut-off for a positive result was proposed to be when two or more of four IDAs are present[20,30]. Highly immunogenic IDAs might still be evident when low titre sera are tested (Fig. 1), suggesting that a cut-off of two IDAs (p37 and p29/30) is appropriate. Crossreactivity to *T. gondii* appears neglible in IBs but this depends on the particular antigen extract and serum dilutions used. If it occurs, crossreactivity is not associated with any of the IDAs[6–9,20].

ELISAs enable more rapid analyses of samples than the IFAT or the IB and so are extremely useful in large-scale screening of cattle herds. For the soluble ELISA, immunostimulatory complex (ISCOM) ELISA, recombinant (r)-ELISA and IFAT-ELISA, absorbance values above the cut-off indicate a positive result. The cut-off is determined from reference sera, often characterized by the IFAT, and is carefully selected to minimize false negative and false positive results. Cut-offs are chosen within 2–3 standard deviations of the mean value given for the negative reference serum group[13,21]. In the CI-ELISA, anti-*Neospora* antibodies compete for an epitope on p65 with a conjugated monoclonal antibody, thereby altering absorbance readings. Based on reference sera, a level of 58% inhibition was proposed as a cut-off[13]. MASTAZYME and AHS ELISAs compare absorbance values with that of a positive control serum and express results as a percent positivity value[26] or ratio[31]. The kinectic ELISA compares antibody-binding kinectics of samples with a positive control serum[8]. Similar to the IFAT and IB, crossreactivity with *T. gondii* has been reported in some ELISAs[3]. MASTAZYME and IDEXX ELISAs are available in kit form from distributors in several countries and have already been used in serological surveys and seroepidemiological studies[20,26,27].

Table 1. Antigens of *Neospora caninum*

Antigen	Localization[a]	Refs
Native		
p37	dg, pv	5,7
p29/30	dg, pv	5,7
Ncp35	cm	9
Ncp29	cm	9
p16/17	rh	5,7
p46	?	5
Ncp43	cm, dg, rh	10
Ncp36	cm, dg	11
p33	dg	12
p65	cm	13
Recombinant		
NCDG1/N57 (NcGRA7)	dg	12,14
NCDG2 (NcGRA6)	dg	15
N54	?	14
recNcp43	cm, dg	10
NcSAG1	cm	9
NcSRS2	cm	9
NcGRA2	?	16
NcGRA1	?	[b]

[a] Abbreviations: cm, cell membrane; dg, dense granule; rh, rhoptry; pv, parasitophorous vacuole; ?, unknown.

[b] Ellis, J. (1999) Gene discovery and prospects for vaccination against *Neospora caninum*. *Proceedings COST-Action 820 Workshop on 'Vaccines against animal coccidioses'* (Gottstein, B. and Hemphill, A., eds), Interlaken, Switzerland.

Table 2. Serological tests for diagnosis of *Neospora caninum*[a]

Test	Antigen	Specificity/Sensitivity[b]	Cut-off	Refs
IFAT	Tachyzoite	82.4–97/85.7–90	1/25–1/640	18,19
Immunoblot	Soluble extract	NS/NS	Refer to text	5–7,20
ELISA	Sonicate[c]	96.5/88.6	V_{max}=0.45	8
ELISA	Soluble extract	100/89–97	A_{492}=0.17–0.40	21
ELISA	ISCOM extract	96/97.6–100	A_{450}=0.20	22,23
CI-ELISA	p65	NS/NS	Refer to text	13
r-ELISA	NCDG1-2, N54	93–96/82–95	A_{450}=0.06–0.14	14,17
IFAT-ELISA	Tachyzoite	96/95	A_{405}=0.77	24
MAT	Tachyzoite	97/100	Refer to text	25
Commercial ELISAs[d]				
MASTAZYME	Tachyzoite	90–100/85–97	PP=15–25%	24,26
IDEXX	Sonicate	87–92/98	S/P=0.5	8,20,27
AHS	Detergent extract	92–100/98	S/P=0.7	27

[a] Abbreviations: ELISA, enzyme-linked immunosorbent assay; CI-ELISA, competitive inhibition ELISA; r-ELISA, recombinant ELISA; IFAT, indirect fluorescent antibody test; ISCOM, immunostimulatory complex; MAT, modified agglutination test; NS, not stated; P, positive control; PP, percent positivity; S, sample.
[b] Expressed as %.
[c] Kinetic ELISA.
[d] Mast Diagnostics, Bootle, Merseyside, UK. IDEXX Laboratories, Westbrook, Maine, USA. Animal Health Service, Drachten, The Netherlands.

An agglutination test has also been introduced into *Neospora* research[25]. After incubation with test sera in microtitre plates, a precipitate of agglutinated tachyzoites indicates a positive result. This is the only serological test listed in Table 2 that does not require secondary antibody conjugates or colorimetric reagents, enabling a range of species to be tested. However, its performance characterisitics will still need to be determined for each species studied, and cut-offs might also vary, as is seen with the agglutination test used for *T. gondii*.

Practical applications and limitations of serology

All these serological tests are useful for identifying sera with moderate to high levels of anti-*Neospora* antibodies (high-titre sera). Examples include sera from infected cows with recrudescing titres during pregnancy[18,32], cows that have aborted as a result of *N. caninum* infection[3] and infected cows or calves during the calving period[33,34]. High-titre sera are often found in clinically affected dogs[35]. Sera with low levels of anti-*Neospora* antibodies (low-titre sera) can be found in a number of circumstances, including cows with fluctuating or declining antibody levels, as occurs several months after parturition or abortion[18], recently seroconverting cows, or calves a number of months postpartum[32]. Low-titre sera are also found in dogs with subclinical infection[3]. In such situations, test performance declines[3,20,31,36]. Thus, there are a number of limitations placed on the use of these serological tests for diagnosing *N. caninum* infection. An appropriate cut-off point is selected for each of these tests and for different applications. The specificity and sensitivity data given in Table 2 are not definitive for *N. caninum*, but are simply values indicated for the nominated cut-off for each test.

The application of ELISAs, including r-ELISAs based on NCDG1 and NCDG2, has revealed discrepancies between tests, especially when low-titre sera are tested[17,31,36], highlighting the importance of selecting appropriate cut-offs for each test and each application. When high-titre sera are tested (which tend to be sera from recently aborted cows) all ELISAs perform well. However, evidence suggests that individual cow serodiagnosis is difficult[36,37] and, therefore, seroepidemiological approaches have been suggested, testing groups of aborted versus unaborted cattle[38,39]. Other difficulties arise from the application: seroepidemiological approaches might need to maximize the sensitivity of the assay (by choosing a low cut-off threshold), whereas diagnostic applications (in the case of abortion diagnosis) might try to maximize specificity (to exclude 'background' reactions to *N. caninum*, which were not causally associated with the abortion).

Serology enables rapid determination of the prevalence of *N. caninum*. Seroprevalences of *N. caninum* are high in many cattle populations, worldwide, and may reach 100% in some herds[3]. In some dog populations, seroprevalences of more than 20% have been reported[40]. These data provide strong evidence on the global distribution and importance of *N. caninum*, which had been previously predicted by histopathology.

A high proportion (80–100%) of seropositive cows transmit infection to their offspring, indicating that vertical transmission is the major mode of infection[20,33,41,42]. This situation appears to predominate in endemically infected herds. In herds that experience epidemics or 'abortion storms', another mode of transmission also appears to operate. This might result from horizontal transmission involving dogs, or an immunosuppressive event leading to the reactivation of a pre-existing, latent infection that results in the transplacental infection of the foetus[4,30] (W. Wouda, PhD thesis, University of Utrecht, The Netherlands, 1998). Serological responses have been shown to differ in endemically and epidemically infected herds[30]. Higher ELISA indices in non-aborting and aborting cows are found in endemic herds compared with epidemic herds. Furthermore, higher IFAT titres are seen in aborting cows in epidemic herds compared with those in endemic herds. This information is consistent with different transmission patterns being in operation. Specifically, vertical transmission in endemic herds might be reflected by higher ELISA indices, which are associated with a latent or chronic infection (ie. release or recrudescence of cytoplasmic or bradyzoite excretory/secretory antigens). Horizontal transmission or immunosuppression in epidemic herds may be reflected by higher IFAT titres, which result from a recent infection, such as oocyst uptake or tachyzoite proliferation as a result of an immunosuppressive event (W. Wouda, op. cit.).

Seropositive cows do not display detectable clinical signs of infection and most deliver healthy, congenitally infected calves. Therefore, seropositivity has a high predictive value for congenital infection in cows but not for abortion (although there is an increased probability of abortion of 2–3-fold compared with seronegative cows[32]). Dogs with clinical disease appear to carry higher levels of anti-*Neospora* antibodies than subclinically infected dogs[3,35], probably reflecting increased antigenic stimulation as a result of parasite proliferation.

Implications for farmers

If *N. caninum* is present in a herd, the farmer can expect infection to be maintained through successive generations (where vertical transmission is the main route of infection), and will need to monitor this using currently available tests. A battery of serological tests might be required (either in parallel or serial interpretation) to achieve an accurate diagnosis. Selective culling strategies may be applied, but this might be impractical in herds with high levels of infection. Where necessary, culling needs to be incorporated into a whole farm management approach. A clear benefit of serology might be in the selection of replacement stock[26]. A farmer can be advised on the serological status of cows or calves before purchase. It has been suggested that an appropriate time to test potential replacement heifer calves should be after parturition[26]. The cut-off threshold for a positive result should be as low as is practical to minimize the likelihood of selecting an uninfected animal. However, further seroepidemiological studies are required to determine the susceptibility of seropositive or seronegative replacement stock to abortion when introduced into herds of differing infection status.

Seroepidemiological and experimental studies support practical control measures that should be adopted to reduce abortion risk in cows. These include protection of feed from dogs (ie. reducing the likelihood of oocyst-bearing faecal contamination), eliminating the practice of feeding raw offal from slaughtered cows or calves to dogs, and preventing or limiting access of dogs to cattle-grazing areas through adequate fencing practices [4,43,44] (W. Wouda, op. cit.). Furthermore, identification of a higher proportion of cows at risk might soon be possible based on the serology of endemic and epidemically infected herds. Farm dogs could also be tested. Those with detectable antibody titres could be periodically treated with drugs (which are effective against tachyzoites) to reduce or prevent oocyst shedding.

Despite some of the advantages of serology, there are a number of practical limitations. Problems with low-titre sera exist, as already discussed. Present serological methods are unable to identify cows at certain stages of infection in endemic and epidemic situations. This occurs in cows where no detectable antibody response exists, such as during seroconversion in epidemic situations, or as a result of fluctuating titres in endemic or epidemic situations. Furthermore, the prognostic limitations of serological tests severely limit the ability to inform farmers of prospective abortion rates.

Future prospects

Further studies are required to identify the different stages of infection in *Neospora*-infected animals. Events such as stage conversion, recrudescence, oocyst uptake, tachyzoite proliferation and release of latent or bradyzoite-specific antigens will become important. Development of tests such as the r-ELISA will also evolve further and be evaluated. A test that can accurately predict the risk of a *Neospor*a-induced abortion in endemic or epidemic situations would be useful for farmers, veterinarians and researchers alike. This might result from improvements in interpretive serology. Alternatively, novel approaches might prove beneficial, such as measuring cell-mediated immune responses with, for example, an interferon γ assay. Investigations on the maternal endocrine environment during pregnancy in infected cows might also prove worthwhile. In either case, it might be possible to identify at-risk cows early on in pregnancy. Ideally, this would enable the veterinarian to apply clinical amelio-ration, thereby enabling foetuses that would otherwise have been aborted to be carried to term.

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References

1 Jenkins, M.C. *et al*. (1999) The relationship of *Hammondia hammondi* and *Sarcocystis mucosa* to other heteroxenous cyst-forming coccidia as inferred by phylogenetic analyses of small subunit ribosomal DNA sequences. *Parasitology* 119, 135–142
2 Marsh, A.E. *et al.* (1998) Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). *J. Parasitol*. 84, 983–991
3 Dubey, J.P. (1999) Recent advances in *Neospora* and neosporosis. *Vet. Parasitol*. 84, 349–367
4 McAllister, M.M. *et al*. (1998) Dogs are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 28, 1473–1478
5 Barta, J.R. and Dubey, J.P. (1992) Characterisation of anti-*Neospora caninum* hyperimmune rabbit serum by western blot analysis and immunoelectron microscopy. *Parasitol. Res.* 78, 689–694
6 Atkinson, R.A. *et al*. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a New South Wales dairy cattle herd. *Aust. Vet. J.* (in press)
7 Bjerkås, I. *et al*. (1994) Identification and characterisation of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. *Clin. Diagn. Lab. Immunol.* 1, 214–221
8 Paré, J. *et al*. (1995) An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. *J. Vet. Diagn. Invest.* 7, 352–359
9 Howe, D.K. *et al.* (1998) The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. *Infect. Immun.* 66, 5322–5328
10 Hemphill, A. *et al.* (1997) Characterisation of a cDNA clone encoding Ncp43, a major *Neospora caninum* tachyzoite surface protein. *Parasitology* 115, 581–590
11 Hemphill, A. *et al.* (1997) Identification and partial characterisation of a 36kDa surface protein on *Neospora caninum* tachyzoites. *Parasitology* 115, 371–380
12 Lally, N.C. *et al.* (1997) A dense granule protein (NCDG1) from *Neospora caninum*. *Mol. Biochem. Parasitol.* 87, 239–243
13 Baszler, T.V. *et al.* (1996) Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 34, 1423–1428
14 Louie, K. *et al.* (1998) Cloning and characterisation of two recombinant *Neospora* protein fragments and their use in serodiagnosis of bovine neosporosis. *Clin. Diagn. Lab. Immunol.* 4, 692–699
15 Liddell, S. *et al.* (1998) Isolation of the cDNA encoding a dense granule associated antigen (NCDG2) of *Neospora caninum*. *Mol. Biochem. Parasitol.* 93, 153–158
16 Ellis, J. *et al.* Isolation, characterisation and expression of a *GRA2* homologue from *Neospora caninum*. *Parasitology* (in press)
17 Lally, N.C. *et al*. (1996) Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.* 3, 275–279
18 Conrad, P.A. *et al.* (1993) Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. *J. Vet. Diagn. Invest.* 5, 572–578

19 Trees, A.J. *et al*. (1993) Prevalence of antibodies to *Neospora caninum* in a population of urban dogs in England. *Vet. Rec*. 132, 125–126

20 Schares, G. *et al*. (1998) The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Vet. Parasitol*. 80, 87–98

21 Osawa, T. *et al*. (1998) A multiple antigen ELISA to detect *Neospora* specific antibodies in bovine sera, bovine foetal fluids, ovine and caprine sera. *Vet. Parasitol.* 79, 19–34

22 Björkmann, C. *et al.* (1994) *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. *Parasite Immunol.* 16, 643–648

23 Björkmann, C. *et al.* (1997) An indirect enzyme linked immunoassay (ELISA) for demonstration of antibodies of *Neospora caninum* in serum and milk of cattle. *Vet. Parasitol.* 68, 251–260

24 Williams, D.J.L. *et al.* (1997) Novel ELISA for detection of *Neospora*-specific antibodies in cattle. *Vet. Rec.* 140, 328–331

25 Packham, A.E. *et al.* (1998) A modified agglutination test for *Neospora caninum* – development, optimisation, and comparison to the indirect fluorescent-antibody test and enzyme linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.* 5, 467–473

26 Williams, D.J.L. *et al*. Evaluation of a commercial ELISA to detect serum antibody to *Neospora caninum* in cattle. *Vet. Rec.* (in press)

27 Paré, J. *et al*. (1995) Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. *J. Vet. Diagn. Invest.* 7, 273–275

28 Björkman, C. and Uggla, A. (1999) Serological diagnosis of *Neospora caninum* infection. *Int. J. Parasitol.* 29, 1497–1507

29 Sasai, K. *et al.* (1998) A chicken anti-conoid monoclonal antibody identifies a common epitope which is present on motile stages of *Eimeria*, *Neospora* and *Toxoplasma*. *J. Parasitol*. 84, 654–656

30 Schares, G. *et al.* (1999) Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. *J. Parasitol.* 85, 688–694

31 Wouda, W. *et al.* (1998) Serodiagnosis of neosporosis in individual cows and dairy herds, a comparative study of three enzyme-linked immunosorbent assays. *Clin. Diagn. Lab. Immunol.* 5, 711–716

32 Paré, J. *et al*. (1997) *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. *J. Parasitol.* 83, 82–87

33 Anderson, M.L. *et al.* (1997) Evidence of vertical transmission of *Neospora* sp. infection in dairy cattle. *J. Am. Vet. Med. Assoc.* 210, 1169–1172

34 Davison, H.C. *et al*. (1999) Herd-specific and age specific seroprevalence of *Neospora caninum* in 14 British dairy herds. *Vet. Rec*. 144, 547–550

35 Barber, J.S. and Trees, A.J. (1996) Clinical aspects of 27 cases of neosporosis in dogs. *Vet. Rec.* 139, 439–443

36 Dubey, J.P. *et al.* (1997) Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. *J. Parasitol.* 83, 1063–1069

37 Schares, G. *et al*. (1999) Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. *Int. J. Parasitol*. 29, 1659–1667

38 Thurmond, M. and Hietala, S. (1995) Strategies to control *Neospora* infection in cattle. *Bovine Practitioner* 29, 60–63

39 Cox, B.T. *et al*. (1998) Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study. *N. Z. Vet. J.* 46, 28–31

40 Barber, J.S. *et al.* (1997) Prevalence of antibodies to *Neospora caninum* in different canid populations. *J. Parasitol.* 83, 1056–1058

41 Paré, J. *et al*. (1996) Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. *Can. J. Vet. Res.* 60, 133–139

42 Björkmann, C. *et al.* (1996) *Neospora* species infection in a herd of dairy cattle. *J. Am. Vet. Med. Assoc.* 208, 1441–1444

43 Harper, P.A.W. (1999) Are your cattle aborting? *Agfact* AO. 9. 58, 1st edn 1994. New South Wales Agriculture

44 McAllister, M.M. (1999) Uncovering the biology and epidemiology of *Neospora caninum*. *Parasitol. Today* 15, 216–217

Tsetse – A Haven for Microorganisms

S. Aksoy

Arthropods are involved in the transmission of parasitic and viral agents that cause devastating diseases in animals and plants. Effective control strategies for many of these diseases still rely on the elimination or reduction of vector insect populations. In addition to these pathogenic organisms, arthropods are rich in microbes that are symbiotic in their associations and are often necessary for the fecundity and viability of their hosts. Because the viability of the host often depends on these obligate symbionts, and because these organisms often live in close proximity to disease-causing pathogens, they have been of interest to applied biologists as a potential means to genetically manipulate populations of pest species. As knowledge on these symbiotic associations accumulates from distantly related insect taxa, conserved mechanisms for their transmission and evolutionary histories are beginning to emerge. Here, Serap Aksoy summarizes current knowledge on the functional and evolutionary biology of the multiple symbionts harbored in the medically and agriculturally important insect group, tsetse, and their potential role in the control of trypanosomiasis.

Tsetse (Diptera: Glossinidae) are the vectors of African trypanosomes, the causative agents of sleeping sickness disease in humans, as well as various diseases in animals. Recently, tsetse-transmitted diseases have been on the rise, causing severe economic hardships in many already stressed communities. The extensive antigenic variation the parasites display in their mammalian host has hampered efforts to develop effective vaccines, and disease management strategies currently rely on the treatment of infected hosts by chemotherapy and on reduction of tsetse challenge by eradication or suppression approaches. However, the success of active surveillance attempts requires the development of improved methods with greater diagnostic sensitivities than the traditional detection techniques[1]. The trypanocidal drugs used for treatment are expensive and highly toxic, with adverse side effects; in addition, their efficacy has recently been challenged in the presence of increasing parasite drug resistance detected in patients[2]. Efforts to control vector populations have been based largely on ground and aerial spraying, as well as the direct application of insecticides to cattle in farming communities. Recently, traps and targets have been used extensively to reduce vector challenge, but reports on their sustainability have been mixed because their long-term success relies heavily on community participation, awareness and resources. In geographically isolated areas, such as the island of Zanzibar, eradication attempts using the 'sterile insect technique' (SIT) have been successful, and have resulted

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1.7.2. The situation in New Zealand

There was no serological testing available in New Zealand for *N caninum* at the commencement of my studies and the interpretation of serological data for the diagnosis of abortions was still under investigation overseas (Conrad *et al.* 1993, Barr *et al.* 1995). The goal was to introduce serological diagnostic testing for *N caninum*, initially utilising a commercially produced test (IFAT) and then to validate it in New Zealand. Thereafter, an ELISA was standardised as it was thought to be more suitable for sero-epidemiological studies.

Reichel, MP and Drake, JM (1996)

The diagnosis of *Neospora* abortions in cattle

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The diagnosis of *Neospora* abortions in cattle

M.P. Reichel*† and J.M. Drake*

Abstract

An indirect immunofluorescent antibody test (IFAT) and an enzyme-linked immunosorbent assay (ELISA) for specific anti-*Neospora* antibodies in bovine sera and foetal fluids were compared with histological examination results on aborted foetal material. The agreement between serological and histological examination results was poor, while the two serological tests showed a high degree of agreement. Serological testing of diagnostic serum samples and foetal fluids suggests that the prevalence of anti-*Neospora* antibodies in cattle which recently aborted is around 40%, in line with previous estimates of the number of abortions in dairy cattle caused by *Neospora* sp. A sero-epidemiological approach to the diagnosis of *Neospora* abortions in cattle may be suggested from these data. (New Zealand Veterinary Journal 44, 151–154, 1996.)

Introduction

Neospora caninum, an apicomplexan protozoan parasite, was first recognised as the cause of protozoan infection in dogs[1]. It has since been found to infect naturally a variety of different host species, including sheep, cattle, horses, goats and, under experimental conditions, laboratory animals[2]. Clinical disease, however, is mainly seen in dogs and cattle. In dogs, disease commonly presents as an encephalitis in animals less than 1 year of age, with neuromuscular deficiency of the hindlimbs a common clinical sign[2]. *Neospora caninum* has been found to be a cause of abortion in dairy cattle, and has been described in the United States, Canada, Great Britain and in Australia[3][4][5][6][7]. In New Zealand, it is estimated to be the cause of abortion in up to 40% of diagnosable submissions sent to animal health laboratories[8]. Average abortion rates due to *Neospora* have been estimated at 7% of pregnancies, compared with 4% due to other causes[9], but may be as high as 33% in individual affected herds. With the New Zealand seasonal calving system, abortions tend to occur most frequently in April and May[10]. Diagnosis is based on characteristic histopathological changes, detecting *Neospora* in foetal tissues and, recently, an indirect fluorescent antibody test (IFAT)[11] on foetal fluids and/or maternal sera. Very little information, however, has been collected on the epidemiology of the parasite in this country, and serological testing on a sample of dairy cattle sera should allow for an assessment of the prevalence of anti-*Neospora* antibodies in the cattle population. Enzyme-linked immunosorbent assay (ELISA) tests have been developed with crude tachyzoite derived antigen and are expected to facilitate large scale sero-epidemiological studies[12].

The complete life cycle of *Neospora* sp. is not yet known. Aspects that are not yet understood include the mode, as well as the rate of, infection. Abortions due to *Neospora* may occur in clusters[10][13] but, as with the closely related organism *Toxoplasma gondii*, which causes abortions in sheep, there is probably a low incidence of infection occurring all the time. To what extent this occurs with *Neospora* sp. has yet not been determined. Observations on naturally infected cows have shown that cattle may abort *Neospora*-infected calves and in subsequent pregnancies deliver calves with congenital infections and neurological signs[14]. Whether abortions occur during subsequent gestations under natural conditions is uncertain. This communication reports on initial experiences with serological tests for *Neospora caninum* in routine diagnostic submissions in our laboratory and discusses the relative merits of diagnostic tests (serology and histology) and their diagnostic value.

Sera/fluids from 656 cattle, from submissions sent to the Central Animal Health Laboratory between January and August 1995 for the investigation of abortion outbreaks, were tested for specific anti-*Neospora* antibodies using the IFAT (VMRD, Pullman, USA). A positive reaction was recorded if the typical peripheral staining pattern of the tachyzoites was observed[11](Figure 1). For the ELISA, microtitre plates were coated in the odd columns with *Neospora caninum* antigen (NC-1 strain, VMRD, Pullman, USA; 338.5 mg/ml) and in even columns with an equal volume of carbonate buffer only. Seventy-six sera (27 positive for *Neospora* in the ELISA/IFAT, 49 negative in the ELISA/IFAT) were also tested for *Toxoplasma gondii* antibodies by latex agglutination (Toxo-reagentEiken, Eiken, Chemical Co., Tokyo). Statistical analysis of data (correlation, chi squared test) was carried out using Microsoft Excel 5.0.

Results and Discussion

Enzyme-linked immunosorbent assay and IFAT test results for sera and foetal fluids showed a high degree of correlation on a logarithmic scale between the respective results, as shown in Figure 2, with increasing titres in the IFAT generally associated with higher corrected absorbances. With a titre of 200 in the IFAT ("gold standard") as the discriminatory threshold

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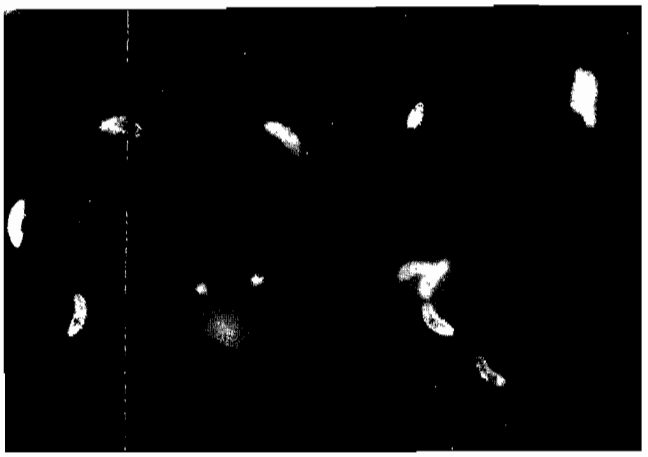

Figure 1. Immunofluorescence of *Neospora caninum* tachyzoites in the IFAT of a positive bovine serum.

signifying negativity or positivity, a negative to positive threshold in the ELISA of a corrected absorbance of 0.15 (at 450 nm) is suggested from these data. The level of agreement (measured as κ = kappa)[15] between IFAT and ELISA results was a high $\kappa = 0.79$ (n=656). Seropositivity for *Toxoplasma gondii* did not differ significantly between *Neospora* positive and negative sera ($p>0.05$) and the reaction was generally at low titres, which should not have interfered with either the ELISA or IFAT, as higher dilutions were used in these assays.

Histological examination results were only available for tissues corresponding to 119 of the foetal/maternal sera/fluids. The histological and serological results showed generally poor agreement, when comparing the histology and ELISA results ($k = 0.37$), and histology and IFAT results ($k = 0.39$) (Table I). Enzyme-linked immunosorbent assay and IFAT results for this series showed again a high level of agreement ($k = 0.96$). The lack of agreement between serological and histological results may be due to a number of reasons. Serologically negative, histologically positive cases may be caused by immunoincompetent foetuses of early gestational age that have not been able to produce appreciable levels of anti-*Neospora* antibody before they were aborted. This explanation is supported by recent observations in California where the sensitivity of foetal *Neospora*-serology was directly related to the gestational age of the foetus, which was low (21%) in calves aborted up to 5 months gestational age, reaching 93% between 8 and 9 months of gestation[16]. Serologically positive yet histologically negative cases may be caused by a failure to detect small focal lesions caused by *Neospora* or the advanced state of autolysis of the examined tissues. Also, serologically positive dams do not always abort their foetuses and other causal agents may be implicated in the abortion. The causality of abortions in cattle is notoriously difficult to diagnose and the diagnostic rate may not exceed 34% at best[17]. Diagnosis of *Neospora* infections may be difficult on the basis of necropsy

Table I. Level of agreement (κ) between the results of an IFAT, an ELISA and histological examination for *Neospora* infection (n=119)

	IFAT	ELISA	Histology
IFAT	1.0		
ELISA	0.96	1.00	
Histology	0.39	0.37	1.00

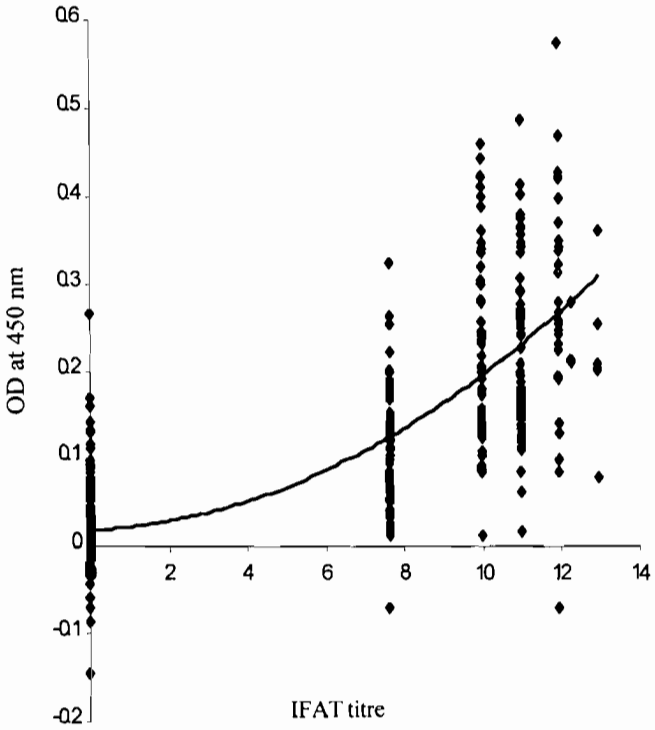

Figure 2. Correlation of titres in the IFAT and absorbance values for the ELISA titres for 656 diagnostic sera.

and histology alone because of the variability of lesions and the low number of parasites present[18], resulting in a lower diagnostic sensitivity for histology than for the serological techniques. Immunohistochemistry (utilising poly- and monoclonal sera) may improve the diagnostic value of histological examination and aid in the detection of *Neospora* in tissue sections[2].

Both IFAT and ELISA have been shown to detect antibodies specific for *Neospora* infection overseas[11][12], with the latter study also suggesting that *Neospora caninum* of dog origin and the *Neospora* species found in cattle are very closely related antigenically, if not identical. The use of a *Neospora* isolate of canine origin (NC-1) as the source of the ELISA antigen should, therefore, not have affected the specificity of the assay. The results obtained in this study show that cross-reactions with a closely related apicomplexan protozoan, *Toxoplasma gondii*, do not occur in New Zealand cattle. This was also supported by the low frequency with which an apical staining pattern was observed in the IFAT in our study, compared with results reported from overseas[11]. A recent study in the USA[19] observed no cross-reaction in serological tests for *Neospora* (IFAT and ELISA) in *Toxoplasma gondii* and *S. hirsuta* infected cattle, but observed

Table II. Comparison of positive and negative serological responses in the *Neospora* ELISA with reactions in the latex agglutination test for *Toxoplasma gondii* (n=76)

	Toxoplasma		
Neospora	**Positive[a]**	**Negative**	**Total**
Positive	22	5	27
Negative	49	0	49
Total	71	5	76

a $p>0.05$.

cross-reactions in *Sarcocystis hominis* and *S. cruzi* infected calves in the ELISA. Transient cross-reactions in the ELISA could also be observed in an uninfected calf. The IFAT titres in those *Sarcocystis* calves remained negative throughout and the serological response in the ELISA may be more a reflection of technical aspects of that ELISA (antigen preparation, plate coating or serum dilution) than of a true cross-reaction.

Neospora abortions have been described in New Zealand since 1991[9]. Immuno-histochemistry has shown *Neospora* to have been present in tissues of a dog dating back to 1972[20], suggesting that the parasite has been in this country for considerable time. Up to 39% of adult cattle which had a recent history of abortion had specific *Neospora* antibodies in this study, a figure similar to the 40% of abortions being caused by *Neospora* recently reported from diagnostic laboratories in this country[8]. While the presence of antibodies does not indicate a causal association *per se*, it suggests that possibility.

Different cut-off titres had been suggested in various serological studies, using either IFAT or ELISA. Patitucci[20], in New Zealand, used an IFAT titre of 1:400 as the determinative cut-off signifying a causal association between *Neospora* and an abortion. Paré *et al.*[11] selected a titre of 1:640 in a Californian study. Trees *et al.*[21] suggested an IFAT titre of ^{3} 1280 as indicative of *Neospora* being the cause of an abortion. In their ELISA evaluation, Paré *et al.*[12] suggest a cut-off value of 0.45 for their assay, but conclude that "practical significance of a seropositive or seronegative result, however, remains unclear until further epidemiological information on infection outcome becomes available". The difficulties of diagnosing *Neospora* abortions based on individual serological results are illustrated by a recent report from the United Kingdom by Dannatt *et al.*[22] who reported high IFAT titres in nine out of ten dairy cattle which had aborted, but also from 48 of 85 cows from the same herd that didn't. In our study, it was decided to use a low "cut-off' value of 1:200 in IFAT, and a net extinction of 0.15 in ELISA to maximise sensitivity of the serological assays for acute sera, especially when it was observed that titres (IFAT) may fall from as high as 1:4000 to 1:200 within 2 months after an abortion "storm" (unpublished observations).

The lack of agreement between histology and serology results for diagnostic samples and observations on affected farms (unpublished observations) suggest that an alternative approach to diagnosis may be needed, as has been proposed by Thurmond and Hietala[23]. Exposure to *Neospora* would be assessed on a farm by serologically screening a statistically valid number of recently aborted cattle for specific anti-*Neospora* antibodies and a similar group of cattle which have not aborted. Significant differences (odds ratios, relative risk) between the two groups will indicate whether a relationship between the abortions and *Neospora* infection exists.

Further serological studies, such as sequential serological testing of seropositive and seronegative cows in a herd that has experienced *Neospora* abortions, are in progress. This should give some indication of sero-conversion rates in infected herds, and the changes (and possible exacerbation) of a serological response in infected animals at subsequent pregnancies, as has been observed by others[24]. The identification of infected and non-infected herds by serological means and an investigation of possible risk factors may lead to recommendations for the management of infected herds, which would attempt to minimise further abortion losses. While the life cycle of *Neospora* sp. remains unclear, the route of infection in cattle also remains a mystery. Overseas studies[25] have suggested that congenital infection could be the most frequent route of infection in herds exposed to *Neospora*, a mode of transmission which would not require a definitive host for the perpetuation of the life cycle. In that case, disease management could be based on the serological identification of positive animals and their elimination from the herd. Vaccination, as used in *Toxoplasma* control[26], may be an alternative approach to control.

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References

(1) Bjerkas I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Zeitschrift für Parasitenkunde 70, 271–4, 1984.
(2) Dubey JP, Lindsay DS. Neosporosis. Parasitology Today 9, 452–8, 1993.
(3) Anderson ML, Blanchard PC, Barr BC, Dubey JP, Hoffman RL, Conrad PA. Neospora-like protozoan infection as a major cause of abortion in California dairy cattle. Journal of the American Veterinary Medical Association 198, 241–4, 1991.
(4) Barr BC, Anderson ML, Blanchard PC, Daft B, Kinde H, Conrad PA. Bovine fetal encephalitis and myocarditis associated with protozoal infection. Veterinary Pathology 27, 354–61, 1990.
(5) Thilsted JP, Dubey JP. Neosporosis-like abortions in a herd of dairy cattle. Journal of Veterinary Diagnostic Investigation 1, 205–9, 1989.
(6) Otter A, Jeffrey M, Griffiths IB, Dubey JP. A survey of the incidence of *Neospora caninum* infection in aborted and stillborn bovine fetuses in England and Wales. Veterinary Record 136, 602–6, 1995.
(7) Obendorf DL, Mason R. *Neospora caninum* infection detected in a bovine aborted foetus. Australian Society of Veterinary Pathology Report 28, 36–7, 1990.
(8) Thornton R. Bovine abortions — Laboratory diagnosis 1991. Surveillance 19 (4), 24, 1992.
(9) Thornton RN, Thompson EJ, Dubey JP. *Neospora* abortion in New Zealand cattle. New Zealand Veterinary Journal 39, 129–33, 1991.
(10) Thornton RN, Gajadhar A, Evans J. *Neospora* abortion epidemic in a dairy herd. New Zealand Veterinary Journal 40, 190–1, 1994.
(11) Paré J, Hietala SK, Thurmond MC. Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. Journal of Veterinary Diagnostic Investigation 7, 273–5, 1995.
(12) Paré J, Hietala SK, Thurmond MC. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. Journal of Veterinary Diagnostic Investigation 7, 352–9, 1995.
(13) Anderson ML, Barr BC, Conrad PA, Thurmond M, Picanso J, Dubey JP. Bovine protozoal abortions in California. Bovine Practitioner 26, 102–4, 1991.
(14) Barr BC, Conrad PA, Breitmeyer R, Sverlow K, Anderson ML, Reynolds J, Chauvet AE, Dubey JP, Ardans AA. Congenital *Neospora* infection in calves born from cows that have previously aborted *Neospora*-infected fetuses: Four cases (1990–1992). Journal of the American Veterinary Medical Association 202,113–17, 1993.
(15) Martin SW, Meek AH, Willeberg P. Measuring agreement. In: Martin SW, Meek AH, Willeberg P (eds). Veterinary Epidemiology. Pp 73–76. Iowa State University Press, Ames, 1987.
(16) Barr BC, Anderson ML, Sverlow KW, Conrad PA. Diagnosis of bovine fetal *Neospora* infection with an indirect fluorescent antibody test. Veterinary Record 137, 611–3, 1995.
(17) Murray RD. A field investigation of causes of abortion in dairy cattle. Veterinary Record 127, 543–7, 1990.
(18) Barr BC, Conrad PA, Dubey JP, Anderson ML. *Neospora*-like encephalomyelitis in a calf: Pathology, ultrastructure and immunoreactivity. Journal of Veterinary Diagnostic Investigation 3, 39–46, 1991.

(19) Dubey JP, Lindsay DS, Adams, DS, Gay JM, Baszler TV, Blagburn BL, Thulliez, P. Serologic responses of cattle and other animals infected with *Neospora caninum*. American Journal of Veterinary Research 57, 329–336, 1996.
(20) Patitucci AN. *Neospora* and abortion in New Zealand dairy cattle. Unpublished MPhil thesis. Massey University, Palmerston North, 1994.
(21) Trees AJ, Guy F, Low JC, Roberts D, Buxton D, Dubey JP. Serological evidence implicating *Neospora* species as a cause of abortions in British cattle. Veterinary Record 134, 405–7, 1994.
(22) Dannatt L, Guy F, Trees AJ. Abortion due to *Neospora* species in a dairy herd. Veterinary Record 137, 566–7, 1995.
(23) Thurmond M, Hietala S. Strategies to control *Neospora* infection in cattle. Bovine Practitioner 29, 60–3, 1995.
(24) Conrad PA, Sverlow K, Anderson M, Rowe J, BonDurant R, Tuter G, Breitmeyer R, Palmer C, Thurmond M, Ardans A, Dubey JP, Duhamel G, Barr B. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. Journal of Veterinary Diagnostic Investigation 5, 572–8, 1993.
25) Paré J, Thurmond M, Hietala S. Congenital *Neospora* infection in dairy cattle. Veterinary Record 134, 531–2, 1994.
(26) Wilkins MF, O'Connell E. Effect on lambing percentage of vaccinating ewes with *Toxoplasma gondii*. New Zealand Veterinary Journal 31, 181–2, 1983.

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1.7.3. Discussion and Conclusions

While there was excellent agreement between IFAT and ELISA results, serological results did not correlate well with histological test results. This may have several reasons: others (Barr *et al.* 1995) have shown that gestational age influences the sensitivity of serological assays. Early gestational calves may not yet have acquired immune-competence, and so may not have developed antibodies against *N caninum*. Also, in most cases, vertical transmission appears to result in live, clinically normal, yet *N caninum*-infected calves (Paré *et al.* 1994, Schares *et al.* 1998). If these are aborted for other reasons this may falsely be diagnosed as having been due to neosporosis.

Other workers validated their serological tests against sera of animals with diagnosed *N caninum* abortions and non-affected controls (Paré *et al.* 1995a) or experimental infections (Conrad *et al.* 1993). Others again used similar approaches as in the current paper and validated the serological assay against results obtained in a reference assay, mostly the IFAT (Björkman *et al.* 1997, Williams *et al.* 1997). Sometimes it was also the combination of these approaches, where specificity was assessed with sera from experimental infection with other apicomplexan parasites and sensitivity against a panel of sera defined by another assay (Schares *et al.* 2000) or combinations of other serological assays and histology and immunohistochemistry (Baszler *et al.* 2001).

Subsequent analyses of the performance characteristics of that ELISA and a commercially available ELISA (IDEXX) were undertaken when a larger set of sera were available for comparative analyses. The performance characteristics of the ELISAs were compared against the IFAT as the “gold standard”. The analyses confirmed the

cut-off threshold for the in-house ELISA, but suggested lower ones for the commercial ELISA:

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An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle

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Abstract

Aims: To analyse the performance characteristics (sensitivity/specificity) of two enzyme-linked immunosorbent assays (ELISA) against the indirect fluorescent antibody test (IFAT).

Methods: A total of 1199 sera were tested in two ELISAs and the IFAT and results analysed utilising software that performed a receiver-operating characteristic (ROC) analysis.

Results: Sensitivity and specificity for the two ELISAs were calculated for a range of different cut-offs. Minimal misclassification was achieved at cut-offs that, in the case of the Central Animal Health Laboratory (CAHL)-ELISA were in line with previously published cut-off values. In the case of the IDEXX-ELISA lower cut-off values than suggested by the manufacturer were calculated.

Conclusions: ROC-analysis resulted in optimised, as compared to the IFAT, cut-off thresholds for the CAHL and IDEXX-ELISA which can be further adjusted depending on the purpose of the investigation.

Keywords: *Neospora caninum*; Cattle; Protozoa; Serology; ROC-analysis; IFAT; ELISA

1. Introduction

Neosporosis is increasingly recognised around the world as an important cause of abortion in cattle and, to a lesser degree of clinical disease in dogs and has been the subject of a number of reviews recently (Dubey, 1999; Reichel, 2000; Antony and Williamson, 2001). Diagnosis

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is based on histopathology and immunohistochemistry on the aborted foetus, but in many cases, when foetal tissues are not available, on the basis of serology. Serological diagnosis has advanced considerably from the early development of indirect fluorescent antibody tests (IFAT) (Dubey et al., 1988) and enzyme-linked immunosorbent assays (ELISA) (Björkman et al., 1997; Paré et al., 1995; Williams et al., 1997). Electrophoretic immunoblotting and the polymerase chain reaction are also employed in diagnostic cases, but generally not on a routine basis (Payne and Ellis, 1996).

There appears to be general agreement about the diagnosis of individual abortion cases (Conrad et al., 1993; Barr et al., 1995), in sero-epidemiological approaches however, there is still discussion about the meaning and usefulness of individual titres (Reichel, 2000).

In New Zealand, an ELISA, developed at the Central Animal Health laboratory (CAHL) for the detection of anti-*Neospora* antibodies, was originally validated against the IFAT and histopathological results from abortion cases. The assay showed good correlation with the other serological assay, but not with histological results (Reichel and Drake, 1996). Since then this ELISA (CAHL-ELISA) has been extensively used in sero-epidemiological studies in New Zealand (Pfeiffer et al., 1998; Reichel, 1998; Schares et al., 1999). In the latter paper, the performance of the ELISA was also compared with that of a number of other serological assays. These included the IDEXX-ELISA (IDEXX Laboratories) (based on the ELISA by Paré (Paré et al., 1995)) and the MAST Diagnostics ELISA (MAST Diagnostics Ltd., Liverpool, UK), which is based on the ELISA developed by Williams et al. at Liverpool (Williams et al., 1997), the IFAT and Western blotting. While all serological tests diagnosed an association between the abortions in a New Zealand dairy herd and *Neospora* infection, the differences between these serological assays in identifying the infection status of individual cows were of concern.

In the current work, a large number of cattle sera were tested in both IDEXX- and CAHL-ELISA. Results of the IFAT, which is regarded the “gold-standard” of serological diagnosis, were deemed to determine the true infection status of the animals. ELISA values were subjected to receiver-operating characteristic (ROC) analysis (Greiner et al., 1995) and the performance characteristics of the assays (sensitivity/specificity, optimal cut-off) were determined.

2. Materials and methods

2.1. Sera

A total of 1199 cattle sera were obtained from whole-herd bleeds of three commercial New Zealand dairy herds, which had experienced *Neospora* abortion outbreaks. Sera from all 306 cattle were obtained from one herd (Herd 1) where 16 (5.2%) out of the total milking herd had aborted. The other dairy herd (Herd 2) experienced *Neospora* abortions in 17 (5.3%) out of 320 milking cows. A total of 241 sera from that herd were available for this study. A further 652 sera were obtained from a whole-herd bleed (Herd 3) where 11.3% of cows had aborted due to *Neospora caninum*. The herd bleeds were performed within 1 month of the abortions occurring.

2.2. Serological assays

Sera were tested in the IFAT and ELISA (CAHL-ELISA) as previously described (Reichel and Drake, 1996) and in a commercial ELISA (IDEXX Laboratories Inc., Westbrook, Maine, USA). A total of 1199 sera were available for the comparison between the CAHL-ELISA and the IFAT and a subset of 306 of those sera (Herd 1) were also tested in the IDEXX-ELISA. Positive results in the IFAT at either $\geq$200 or $\geq$600 serum dilution were regarded as the "gold-standard" positive diagnosis and the resultant data pairs analysed.

2.3. Data analysis

Two-graph receiver-operating characteristic (TG-ROC) analysis was used to assess the diagnostic performance of both ELISA tests against the IFAT results (Greiner et al., 1995). A cut-off value was estimated from the TG-ROC plot, using the non-parametric method, as the optical density (OD)-value (d_0) where sensitivity and specificity were equal (θ_0). The 90% intermediate range was calculated which defines OD-values outside this range as being at least 90% accurate. The cut-off value that minimises misclassification costs (MCT) was estimated using the IFAT prevalence in the sample population (Greiner, 1996). Sensitivity and specificity were calculated for cut-offs based on the manufacturer's recommendations, d_0 based on the TG-ROC plot and for minimised MCT.

While the above analyses were conducted using 1199 and 306 sera for the comparison of the IFAT with CAHL- and IDEXX-ELISA, respectively, a direct comparison between both ELISA tests was conducted using the 306 sera (Herd 1) for which both sets of ELISA results were available. The permutation test described by (Venkatraman and Begg, 1996) was used to test the null hypothesis that the curves of the two diagnostic tests are identical at all possible cut-off values. The Bland–Altman plot was used to visualise the differences between paired OD values at different average OD levels (Bland and Altman, 1986). The areas under the curve were calculated using the Mann-Whitney rank sum test as a test statistic. A 95% confidence intervals were obtained using the 5th and 95th percentile of 2000 bootstrapped replications as described by (Efron and Tibshirani, 1996) and implemented in the computer software *Computational methods for diagnostic tests* (CMDT, FU Berlin, Germany).

3. Results

3.1. CAHL-ELISA versus IFAT

3.1.1. Positive equals IFAT $\geq$200

Out of a total of 1199 sera available for this comparison, 403 sera were classified by the IFAT as positive, 796 as negative (Table 1).

Non-parametric analyses of the data suggested a cut-off OD of $d_0 = 0.12$ for the ELISA, resulting in equal (θ_0) sensitivity and specificity of 81%. The intermediate range ranged from an OD of 0.06–0.16 (Fig. 1).

Table 1
Sensitivity (Se) and specificity (Sp) of two enzyme-linked immunosorbent assays (compared against the "gold-standard" of the indirect fluorescent antibody test (IFAT) at published and "minimised cost" cut-offs ($n = 306$ or 1199, respectively)

Tests	IFAT		Cut-off		IFAT ≥ 200		IFAT		Cut-off		IFAT ≥ 600	
	Positive	Negative	Method	Level for positive	Se	Sp	Positive	Negative	Method	Level for positive	Se	Sp
IDEXX	92	214	Manufacturer	>0.5	0.5	0.98	47	259	Manufacturer	>0.5	0.77	0.95
CAHL	403	796	Reichel and Drake, 1996	>0.15	0.76	0.88	227	972	Reichel and Drake, 1996	>0.15	0.91	0.80
IDEXX	92	214	Min misclassification	>0.21	0.79	0.92	47	259	Min misclassification	>0.22	0.94	0.83
CAHL	403	796	Min misclassification	>0.15	0.76	0.88	227	972	Min misclassification	>0.21	0.86	0.90
IDEXX	92	214	Sensitivity = specificity	>0.11	0.81	0.81	47	259	Sensitivity = specificity	>0.28	0.87	0.87
CAHL	403	796	Sensitivity = specificity	>0.12	0.81	0.81	227	972	Sensitivity = specificity	>0.19	0.87	0.87

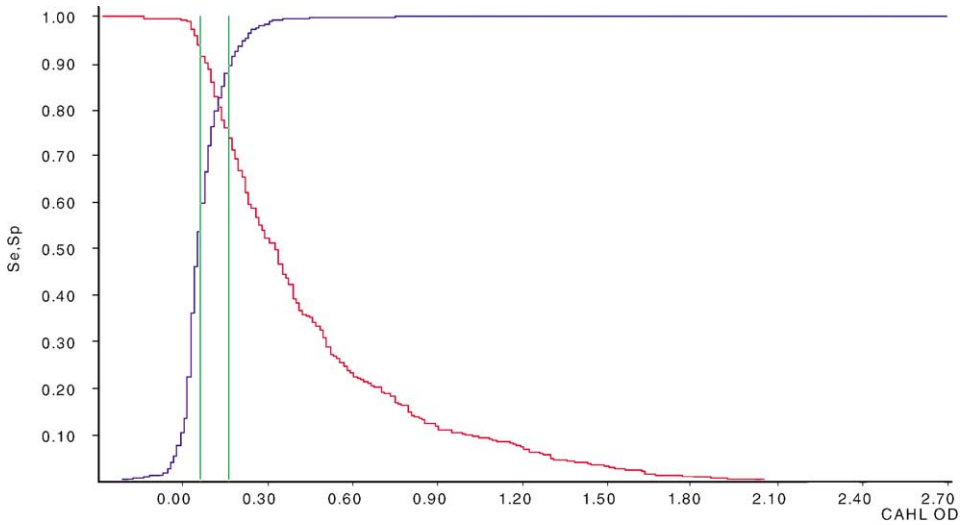

Fig. 1. Non-parametric results for sensitivity (red) and specificity (blue) and intermediate range (green) for Se/Sp ≥0.9 for 1199 sera tested in the CAHL-ELISA compared to the IFAT gold-standard (1:200).

If MCT were minimised (Fig. 2), an optimal cut-off with an OD = 0.15 (equal to the published cut-off) in the CAHL-ELISA was suggested by the data, resulting, at the cut-off in sensitivity of 76%, specificity of 88% (Table 1).

3.1.2. Positive equals IFAT ≥600

At an IFAT titre ≥600 as the gold-standard positive result, 227 sera were classified by the IFAT as positive, 972 as negative (Table 1).

At the published cut-off value for the CAHL-ELISA, sensitivity was 91%, specificity 80%. Non-parametric analyses of the data suggested a cut-off of $d_0 = 0.19$ for the CAHL-ELISA, resulting in equal (θ_0) sensitivity and specificity of 87%.

If MCT were minimised, an optimal cut-off ≥0.21 in the CAHL-ELISA was suggested by the data, resulting in sensitivity at the cut-off of 86%, specificity of 90% (Table 1).

3.2. IDEXX-ELISA versus IFAT

3.2.1. Positive equals IFAT ≥200

Out of the 306 sera analysed in this comparison, 92 sera were classified by the IFAT, using this threshold value, as positive, 214 as negative (Table 1).

The published cut-off value of a sample/positive (S/P) ratio of 0.5 resulted in sensitivity of 50%, specificity of 98%. Non-parametric results suggested a cut-off of $d_0 = 0.11$ for the ELISA, resulting in equal (θ_0) sensitivity and specificity of 81%. The intermediate range ranged from an S/P ratio of 0.03–0.19 (Fig. 3).

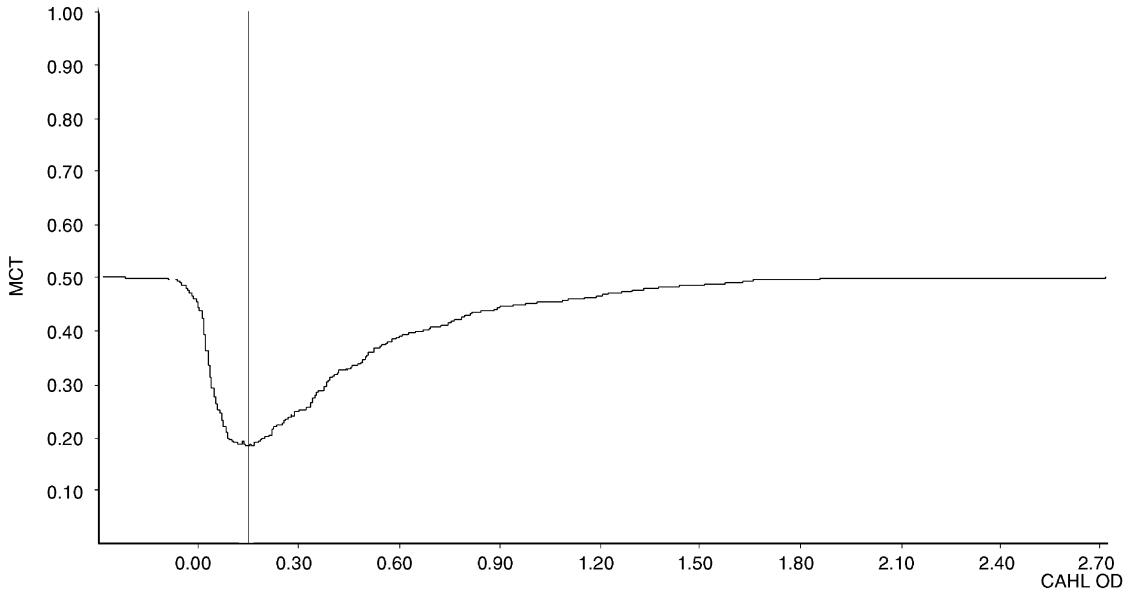

Fig. 2. Minimal misclassification cost (MCT) for 1199 sera tested in the CAHL-ELISA (compared with the gold-standard of a titre of $\geq$1:200 in the IFAT).

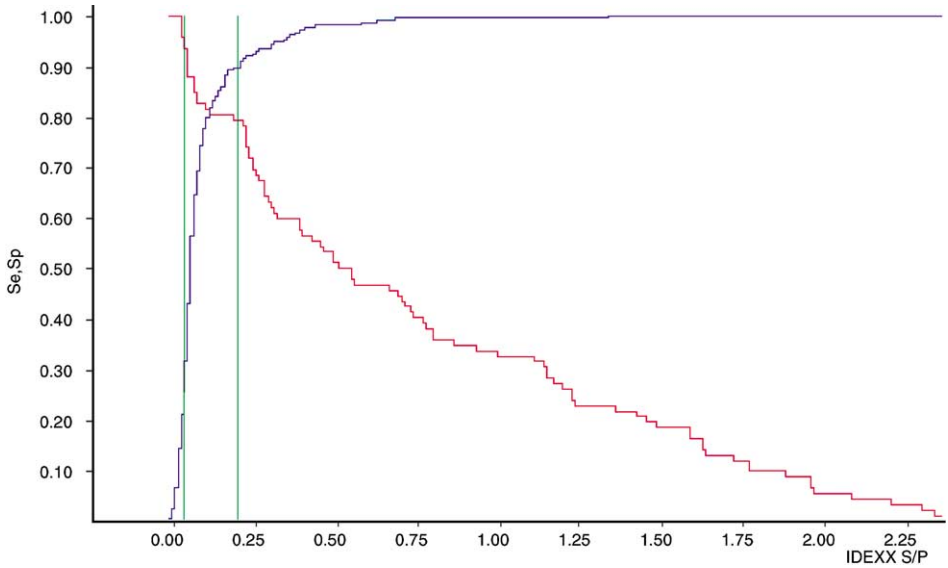

Fig. 3. Non-parametric results for sensitivity (red) and specificity (blue) and intermediate range (green) for Se/Sp ≥0.9 for 306 sera tested in IDEXX-ELISA compared to the IFAT gold-standard (≥200).

If MCT were minimised (Fig. 4), an optimal cut-off with a S/P ratio ≥0.21 in the IDEXX-ELISA was suggested by the data, resulting in sensitivity at the cut-off of 79%, specificity of 92%.

3.2.2. Positive equals IFAT ≥600

At this cut-off value for the IFAT, 47 sera were classified as positive, 259 as negative (Table 1).

The published cut-off value of a S/P ratio of 0.5 resulted in sensitivity of 77%, specificity of 95%. Non-parametric results suggested a cut-off of $d_0 = 0.28$ for the ELISA, resulting in equal sensitivity and specificity of $\theta_0 = 87\%$.

If MCT were minimised, an optimal cut-off with an S/P ratio ≥0.22 in the IDEXX-ELISA was suggested by the data, resulting in sensitivity at the cut-off of 94% and a specificity of 83%.

3.3. Direct comparison of CAHL and IDEXX-ELISA

The permutation test for IFAT ≥ 200 using 1000 samples and a bin width of 4 resulted in a reference distribution with a mean E-value of 1588 compared with an observed value of $E = 4340$ reflecting a P-value of 0.03. With IFAT ≥ 600, the results were a mean $E = 2282$, observed $E = 1054$ and $P = 0.01$. The area under the curve (AUC) for the IDEXX at IFAT cut-off ≥200 was 0.87 (95% CI 0.82–0.92) versus 0.76 (95% CI 0.7–0.83) for the CAHL-ELISA.

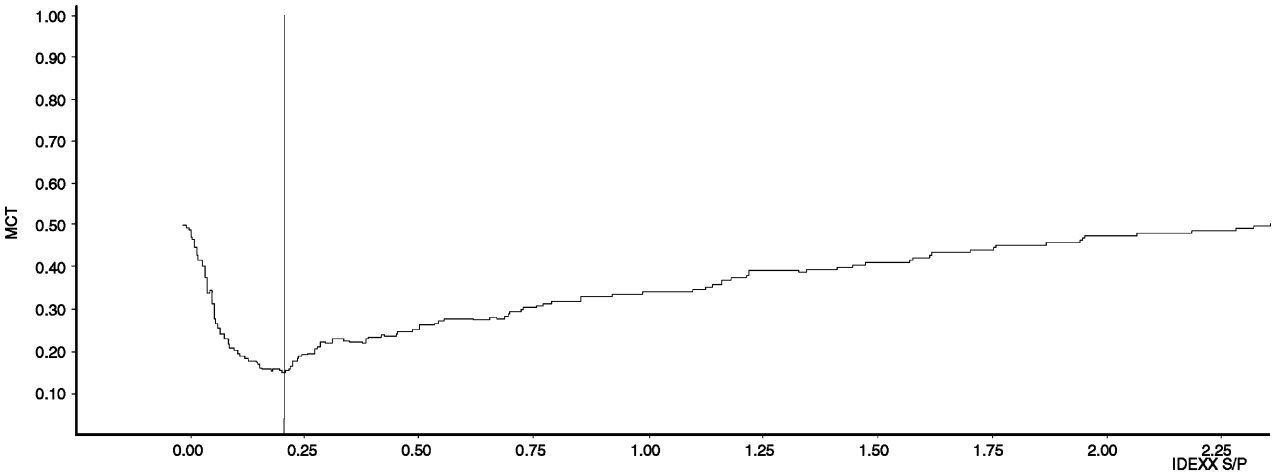

Fig. 4. Minimal misclassification cost (MCT) for 306 sera tested in the IDEXX-ELISA (compared with the gold-standard of a titre of $\geq$1:200 in the IFAT).

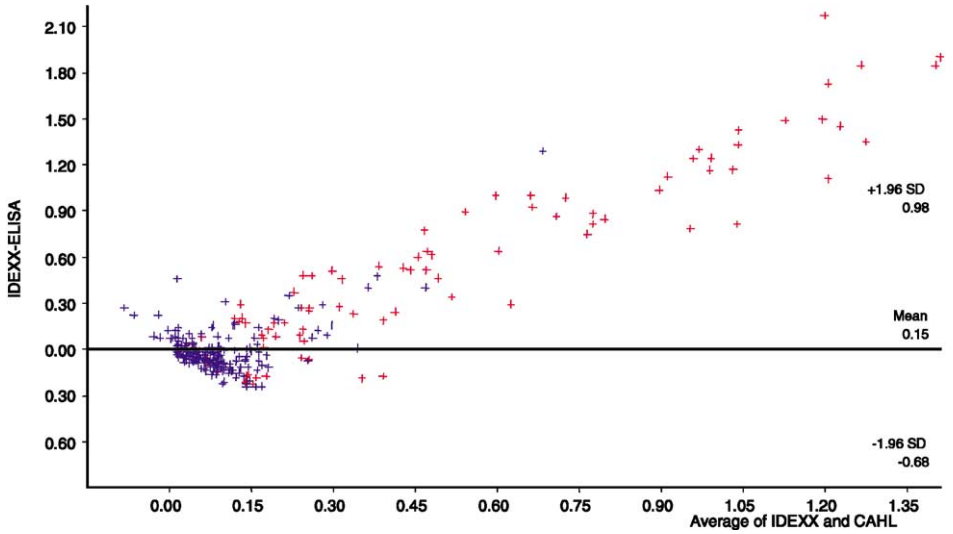

Fig. 5. Bland–Altman plot (blue and red crosses represent IFAT negative and positive sera, respectively based on an IFAT cut-off at 1:200).

The Bland–Altman plot is shown in Fig. 5. There is fairly good agreement between both ELISAs at low OD values, however, with increasing average OD, the IDEXX-ELISA tends to result in higher OD values than the CAHL-ELISA.

4. Discussion

The IFAT is still regarded as the gold-standard test for the serology of *N. caninum* infection in cattle and dogs (Dubey and Lindsay, 1996). A positive IFAT result at the 1:200 serum dilution is generally considered to be indicative of infection (Dubey et al., 1997) and the sensitivity and specificity of the IFAT have been cited as between 85.7 and 90% and between 82.4 and 97%, respectively (Atkinson et al., 2000). However, some researchers have used serum dilutions as low as 1:25 in the IFAT to indicate infection with *N. caninum* (Schares et al., 1999) and for foetal serology, IFAT dilutions of 1:80 are generally considered to be specific (Barr et al., 1995). In the case of abortion diagnosis, IFAT titres of 1:640 have been suggested as appropriate cut-offs (Conrad et al., 1993).

In a previous paper, the optimal cut-off value in the CAHL-ELISA had been determined on the basis of a comparison with the IFAT and histological data from abortion case material (Reichel and Drake, 1996). However, the number of serum samples and herds was limited in that comparison. The results from the present study, with a much larger dataset of sera available, however, are generally in line with the previous determination of an optimal cut-off value for this assay. The analysis of the serological data suggests that at the published cut-off (of 0.15 or 15% positivity), MCT are minimised, when the CAHL-ELISA is compared against a gold-standard result for positivity of an IFAT titre of 200. Sensitivity at that cut-off

(compared to the IFAT) is then estimated to be 76%, specificity lies at 88%. Compared to an IFAT titre of 600, the optimal cut-off (min MCT) is suggested at 0.21 with sensitivity at 86%, specificity at 90%.

It was unexpected for the optimised cut-off thresholds for the IDEXX-ELISA to deviate so considerably in the ROC-analyses from the vendor-suggested thresholds. MCT estimates are dependent on the prevalence of disease/infection in a given dataset, and this will therefore have influenced the threshold suggested by the analysis. However, the θ_0 cut-off values (which are free from that bias), where sensitivity equals specificity, are also consistently considerably lower than the cut-off suggested by the manufacturer.

The intermediate range, outside which either sensitivity or specificity reach values larger than 90%, is much smaller in the case of the CAHL-ELISA than in the case of the IDEXX-ELISA, suggesting better discrimination between positive and negative serum populations by the former assay. This better discrimination is also supported by the steeper slope of the TG-ROC curve for both, sensitivity and specificity in the case of the CAHL-ELISA.

At a S/P ratio of 0.5, the IDEXX-ELISA performance characteristics are cited as 98% for sensitivity and between 87 and 92% for specificity (Paré et al., 1995; Atkinson et al., 2000). However, in this comparison of IDEXX-ELISA with IFAT results, the CDMT optimised thresholds suggested minimal MCT at a cut-off of a S/P ratio of 0.21 or 0.22, depending on whether an IFAT titre of 200 or 600 is used as the reference value, giving IFAT prevalences of 34 and 19%, respectively. At the vendor-suggested threshold of an S/P ratio of 0.5, only the comparison with the IFAT titre of 600 resulted in reasonable performance characteristics with an estimated sensitivity for the assay of 77% and a specificity of 95%. This cut-off may hence be usefully applied in abortion diagnoses, where high specificity is required. In sero-epidemiological investigations, where a high sensitivity might be desired, however, a lower cut-off, such as 0.22 might be suggested from the present study.

References

Antony, A., Williamson, N.B., 2001. Recent advances in understanding the epidemiology of *Neospora caninum* in cattle. New Zealand Vet. J. 49, 42–47.

Atkinson, R., Harper, P.A.W., Reichel, M.P., Ellis, J.T., 2000. Progress in the serodiagnosis of *Neospora caninum* infections in cattle. Parasitol. Today 16, 110–114.

Barr, B.C., Anderson, M.L., Sverlow, K.W., Conrad, P.A., 1995. Diagnosis of bovine fetal *Neospora* infection with an indirect fluorescent antibody test. Vet. Rec. 137, 611–613.

Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical assessment. Lancet 1, 307–310.

Conrad, P.A., Sverlow, K., Anderson, M., Rowe, J., BonDurant, R., Tuter, G., Breitmeyer, R., Plamer, C., Thurmond, M., Ardans, A., Dubey, J.P., Duhamel, G., Barr, B., 1993. Detection of serum antibody response in cattle with natural or experimental *Neospora* infections. J. Vet. Diagn. Invest. 5, 572–578.

Dubey, J.P., 1999. Recent advances in *Neospora* and neosporosis. Vet. Parasitol. 84, 349–367.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 93, 1259–1263.

Dubey, J.P., Jenkins, M.C., Adams, D.S., McAllister, M.M., AndersonSprecher, R., Baszler, T.V., Kwok, O.C.H., Lally, N.C., Björkman, C., Uggla, A., 1997. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. J. Parasitol. 83, 1063–1069.

Dubey, J.P., Lindsay, D.S., 1996. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67, 1–59.

Efron, B., Tibshirani, R., 1996. An Introduction to the Bootstrap: Monographs on Statistics and Applied Probability, 1st Edition. Chapman & Hall, New York.

Greiner, M., 1996. Two-graph receiver operating characteristic (TG-ROC): update version supports optimisation of cut-off values that minimise overall misclassification costs. J. Immunol. Methods 191, 93–94.

Greiner, M., Sohr, D., Göbel, P., 1995. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J. Immunol. Methods 185, 123–132.

Paré, J., Hietala, S.K., Thurmond, M.C., 1995. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J. Vet. Diagn. Invest. 7, 352–359.

Payne, S., Ellis, J., 1996. Detection of *Neospora caninum* DNA by the polymerase chain reaction. Int. J. Parasitol. 26, 347–351.

Pfeiffer, D.U., Wichtel, J.J., Reichel, M.P., Williamson, N.B., Teague, W.R., Thornton, R.N., 1998. Investigations into the epidemiology of *Neospora caninum* infection in dairy cattle in New Zealand: dairy cattle and industry sessions. In: Proceedings of the 75th Jubilee New Zealand Veterinary Association Annual Conference on Continuing Education. Massey University, Rotorua, pp. 279–292.

Reichel, M.P., 2000. *Neospora caninum* infections in Australia and New Zealand. Aust. Vet. J. 78, 258–261.

Reichel, M.P., 1998. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. New Zealand Vet. J. 46, 38.

Reichel, M.P., Drake, J.M., 1996. The diagnosis of *Neospora* abortions in cattle. New Zealand Vet. J. 44, 151–154.

Schares, G., Conraths, F.J., Reichel, M.P., 1999. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. Int. J. Parasitol. 29, 1669–1676.

Schares, G., Rauser, M., Zimmer, K., Peters, M., Wurm, R., Dubey, J.P., de Graaf, D.C., Edelhofer, R., Mertens, C., Hess, G., Conraths, F.J., 1999. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. J. Parasitol. 85, 688–694.

Venkatraman, E.S., Begg, C.B., 1996. A distribution-free procedure for comparing receiver operating characteristic curves from a paired experiment. Biometrika 83, 835–848.

Williams, D.J.L., McGarry, J., Guy, F., Barber, J., Trees, A.J., 1997. Novel ELISA for detection of *Neospora*-specific antibodies in cattle. Vet. Rec. 140, 328–331.

1.8. Sero-epidemiology

1.8.1. Sero-prevalence

There was limited information available internationally on the prevalence of *N caninum* infection in dog and cattle populations (Lindsay *et al.* 1990, Trees *et al.* 1993, Barber *et al.* 1997a), but none from New Zealand. Information about the prevalence of *N caninum* infection in New Zealand dog and cattle populations however would provide an estimate of the size of the problem, which would help with planning ways to deal with the infection.

Further work therefore assessed the national prevalence of *N caninum* infection in New Zealand dairy (Reichel 1998) and beef (Tennent-Brown *et al.* 2000) cattle, and in the dog population (Reichel 1998):

Reichel, MP (1998)

Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs

New Zealand Veterinary Journal **46**, 38

Correspondence

Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs

(New Zealand Veterinary Journal 46, 38, 1998)

Neospora caninum is a major cause of disease in cattle and dogs, manifesting with abortions in cattle, and hind limb paresis in mostly young dogs[1]. Previous reports from New Zealand suggest that around 30% of bovine abortions may be due to *Neospora*[2][3] and that about 40% of recently aborted dairy cows have antibodies against *Neospora*[4].

In an effort to establish the national prevalence of *Neospora* infection, 400 sera from dairy cattle (dating from 1985 and 1995), selected to be representative of the distribution of dairy cattle in New Zealand and which did not have a history of recent abortion, were tested in ELISA for *Neospora* antibodies as previously described[4].

The serological testing of that serum bank showed that between 6.75% (95% CI ± 2.4%) in 1995 and 8.5% (95% CI ± 2.7%) in 1985 of New Zealand dairy cattle had antibodies against *N. caninum* (Table I).

The serological reactions in sera from 1985 precede previous diagnoses of *Neospora* as the cause of abortions in New Zealand cattle by a few years[2]. The serological testing results from 1985 and 1995 show the seroprevalence in non-aborted dairy cattle stable at between 8.5% and 6.75%. It is also likely that *Neospora* has been affecting New Zealand cattle for a considerably longer period of time, similar to the situation in Australia, where the earliest recorded case of neosporosis in cattle dates from 1974[5]. In dairy herds experiencing abortion storms in New Zealand, the within herd seroprevalence is higher than the national seroprevalence, generally about 30%[6][7]. This suggests that *Neospora* infection may only be found in some dairy herds and that the majority of dairy herds may be totally uninfected. More work is needed in order to collect further information about the distribution of *Neospora* infection, within herds and nationally. Similarly, no information on the prevalence of *Neospora* infections and abortions in beef cattle in New Zealand is available.

Two hundred randomly selected blood samples of clinically unaffected dogs were tested in a commercially available indirect fluorescent antibody test for *Neospora* antibodies (Veterinary Medical Research and Development (VMRD) Inc., Pullman, WA, USA). Twenty-two percent (95% CI ± 5.7%) had a positive reaction at the 1:40 and 9% (95% CI ± 3.9%) at the 1:200 dilution (Table I). Some authors[1] report that the higher titres are usually associated with clinical neosporosis in dogs, yet these dogs showed no clinical signs. A similar seroprevalence in clinically unaffected dogs has been reported from dogs in Australia[8], the United Kingdom and Denmark[9][10], but it was lower in dogs from the Falkland Islands[8], the United States and Sweden[11][12]. While the study in Denmark suggests that an association with cats may be responsible for *Neospora* infection in dogs, other overseas work indicates that dogs and cats are not the putative definitive host of *Neospora* in which the sexual cycle is completed[1]. In contrast to the evidence which suggests that transmission of *Neospora* infection in cattle is mainly via the congenital route[13], serological studies in dogs suggest that in this species the infection may be acquired mainly via horizontal transmission, as evidenced by the increase in age-specific seroprevalence[14].

Table I. Numbers of samples with anti-*Neospora* antibodies from a serum bank of 400 New Zealand dairy cattle, dating from 1985 and 1995, and 200 dogs from 1995 (95% CI in brackets)

Year	Number tested	Positive		Negative
Cattle				
1985	400	8.5% (± 2.7)*		91.5%
1995	400	6.75% (± 2.4)*		93.2%
Dogs		1:40	1:200	
1995	200	22% (± 5.7)	9% (± 3.9)	78%

* $p > 0.05$.

References

(1) Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Veterinary Parasitology 67, 1–59, 1996.

(2) Thornton RN, Thompson EJ, Dubey JP. *Neospora* abortion in New Zealand cattle. New Zealand Veterinary Journal 39, 129–33, 1991.

(3) Thornton R. Bovine abortions — Laboratory diagnosis 1991. Surveillance 19, 24, 1992.

(4) Reichel MP, Drake JM. The diagnosis of *Neospora* abortions in cattle. New Zealand Veterinary Journal 44, 151–4, 1996.

(5) Dubey JP, Hartley WJ, Lindsay DS. Congenital *Neospora caninum* infection in a calf with spinal cord anomaly. Journal of the American Veterinary Medical Association 197, 1043–4, 1990.

(6) Thornton RN, Gajadhar A, Evans J. *Neospora* abortion epidemic in a dairy herd. New Zealand Veterinary Journal 42, 190–1, 1994.

(7) Cox BT, Reichel MP, Griffiths LM. Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: A case study. New Zealand Veterinary Journal 46, 28–31, 1998.

(8) Barber JS, Gasser RB, Ellis J, Reichel MP, McMillan D, Trees AJ. Prevalence of serum antibodies to *Neospora caninum* in different canid populations. Journal of Parasitology 83, 1056–81, 1997.

(9) Trees AJ, Guy F, Tennant BJ, Balfour AH, Dubey JP. Prevalence of antibodies to *Neospora caninum* in a populaton of urban dogs in England. Veterinary Record 132, 125–6, 1993.

(10) Rasmussen K, Jensen AL. Some epidemiologic features of canine neosporosis in Denmark. Veterinary Parasitology 62, 345–9, 1996.

(11) Lindsay DS, Dubey JP, Upton SJ, Ridley RK. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. Journal of the Helminthological Society of Washington 57, 86–8, 1990.

(12) Björkman C, Lunden A, Uggla A. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in Swedish dogs. Acta Veterinaria Scandinavica 35, 445–7, 1994.

(13) Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Canadian Journal of Veterinary Research 60, 133–9, 1996.

(14) Barber JS, van Ham L, Polis I, Trees AJ. Seroprevalence of antibodies to *Neospora caninum* in Belgian dogs. Journal of Small Animal Practice 38, 15–6, 1997.

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Prevalence of *Neospora* antibodies in beef cattle in New Zealand

New Zealand Veterinary Journal **48**, 149-150

Short Communication

Prevalence of *Neospora* antibodies in beef cattle in New Zealand

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Abstract

Aim: To estimate the prevalence of *Neospora* infection in a sample of New Zealand beef cattle.
Methods: The prevalence of *Neospora caninum* infection in New Zealand beef cattle was estimated by collecting blood at slaughter from 499 beef cattle from 40 different farms at 2 slaughter plants in the North Island and 1 in the lower South Island. Sera were tested using an ELISA against *Neospora* tachyzoite antigen.
Results: The prevalence of seropositive cattle was 2.5% (n=120), 3.6% (n=166) and 2.3% (n=213) at the plants surveyed, the overall prevalence being 2.8%. The serologically positive cattle came from 9 farms, 3 of which had more than 1 positive animal. The highest prevalence recorded amongst animals from 1 farm was 4/13 (31%), in a group of young steers.
Conclusion: Neosporosis appears to be present at a lower level in the New Zealand beef cattle population than in the New Zealand dairy cattle population. Nevertheless, from the high seroprevalence evident amongst young cattle on 1 farm, we suggest that *Neospora* may be a cause of infertility in beef cattle in this country.
Keywords: *Neospora*, epidemiology, serology, ELISA, beef cattle.

(New Zealand Veterinary Journal 48, 149-50, 2000)

Introduction

Neospora caninum is a protozoan parasite that has only relatively recently been identified as a cause of animal disease; it was recognised as a cause of disease in dogs in 1984 (Bjerkås *et al*., 1984) and bovine abortion in 1988 (Dubey *et al*., 1989). Re-examination of archived material from overseas, however, has identified *Neospora* in canine tissue dating from 1957 (Dubey and Lindsay, 1996) and in bovine tissue from 1974 (Dubey *et al.*, 1990), indicating that it is not a new disease. Retrospective studies of dogs in New Zealand have shown that it has been present since at least 1972 (Patitucci *et al.*, 1997). In New Zealand, *N. caninum* has been identified as the cause of at least 38% of diagnosed bovine abortions (Thornton, 1992). Neosporosis may present as only one or a few cows in a herd aborting, ranging up to abortion storms in which as many as 30% of cows in a herd may abort (Thornton *et al.*, 1991). Seroprevalence in non-aborting dairy cows was estimated to be 6.75% in 1985 and 8.5% in 1995 (Reichel, 1998), indicating that it is a relatively common infection and has a stable prevalence in the New Zealand dairy cattle population.

Neospora caninum is documented as a cause of abortion in beef cattle overseas (Boulton *et al.*, 1995, Dubey *et al.*, 1992, Hoar *et al.*, 1996, Venturini *et al.*, 1995), but survey work in this country to date has been principally carried out on dairy cattle because they predominate in abortion submissions to animal health laboratories. Consequently, there is no specific information on the prevalence of *Neospora* infection or abortion in New Zealand beef cattle, and there are no documented cases of *Neospora*-induced abortion storms in beef herds in this country. The primary aim of this study was to estimate the seroprevalence of *Neospora* infection in a small sample of New Zealand beef cattle.

Materials and Methods

A total of 499 blood samples were collected from beef cattle at the time of slaughter from 3 beef slaughter plants. Two were situated in the North Island and processed cattle from throughout the North Island, and the other was in Invercargill, in the lower South Island, which processed cattle from Southland and Otago. Lines containing at least 5 animals were selected for sampling, and 5-20 individual samples per farm were taken. The sex of individuals, and their approximate age determined from the pattern of eruption of permanent incisor teeth, were recorded. Samples were collected in April and December 1998. Serum was analysed for anti-*Neospora* antibodies using an indirect ELISA as previously described (Reichel and Drake, 1996). A corrected absorbance value of 0.15 or greater indicated a positive result, from which infection with *Neospora* was inferred.

Results

The majority of anti-*Neospora* antibody titres were low; only 14 animals returned a positive result. The prevalence of seropositive results at the 2 North Island plants was 2.5% (3/120) and 3.6% (6/166), and in Invercargill was 2.3% (5/213); the estimated overall prevalence of *Neospora* infection in beef cattle in this study was 2.8% (14/499). The serologically positive cattle came from 9 farms. Of these, 3 had more than 1 animal that was serologically

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positive, including 1 farm from which 4/13 (31%) young steers sampled were positive. The animals from this particular farm were identified on the plant's records, compiled by plant staff at the time of slaughter, as Simmental, Charolais, or Angus-cross. Consequently, it was considered unlikely that this high prevalence was influenced by congenital transfer from dairy cows.

Discussion

Neosporosis is an important cause of abortion and reproductive inefficiency in dairy cattle in New Zealand, but little is known about its potential significance in beef cattle. Identification of beef cattle seropositive for *Neospora,* and the high seroprevalence evident amongst young steers on 1 farm in this study, raises the possibility that this organism may be playing some role in beef cattle infertility in this country.

It has been shown that the seroprevalence of *Neospora* infection in dairy cattle in New Zealand is between 6.75% and 8.50% (Reichel, 1998), using the same assay that was used in the present study. However, the dairy cattle tested by Reichel (1988) were all mature lactating cows, whereas a large proportion of the beef cattle sampled in the present study were younger prime steers, 1.5 to 2 years of age. Comparison between these 2 groups is, therefore, difficult. Nevertheless, the low seroprevalence (2.8%) recorded for beef cattle in the present study might reflect a difference in the epidemiology of neosporosis between dairy and beef cattle. One problem with serology for this infection is that titres may decline from high levels to around threshold levels over 1 to 2 months (Cox *et al.*, 1998), which may result in a serological survey underestimating the true prevalence of infection.

Dogs have recently been shown to be definitive hosts for *Neospora caninum* (McAllister *et al.*, 1998) and cattle may presumably become infected by ingesting oocysts shed by infected dogs. The only other known route of infection is via congenital transmission, by which as many as 80-90% of calves from infected cows become infected (Paré *et al.*, 1996). There is no reason to suppose that there is a difference in the rate of congenital infection between dairy and beef cattle, so any difference in the prevalence of infection between breeds would presumably be due to differences in the rate of infection by other means, such as ingestion of oocysts.

The lower prevalence of seropositive results evident in beef cattle in this survey, compared to dairy cattle in the study of Reichel (1988), may reflect differences in management between dairy and beef farms; possible reasons for lower exposure to infection on beef farms remain to be determined. One hypothesis is that the more regular stock movement on dairy farms may result in dogs more frequently moving over pastures on dairy farms than on beef farms. This could increase the chance of infected dogs defecating on pasture, and more regular cattle movement over dog faeces could result in more widespread contamination of dairy farms. Nevertheless, dogs are kept on the majority of beef farms in New Zealand, and the reasons why *Neospora* has not yet been identified as a cause of abortion and that no *Neospora*-induced abortion storms have been reported in beef cattle in this country, remain an enigma.

Because of the limited number of positive results in this study, it is not possible to draw any conclusions about the epidemiology of *Neospora* in beef cattle from these results. There were too few positive results to measure differences in age-specific prevalence, which can indicate whether vertical transmission is the predominant means of infection or whether there is a significant component of horizontal transmission. However, it is clear that *N. caninum* infection is not limited to the dairy industry in New Zealand. Causes of reproductive failure are often difficult to identify in beef cattle because of the extensive nature of most beef farming systems. We suggest from the results of this preliminary study that *Neospora* may be less of a problem in the beef industry than it is in the dairy industry, but even so, it may still be an economically important disease. Further studies are required to determine whether *Neospora* is a significant cause of abortion and reproductive failure in the beef cattle population in New Zealand, and a more formally structured comparison of beef and dairy cattle would be useful to confirm these preliminary findings and to identify differences in risk factors for neosporosis between dairy and beef cattle.

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References

Bjerkås I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Zeitschrift für Parasitenkunde 70, 271-4, 1984.

Boulton JG, Gill PA, Cook RW, Fraser GC, Harper PAW, Dubey JP. Bovine *Neospora* abortion in north-eastern New South Wales. Australian Veterinary Journal 72, 119-20, 1995.

Cox BT, Reichel MP, Griffiths LM. Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study. New Zealand Veterinary Journal 46, 28-31, 1998.

Dubey JP, Hartley WJ, Lindsay DS. Congenital *Neospora caninum* infection in a calf with a spinal cord anomaly. Journal of the American Veterinary Medical Association 197, 1043-4, 1990.

Dubey JP, Janovitz EB, Skowronek AJ. Clinical neosporosis in a 4-week-old Hereford calf. Veterinary Parasitology 43, 137-41, 1992.

Dubey JP, Leathers CW, Lindsay DS. *Neospora caninum*-like protozoon associated with fatal myelitis in newborn calves. Journal of Parasitology 75, 146-8, 1989.

Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Veterinary Parasitology 67, 1-59, 1996.

Hoar BR, Ribble CS, Spitzer CC, Spitzer PG, Janzen ED. Investigation of pregnancy losses in beef cattle herds associated with *Neospora* sp. infection. Canadian Veterinary Journal 37, 364-6, 1996.

McAllister MM, Dubey JP, Lindsay DS, Jolley WR, Wills RA, McGuire AM. Dogs are definitive hosts of *Neospora caninum*. International Journal for Parasitology 28, 1474-8, 1998.

Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Canadian Veterinary Journal 60, 133-9, 1996.

Patitucci AN, Alley MR, Jones BR, Charleston WAG. Protozoal encephalomyelitis of dogs involving *Neospora caninum* and *Toxoplasma gondii* in New Zealand. New Zealand Veterinary Journal 45, 231-5, 1997.

Reichel MP. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. New Zealand Veterinary Journal 46, 38, 1998.

Reichel MP, Drake JM. The diagnosis of *Neospora* abortions in cattle. New Zealand Veterinary Journal 44, 151-4, 1996.

Thornton R. Bovine abortions - laboratory diagnosis 1991. Surveillance 19(4), 24, 1992.

Thornton RN, Thompson EJ, Dubey JP. *Neospora* abortion in New Zealand cattle. New Zealand Veterinary Journal 39, 129-33, 1991.

Venturini L, Lorenzo CD, Venturini C, Romero J, Di Lorenzo C. Antibodies to *Neospora* sp. in aborted cows. Veterinaria Argentina 12, 167-70, 1995.

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1.8.2. Discussion and Conclusions

This sero-prevalence work produced useful “baseline” data for these host populations, and demonstrated differences between dairy and beef cattle populations.

The dog sero-prevalence reported in my own study was in line with reported *N caninum* sero-prevalence in Australia, Belgium, Tanzania, the United Kingdom and Uruguay, but higher than in the Falkland Islands, Kenya and Sweden and the US (Lindsay *et al.* 1990, Trees *et al.* 1993, Björkman *et al.* 1994, Barber *et al.* 1997a, Barber *et al.* 1997b).

The sero-prevalence in the New Zealand dairy cattle population was about 3 times higher than in the beef cattle population tested, suggesting that the former population is at greater risk of *N caninum* abortion than the latter. This would be in line with anecdotal reports, suggesting that *N caninum* abortion epidemics are rarely reported from beef cattle, although they have been reported in almost equal numbers from beef and dairy cattle in Australia (Boulton *et al.* 1995). Overseas sero-prevalence data in cattle show a very large range, from nationally 2% in Sweden (Björkman *et al.* 2000) to 7% to 45% herd sero-prevalence in the UK (Davison *et al.* 1999a) to nationally 11.5% in Switzerland (Gottstein *et al.* 1998). In aborted herds in Australasia, the within herd sero-prevalence reported tends to be around 30-35% (Thornton *et al.* 1994, Patitucci *et al.* 1999, Atkinson *et al.* 2000b).

1.8.3. Case studies and Transmission patterns

At the time of the investigation there was only scant knowledge (from few animals and experimental infections only) of the kinetics of serological responses to *N caninum* infection (Conrad *et al.* 1993). Field investigations were needed to obtain data from a larger number of animals that had been infected naturally. The first case study (Cox *et al.* 1998) defined the duration of peak titres in one serological test, the IFAT, established the within-herd prevalence and made an assessment of the usefulness of serological testing for the diagnosis of *Neospora* abortions in the field. Sero-epidemiological approaches to *Neospora* abortion diagnosis were demonstrated to offer advantages over the traditional, individual abortion case diagnosis.

Some overseas data indicated problems with the use of serological assays in the diagnosis of *N caninum* infection/abortions (Dubey *et al.* 1997). Overseas work also indicated that vertical transmission was responsible for the majority of new infections in cattle (Björkman *et al.* 1996, Paré *et al.* 1996, Schares *et al.* 1998), but no information existed in New Zealand about the mode of transmission. In a case study (Schares *et al.* 1999) involving a dairy herd of several hundred cows, which had experienced an abortion outbreak, the two serological tests established were compared with a series of serological diagnostic tests (two ELISA and Western Blot). All tests were evaluated for their utility in diagnosing *Neospora* abortion outbreaks. As in the first case study (Cox *et al.* 1998), sero-epidemiological approaches to diagnosis proved more useful than individual case diagnosis.

In accordance with studies carried out overseas, this study also analysed the relative importance of vertical versus horizontal transmission of the parasite. The evidence

suggested that horizontal transmission patterns exist (and predominate) in the New Zealand dairy situation, and that vertical transmission patterns only play a minor role.

Cox, BT, **Reichel, MP** and Griffiths, LM. (1998)

Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: A case study

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Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: A case study

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Abstract

Aim. To describe the kinetics of serological titres after an abortion outbreak in April-May 1995 due to *Neospora caninum* affected 17 dairy cows in a herd of 320.

Methods. Thirty-five cows, that had either aborted, carried mummified calves, were not pregnant or calved normally were bled several times at regular intervals and the sera tested for *Neospora* antibodies in the indirect fluorescence antibody test (IFAT).

Results. Maximal IFAT titres of up to 1:4000 occurred within 6 weeks of the abortion outbreak, decreased over the next 2 months to ≤ 1:200 and remained at this level until the next scheduled bleed a further 2 months later. A rise in titres was subsequently observed in the cows that had aborted or were not pregnant (at the time of the abortions) or had carried mummified foetuses. Seroconversion was also observed in some of the control cows, which had, up until then, remained seronegative. A dog and cat in contact with the cows in the herd investigated were, however, negative in the IFAT.

Conclusions. Maximal serological titres in *Neospora* abortions are observed within weeks of the abortion event and then quickly return to very low levels. Subsequently, a recrudescence of titres can be observed in infected cows during the next pregnancy, without it being associated with repeat abortions. (New Zealand Veterinary Journal 46, 28–31, 1998)

Introduction

Neospora caninum is recognised as a major cause of abortions in cattle worldwide[1]. In New Zealand it has been identified as a cause of close to 40% of bovine abortions in which a diagnosis could be reached[2]. The life cycle of *N. caninum* is poorly understood and the definitive host unknown. The only proven route of transmission is by congenitally acquired infection[3][4][5]. Diagnosis is based on the finding of characteristic lesions in tissues of aborted foetuses, immunohistochemistry and serological testing of the dam and foetus. Recent serological studies have suggested that indirect fluorescent antibody test (IFAT) titres of ≥ 1:200 are specific for *N. caninum* infection[6] but various (higher) titres have been suggested for the diagnosis of abortions[7][8]. There are indications that titres may decay quickly after the abortion event[9], thus the choice of cutoff titre and time of testing appears to be critical for an accurate diagnosis.

The incidence of repeat abortions due to neosporosis under natural conditions is uncertain. In one study, four of 41 cows had a repeat *Neospora* abortion[10]. In contrast, Thornton *et al.*[11] reported few repeat abortions in New Zealand from an analysis of dairy farmer questionnaires, but it was unclear how many aborted cows were rebred. The most common opinion is that repeat abortion is not the rule[1]. Congenital infection of the calf, however, may occur frequently[4].

In this study, the serological response, dynamics of anti-*Neospora* titres and calving performance of cows that had either aborted or calved normally in a herd in which a *Neospora* abortion episode occurred was studied over a 1-year period.

The Abortion Outbreak

An abortion outbreak started in mid-April 1995 on a high-producing 320 cow South Island dairy farm, running mostly Holstein Friesians. Foetal tissue samples from the first abortion were submitted for histological examination to the Lincoln Animal Health Laboratory and lesions suggestive of protozoal abortion were found. There were focal areas of non-suppurative necrosis and gliosis of the brain (Figure 1). There were scattered foci of non-suppurative interstitial myocarditis in the heart as well as infiltrates of mononuclear cells in the endo- and epicardium (Figure 2). Although no protozoan organisms were detected, such lesions were typical of those previously seen in *Neospora* abortion cases. Serological testing for anti-*Neospora* antibodies, using an indirect fluorescent antibody test (IFAT) (Veterinary Medical Research and Development (VMRD) Inc., Pullman, WA, USA), was negative in samples taken from the aborting dam on the day of the abortion.

A further three cows aborted a week later and foetal tissues were examined bacteriologically and histologically. Typical lesions were found once again in the myocardia of the three calves, even when tissues of two calves were too autolysed to allow the brain to be examined. *Pasteurella haemolytica* and *Escherichia coli* were isolated, but the histological changes raised the suspicion of *Neospora caninum* being the cause of the abortions. Titres in the IFAT were 1:200 for one dam and 1:1000 for the other two dams. Abortions continued on the property for a period of 3 weeks into early May 1995, by which time 17 out of the 320 cows had aborted, all at about the same stage of gestation (5–6 months).

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A total of 35 cows were part of the study: ten serologically negative animals, introduced from another property, eleven cows that had aborted, five which had mummified calves, three that were found not to be in-calf and six cows that calved normally. These were sequentially bled in June, August and October 1995, and January, February and April 1996. In February 1996, a random sample of 62 further adult cattle from the farm were bled and tested using the IFAT.

A dog and cat on the farm were bled by venepuncture, and the sera tested in the IFAT for anti-*Neospora* antibodies.

Odds ratios and confidence intervals were calculated using EpiInfo 6.03 (WHO, Geneva).

Results

In June 1995, nine of the 11 aborted cows had IFAT titres ranging from 1:200 (three cows) to 1:4000 (one cow) and two were negative. Two of the six pregnant cows which had carried to full-term had titres in the IFAT of 1:1000 and 1:4000. All the cows with mummified calves had titres ranging from 1:200 to 1:1000 and two of the three empty cows had an IFAT titre of 1:200 (Table I).

By August 1995, the highest IFAT titre in all cows was 1:200, and one cow that had aborted and one cow with a mummified foetus had become serologically negative. Indirect fluorescent antibody test titres did not change much by October, with 1:200 remaining the highest titre. In January 1996, titres had increased to 1:1000 in three cows which had been seropositive since June 1995. Two of them remained at this titre in February and one cow seroconverted to negative. Six of the ten control animals seroconverted to positive in January, with

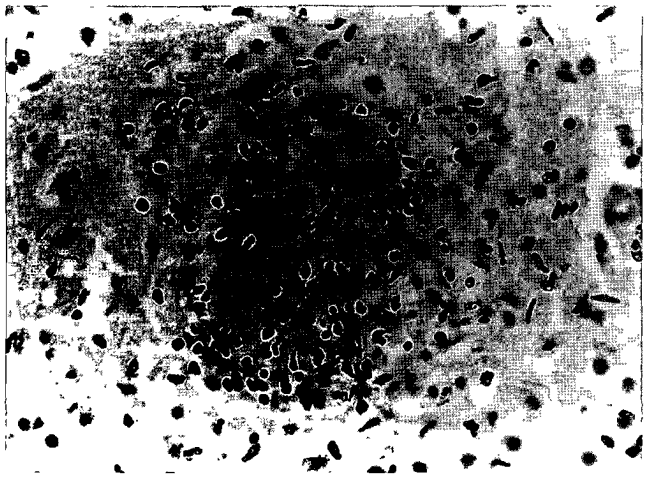

Figure 1. Section of brain showing a focus of necrosis surrounded by mononuclear inflammatory cells and glial cells (H&E, × 800).

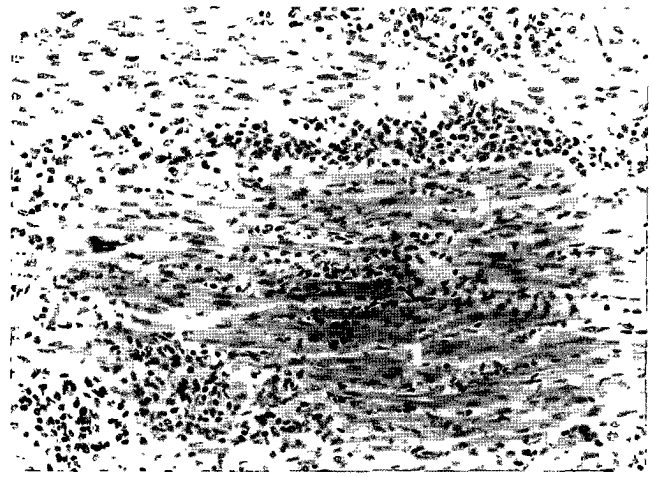

Figure 2. Section of myocardium showing large interstitial infiltrates of mononuclear inflammatory cells (H&E, × 400).

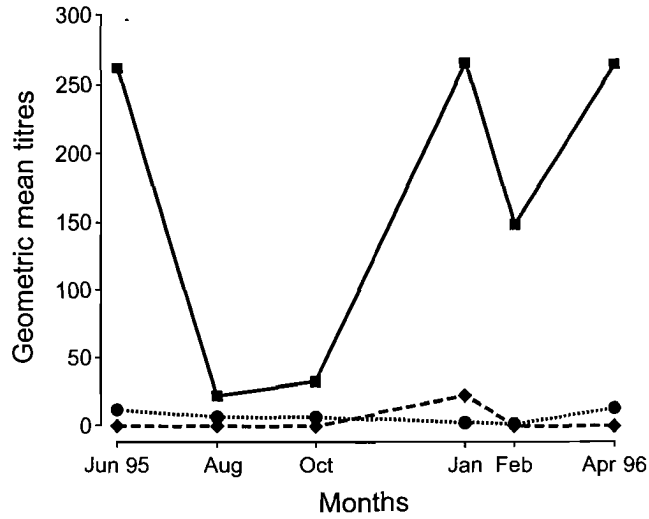

Figure 3. Changes in mean geometric anti-*Neospora* IFAT titres of three groups of cattle in a dairy herd from June 1995 to April 1996 (—■— aborted group, ···●··· in-calf group, – –▲– – control group).

Table I. Number of reactors with anti-*Neospora* antibodies / total number of cows tested in each of three groups of dairy cattle (range of IFAT titres in brackets) on a dairy farm which experienced a *Neospora* abortion outbreak, and odds ratios (OR) (95% CI in brackets) for the aborted/mummified/empty group versus the non-aborted group

Date of bleed	Aborted/Mummified/Empty	Non-aborted	OR	Controls
June 95	17/19 (200-4000)	2/6 (4000)	17 (1.22; 268.77)	0/10
August 95	6/10 (200)	2/6 (200)	3 (0.25; 45.77)	0/7
October 95	6/9 (200)	2/5 (200)	3 (0.2; 52.02)	0/10
January 96	11/11 (200-1000)	1/5 (1000)	NC[a]	6/10 (200)
February 96	10/12 (200-2000)	1/5 (200)	20 (0.96; 1079.14)	1/9 (200)
April 96	8/8 (200)	2/4 (200)	NC	1/10 (200)

a NC = not calculated.

an IFAT titre of 1:200, but five of them reverted to negative at the next testing in February. One control animal remained, however, at an IFAT titre of 1:200 over the next three months. In February, another cow, which previously had a mummified calf (titre 1:1000 in June 1995), had a resurgence of the IFAT titre. After several months at a titre of mainly 1:200, the titre increased to 1:2000, the highest titre observed in all cows bled during that month. In April the titre in only one cow exceeded 1:200. Mean geometric IFAT titres for the aborted group were above 1:200 in June 1995, and again in January and April 1996 (Figure 3). Odds ratios for an association between *Neospora* positivity and the abortions were calculated to be the highest at 17 in June 1995 and 20 in February 1996 (Table I).

Of the 62 additional cows bled in February, 15 (24.2%) had titres in the IFAT (range 1:200–1:1000), resulting in an overall seroprevalence for the herd at that time of 30.7% (95% CI ± 9.6%).

None of the cows in this follow-up study aborted, although two further *Neospora* (histologically and serologically confirmed) abortions occured on the property. One of these cows had been included in the group of 62 cows bled in February (IFAT titre 1:200) and aborted in late April 1996 with an IFAT titre of 1:4000. In addition to the cows, a cat and a dog from the property were also bled, and tested for *Neospora* antibodies in the IFAT, both with negative results.

Discussion

The abortions observed on this dairy farm in April-May 1995 followed the usual pattern for *Neospora*-induced abortions in this country[12]. The serological follow-up study reported here suggests that IFAT titres decline to 1:200 soon after a cow has aborted, in this case in less than 3 months. A decline in titre to similar levels has been previously reported in experimentally infected cows[9]. Cows that aborted, as well as cows which carried to full-term, had titres which are usually thought to be specific for *Neospora*-induced abortions[7][8][13], thus making a causal diagnosis based on individual sera difficult. A *Neospora* outbreak on a dairy farm in California[14] showed a similar pattern of serological reactors in both aborting and non-aborting cows, with only the frequency distribution of titres distinguishing the aborting cows from those that did not abort. A seroepidemiological approach has been suggested by Thurmond and Hietala[15] for the diagnosis of *Neospora* abortions. In the case study presented here, an odds ratio of 17 after the abortion outbreak (at the June bleed), suggests that there was an association between the *Neospora* serological status and abortions.

The estimate of the within-herd seroprevalence is in line with previous estimates for dairy herds experiencing an "abortion storm" in New Zealand[12] and considerably higher than the national prevalence[16]. This suggests that *Neospora* may only be found in some herds and that the majority of dairy herds may be totally uninfected.

A stimulation of an anti-*Neospora* immune response appeared to have occurred in the cows in this study between October 95 and January 96, which suggests that some exposure to *Neospora*-antigen occurred during that time. In some animals which had either aborted or been found not to be in-calf, this led to a resurgence of IFAT titres, with similar mean titres for this group as shortly after the abortions. This exposure to (or reactivation of) a *Neospora* infection, however, did not result in repeat abortions in the cows that were part of the study. It could be speculated that these dams may have carried a congenitally infected calf to full-term, especially where a rise in titres could be observed. Unfortunately, none of the calves were available for serological evaluation.

Californian data suggest that a majority of infections (> 80%) with *Neospora* may be congenitally acquired[4], resulting in clinically normal calves. Whether some of those in turn abort (and if so, why) is not known. Whether congenital infection alone is sufficient to maintain *Neospora* infection in the cattle population is doubtful, although congenitally acquired *Neospora* infection appears to positively influence calfhood mortality up to 90 days[4]. The offspring of two (of originally 34) *Neospora*-positive cows in a Swedish dairy herd made up 45% of the female herd 14 years later, further indicating that the effects of *Neospora* infection may not all be negative and that *Neospora* infection may confer a selective advantage[5].

The one cow (out of the group of 62) in this study which did abort the year after the initial "abortion storm" had an IFAT titre of 1:200 in February 1996 (suggesting it was infected prior to that date) and aborted in April with a titre of 1:4000. In experimental infections, *Neospora*-infected dead foetuses could be removed from their dams as early as 17 days after infection[17]. Other authors, however, report 74 and 101 days from infection to abortion[18], and the abortion in this cow suggests that this is similar under field infections. Alternatively, the abortion in this cow may have been the result of a reactivation of a previously acquired or congenital infection with *Neospora*. Tissue cysts of the closely related organism *Toxoplasma gondii* may persist for several years[19] so this may also occur with *Neospora*. One can only speculate as to the reasons for the reactivation of the *Neospora* infection, but factors such as advancing gestation, stress and BVD virus infection may affect the ability to maintain an effective immune response.

All of the cows with mummified foetuses were seropositive in June 1995, suggesting that *Neospora*-infection may have been the cause of the mummification. Also, two of the three empty cows had low titres, raising the possibility of early reproductive failure being another feature of *Neospora*-infections in dairy cows in New Zealand, as has been reported overseas[20]. Other detrimental effects of *Neospora*-infection which have been noted overseas include premature culling and diminished milk production[21].

The dog and cat on the farm did not have any anti-*Neospora* titres in the IFAT, whereas a considerable number of bloods in a random sample of New Zealand dogs have anti-*Neospora* titres[16]. Overseas work suggests that dogs and cats are not the putative definitive host of *Neospora* in which the sexual cycle is completed[1].

The results of this study and other studies indicate that the serological tests for *Neospora* are most usefully employed as a herd test. Collection of about 20 samples from a herd is recommended, and positive titres of 1:200 or higher provide a definite indication of infection and give an indication of the extent of the infection in the herd. In individual animals, low positive titres of 1:200 indicate infection, but may or may not indicate that an abortion was caused by *Neospora*. Titres at this level are also commonly encountered in animals that do not abort. Titres of 1:1000 or higher at about the time of abortion provide strong evidence that an abortion was caused by *Neospora*. High titres generally decline rapidly after abortion but low positive titres persist for a prolonged time.

Histological findings in foetuses from this and other abortion outbreaks and overseas data[22] suggest that cardiac muscle is frequently the tissue of choice for histopathology, especially when the brain is too autolysed for examination.

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References

(1) Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Veterinary Parasitology 67, 1–59, 1996.
(2) Thornton R. Bovine abortions — Laboratory diagnosis 1991. Surveillance 19, 24, 1992.
(3) Barr BC, Conrad PA, Breitmeyer R, Sverlow K, Anderson ML, Reynolds J, Chauvet AE, Dubey JP, Ardans AA. Congenital *Neospora* infection in calves born from cows that had previously aborted *Neospora*-infected fetuses: four cases (1990-1992). Journal of the American Veterinary Medical Association 202, 113–7, 1993.
(4) Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Canadian Journal of Veterinary Research 60, 133–9, 1996.
(5) Björkman C, Johansson O, Stenlund S, Holmdahl OJM, Uggla A. *Neospora* species infection in a herd of dairy cattle. Journal of the American Veterinary Medical Association 208, 1441, 1996.
(6) Dubey JP, Lindsay DS, Adams DS, Gay JM, Baszler TV, Blagburn BL, Thulliez P. Serologic responses of cattle and other animals infected with *Neospora caninum*. American Journal of Veterinary Research 57, 329–36, 1996.
(7) Dannatt L, Guy F, Trees AJ. Abortion due to *Neospora* species in a dairy herd. Veterinary Record 137, 566–7, 1995.
(8) Trees AJ, Guy F, Low JC, Roberts L, Buxton D, Dubey JP. Serological evidence implicating *Neospora* species as a cause of abortion in British cattle. Veterinary Record 134, 405–7, 1994.
(9) Conrad PA, Sverlow K, Anderson M, Rowe J, BonDurant R, Tuter G, Breitmeyer R, Plamer C, Thurmond M, Ardans A, Dubey JP, Duhamel G, Barr B. Detection of serum antibody response in cattle with natural or experimental *Neospora* infections. Journal of Veterinary Diagnostic Investigation 5, 572–8, 1993.
(10) Anderson ML, Palmer CW, Thurmond MC, Picanso JP, Blanchard PC, Breitmeyer RE, Layton AW, McAllister M, Daft B, Kinde H, Read DH, Dubey JP, Conrad PA, Barr BC. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. Journal of the American Veterinary Medical Association 207, 1206–10, 1995.
(11) Thornton RN, Thompson EJ, Dubey JP. *Neospora* abortion in New Zealand cattle. New Zealand Veterinary Journal 39, 129–33, 1991.
(12) Thornton RN, Gajadhar A, Evans J. *Neospora* abortion epidemic in a dairy herd. New Zealand Veterinary Journal 42, 190–1, 1994.
(13) McNamee PT, Trees AJ, Guy F, Moffett D, Kilpatrick D. Diagnosis and prevalence of neosporosis in cattle in Northern Ireland. Veterinary Record 138, 419–20, 1996.
(14) McAllister MM, Huffman EM, Hietala SK, Conrad PA, Anderson ML, Salman MD. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. Journal of Veterinary Diagnostic Investigation 8, 355–7, 1996.
(15) Thurmond M, Hietala S. Strategies to control neospora infection in cattle. Bovine Practitioner 29, 60-3, 1995.
(16) Reichel MP. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. New Zealand Veterinary Journal 46, 38, 1998.
(17) Barr BC, Rowe JD, Sverlow KW, BonDurant R, Ardans AA, Oliver MN, Conrad PA. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. Journal of Veterinary Diagnostic Investigation 6, 207–15, 1994.
(18) Dubey JP, Lindsay DS, Anderson ML, Davis SW, Shen SK. Induced transplacental transmission of *Neospora caninum* in cattle. Journal of the American Veterinary Medical Association 201, 709–13, 1992.
(19) Dubey JP. Toxoplasma, Neospora, Sarcocystis, and other tissue cyst-forming coccidia of humans and animals. In: Kreier JP (ed). Parasitic Protozoa. Pp 31–6. Academic Press, San Diego, 1993.
(20) Paré J, Thurmond MC, Hietala SK. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. Journal of Parasitology 83, 82–7, 1997.
(21) Thurmond MC, Hietala SK. Culling associated with *Neospora caninum* infection in dairy cows. American Journal of Veterinary Research 57, 1559–62, 1996.
(22) Barr BC, Anderson ML, Blanchard PC, Daft BM, Kinde H, Conrad PA. Bovine fetal encephalitis and myocarditis associated with protozoal infections. Veterinary Pathology 27, 354–61, 1990.

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Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand

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Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand

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Abstract

Serological methods developed to detect *Neospora caninum* specific bovine antibodies were compared using sera from an abortion outbreak on a dairy farm in New Zealand. These methods included four ELISAs, IFAT and immunoblot. The abortions ($n = 16$) on the farm were restricted to one of two groups of the herd which consisted of 194 adult dairy cows. All ELISAs, IFAT and immunoblot (applying stringent cut-off levels) revealed a statistically significant association between seropositivity and abortion among the adult cows from the afflicted group 1. The strength of this association varied between different tests, and different seroprevalences were determined for various animal groups. Among the different ELISAs, the strength of the relationship between single test results was characterised by a considerable variation in the determinants of correlation. Discrepancies in positive–negative classification between the two commercial ELISAs and one of the in-house ELISAs were due mainly to differences in the cut-off selection.

Keywords: Abortion; *Apicomplexa*; Commercial test; Cut-off; ELISA; IFAT; Immunoblot; Neosporosis; *Neospora caninum*; Outbreak

1. Introduction

Neospora caninum is one of the most frequently diagnosed causes of epidemic and endemic bovine abortion [1]. The dog is a definitive host of *N. caninum*, but its importance for the epidemiology of bovine neosporosis is not yet clear [2]. Vertical transmission seems to be of major importance in the spread of *N. caninum*, and most congenital infections result in the birth of apparently healthy calves [3–5].

Neospora caninum infections can be diagnosed using histological and immunohistochemical techniques, such as immunoperoxidase staining (IPX) or by PCR [1, 6]. Recently, a low positive predictive value of foetal histopathology and IPX in diagnosing *N. caninum* abortions has been reported [7]. This underlines the importance of

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approaches to diagnose neosporosis in cattle herds by demonstrating statistical association between seropositivity and abortion [8]. Serological techniques for the specific detection of bovine antibodies to *N. caninum* include IFAT [1, 6], immunoblotting (IB) [4, 9], and several ELISAs [1, 6, 9–12].

The application for which a serological test has been developed strongly influences the cut-off selection: cut-offs meant to detect specific antibodies in aborting or non-aborting *N. caninum*-infected cattle [9] might differ from cut-offs selected to demonstrate association between seropositivity and the abortions [10] or to detect *N. caninum* specific antibodies in foetal fluids [13, 14]. Therefore, the aim of the present study was to compare serological tests with respect to the cut-offs applied. As an example, abortion outbreak sera from a dairy cattle herd from New Zealand were used.

2. Material and methods

2.1. Study population

A dairy herd of 306 milking cows experienced a series of abortions over a period of about 3 weeks during March 1997. The milking cows had been kept in two different lots (groups 1 and 2) for a 3–4 month period prior to the abortions. The abortions ($n = 16$) occurred only in group 1, which consisted of 194 milking cows. In addition to the abortions, four cows were found to be not pregnant after the abortion series, although all cows had been artificially inseminated 4.5–5 months prior to the abortion series, and had been tested pregnant by rectal examination 2 months prior to the abortions. The adult cows were solely pasture fed. No abortions occurred within the group of heifers ($n = 70$). The heifers, all raised from the adult herd, had been grazing away from the property for about 9 months prior to the abortion series. Calves ($n = 69$) raised from the adult herd at least 9 months prior to the abortion series were also grazed separately from the adult milking herd. A dog (male cross-bred border collie, mature age) was kept on the farm. After the abortion series, all animals in the herd and the dog were bled by venipuncture in early April and the sera subjected to testing for *N. caninum* antibodies.

2.2. Serology

2.2.1. ELISAs 1–4

ELISA 1 (IDEXX Laboratories), a test derived from an ELISA originally described by Paré et al. [10], was run according to the manufacturer's instructions, and test results were expressed as S/P ratio based on a positive and a negative control serum. S/P results < 0.50 were classified as negative and ≥ 0.50 as positive.

ELISA 2 (MAST Diagnostics), a test described by Williams et al. [12], was run as recommended by the manufacturer and test results expressed as a percentage of the O.D. readings of a high positive control (percent positivity, PP). A test result > 25 PP was regarded as positive.

ELISA 3 was performed as described by Schares et al. [9]. Test results were expressed as S/P ratio. As cut-off, an S/P of 0.034 was used [9].

ELISA 4 was performed as described by Reichel and Drake [11] using antigen from a commercial supplier (VMRD). Results were expressed as percentage of the O.D. readings of a positive control (percent positivity, PP). Values of ≥ 15 PP were considered positive.

2.2.2. IFAT

Sera were diluted 1:200, 1:600, 1:1000 and 1:2000, and tested on commercially available *N. caninum* (NC1) tachyzoite-coated IFAT slides (VMRD), following the manufacturer's instructions. Only a complete peripheral immunofluorescence of tachyzoites was considered as specific.

2.2.3. Immunoblots

Immunoblots (IBs) were performed as described [4, 9]. The reactivities of the sera with immunodominant tachyzoite antigens of M_r 17, 29, 30, and 37 kDa, as well as the responses to a 33 kDa antigen were recorded [9]. To analyse the IB reactions, the number of immunodominant antigens recognised by a single serum was counted and categorised using five different cut-

off levels (levels 1–5). The cut-offs differed in the minimum number of recognised immunodominant bands (1–5) required to consider a single serum as positive.

2.3. Analysis of data

2.3.1. Differences in seroprevalences between groups

The statistical significance of differences in the seroprevalences was tested using χ^2 (Yates corrected) or Fisher exact tests as appropriate. P values ≤ 0.05 were interpreted as significant.

2.3.2. Association between seropositivity and abortion

The distribution of seropositivity among aborting and non-aborting pregnant cows in group 1 ($n = 190$) was tested using χ^2 (Yates corrected) or Fisher exact tests. Four non-pregnant cows were excluded from the analysis. The strength of statistically significant associations between *N. caninum* seropositivity and abortion was estimated by calculating odds ratios (OR). A P value ≤ 0.05 was considered to indicate a significant association, and OR with a lower limit of the 95% confidence interval (CI) > 1 was interpreted as an indication of significant strength of the association.

2.3.3. Homogeneity of OR

To test the hypothesis of homogeneity of OR, the Breslow–Day test was used. $P < 0.05$ was regarded as an indication of a significant difference between OR.

2.3.4. Regression analysis

ELISA results obtained with the different tests were plotted pair-wise. To analyse whether the results of two ELISAs were correlated, a regression analysis was performed to determine the Pearson correlation coefficient r and the significance of correlation ($P < 0.05$) using a computer program (Statistica 5.0; Stat Soft). The strength of the relationship between results of different ELISAs was expressed as r^2 (coefficient of determination). If r^2 was lower than 0.5, the strength of relationship between the results was considered to be low. A r^2-value between 0.5 and 0.7 indicated moderate and a r^2-value > 0.7 high strength of relationship.

2.3.5. Two-graph receiver operating characteristic (TG-ROC)

Based on the positive–negative classifications of ELISA 1 or 2, the sensitivity and specificity of ELISAs 3 and 4 were described as a function of the cut-off selected in the respective test. The cut-offs d_0 at which ELISA 3 or 4 had equal sensitivity and specificity (θ_0) relative to ELISA 1 or 2 were determined by a TG-ROC analysis using non-parametric methods [15].

3. Results

3.1. Seroprevalence as determined by ELISAs, IFAT and IB

The seroprevalences determined by ELISAs 1–4 differed considerably (Fig. 1A). Using ELISAs 1–3, the seroprevalences in group 1 were significantly higher than in the other groups (group 2, heifers, calves). In contrast, ELISA 4 detected nearly equal seroprevalences in groups 1 and 2, which were significantly higher than those in heifers and calves.

The seroprevalences determined by IFAT (Fig. 1B) and IB (Fig. 1C) depended markedly on the cut-offs used. An IFAT cut-off of 1:200 led to significant differences between group 1 and group 2, or between group 1 and the heifers (Fig. 1B). The differences between calves and the adults in group 1 or group 2 were not significant. Using cut-off titres of 1:600 or 1:1000, the IFAT detected significantly higher seroprevalences in group 1 than in the other groups (group 2, heifers, calves). With a cut-off titre of 1:2000, the seroprevalence in none of the groups differed significantly from 0% (Fig. 1B).

In the IB (Fig. 1C), the seroprevalences among adults from groups 1 and 2 decreased with increasing cut-off (defined by the minimum number of recognised immunodominant antigens). With increasing cut-off, the differences in seroprevalences between group 1 and group 2 increased.

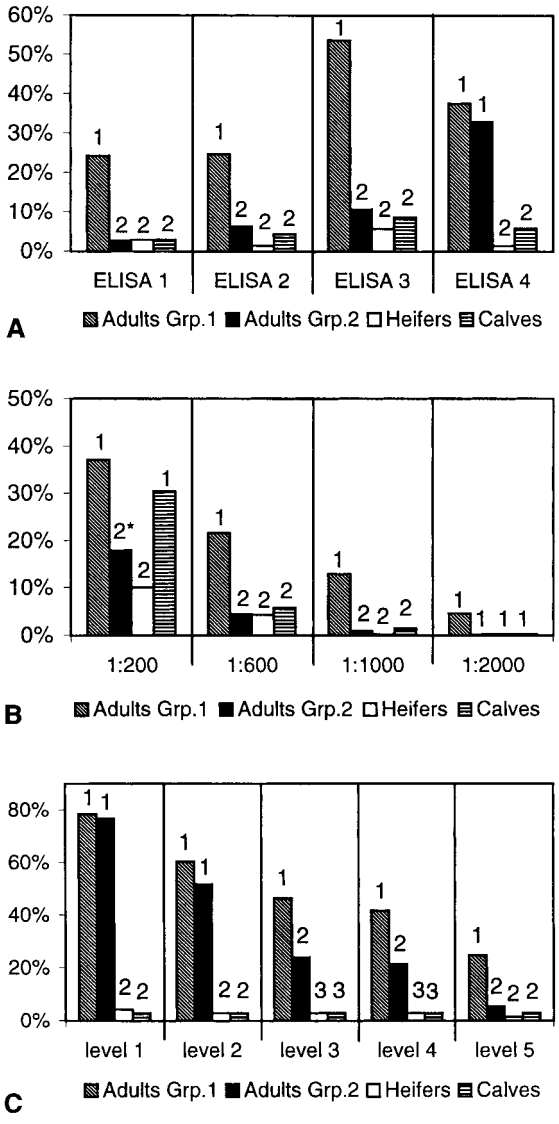

Fig. 1. Prevalence of antibodies against *N. caninum* as determined by four ELISAs (A), IFAT (B) and immunoblot (C) in various animal groups (adults in group 1, adults in group 2, heifers, calves). The IFAT was performed using four different positive cut-off titres: 1:200, 1:600, 1:1000, and 1:2000. Immunoblot results were classified using five different cut-off levels, 1–5 (one to five immunodominant antigens recognised). Significant differences (Yates corrected χ^2, $P \leq 0.05$) between the seroprevalences in various animal groups are indicated by different numbers. (2*) The seroprevalence (IFAT, cut-off 1:200) among the adults in group 2 is not significantly different from that among the calves, but significantly different from the seroprevalence among adults from group 1.

The difference was statistically significant only when cut-off levels ≥3 were applied. The seroprevalences observed in heifers and calves were in no case significantly different from 0%. When the most stringent cut-off level was applied in IB, the prevalence observed in the adults from group 2 was no longer significantly different from those observed in heifers and calves.

A serum of the dog living on the farm prior to and during the abortions was tested negative by IFAT (titre < 1:50).

3.2. Association between abortion and seropositivity, positive and negative predictive values of tests

Among the pregnant cows of group 1, there was a statistical association between seropositivity and the abortions which was detected by all but one of the tests (IB, cut-off level 1). The strength of the association (expressed as OR) varied between the tests (Table 1). Differences between OR determined for the four ELISAs or for different cut-offs in the IFAT and IB were not statistically significant when tested by the Breslow–Day test. Based on the hypothesis that all abortions had been caused by *N. caninum*, negative and positive predictive values (NPV and PPV, respectively) were calculated (Table 1).

3.3. Association between seropositivity of dams and their daughters

With none of the tests could a significant association between seropositivity of dams and their daughters (heifers or calves) be demonstrated (Table 2).

In all but one of the tests the proportions of calves and heifers born by seropositive mothers were low. Using the IFAT with a cut-off of 1:200, 60% of the calves and 23.5% of the heifers born by seropositive mothers were seropositive themselves. However, also considerable proportions of calves (33.3%) and heifers (14.3%) born by seronegative mothers were tested positive. In all other tests, only small proportions of calves and heifers born by seronegative mothers

Table 1
Association between abortion and seropositivity as determined by four ELISAs, IFAT using cut-offs from 1:200 to 1:2000, and immunoblot (IB) with different cut-off levels 1–5 (one to five immunodominant antigens recognised)[a]

Test		OR (95% CI)	*P*	NPV	PPV
ELISA					
	1	69.2 (9.0, 1457)	< 0.0001[b]	0.99	0.33
	2	69.2 (9.0, 1457)	< 0.0001[b]	0.99	0.33
	3	15.0 (2.0, 311)	0.002[c]	0.98	0.15
	4	33.3 (4.4, 694)	< 0.0001[c]	0.99	0.22
IFAT	Cut-off				
	≥1:200	31.6 (4.2, 657)	< 0.0001[b]	0.99	0.21
	≥1:600	15.0 (4.1, 59.9)	< 0.0001[c]	0.97	0.29
	≥1:1000	27.3 (7.3, 109)	< 0.0001[c]	0.97	0.46
	≥1:2000	14.2 (2.6, 80.2)	< 0.0001[c]	0.93	0.50
IB	Cut-off				
	Level 1	NS	0.2009[b]	0.98	0.10
	Level 2	10.9 (1.5, 225)	0.0112[c]	0.99	0.13
	Level 3	20.3 (2.7, 420)	0.0002[c]	0.99	0.17
	Level 4	25.2 (3.4, 522)	0.0004[c]	0.99	0.19
	Level 5	66.6 (8.7, 1400)	< 0.0001[b]	0.99	0.32

[a]The strength of association is expressed as odds ratio (OR) with the 95% confidence intervals (CI). Analysis is restricted to cows from group 1 ($n = 190$). NPV and PPV (negative and positive predictive value) are calculated for each test.
[b] Fisher exact test.
[c] Yates-corrected χ^2.

were seropositive. The only test which detected no seropositive calves or heifers born by seronegative mothers was the IB employing cut-off levels 1–4.

3.4. Correlation between different ELISAs

To determine whether the results of the ELISAs were correlated, regression analyses were conducted using the data from all adult cows (Fig. 2). High coefficients of determination were observed between ELISAs 1 and 3 and between ELISAs 1 and 2. A moderate strength of relationship was observed when ELISA 2 was tested vs ELISA 3. The strength of the relationship was always low when ELISA 4 was tested vs any other ELISA.

When the results of ELISA 1 were plotted against those of ELISA 2, the intersection of lines representing the respective ELISA cut-offs was near to the regression line (Fig. 2A). By contrast, the distances between the respective cut-off line intersections and the regression lines were greater in the scatterplots of ELISA 3 vs ELISA 1 or 2 (Fig. 2B, C), as well as in the scatterplots of ELISA 4 vs ELISA 1–3 (Fig. 2D, E, F).

Table 3
Results of analyses to adapt the cut-offs of in-house tests (ELISAs 3 and 4) to the cut-offs of ELISAs 1 or 2 (commercial tests)[a]

Gold standard	ELISA 3 (0.034 S/P[c])[b]	ELISA 4 (15.0 PP[d])[b]
ELISA 1	$d_0 = 0.19$ S/P[c]	$d_0 = 15.8$ PP[d]
	$\theta = 93.9\%$	$\theta = 79.0\%$
ELISA 2	$d_0 = 0.14$ S/P[c]	$d_0 = 13.7$ PP[d]
	$\theta = 87.6\%$	$\theta = 71.0\%$

[a]ELISA 1 or ELISA 2 were used as gold standards. By two-graph receiver operating characteristic (TG-ROC) analyses the cut-offs (d_0) were determined at which ELISA 3 or 4 had an equal sensitivity and specificity (θ_0) relative to the respective gold standard.
[b]Published cut-off.
[c]S/P = sample to positive ratio (see Material and methods).
[d]PP = percent positivity (see Material and Methods).

Table 2
Proportion of seropositive daughters born by seropositive (M+) or seronegative mothers (M−)[a]

	ELISA				IFAT				IB				
	1	2	3	4	≥1:200	≥1:600	≥1:1000	≥1:2000	Level 1	Level 2	Level 3	Level 4	Level 5
Calves													
M+	0.0 (0/9)	8.3 (1/12)	10.3 (3/29)	10.5 (2/19)	60.0 (9/15)	0.0 (0/5)	0.0 (0/3)	0.0 (0/1)	3.8 (2/53)	5.0 (2/40)	8.0 (2/25)	8.7 (2/23)	9.1 (1/11)
M−	3.7 (2/54)	3.9 (2/51)	8.1 (3/34)	4.5 (2/44)	33.3 (16/48)	6.9 (4/58)	1.7 (1/60)	0.0 (0/62)	0.0 (0/10)	0.0 (0/23)	0.0 (0/38)	0.0 (0/40)	2.0 (1/52)
Heifers													
M+	22.2 (2/9)	8.3 (1/12)	13.0 (3/23)	4.3 (1/23)	23.5 (4/17)	0.0 (0/5)	0.0 (0/1)	0.0 (0/1)	4.7 (2/43)	6.3 (2/32)	9.5 (2/21)	10.0 (2/20)	12.5 (1/8)
M−	2.3 (1/43)	0.0 (0/40)	3.4 (1/29)	0.0 (0/29)	14.3 (5/35)	6.4 (3/47)	0.0 (0/51)	0.0 (0/51)	0.0 (0/9)	0.0 (0/20)	0.0 (0/31)	0.0 (0/32)	0.0 (0/44)

[a]Calves ($n = 63$), heifers ($n = 52$), and their mothers were analysed by four ELISAs, the IFAT using cut-offs from 1:200 to 1:2000, and the immunoblot (IB) with different cut-off levels 1–5 (one to five immunodominant antigens recognised).

3.5. Optimisation of cut-offs used in ELISAs 3 and 4

Two-graph receiver operating characteristic analyses (Table 3) were conducted to optimise cut-offs used in the in-house tests (ELISAs 3 and 4) with respect to the positive–negative classifications of ELISAs 1 and 2. If ELISA 1 was used as gold standard, the optimised cut-off (d_0) for ELISA 3 was reached at a S/P of 0.19. If ELISA 2 was used as gold standard, the optimised cut-off (d_0) for ELISA 3 was reached at a S/P of 0.14. ELISA 4 gave optimum results to ELISA 1 using a cut-off of $d_0 = 15.8$ PP. When ELISA 2 was used as gold standard, ELISA 4 had equal sensitivity and specificity at a cut-off of $d_0 = 13.7$ PP.

4. Discussion

In the present study, various serological tests were compared using sera from an abortion outbreak. Regarding the following aspects, the results of most of tests agreed: (i) Among the adult cows from group 1, all ELISAs, IFAT and IB (applying stringent cut-off levels) revealed a statistical association between seropositivity and the abortions. (ii) Among heifers and calves, all tests except the IFAT (using the lowest cut-off) detected only a low seroprevalence. (iii) The results of none of the tests led to statistically significant association between the serological status of dams and that of their daughters. Together with previous experiences which demonstrated that *N. caninum* is congenitally transmitted rather efficiently [3, 4, 16], these results may indicate that the serological reactions and the abortions observed among the adults from group 1 were due to recent postnatal infection, and that calves and heifers grazing away from the property had not been exposed to the parasite. Using the IFAT, however, no evidence was found that the dog on the farm was infected with *N. caninum* and had been the source of infection for the cattle. However, it is known that not all dogs shedding oocysts develop a detectable antibody response to the parasite [2].

In most of the tests used, the seroprevalences in group 2 were significantly lower than in group 1. However, the seroprevalences in group 2 as recorded by ELISA 4 or IB (cut-off level 1 and 2) might indicate that dams from this group were also infected with *N. caninum*. Given that group 2 was also exposed to *N. caninum*, one or more parameters of this exposure (e.g. infection dose) might have been different as compared with group 1, since abortions occurred only in the latter group of adults. It is also possible that some of the serological reactions observed in group 2

by ELISA 4 or IB (cut-off level 1 and 2) represent false-positive reactions. However, in the analyses to determine associations between the serological status of dams and their daughters, the IB (cut-off levels 1–4) was the only test in which no indications for false-positive reactions were observed (seronegative mothers never had seropositive daughters).

Among the adult cows from group 1, the strength of the association between seropositivity and abortion (expressed as OR) varied between the different tests. Also, the PPV (calculated on the basis of the hypothesis that all abortions were caused by *N. caninum*) varied, while the NPV was rather stable. In the IFAT and the IB, the PPV generally increased with increasing cut-off. This is in accord with earlier observations in herds with *N. caninum*-associated abortions where dams that had aborted often showed stronger antibody responses to the parasite than non-aborting dams [9, 17–19]. Therefore, among infected cattle, tests with more stringent cut-offs might better discriminate between aborting and non-aborting dams, as more stringent cut-offs reduce the number of 'false positive' results.

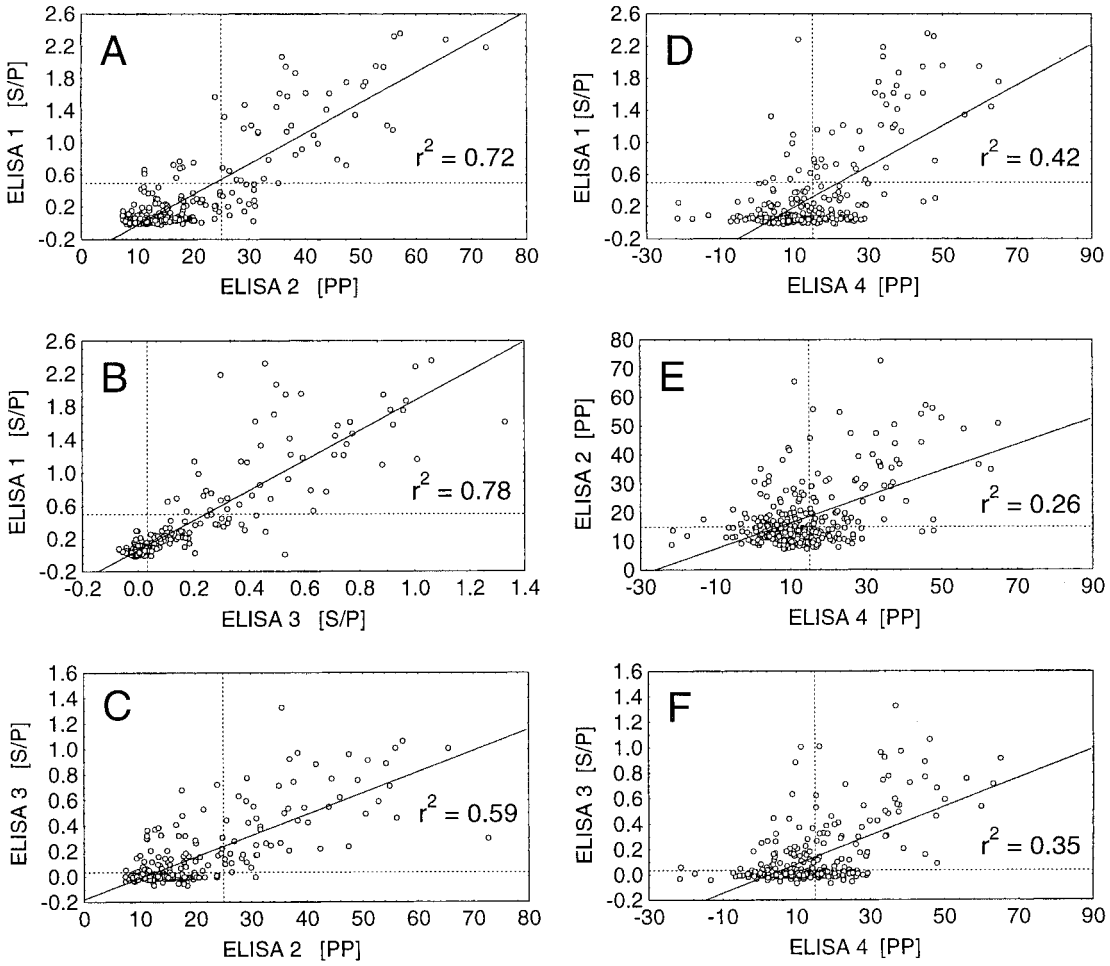

Fig. 2. Results on sera from adults in group 1 and group 2 of ELISAs 1–4 are plotted to each other (A–F). ——— = regression line; - - - = cut-offs of the respective ELISA. The strength of the relationship is expressed as coefficient of determination (r^2).

The cut-off used in the in-house ELISA 3 was selected to maximise sensitivity [9], i.e. it was less stringent. Therefore, it was not unexpected that this cut-off was not ideal (too low) for diagnosing *N. caninum*-associated abortions, as evidenced by the low strength of the association between seropositivity and abortion and by a low PPV. ELISAs 1 and 2 seem to be better optimised for this purpose. Regression analysis demonstrated that the results for single sera of ELISA 3 were highly correlated to those of ELISA 1 and moderately to those of ELISA 2. Therefore, for ELISA 3, differences in the positive–negative classification based on ELISAs 1 or 2 seem to be due mainly to differences in the cut-off selection. This is confirmed by the observation that in the respective scatterplots of the results of ELISA 3 vs the results of ELISAs 1 and 2, there is a marked distance between the intersection of the cut-off lines and the regression line. By contrast, the cut-off line intersection of ELISA 1 vs ELISA 2 is very close to the respective regression line. This might indicate that the cut-offs for ELISAs 1 and 2 have been selected according to similar criteria. This is in contrast to findings of an other working group comparing ELISAs 1 and 2 [20]. However, using a different set of sera or another batch of ELISA might influence the results. Using TG-ROC analyses, cut-offs for ELISA 3 were determined which were optimised to the positive–negative classifications made by ELISA 1 or ELISA 2. As expected, these optimised cut-offs were considerably higher than the cut-off originally used in ELISA 3.

Like ELISA 3, in-house ELISA 4 also had a lower PPV compared with the commercial tests, ELISAs 1 and 2. However, in ELISA 4, differences in the positive–negative classification of sera by ELISAs 1 and 2 do not seem to be due to differences in the cut-off selection. This is demonstrated by TG-ROC analyses (cut-offs determined are close to the original cut-off) and by a generally low strength of relationship between ELISA 4 and the other tests. These differences might be explained by other differences in the ELISA protocols (e.g. the antigen used) [9–12].

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References

[1] Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. Vet Parasitol 1996;67:1–59.
[2] McAllister MM, Dubey JP, Lindsay DS, Jolley WR, Wills RA, McGuire AM. Dogs are definitive hosts of *Neospora caninum*. Int J Parasitol 1998;28:1473–8.
[3] Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Can J Vet Res 1996;60:133–9.
[4] Schares G, Peters M, Wurm R, Bärwald A, Conraths FJ. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet Parasitol 1998;80:87–98.
[5] Björkman C, Johansson O, Stenlund S, Holmdahl OJM, Uggla A. *Neospora* species infection in a herd of dairy cattle. J Am Vet Med Assoc 1996;208:1441–4.
[6] Hemphill A. The host–parasite relationship in neosporosis. Adv Parasitol 1999;43:48–104.
[7] Thurmond MC, Hietala SK, Blanchard PC. Predictive values of fetal histopathology and immunoperoxidase staining in diagnosing bovine abortion caused by *Neospora caninum* in a dairy herd. J Vet Diagn Invest 1999;11:90–4.
[8] Thurmond M, Hietala S. Strategies to control *Neospora* infection in cattle. Bovine Pract. 1995;29:60–3.
[9] Schares G, Rauser M, Zimmer K et al. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. J Parasitol, 1999;85:688–694.
[10] Paré J, Hietala SK, Thurmond MC. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J Vet Diagn Invest 1995;7:352–9.
[11] Reichel MP, Drake JM. The diagnosis of *Neospora* abortions in cattle. New Zealand Vet J 1996;44:151–4.
[12] Williams DJL, McGarry J, Guy F, Barber J, Trees AJ. Novel ELISA for detection of *Neospora*-specific antibodies in cattle. Vet Rec 1997;140:328–31.
[13] Barr BC, Anderson ML, Sverlow KW, Conrad PA. Diagnosis of bovine fetal *Neospora* infection with an

indirect fluorescent antibody test. Vet Rec 1995;137:611–3.

[14] Wouda W, Dubey JP, Jenkins MC. Serological diagnosis of bovine fetal neosporosis. J Parasitol 1997;83:545–7.

[15] Greiner M. Two-graph receiver operating characteristics (TG-ROC): a Microsoft-EXCEL template for the selection of cut-off values in diagnostic tests. J Immunol Methods 1995;185:145–6.

[16] Anderson ML, Reynolds JP, Rowe JD et al. Evidence of vertical transmission of *Neospora* sp. infection in dairy cattle. J Am Vet Med Assoc 1997;210:1169–72.

[17] Waldner CL, Janzen ED, Ribble CS. Determination of the association between *Neospora caninum* infection and reproductive performance in beef herds. J Am Vet Med Assoc 1998;213:685–90.

[18] Dubey JP, Jenkins MC, Adams DS et al. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. J Parasitol 1997;83:1063–9.

[19] McAllister MM, Huffman EM, Hietala SK, Conrad PA, Anderson ML, Salman MD. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. J Vet Diagn Invest 1996;8:355–7.

[20] Wouda W, Brinkhof J, van Maanen C, de Gee ALW, Moen AR. Serodiagnosis of neosporosis in individual cows and dairy herds: a comparative study of three enzyme-linked immunosorbent assays. Clin Diagn Lab Immunol 1998;5:711–6.

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A longitudinal study of *Neospora caninum* infection on a dairy farm in New Zealand

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Abstract

A 600-cow New Zealand dairy herd experienced an abortion storm in 1997 and was monitored (blood sampling at about 3-month intervals) from May 1997 until January 1999. Abortion risk reached 9% in 1997 and was highest in heifers at 19%. The abortion risk decreased in 1998 to 3.2% (still somewhat higher than during the years prior to the outbreak). The serological reaction pattern for *Neospora caninum* showed an association with abortion risk only around the time of the 1997 outbreak when seropositive cows were 4.2 times more likely to abort than negative ones. Over the whole study period, only 27% of cows that were sampled on all nine visits always tested negative. Offspring from dams which had positive tests for *Neospora caninum* were 2.4 times more likely to abort than those from dams testing consistently negative. Controlling for age and breed, seropositive cows produced more milk than those that were consistently negative. Infection might have been present endemically within this herd prior to the epidemic, but in 1997 an additional factor appeared to have triggered the outbreak.

Keywords: *Neospora caninum*; Cattle; Parasitological disease; Abortion; Production; Serology

1. Introduction

Neospora caninum infection is an important cause of abortion in dairy cattle in many countries with intensive dairy production (Dubey, 1999). The epidemiology of the infection is still poorly understood. Transmission occurs vertically from cow to calf

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(Björkman et al., 1996; Paré et al., 1996; Schares et al., 1998) and has been postulated to occur post-natally through indirect contact (Schares et al., 1999). Definitive hosts such as dogs (McAllister et al., 1998) or other unidentified species might cause new outbreaks and an association between the presence of dogs and abortion outbreaks has been demonstrated (Paré et al., 1998; Wouda et al., 1999; Barling et al., 2000). The relative importance of the different transmission paths is not known and is likely to vary between different cattle-management systems as well as habitats.

Over the last 10 years, *N. caninum* abortion outbreaks have been described in New Zealand (Thornton et al., 1991, 1994; Cox et al., 1998; Patitucci et al., 1999) and are now considered a major problem affecting dairy herds.

This paper describes an investigation into the epidemiology of *N. caninum* infection on a dairy farm in New Zealand.

2. Materials and methods

2.1. Herd history

In the first half of 1997, an abortion outbreak occurred in a spring-calving 600-cow crossbred Friesian/Jersey dairy herd in the North Island of New Zealand. The last recorded outbreak in that herd had occurred >10 years previously. At the time of the abortions in 1997, serum antibodies to *N. caninum* were identified in some of the cows involved. Abortions were confirmed by negative pregnancy diagnosis during June and July 1998 in the current study. Histopathologic diagnosis of neosporosis was confirmed in four of six aborted foetuses submitted to the local Ministry of Agriculture diagnostic laboratory. No other bacterial or viral cause of abortion was identified.

Eleven leased cows were introduced to the herd in 1996/1997. Ten of these animals originated from a herd with a history of abortions possibly associated with *N. caninum* infection. No history was available on the herd from which the remaining cow was leased. Yearling Murray Grey bulls were purchased every year from the same breeder.

2.2. Data collection

Blood samples were collected from bulls, steers, calves (<12 months of age), heifers (12 to <24 months of age) and cows ($\geq$1 parity) in May, June, July, and October of 1997, January, May, June, and November of 1998 and in January 1999. These samplings included all animals present in the herd on the respective occasions. An ELISA test developed at Wallaceville Animal Health Laboratory was used to test the samples for the presence of antibodies to *N. caninum* (Reichel and Drake, 1996). ELISA values $\geq$0.15 corrected absorbance at 450 nm were classified as positive. The cut-off for this test was developed using indirect fluorescent antibody test (IFAT) results as a gold standard, yielding relative sensitivity and specificity values of 92% (unpublished data). Blood samples were taken from three dogs on the farm during June 1997 and tested for *N. caninum* antibodies using the IFAT as previously described (Reichel, 1998). A positive result in the IFAT for dogs at a 1:50 serum dilution is regarded as specific (Dubey and Lindsay, 1996).

2.3. Data analysis

Cow histories (age, family relationship, milk-production parameters (milk volume, fat, protein, and solids), time to culling) were obtained from a database generated using the dairy cattle-herd management software DairyWIN (Livestock Improvement, Hamilton, New Zealand). The data were manipulated using the database management software Microsoft Access 2000 (Microsoft, Redmond, WA). The relational database underlying DairyWIN was used to identify dams and their offspring which were included in the samplings, so that it was possible to assess the association between serological status of the dam and seroreactivity or abortion risk of the offspring.

Confidence intervals of proportions were calculated using Wilson's method as described by Newcombe and Altman (2000). Relative risks and their 95% confidence limits were used to quantify the strength of the association between abortion risk, seropositivity and categorical risk factors using the methods described in Morris and Gardner (2000). Significance statistics for chi-squared and Mann-and-Whitney *U*-tests were calculated using the Monte Carlo method available in SPSS for Windows 10.0 (SPSS, Chicago, IL) based on 10 000 samples (Mehta and Patel, 1996). The Kaplan–Meier product-limit method was used to conduct survival analyses of time from birth until culling. The resulting survival curves were compared using the log-rank test. The temporal pattern of serological responses in relation to abortion date was analysed using loess regression (a non-parametric method for estimating locally weighted regression lines). The relationship between serological-test result and milk-production parameters was examined using a linear mixed model for longitudinal data (Verbeke and Molenberghs, 2000). Analyses were conducted separately for milk volume, fat, protein and solids as dependent variables. In these models, seropositivity (positive on at least one test vs. never positive), herd-test number within lactation (ranging between 1 and 4), lactation year (1996/1997, 1997/1998 and 1998/1999), breed category ($\leq$50% vs. >50% Jersey) and age group (2–4 years vs. 5–8 years vs. 9–13 years) were used as fixed effects. Main effects and their first-order interaction terms were included, if the type-3 test of the fixed effects was significant at $P \leq 0.05$. The denominator degrees of freedom for the *F*-tests were estimated using Satterthwaite's approximation to take into account the unbalanced nature of the data. Herd-test number was also used as a repeated-measures effect, for each cow nested within lactation year. The compound-symmetry covariance structure was chosen to model the covariance between observations. Least-squares means (which express the mean estimates of the effect categories adjusted for other effects in the model) are presented. The procedures PROC MIXED and PROC LOESS in SAS for Windows version 8.0 (SAS Institute, Cary, NC) were used for the repeated-measures ANOVA and the loess-regression analysis, respectively. $P \leq 0.05$ was used to indicate statistical significance.

3. Results

3.1. Descriptive analysis

A total of 6341 blood samples from 1037 female and from 35 male cattle were included in this analysis. Over the lactation periods 1996/1997 and 1997/1998, 90 abortions were

recorded in 858 adult cows at risk (1474 cow years), an abortion risk of 6.1% (95% CI: 5.0–7.4%) per cow year. One cow aborted in two successive lactation years.

Two of the three dogs were positive in the IFAT with titres of 1/50. Examination of faeces from one of the positive dogs revealed coccidial oocysts.

Seventy-seven heifers were leased to replace culled cows and 38 aborting heifers were retained after the abortion outbreak in 1997. Of the 10 leased cows coming from a property with abortion history likely to be associated with *N. caninum*, two were seropositive in May 1997 and one subsequently aborted.

3.2. Temporal pattern of abortion occurrence and serological reactivity

The herd experienced an abortion outbreak during the lactation year 1996/1997. Until then, abortion risk had been between 2 and 4% per year. Sixty-seven abortions amongst 745 animals at risk (9% abortion risk; 95% CI: 7.1, 11.3%) associated with the 1996/1997 outbreak began on 17 February 1997 and continued until 18 July 1997. The peak incidence was 28 abortions occurring during the first half of April. In 1998, only 23 abortions amongst 729 animals at risk (3.2% abortion risk; 95% CI: 2.1, 4.7%) occurred in the entire comparable 5-month period.

Calves born in 1996 and heifers born in 1995 showed a high seroprevalence during the May 1997 sampling (Table 1). Seroprevalence declined in both groups during June and July 1997, but increased again in both groups at the October 1997 sampling (which included calves born during this year). In the following year, seroprevalence remained low in calves in contrast to heifers, which experienced another peak in prevalence in June 1998. Cows had seroprevalences around 30% during the May and June samplings in 1997 and 1998.

The distribution of optical density values for each of the sampling months varied widely around the cut-off value of 0.15 (Fig. 1). Variability was reduced during July 1997, November 1998 and January 1999. The pattern of serological reactivity relative to the abortion date of individual heifers and cows is presented in Fig. 2. This scatter plot of

Table 1
Percentage seroprevalence of *N. caninum* in a NZ dairy herd stratified by stock class and sampling month (*n*: number sampled)

Sampling month	Calves (age: <12 months)		Heifers (age: 12 to <24 months)		Cows (≥1 parity)	
	% Positive	*n*	% Positive	*n*	% Positive	*n*
May 1997	53	123	53	19	28	473
June 1997	27	112	42	95	34	445
July 1997	15	111	21	174	11	443
October 1997	36	193	42	162	32	431
January 1998	3	59	nt[a]	nt[a]	48	590
May 1998	8	145	26	99	32	497
June 1998	13	145	49	99	31	509
November 1998	7	139	20	96	9	488
January 1999	4	70	6	50	7	578

[a] Not available for testing.

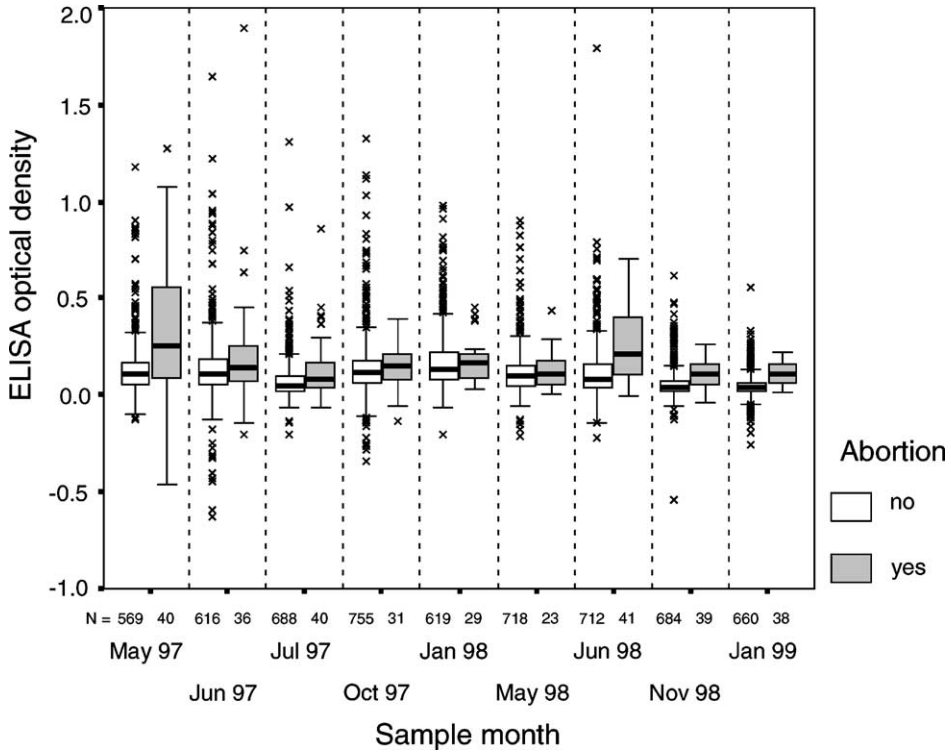

Fig. 1. Box-and-whisker plot for optical density distributions by sample month and abortion status of heifers and cows in a New Zealand dairy herd (1997–1999).

optical density is from sequentially sampled animals. While visual inspection suggests the presence of three peaks occurring at annual intervals before, at and after abortion, the flat loess-regression line indicates that this pattern is not supported by the majority of serological results in aborting heifers and cows. Inspection of the reactivity pattern in individual animals indicated that these apparently repeated annual peaks did not occur in the same animals.

In a comparison of the temporal pattern of serological reactivity between aborting and non-aborting heifers and cows, it appears that those which aborted in one of the two years had higher titres than the ones that did not (particularly during May 1997, June 1998 and January 1999; Fig. 1). During the remaining months, the difference between the two groups of heifers and cows still existed but was much reduced.

Cows and heifers with an ELISA optical density $\geq$0.15 at the May 1997 sampling were four times more likely to abort during 1997 as cows that had a lower optical density (Table 2). During the following sampling months, the risk of a seropositive cow or heifer having aborted or going to abort reduced substantially and none of these relative risks is significant.

The number of serological reactions above the cut-off value increased with the number of repeated samples taken from individual animals (Table 3). However, amongst cows with

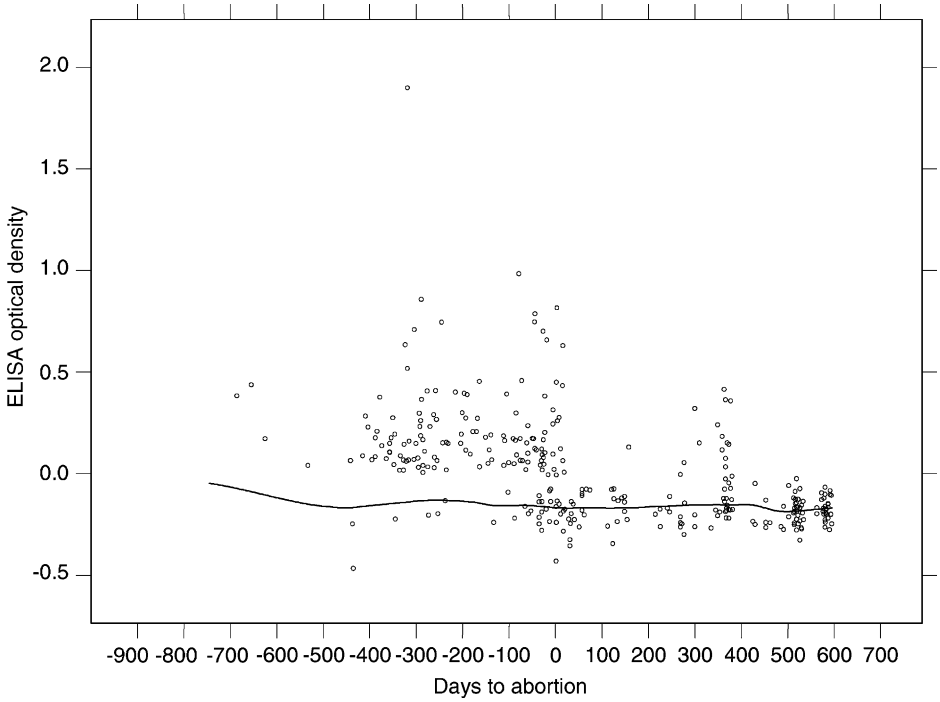

Fig. 2. Scatter plot of seroreactivity relative to abortion date of heifers and cows including loess-smoothed regression line for a New Zealand dairy herd (1997–1999).

between six and nine longitudinal samples, between 18% (eight samples) and 35% (six samples) were consistently negative on all samples. Seroconversion risk between May 1997 and January 1999 in this study was 55% (95% CI: 51, 60) when aggregating 409 animals from all age groups that had tested negative during the May sampling and were re-sampled at least once during that period.

3.3. Abortion risk, serological status, age and culling

The abortion risk was highest in heifers in 1997 at 19% compared with the other ages ($\chi^2 = 45.3$, 8 d.f., Monte Carlo $P < 0.0001$). During the 1998 gestation, there was no increase in abortion risk with age ($\chi^2 = 7.0$, 8 d.f., Monte Carlo $P = 0.52$; Fig. 3). Cows and heifers that aborted during 1997 and 1998 had a median survival time from birth to culling of 1680 days (95% CI: 796, 2564) and non-aborting animals of 3402 days (95% CI: 3195, 3609) (Fig. 4; log-rank test for equality of survival distributions ($\chi^2 = 27.6$, 1 d.f., $P < 0.001$)). Of the cows and heifers which aborted, about 80% were culled within 100 days after the abortion. There was no difference between the survival curves for age at culling between heifers and cows without any positive serological test result and those with at least one (log-rank test: $\chi^2 = 0.14$, 1 d.f., $P = 0.7$).

Table 2
Abortion status of heifers and cows (May 97–January 1998 aborted in 1997, May 1998–January 1999 aborted in 1998) by their serological status for *N. caninum* in a New Zealand dairy herd

Sampling month	ELISA result	Abortion risk		Total	Relative risk	
		%	*N*		RR	95% CI
May 1997	+	10.6	15	141	4.2	1.9, 9.3
	−	2.6	9	351		
June 1997	+	3.2	6	189	1.1	0.4, 3.0
	−	2.8	10	351		
July 1997	+	4.8	4	83	2.0	0.7, 5.9
	−	2.4	13	534		
October 1997	+	2.4	5	205	1.6	0.5, 5.1
	−	1.5	6	388		
January 1998	+	1.8	5	282	0.9	0.3, 3.0
	−	1.9	6	308		
May 1998	+	3.1	5	160	1.8	0.5, 5.7
	−	1.8	6	337		
June 1998	+	1.9	3	156	Not estimated	Not estimated
	−	0	0	353		
November 1998	+	2.3	1	43	5.2	0.5, 55.9
	−	0.4	2	445		
January 1999	+	3.6	1	28	8.1	0.8, 86.7
	−	0.4	2	454		

3.4. Status of dam

Samples from both dam and offspring were available for 571 calves, heifers and cows. The risk of being serologically positive at least once in calves, heifers and cows was not

Table 3
Percent of seropositive samples for *N. caninum* per cow by total number of samples taken per cow (one New Zealand dairy herd, 1997–1999)

Number of samples collected per cow	Positive samples per cow										Number of cows
	0	1	2	3	4	5	6	7	8	9	
1	77	23									134
2	50	33	17								6
3	35	30	22	13							63
4	58	27	7	5	3						108
5	38	20	14	13	11	5					96
6	35	39	11	5	3	3	5				66
7	30	25	15	13	7	5	3	2			121
8	18	21	21	13	11	5	6	2	1		136
9	27	20	14	14	12	5	2	4	2	0	307

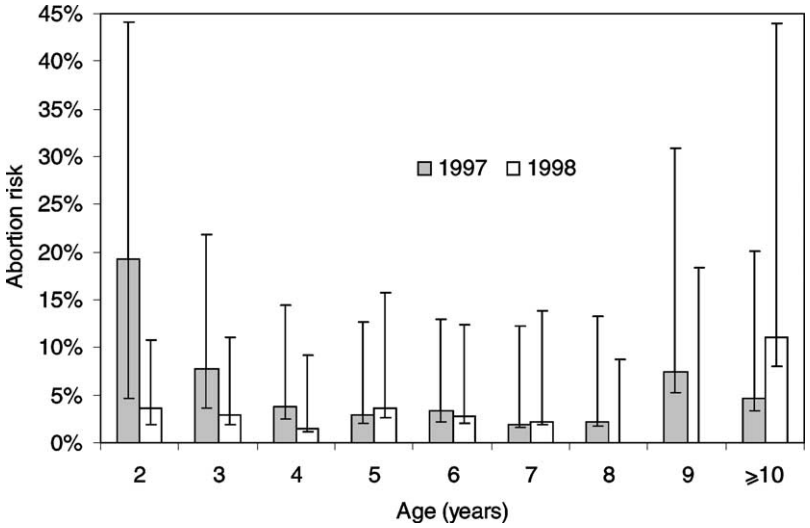

Fig. 3. Bar chart of abortion risk (including 95% confidence intervals) in heifers and cows by age and year of lactation for a New Zealand dairy herd (1997–1999).

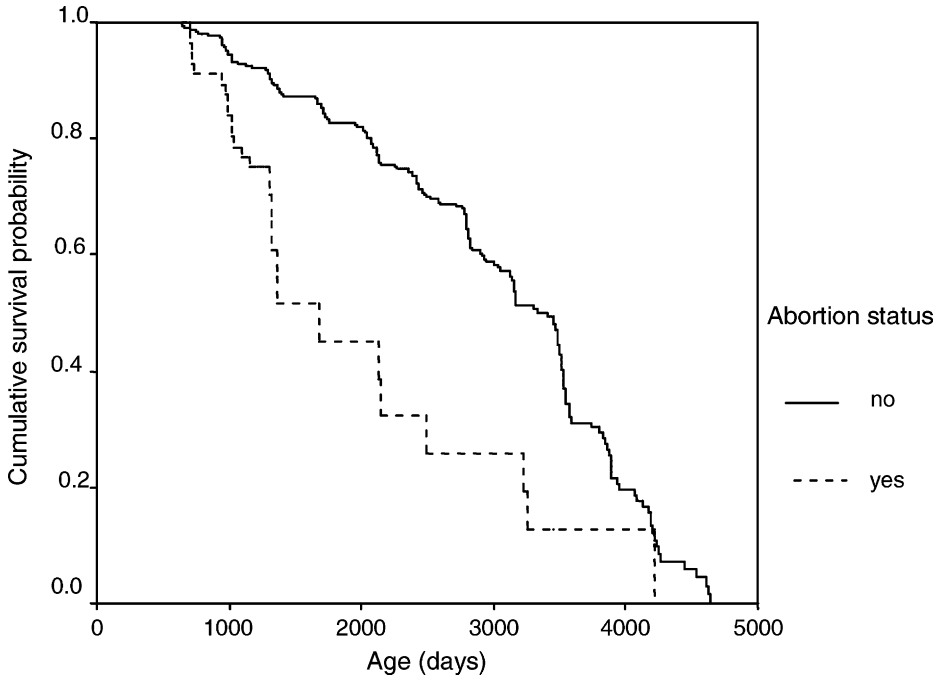

Fig. 4. Kaplan–Meier survival curves of time from birth to culling for aborting and non-aborting heifers and cows from a New Zealand dairy herd (1997–1999).

associated with the serological status of the dam (RR = 1.14, 95% CI: 0.98, 1.3). Offspring from aborting dams were 1.6 times (95% CI: 0.64, 4.04) more likely to abort as offspring from non-aborting dams. However, heifers and cows born from a dam which had ever been positive had 2.4 times the risk (95% CI: 1.2, 5.0) of aborting as those from consistently seronegative dams. Seropositive offspring had dams with a higher number of positive tests than seronegative offspring (Mann–Whitney U: $z = -2.4$, 2-sided Monte Carlo $P = 0.02$). The same comparison between aborting and non-aborting offspring showed a trend towards statistical significance (Mann–Whitney U: $z = -1.9$, 2-sided Monte Carlo $P = 0.07$).

3.5. Milk production

After inclusion of indicator variables for amount of Jersey genetics, age category, herd test number and lactation year, the linear mixed models included significant fixed effects for serological test category as a main effect as well as its interaction with lactation year in the case of the dependent variables milk volume and protein. With milk fat and solids as the dependent variable, the main effect of serological test category was not significant (Table 4).

Table 4
Least-squares means for linear mixed models of milk-production parameters (one for each parameter) including type-3 tests of main effect serological status category and its interaction with lactation year (other fixed effects in the models include breed category, age group and herd test number)

Milk parameter (kg)	Interaction with lactation year	Serological test category				Type-3 tests of fixed effects		
		Positive at least once		Never positive		F	d.f.	P
		Mean	S.D.	Mean	S.D.			
Volume	–	15.2	0.22	14.8	0.12	6.0[a]	1, 1480	0.01
	1996/1997	15.6	0.14	15.5	0.22	2.9[b]	2, 1479	0.06
	1997/1998	15.1	0.13	14.3	0.19			
	1998/1999	14.8	0.13	14.7	0.21			
Fat	–	0.76	0.004	0.75	0.005	0.8[a]	1, 1437	0.38
	1996/1997	0.80	0.006	0.80	0.009	3.9[b]	2, 1437	0.02
	1997/1998	0.76	0.005	0.74	0.008			
	1998/1999	0.72	0.006	0.73	0.009			
Protein	–	0.56	0.003	0.55	0.004	6.2[a]	1, 1456	0.01
	1996/1997	0.59	0.005	0.59	0.007	4.1[b]	2, 1456	0.02
	1997/1998	0.54	0.004	0.51	0.006			
	1998/1999	0.55	0.004	0.55	0.007			
Solids	–	1.32	0.006	1.30	0.009	2.7[a]	1, 1440	0.1
	1996/1997	1.38	0.001	1.38	0.02	4.3[b]	2, 1440	0.01
	1997/1998	1.30	0.009	1.25	0.01			
	1998/1999	1.27	0.01	1.28	0.02			

[a] Test for main effect "serological test result category".
[b] Test for interaction between "serological test category" and "lactation year".

4. Discussion

Identification of the cause of an abortion outbreak can be a difficult task, if aborted foetuses are not available for pathological examination. In the current investigation, serological testing was used as an indicator of infection with *N. caninum*. As with all serological tests currently available for *N. caninum* diagnosis, the ELISA test used in this study has limitations with respect to its performance parameters. It still is a useful tool for an epidemiological investigation into possible causes of an outbreak, because there are no alternatives. But it is important to remember that it will classify 8% of uninfected animals as false-positives, and amongst infected animals it will diagnose 8% as false-negatives if interpreted as single samples. If one then applies aggregated parallel interpretation to multiple test results for the same animal (as was done with some of the following analyses) the number of false-negatives will be reduced, whereas the number of false-positives increases.

A likely causal link between serological status and abortion based on a relative risk of 4.2 could only be inferred from data collected in May 1997, which was when the abortion outbreak occurred. This is consistent with reports from The Netherlands and Switzerland, which report a 3- to 4-fold increase in abortion risk in seropositive cows (Moen et al., 1998; Sager et al., 2001) and a previous serological investigation of a *Neospora* abortion outbreak in NZ using the IFAT which found a clear association between abortion and serological status only immediately following the abortion storm (Cox et al., 1998). Serological reactions at the given cut-off value on any other sampling occasion did not show a significant association, but the relative risk was around or above 2 during many of the sampling months. Hence, a positive serological reaction to this ELISA is a poor predictor of abortion occurrence, except shortly after the abortion event. In the current study, it is also notable that a number of abortions occurred without the cow reacting positively. In general, the serological reactivity patterns in the current study confirm its and others' (Cox et al., 1998; Schares et al., 1999; Anderson et al., 2000) conclusion that serological tests cannot be used to establish that *N. caninum* caused an abortion in an individual animal. But those authors did emphasise test usefulness at the herd level. Therefore, the presence of histopathologic neosporosis lesions in four of six foetuses examined together with the increased risk of seropositivity in aborted cows allows the conclusion that this outbreak was caused by *N. caninum*.

One of the main objectives of an outbreak investigation is to identify potential sources of infection; this might allow prevention of such events in the future. The importance of identification of cause and source cannot be emphasised enough, because such outbreaks are extremely traumatic experiences for farmers. In the current outbreak, hypotheses include that in the presence of endemic *N. caninum* infection within the herd some other factor may have caused clinical expression of the infection (Guy et al., 2001). Others have speculated about possible involvement of immunosuppressive agents, such as infection with bovine pestivirus (Cox et al., 1998). Recently, an association between *N. caninum* infection and bovine viral diarrhoea virus (BVDV) infection was shown in Sweden (Björkman et al., 2000). Also, other workers have shown immunomodulation of the cell-mediated response in mid-gestation, which might be sufficient to permit recrudescence of the parasite, and infection of the foetus (Innes et al., 2001). Alternatively, *N. caninum*

infection could have been introduced recently into this herd by, e.g., the leased heifers (which had high serological reactivity) or another vector. But considering the high prevalence in calves that were unlikely to have been exposed to the leased heifers in May 1997 (because they were kept on another property, as is common farming practice in NZ), it seems unlikely that the heifers were the source of infection. It is possible though that both heifers and calves had been exposed to an external source during early 1997, such as a farm dog or a wild animal shedding oocysts of *N. caninum*. Comparing both scenarios, the recrudescence of endemic infection seems to be the most likely cause of this outbreak.

Instead of interpreting the serological results purely using seropositivity (because that is based on a cut-off value set to produce a compromise between sensitivity and specificity), additional epidemiological information can be gained from examining the serological reactivity patterns. Fig. 2 shows that serological reaction levels in aborting cows did not have a clear pattern, because optical densities varied considerably longitudinally for the same animals as well as at abortion time between animals. This finding suggests considerable variation in individual animals' ability to respond immunologically to infection. Paré et al. (1997) found that in a dairy herd with endemic infection, 87.5% of seropositive cows were seropositive (based on monthly blood samples) throughout their pregnancy. In the current study, animals were sampled at much longer intervals during their pregnancy; therefore, results could not be directly compared with the above finding. Waldner et al. (2001) sero-sampled a Canadian dairy herd four times at about 6-month intervals following an abortion outbreak. They report a decrease in seroprevalence from >80% immediately after the abortion outbreak down to <50% in the following spring. Sager et al. (2001) also reported changes in seropositivity in both directions when sampling 3551 cows twice at 3- to 12-month intervals. However, fluctuating serological titres have been observed in another New Zealand study (Cox et al., 1998) using the IFAT. It also has been reported that IFAT titres are higher than ELISA responses in epidemically affected herds possibly explaining the fluctuating serological responses observed in the current study (Schares et al., 1999).

Paré et al. (1997) reported a seroconversion rate of 8.5/100 cows/year in a herd with endemic infection. On conversion of this estimate into a risk (13% over 1.5 years), it would result in a lower risk estimate than that obtained in the current study (55% over 1.5 years). These data are difficult to interpret because seropositivity in individual animals fluctuated considerably, i.e. animals which already had been infected could well have responded negatively during the May 1997 sampling.

There was an increased risk of abortion in offspring from seropositive dams, but there was no association between the serological status of the offspring and dam. Anderson et al. (1997) found (in their prospective study) that all calves from 25 seropositive heifers were seropositive for *N. caninum* infection in pre-colostral blood samples. A similar result was described by Paré et al. (1996). Such findings suggest vertical transmission from dam to offspring. In a study of herds with abortion outbreaks, the lack of an association between seropositivity of daughter and dam was interpreted by Thurmond et al. (1997) as an indication of infection having occurred post-natally. No pre-colostral samples were collected in our study, but there was no indication of a link between serological status of dam and offspring. If dams and offspring both had been exposed to infection during 1997, there should not have been a difference in seropositivity/abortion risk between both

groups (assuming random mixing within the herds). This would present another indication that infection was likely to have been present within this herd before 1997, and thereby gave rise to some level of vertical transmission.

Dogs are a definitive host for *N. caninum* (McAllister et al., 1998), and epidemiological evidence has identified the presence of dogs as a risk factor in abortion epidemics (Wouda et al., 1999). Two of the farm dogs appeared infected with *N. caninum* in 1997, as evidenced by their positive serological reaction in the IFAT. But given that the baseline seroprevalence for NZ dogs has been reported to be as high as 22% (Reichel, 1998), this is a relatively common occurrence and therefore, unlikely to be an indication of a causal linkage. One of the farm dogs appeared to be shedding coccidial oocysts; however, these oocysts could not be identified. It is notable that oocyst output in dogs in all experimental studies has been low, and dogs therefore are unlikely to cause abortion outbreaks.

Analysis of the milk-production data indicates that seropositive cows were higher producers. It is unlikely that this was causally related to the serological status; perhaps the farmer was inclined to keep aborting cows if they were of higher genetic/production value. A consequence of this management decision would be that in the presence of long-term endemic herd infection and vertical transmission, farmers will unknowingly contribute to maintaining the infection within their herds. The survival analysis of the culling data could not test this hypothesis, because testing for such an effect would require detailed abortion records over a longer period of time.

5. Conclusions

The serological and abortion history of this herd suggests that the classic epidemic *N. caninum* abortion outbreak pattern observed in 1997 was the result of a combination of endemic infection presence and an additional factor resulting in increased abortion risk in this herd. Such an additional factor could have been a stressor having an immunocompromising effect (such as an infectious agent, which could have been introduced by the heifers or bull).

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References

Anderson, M.L., Reynolds, J.P., Rowe, J.D., Sverlow, K.W., Packham, A.E., Barr, B.C., Conrad, P.A., 1997. Evidence of vertical transmission of *Neospora* sp. infection in dairy cattle. J. Am. Vet. Med. Assoc. 210, 1169.

Anderson, M.L., Andrianarivo, A.G., Conrad, P.A., 2000. Neosporosis in cattle. Anim. Reprod. Sci. 60, 417–431.

Barling, K.S., Sherman, M., Peterson, M.J., Thompson, J.A., McNeill, J.W., Craig, T.M., Adams, L.G., 2000. Spatial associations among density of cattle, abundance of wild canids, and seroprevalence to *Neospora caninum* in a population of beef calves. J. Am. Vet. Med. Assoc. 217, 1361–1365.

Björkman, C., Johansson, O., Stenlund, S., Holmdahl, O.J.M., Uggla, A., 1996. *Neospora* species infection in a herd of dairy cattle. J. Am. Vet. Med. Assoc. 208, 1441.

Björkman, C., Alenius, S., Manuelsson, U., Uggla, A., 2000. *Neospora caninum* and bovine virus diarrhoea virus infections in Swedish dairy cows in relation to abortion. Vet. J. 159, 201–206.

Cox, B.T., Reichel, M.P., Griffiths, L.M., 1998. Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study. NZ Vet. J. 46, 28–31.

Dubey, J.P., 1999. Recent advances in *Neospora* and neosporosis. Vet. Parasitol. 84, 349–367.

Dubey, J.P., Lindsay, D.S., 1996. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67, 1–59.

Guy, C.S., Williams, D.J.L., Smith, R., McKay, J., Guy, F., McGarry, J.W., Trees, A.J., 2001. Protective immunity in bovine neosporosis. WAAVP. Stresa:17.

Innes, E.A., Wright, S.E., Maley, S., Rae, A., Schock, A., Kirvar, E., Bartley, P., Hamilton, C., Carey, I.M., Buxton, D., 2001. Protection against vertical transmission in bovine neosporosis. Int. J. Parasitol. 31, 1523–1534.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.

Mehta, C.R., Patel, N.R., 1996. SPSS Exact Tests 7.0 for Windows. SPSS, Inc., Chicago, IL.

Moen, A.R., Wouda, W., Mul, M.F., Graat, E.A.M., van Werven, T., 1998. Increased risk of abortion following *Neospora caninum* abortion outbreaks: a retrospective and prospective cohort study in four dairy herds. Theriogenology 49, 1301–1309.

Morris, J.A., Gardner, M.J., 2000. Epidemiological studies. In: Altman, D.G., Machin, D., Bryant, T.N., Gardner, M.J. (Eds.), Statistics with Confidence. British Medical Journal Books, London, UK, pp. 57–72.

Newcombe, R.G., Altman, D.G., 2000. Proportions and their differences. In: Altman, D.G., Machin, D., Bryant, T.N., Gardner, M.J. (Eds.), Statistics with Confidence. British Medical Journal Books, London, UK, pp. 45–56.

Paré, J., Thurmond, M.C., Hietala, S.K., 1996. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. Can. J. Vet. Res. 60, 133–139.

Paré, J., Thurmond, M.C., Hietala, S.K., 1997. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. J. Parasitol. 83, 82–87.

Paré, J., Fecteau, G., Fortin, M., Marsolais, G., 1998. Seroepidemiologic study of *Neospora caninum* in dairy herds. J. Am. Vet. Med. Assoc. 213, 1595–1598.

Patitucci, A.N., Charleston, W.A.G., Alley, M.R., O'Connor, R.J., Pomroy, W.E., 1999. Serological study of a dairy herd with a recent history of *Neospora* abortion. NZ Vet. J. 47, 28–30.

Reichel, M.P., 1998. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. NZ Vet. J. 46, 38.

Reichel, M.P., Drake, J.M., 1996. The diagnosis of *Neospora* abortions in cattle. NZ Vet. J. 44, 151–154.

Sager, H., Fischer, I., Furrer, K., Strasser, M., Waldvogel, A., Boerlin, P., Audige, L., Gottstein, B., 2001. A Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. Vet. Parasitol. 102, 1–15.

Schares, G., Peters, M., Wurm, R., Barwald, A., Conraths, F.J., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol. 80, 87–98.

Schares, G., Conraths, F.J., Reichel, M.P., 1999. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. Int. J. Parasitol. 29, 1669–1676.

Schares, G., Rauser, M., Zimmer, K., Peters, M., Wurm, R., Dubey, J.P., de Graaf, D.C., Edelhofer, R., Mertens, C., Hess, G., Conraths, F.J., 1999. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. J. Parasitol. 85, 688–694.

Thornton, R.N., Thompson, E.J., Dubey, J.P., 1991. *Neospora* abortion in New Zealand cattle. NZ Vet. J. 39, 129–133.

Thornton, R.N., Gajadhar, A., Evans, J., 1994. *Neospora* abortion epidemic in a dairy herd. NZ Vet. J. 42, 190–191.

Thurmond, M.C., Hietala, S.K., Blanchard, P.C., 1997. Herd-based diagnosis of *Neospora caninum*-induced endemic and epidemic abortion in cows and evidence for congenital and postnatal transmission. J. Vet. Diagn. Invest. 9, 44–49.

Verbeke, G., Molenberghs, G., 2000. Linear Mixed Models for Longitudinal Data. Springer, New York.

Waldner, C.L., Henderson, J., Wu, J.T., Breker, K., Chow, E.Y., 2001. Reproductive performance of a cow–calf herd following a *Neospora caninum*-associated abortion epidemic. Can. Vet. J. 42, 355–360.

Wouda, W., Dijkstra, T., Kramer, A.M.H., van Maanen, C., Brinkhof, J.M.A., 1999. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. Int. J. Parasitol. 29, 1677–1682.

1.8.4. Discussion and Conclusions

The three case studies established sero-epidemiological approaches as the appropriate tool for the diagnosis of *N caninum* abortion epidemics in dairy cattle. In all three cases, the calculation of odds-ratios or relative risks established an association between abortion and the infection status of the cow, while individual cow serology proved to be subject to considerable variation over time. Serological titres were higher immediately after the abortion event (Cox *et al.* 1998) and declined within two months. This has been reported by others after natural and experimental infection (Conrad *et al.* 1993). Long-term monitoring established considerable changes in the serological status of individual cows (Pfeiffer *et al.* 2002), thus also suggesting that individual cow testing at, even, several time points is fraught with difficulty. Others have reported more stable serological responses in cows (Thurmond and Hietala 1997). Even the application of several serological tests did not secure a more reliable diagnosis in individual cows (Schares *et al.* 1999). It did, however, suggest that in New Zealand post-natal transmission might be more important than vertical transmission, which is pre-dominantly reported from overseas (Paré *et al.* 1994, Björkman *et al.* 1996, Schares *et al.* 1998, Davison *et al.* 1999b). In more recent years, less efficient vertical transmission has also been reported from overseas (Bergeron *et al.* 2000, Dyer *et al.* 2000)

With the increase in knowledge about the prevalence of neosporosis in New Zealand and a better understanding of the epidemiology in that country and overseas, further thoughts were directed at what efforts could be made at controlling infection with *N caninum*. A review paper on control options for *N caninum* was accepted for publication in the *New Zealand Veterinary Journal*:

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Control options for *Neospora caninum* infections in cattle – current state of knowledge

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Scientific Article

Control options for *Neospora caninum* infections in cattle – current state of knowledge

MP Reichel*§ and JT Ellis†

Abstract

This article reviews control options for *Neospora caninum* infection and abortion in cattle, drawing on published literature and the authors' own research in this field. Apart from the successful use of embryo transfer to prevent congenital infection in calves born to infected cows, there are currently no accepted control methods for the prevention of abortions in cattle. The epidemiological data at hand suggest that concomitant infections with bovine pestivirus increase the risk of abortion significantly and that these infections, for which effective vaccines exist, should therefore be controlled. While vertical transmission appears to be the major route of infection in cattle, there is also a role for postnatal transmission, involving a definitive host. Presently, the control of dogs and their access to bovine tissues, particularly potentially infected placentae and other foetal tissues, appear to be the most prudent control methods. There are some indications that vaccination against *N. caninum* may aid in the prevention of abortions. Suggestions for control options are limited by our current lack of actual experiences with control strategies. Further practical fieldwork is needed in this area.

KEY WORDS: *Neospora caninum, abortion, cattle, epidemiology, immunology, control*

Key Points

- *Neospora caninum* is recognised as a major cause of abortions in (dairy) cattle around the world.
- Control options have rarely been explored and hitherto only embryo transfer has been successfully applied, resulting in uninfected calves born from infected dams.
- Treatment appears to be uneconomical in cattle, but better knowledge of the interaction between parasite and host during pregnancy might yet identify a period for strategic, preventive treatment of short duration.
- Test-and-cull, and replacement policies hinge on highly sensitive assays for which cut-off thresholds may need to be redefined.
- Infection with bovine pestivirus appears to increase the risk of *Neospora* infection threefold, hence control of pestivirus infection by means of vaccination seems indicated where cattle are at high risk from *Neospora* infection.
- Recent research indicates an association between *Neospora* abortions and dogs on farms – it seems prudent to advocate that dogs and cattle should not mix on dairy farms.

Introduction

Neospora caninum is a protozoan parasite that was first described in a litter of dogs in Norway in 1984 (Bjerkås et al 1984). Today it is recognised worldwide as an infection, predominantly, of dogs and cattle (Dubey and Lindsay 1996). Other species such as sheep, goats, deer and horses (Dubey and Porterfield 1990; Dubey et al 1990b; Barr et al 1992; Woods et al 1994) have also infrequently been reported to be naturally infected. Infection has been reported from retrospective examination of stored tissues from dogs and cattle, dating back to 1957 (Dubey 1992) and 1974 (Dubey et al 1990a), respectively.

Since its recognition as a new parasite entity, *N. caninum* has emerged as a major cause of abortion in cattle. The economic impact of *N. caninum* infection in cattle has been estimated at AUS$85 million per annum for the dairy and AUS$25 million for the beef cattle industry in Australia (Ellis 1997), and NZ$17.8 million for the dairy industry in New Zealand (Pfeiffer et al 1998). In comparison, Dubey (1999b) summarised the economic losses in California to be US$35 million/year. The true costs are probably higher, since these calculations only took account of losses in animal and milk production after abortion outbreaks of epidemic proportions. Sporadic and low-level endemic abortions

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BALB/c	Bagg albino inbred mice
BVDV	Bovine viral diarrhoea virus
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-4	Interleukin 4
NcSAG1	*N. caninum* surface antigen 1
NcSRS2	*N. caninum* surface antigen-1 related sequence 2
Th1	T cell helper 1

also occur (Thornton et al 1991; Boulton et al 1995; Wouda et al 1997), but their costs and relative importance are less well defined.

Cattle with serum antibody responses to *N. caninum* are thought to be chronically infected. A study from the United States of America reported a decrease in milk yield in seropositive cattle (Thurmond and Hietala 1997). Recently, Barling et al (2000a) demonstrated that seropositivity was associated with significant reductions in average daily weight gain and liveweight at slaughter. Serologically positive animals also had higher veterinary treatment costs and showed significantly lower economic returns (Barling et al 2000a). Trees et al (1999), in their review, discussed additional costs such as early foetal death, stillbirth, neonatal death, and reduced breeding value. Early culling would further increase the cost of *N. caninum* infection to primary producers (Thurmond and Hietala 1996). Because of our poor knowledge of their relative contribution, the total cost of these factors remains to be quantified.

More than 10 years have passed since the realisation that *N. caninum* is a major cause of abortion in cattle and, less frequently, of disease in dogs. The general structure of the life cycle was elucidated recently and some of the modes of transmission clarified. Dogs were demonstrated to be a definitive host of *N. caninum*. Oocysts of *N. caninum* were excreted by experimentally infected dogs, if only in low numbers (McAllister et al 1998; Lindsay et al 1999a). Vertical transmission of infection occurs both in dogs and cattle (Paré et al 1994; Barber et al 1998). In the latter species it appears to be the main pathway for infection from one cow to the next (Paré et al 1996; Schares et al 1998). However, epidemiological investigations point to postnatal infections with *N. caninum* as the cause of abortion storms (McAllister et al 1996). Antony and Williamson (2001) and other authors (Thornton et al 1994; Schares et al 1999) have suggested that postnatal infection occurred more often in New Zealand than is reported from other parts of the world.

Diagnosis and diagnostic techniques have been improved and optimised (Atkinson et al 2000). Control of *Neospora* infection or prevention of abortions is the critical issue for primary producers. Control or treatment options have been discussed in the literature, but rarely tried in the field. This paper discusses possible strategies for the control of *N. caninum* infection and/or abortions in cattle in the context of the current state of knowledge about the parasite, its life cycle and epidemiology, and makes recommendations for its control.

Control of infection

Treatment options

Presently, only dogs with clinical signs of neosporosis receive treatment. Barber and Trees (1996) reviewed 27 clinical cases and their treatment. In vitro data suggest that a number of chemicals might be effective (Lindsay and Dubey 1989b; Lindsay et al 1994, 1996, 1997), and in dogs the drugs of choice appear to be clindamycin (Dubey et al 1995) and potentiated sulphonamides.

In vivo data for cattle are few. Gottstein et al (2001) recently presented data that suggest that the severity of clinical outcome of experimental infection with $2x10^8$ tachyzoites of *N. caninum* in calves could be modulated by treatment with toltrazuril and ponazuril at 20 mg/kg/day. However, the economics of treating cattle are questionable. The fact that treatment could only be used as a preventive measure and hence be long-term, might do little to curb an ongoing abortion epidemic. Additionally, it would result in considerable and likely unacceptable milk or meat residues or withdrawal periods, which would be further restrictive. Also, the treatment was directed at a tachyzoite challenge, whereas it is doubtful that encysted bradyzoites are as susceptible to chemical treatment.

Guy et al (2001) reported a rise in antibody response in pregnant cows, and thought this was due to recrudescence of the parasite resulting in congenital infection of the calves. Innes et al (2001b) demonstrated a transient immunosuppression during gestation. Thus, chemical treatment of very limited duration during pregnancy might be efficacious, assisting the dam's immune system during this period of immunosuppression. The aim of such treatment would be to prevent recrudescence of the parasite. However, just when in relation to stage of pregnancy such a treatment would need to be administered to be effective is uncertain and more research is needed before the practicalities of this option can be evaluated.

Control of concomitant infections

It has been reported that *N. caninum* infection on its own increases the risk of abortion in cattle by a factor of at least three (Moen et al 1998; Wouda et al 1998). In addition, several reports suggest that concomitant infection of *N. caninum*-infected cows with bovine pestivirus increases the risk of *Neospora* abortions a further threefold (Pfeiffer et al 2000). A Swedish study (Björkman et al 2000) has also reported a significant association between bovine viral diarrhoea virus (BVDV)-infection in cattle, *Neospora*, and abortion risk. Conversely, a study from Spain could not find any increased risk of abortion in dairy cows that were infected with both BVDV and *N. caninum* (Mainar-Jaime et al 2001). It is hypothesised that infection with BVDV or other potentially immunosuppressive agents such as mycotoxins might lead to or facilitate a recrudescence of a chronic *N. caninum* infection. Recrudescence could result in parasitaemia, and then transplacental infection of the foetus, foetopathy, and ultimately abortion. Depending on the timing of the parasitaemia (Guy et al 2001), it may also result in congenitally-infected asymptomatic calves.

Effective vaccines for the control of bovine pestivirus exist (Brownlie et al 1995). However, there are currently no reports of the effect of controlling pestivirus-infection, whether by vaccination or other means of control (Mainar-Jaime et al 2001), on the incidence of *N. caninum* infections/abortions in cattle. Such an approach might decrease the risk of abortion due to *N. caninum*, and further research is clearly warranted in this area.

Control of the route of transmission

Vertical transmission

There is a large body of evidence suggesting the predominant route of infection with *N. caninum* is via vertical transmission, namely from dam to daughter in utero. Studies in several countries have provided strong evidence that this mode of transmission may be very efficient (Björkman et al 1996; Paré et al 1996; Schares et al 1998; Davison et al 1999), resulting in infection of more than 80%, and up to 95%, of offspring of seropositive dams. Some

more recent studies have reported that the efficiency of vertical transmission may be lower than 50% (Bergeron et al 2000; Dyer et al 2000).

Test and cull

It has been proposed that infection and hence abortion could be controlled by testing cows and culling those that are seropositive. Culling infected cattle and replacing them with uninfected animals hinges on the ability of serological tests to accurately identify animals according to their true infection status. Several serological tests have been validated over recent years and these have been recently reviewed by Atkinson et al (2000). Care must be taken to select the performance characteristics of any test for maximum sensitivity, in order that all infected individuals are removed from the herd and only non-infected replacements bought in. Serological responses are highest at the time of abortion and then decline (Conrad et al 1993; Cox et al 1998). Commercial tests are optimised for the diagnosis of *N. caninum* abortions, and tend to use more conservative (higher), highly specific threshold values. For a test-and-cull-policy these thresholds would have to be adjusted downwards to maximise sensitivity, so that all infected cattle in a herd could be reliably identified (Reichel and Pfeiffer 2002).

Testing and culling, as proposed and modelled by Thurmond and Hietala (1995), and French et al (1999), may be an option for herds that have a high seroprevalence of infection, if the predominant mode of transmission is vertical. Even in herds where the seroprevalence exceeds 20%, as is frequently reported (Jensen et al 1999), it should be possible to replace culled animals with non-infected animals over a number of years, provided there is no postnatal transmission.

The general consensus is that repeat abortions are a rare event in cattle and that <5% of cows that have aborted once abort again (Innes et al 2000). There is also experimental evidence suggesting that previous infection with *N. caninum* may protect foetuses against an otherwise lethal challenge with *N. caninum* tachyzoites (Williams et al 2000; Innes et al 2001b). It is unlikely that chronically infected dams that have previously aborted will abort again. Thus, culling may eliminate those animals from the herd that have established a strong immunity. If postnatal transmission is thought to present a high risk in the herd, it may be advisable to keep such cows in the herd, as they provide the strongest protection against subsequent challenge from the parasite. The benefits of retaining those animals in the herd in terms of protecting against abortions, however, need to be carefully balanced against the known lower production (e.g. milk) of infected cows (Thurmond and Hietala 1997; Barling et al 2000a).

Embryo transfer

Congenitally infected calves appear to become infected late rather than early in gestation. Recrudescence of *N. caninum* infection leading to infected calves also occurred late in gestation in some of the experiments reported by Guy et al (2001). Protecting calves from congenital infection by embryo transfer into uninfected recipient cows has been tried successfully. This resulted in seronegative offspring born to seropositive, infected dams (Baillargeon et al 2001). As this is an expensive way of breaking the life cycle of *N. caninum*, it will most likely be reserved for valuable dams and their embryos.

Postnatal transmission

Control of dogs and their access to bovine tissues

While the main route of bovine infection appears to be vertical, transmission between cattle and their offspring by means of foetal fluids, and from cattle to dogs via ingestion of infected tissues, has been suggested. There is epidemiological evidence linking the presence of dogs to abortion epidemics in cattle.

McAllister et al (1998) demonstrated that dogs are a definitive host for *N. caninum*. While many cattle appear to be born congenitally infected, and hence may not get infected by oocysts produced by a definitive host, there is evidence that point-source infections may have caused some of the abortion epidemics that have been recorded (Thornton et al 1994; McAllister et al 1996). Mathematical modelling also supports the notion that vertical transmission alone is not sufficient to maintain infection in a population (French et al 1999). Investigations in North America and Europe suggest an association between serological status or infection of cattle and the presence and density of domestic and wild canids (Paré et al 1998; Wouda et al 1999; Barling et al 2000b). It thus seems prudent to restrict numbers of dogs and their association with cattle and access to bovine tissues, especially foetal, placental and aborted tissues. Dijkstra et al (2001) demonstrated that dogs shed *N. caninum* oocysts after they had been fed placentae from cattle naturally infected with *N. caninum*. However, others (Bergeron et al 2001) have argued that the presence of *N. caninum* in the bovine placenta is rare. The dogs in the study by Dijkstra et al (2001), and some dogs in the earlier transmission experiments conducted by McAllister et al (1998) and Lindsay et al (1999a), did not seroconvert, and hence were impossible to identify as a potential source of infection for cattle. This is not dissimilar to the situation with *Toxoplasma gondii*, in which not all infected cats seroconvert (Dubey et al 1977; Ruiz and Frenkel 1980). This suggests that all dogs must be considered a potential source of *N. caninum* infection for cattle, whether or not they are serologically positive.

The role of colostrum as a potential source of infection for cattle and dogs is equivocal. Infection in calves was based on demonstration of the parasite (Uggla et al 1998), or on the production of antibodies (Davison et al 2001), whereas other authors (Dijkstra et al 2001) provided no evidence that dogs may be infected in this way.

Dijkstra et al (2002ab) have reported epidemiological associations between abortions and postnatal infection, and the presence/arrival of dogs on farms. In controlled epidemiological studies, access by dogs to foetal fluids and placental tissue appeared to be linked to postnatal transmission in cattle. However, little direct evidence exists that cows may be infected in any way other than by the well-documented vertical route of transmission. Trees et al (2001) reported experiments in which three cows were each fed approximately 600 *N. caninum* oocysts but apparently did not become infected. The number of oocycts shed by experimentally infected dogs always remained very low (McAllister et al 1998; Lindsay et al 1999a) and it is difficult to understand how such low faecal shedding of oocysts could cause the abortion outbreaks observed in cattle.

Vaccination approaches

A vaccine that is claimed to aid in the control of abortions associated with *N. caninum* in cattle (Bovilis Neoguard, Intervet, Auckland, New Zealand) has recently been released and is currently the subject of field trials in New Zealand. However, to date there are no published data on which its efficacy can be assessed.

Previous attempts to formulate a vaccine against *N. caninum* have met with limited success (Dubey 1999a). It is generally considered important in the control of protozoal infections to elicit an effective T cell helper-1 (Th1) -type response, which is evident in experimental *N. caninum* infections in cattle (Williams et al 2000). However, a very strong Th1-type response might be incompatible with a successful outcome for pregnancy (Raghupathy 1997), as it may lead to rejection of the developing foetus. Thus, the generation of a strong and successful Th1-response by vaccination needs to be compatible with the successful continuation and completion of gestation in the vaccinated dam. Successful vaccines have been introduced for some coccidian infections, such as the live attenuated vaccine Toxovax™ for *T. gondii* and the protective immunity-inducing antigens in the case of *Eimeria* (Wallach 1997). These might be examples of the direction to take in the development of a potential vaccine for *N. caninum*.

Several experimental studies (Williams et al 2000; Innes et al 2001b) and reports from the field (McAllister et al 2000) support the view that protective immunity develops in chronically infected dams. This is further supported by observations that repeat abortions due to *N. caninum* in cattle are rare (Anderson et al 1995). It has also been demonstrated that the time of challenge with *N. caninum* in relation to stage of gestation is an important determinant of the outcome of infection. Infection at 70 days gestation in naïve cows reliably resulted in foetopathy, whereas infection at 30 weeks resulted in congenitally infected, clinically asymptomatic calves (Williams et al 2000; Innes et al 2001b). Cows chronically infected with *N. caninum* and challenged at 10 weeks gestation did not abort (Guy et al 2001). One might therefore expect that vaccination could be successful in either preventing abortions or infection, or both.

Most research to date has been conducted using animal models such as dogs, mice, sheep and cows, and as some of those models, especially those involving non-target species, might not reflect the natural situation, results should be treated with caution.

Liddell et al (1999) vaccinated female BALB/c mice with a crude *N. caninum* tachyzoite lysate preparation co-administered with ImmuMAXSR™ adjuvant. The mice were subsequently mated, and pregnant dams were challenged with *N. caninum* tachyzoites at 10–12 days gestation. Results demonstrated that this single inoculation appeared to confer complete protection against vertical transmission of infection to the offspring. All pups in the experimental group were free from parasitic infection. No results have yet been reported on the efficacy of this vaccine formulation in cattle. Baszler et al (2000) examined the possibility of vaccination of BALB/c mice with a soluble *N. caninum* antigen either trapped in nonionic surfactant vesicles or formulated with Freunds Complete Adjuvant. This approach, however, resulted in exacerbation of encephalitis and neurological disease in the mice. Observations were characterised by increased antigen specific interleukin-4 (IL-4) secretion and increased IgG1:IgG2a ratios in vivo, possibly suggesting an inappropriate immune response was generated in that experiment.

Andrianarivo et al (1999) tested four different adjuvants and a killed whole *N. caninum* tachyzoite preparation for immunogenicity in cattle, and compared these with responses to experimentally-induced infection with culture-derived tachyzoites. The humoral immune responses, determined by indirect fluorescent antibody test (IFAT) titres, were always significantly lower in immunised cattle than the experimentally infected cattle. Cell-mediated responses were studied in two adjuvant groups immunised with the tachyzoite preparation; POLYGEN™-adjuvant-immunised cattle produced interferon (IFN) levels similar to those of infected animals.

Andrianarivo et al (2000) also studied the effect of a killed *N. caninum* tachyzoite preparation in pregnant cattle using a POLYGEN™ adjuvant. Heifers were immunised at 35 and 65 days gestation, and then 4 weeks later challenged with an intravenous or intramuscular inoculation of tachyzoites. Immunised heifers developed both humoral and cell-mediated immune responses, characterised by an increase in production of IgG1 and IFN-γ, respectively. Following a challenge with *N. caninum* tachyzoites, there was no significant cell-mediated immune response. Since all foetuses from control and experimental cattle developed lesions characteristic of *N. caninum* infection, it was concluded that this formulation did not prevent foetal infection in pregnant cattle.

Choromanski and Block (2000) formulated vaccine preparations from *N. caninum* tachyzoites using Havlogen and Bay R1005 adjuvants. Initial inoculations were followed by booster vaccinations 2 weeks later. Experimental heifers had greatly increased IFAT antibody titres, but were not challenged with *N. caninum*. There is some anecdotal evidence of efficacy of this tachyzoite formulation after a series of three inoculations. However, unvaccinated controls were not kept in the herd, making it difficult to draw conclusions from the study (Wren 2000).

Nishikawa et al (2001) used live vaccinia virus vectors to deliver NcSRS2 and NcSAG1 *N. caninum* antigens to pregnant mice. Vertical transmission of the parasite to foetuses was prevented. These workers also demonstrated the immunogenicity of their construct in dogs (Nishikawa et al 2000). Many advantages exist for live antigen delivery systems, such as ease and relatively low cost of production. Little debate has occurred, however, over the suitability of live vectors for the delivery of *N. caninum* antigens to cattle. No doubt this will occur in coming years.

Different isolates of *N. caninum* appear to have different biological characteristics, including differences in their ability to cause pathological change. Prior infection with a less virulent strain protected mice against an otherwise lethal challenge with NC-Liverpool (Atkinson et al 1999). Temperature-sensitive mutants of *N. caninum* (Lindsay et al 1999b) were able to protect mice from a potentially lethal challenge with tachyzoites. Following data presented by Williams et al (2000) and Innes et al (2001b), in which prior natural chronic infection with *N. caninum* protected foetuses against a lethal challenge, one could envisage the use of a live attenuated vaccine to prevent abortion, such as is the case with *T. gondii* and vaccination with Toxovax™. However, "vaccination" with Toxovax™ did not protect sheep foetuses from a lethal challenge of *N. caninum* (Innes et al 2001a). In contrast, "vaccination" with *N. caninum* provided protection from a lethal challenge with *T. gondii* when ewes were challenged with a moderately virulent strain but not when challenged with a highly virulent strain (Lindsay et al 1998).

The majority of vaccine preparations used in immunogenicity trials thus far have been based on whole-cell or cell-lysate formulations. It is questionable whether vaccines of this type are economical and effective to produce. In vitro culture of *N. caninum* tachyzoites is a laborious and expensive process (Lindsay

and Dubey 1989a). Economic factors may prevent the large-scale development of whole-cell or cell-lysate vaccines, especially in a global context.

Recombinant antigens of *N. caninum* have been produced, but have mainly been used in the serological diagnosis of infection (Lally et al 1996; Dubey et al 1997; Jenkins et al 1997). Future work might be directed at establishing their utility as vaccine candidates.

Conclusions

Although knowledge on the biology and epidemiology of *N. caninum*, and recognition of it as an abortificant, has grown over the last 10 years, there is still a paucity of practical field experience with methods for prevention of infection and foetal loss. The only proven method for prevention of vertical transmission is via embryo transfer, which would likely be reserved for a small number of highly valuable *N. caninum*-infected cows.

A small number of epidemiological investigations suggest that bovine pestivirus infections significantly increase the risk of abortion in *N. caninum*-infected cattle. Efficacious vaccines for the control of pestivirus infection exist and their use is advocated in situations where there is a high risk of both bovine pestivirus and *N. caninum* infection.

Some epidemiological investigations of *N. caninum* abortions point to postnatal infections as the cause of abortion storms. Antony and Williamson (2001), Schares et al (1999) and Thornton et al (1994) suggest that this is more often the case in New Zealand than is reported from other parts of the world. Testing for *N. caninum* infection and culling of infected animals may be less successful in New Zealand than in parts of the world where vertical transmission predominates.

In order to control postnatal transmission, the interaction of dogs with pregnant cattle, and their access to bovine placentae and foetal tissues should be controlled. All dogs should be presumed a potential source of infection for cattle, as serodiagnosis of the infection status of individual dogs is fraught with difficulty. As a considerable number of dogs experimentally infected with *N. caninum* did not seroconvert, it may be prudent to eliminate, as far as is practicable, all dogs from the vicinity of cattle that may be at risk.

References

Anderson ML, Palmer CW, Thurmond MC, Picanso JP, Blanchard PC, Breitmeyer RE, Layton AW, McAllister M, Daft B, Kinde H, Read DH, Dubey JP, Conrad PA, Barr BC. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. *Journal of the American Veterinary Medical Association* 207, 1206–10, 1995

Andrianarivo AG, Choromanski L, McDonough SP, Packham AE, Conrad PA. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *International Journal for Parasitology* 29, 1613–25, 1999

Andrianarivo AG, Rowe JD, Barr BC, Anderson ML, Packham AE, Sverlow KW, Choromanski L, Loui C, Grace A, Conrad PA. A POLYGEN-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following i.v./i.m. experimental tachyzoite challenge. *International Journal for Parasitology* 30, 985–90, 2000

Antony A, Williamson NB. Recent advances in understanding the epidemiology of *Neospora caninum* in cattle. *New Zealand Veterinary Journal* 49, 42–7, 2001

Atkinson R, Harper PAW, Ryce C, Morrison DA, Ellis JT. Comparison of the biological characteristics of two isolates of *Neospora caninum*. *Parasitology* 118, 363–70, 1999

Atkinson R, Harper PA, Reichel MP, Ellis JT. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. *Parasitology Today* 16, 110–4, 2000

Baillargeon P, Fecteau G, Paré J, Lamothe P, Sauve R. Evaluation of the embryo transfer procedure proposed by the International Embryo Transfer Society as a method of controlling vertical transmission of *Neospora caninum* in cattle. *Journal of the American Veterinary Medical Association* 218, 1803–6, 2001

Barber JS, Trees AJ. Clinical aspects of 27 cases of neosporosis in dogs. *Veterinary Record* 139, 439–43, 1996

Barber JS, Trees AJ, Johnson AM. Naturally occurring vertical transmission of *Neospora caninum* in dogs. *Proceedings of the 10th International Congress of Protozoology* 28, 57–64, 1998

Barling KS, McNeill JW, Thompson JA, Paschal JC, McCollum FT 3rd, Craig TM, Adams LG. Association of serologic status for *Neospora caninum* with postweaning weight gain and carcass measurements in beef calves. *Journal of the American Veterinary Medical Association* 217, 1356–60, 2000a

Barling KS, Sherman M, Peterson MJ, Thompson JA, McNeill JW, Craig TM, Adams LG. Spatial associations among density of cattle, abundance of wild canids, and seroprevalence to *Neospora caninum* in a population of beef calves. *Journal of the American Veterinary Medical Association* 217, 1361–5, 2000b

Barr BC, Anderson ML, Woods LW, Dubey JP, Conrad PA. *Neospora*-like protozoal infections associated with abortion in goats. *Journal of Veterinary Diagnostic Investigation* 4, 365–7, 1992

Baszler TV, McElwain TF, Mathison BA. Immunization of BALB/c mice with killed *Neospora caninum* tachyzoite antigen induces a type-2 immune response and exacerbates encephalitis and neurological disease. *Clinical and Diagnostic Laboratory Immunology* 7, 893–8, 2000

Bergeron N, Fecteau G, Paré J, Martineau R, Villeneuve A. Vertical and horizontal transmission of *Neospora caninum* in dairy herds in Quebec. *Canadian Veterinary Journal* 41, 464–7, 2000

Bergeron N, Girard C, Paré J, Fecteau G, Robinson J, Baillargeon P. Rare detection of *Neospora caninum* in placentas from seropositive dams giving birth to full-term calves. *Journal of Veterinary Diagnostic Investigation* 13, 173–5, 2001

Bjerkås I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Zeitschrift für Parasitenkunde* 70, 271–4, 1984

Björkman C, Johansson O, Stenlund S, Holmdahl OJM, Uggla A. *Neospora* species infection in a herd of dairy cattle. *Journal of the American Veterinary Medical Association* 208, 1441, 1996

Björkman C, Alenius S, Manuelsson U, Uggla A. *Neospora caninum* and bovine virus diarrhoea virus infections in Swedish dairy cows in relation to abortion. *The Veterinary Journal* 159, 201–6, 2000

Boulton JG, Gill PA, Cook RW, Fraser GC, Harper PAW, Dubey JP. Bovine *Neospora* abortion in north-eastern New South Wales. *Australian Veterinary Journal* 72, 119–20, 1995

Brownlie J, Clarke MC, Hooper LB, Bell GD. Protection of the bovine fetus from bovine viral diarrhoea virus by means of a new inactivated vaccine. *Veterinary Record* 137, 58–62, 1995

Choromanski L, Block W. Humoral immune responses and safety of experimental formulations of inactivated *Neospora* vaccines. *Parasitology Research* 86, 851–3, 2000

Conrad PA, Sverlow K, Anderson M, Rowe J, BonDurant R, Tuter G, Breitmeyer R, Plamer C, Thurmond M, Ardans A, Dubey JP, Duhamel G, Barr B. Detection of serum antibody response in cattle with natural or experimental *Neospora* infections. *Journal of Veterinary Diagnostic Investigation* 5, 572–8, 1993

Cox BT, Reichel MP, Griffiths LM. Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study. *New Zealand Veterinary Journal* 46, 28–31, 1998

Davison HC, Otter A, Trees AJ. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *International Journal for Parasitology* 29, 1683–9, 1999

Davison HC, Guy CS, McGarry JW, Guy F, Williams DJ, Kelly DF, Trees AJ. Experimental studies on the transmission of *Neospora caninum* between cattle. *Research in Veterinary Science* 70, 163–8, 2001

Dijkstra T, Eysker M, Schares G, Conraths FJ, Wouda W, Barkema HW. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. *International Journal for Parasitology* 31, 747–52, 2001

Dijkstra T, Barkema HW, Hesselink JW, Wouda W. Point source exposure of cattle to *Neospora caninum* consistent with periods of common housing and feeding and related to the introduction of a dog. *Veterinary Parasitology* 105, 89–98, 2002a

Dijkstra T, Barkema HW, Eysker M, Hesselink JW, Wouda W. Natural transmission routes of *Neospora caninum* between farm dogs and cattle. *Veterinary Parasitology* 105, 99–104, 2002b

Dubey JP. Neosporosis - a newly recognized protozoal infection. *Comparative Pathology Bulletin* 24, 4–6, 1992

Dubey JP. Recent advances in *Neospora* and neosporosis. *Veterinary Parasitology* 84, 349–67, 1999a

Dubey JP. Neosporosis in cattle: biology and economic impact. *Journal of the American Veterinary Medical Association* 214, 1160–3, 1999b

Dubey JP, Lindsay DS. A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology* 67, 1–59, 1996

Dubey JP, Porterfield ML. *Neospora caninum* (Apicomplexa) in an aborted equine fetus. *Journal of Parasitology* 76, 732–4, 1990

Dubey JP, Christie E, Pappas PW. Characterization of *Toxoplasma gondii* from the feces of naturally infected cats. *The Journal of Infectious Diseases* 136, 432–5, 1977

Dubey JP, Hartley WJ, Lindsay DS. Congenital *Neospora caninum* infection in a calf with spinal cord anomaly. *Journal of the American Veterinary Medical Association* 197, 1043–4, 1990a

Dubey JP, Hartley WJ, Lindsay DS, Topper MJ. Fatal congenital *Neospora caninum* infection in a lamb. *Journal of Parasitology* 76, 127–30, 1990b

Dubey JP, Metzger FL Jr, Hattel AL, Lindsay DS, Fritz DL. Canine cutaneous neosporosis: clinical improvement with clindamycin. *Veterinary Dermatology* 6, 37–43, 1995

Dubey JP, Jenkins MC, Adams DS, McAllister MM, AndersonSprecher R, Baszler TV, Kwok OCH, Lally NC, Björkman C, Uggla A. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. *Journal of Parasitology* 83, 1063–9, 1997

Dyer RM, Jenkins MC, Kwok OC, Douglas LW, Dubey JP. Serologic survey of *Neospora caninum* infection in a closed dairy cattle herd in Maryland: risk of serologic reactivity by production groups. *Veterinary Parasitology* 90, 171–81, 2000

Ellis JT. *Neospora caninum*: prospects for diagnosis and control using molecular methods. In: Shirley MW, Tomley FM, Freeman BM (eds). *Proceedings of the VII International Coccidiosis Conference and EU COST820 Workshop*. Pp 80–81. Oxford Institute for Animal Health, Oxford, 1997

French NP, Clancy D, Davison HC, Trees AJ. Mathematical models of *Neospora caninum* infection in dairy cattle: transmission and options for control. *International Journal for Parasitology* 29, 1691–704, 1999

Gottstein B, Kritzner S, Cannas A, Sager H, Greif G. Toltrazuril and Ponazuril for treatment of experimental murine and bovine neosporosis. *Proceedings of the 18th International Conference of the World Association for the Advancement of Veterinary Parasitology*. B9, 2001

Guy CS, Williams DJL, Smith R, McKay J, Guy F, McGarry JW, Trees AJ. Protective immunity in bovine neosporosis. *Proceedings of the 18th International Conference of the World Association for the Advancement of Veterinary Parasitology*. B10, 2001

Innes EA, Buxton D, Maley S, Wright S, Marks J, Esteban I, Rae A, Schock A, Wastling J. Neosporosis. Aspects of epidemiology and host immune response. *Annals of the New York Academy of Sciences* 916, 93–101, 2000

Innes EA, Lunden A, Esteban I, Marks J, Maley S, Wright S, Rae A, Harkins D, Vermeulen A, McKendrick IJ, Buxton D. A previous infection with *Toxoplasma gondii* does not protect against a challenge with *Neospora caninum* in pregnant sheep. *Parasite Immunology* 23, 121–32, 2001a

Innes EA, Wright SE, Maley S, Rae A, Schock A, Kirvar E, Bartley P, Hamilton C, Carey IM, Buxton D. Protection against vertical transmission in bovine neosporosis. *International Journal for Parasitology* 31, 1523–34, 2001b

Jenkins MC, Wouda W, Dubey JP. Serological response over time to recombinant *Neospora caninum* antigens in cattle after a neosporosis-induced abortion. *Clinical and Diagnostic Laboratory Immunology* 4, 270–4, 1997

Jensen AM, Björkman C, Kjeldsen AM, Wedderkopp A, Willadsen C, Uggla A, Lind P. Associations of *Neospora caninum* seropositivity with gestation number and pregnancy outcome in Danish dairy herds. *Preventive Veterinary Medicine* 40, 151–63, 1999

Lally NC, Jenkins MC, Dubey JP. Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. *Clinical and Diagnostic Laboratory Immunology* 3, 275–9, 1996

Liddell S, Jenkins MC, Collica CM, Dubey JP. Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. *Journal of Parasitology* 85, 1072–5, 1999

Lindsay DS, Dubey JP. Evaluation of anti-coccidial drugs' inhibition of *Neospora caninum* development in cell cultures. *Journal of Parasitology* 75, 990–2, 1989a

Lindsay DS, Dubey JP. In vitro development of *Neospora caninum* (Protozoa: Apicomplexa) from dogs. *Journal of Parasitology* 75, 163–5, 1989b

Lindsay DS, Rippey NS, Cole RA, Parsons LC, Dubey JP, Tidwell RR, Blagburn BL. Examination of the activities of 43 chemotherapeutic agents against *Neospora caninum* tachyzoites in cultured cells. *American Journal of Veterinary Research* 55, 976–81, 1994

Lindsay DS, Butler JM, Rippey NS, Blagburn BL. Demonstration of synergistic effects of sulfonamides and dihydrofolate reductase/thymidylate synthase inhibitors against *Neospora caninum* tachyzoites in cultured cells, and characterization of mutants resistant to pyrimethamine. *American Journal of Veterinary Research* 57, 68–72, 1996

Lindsay DS, Butler JM, Blagburn BL. Efficacy of decoquinate against *Neospora caninum* tachyzoites in cell cultures. *Veterinary Parasitology* 68, 35–40, 1997

Lindsay DS, Lenz SD, Dykstra CC, Blagburn BL, Dubey JP. Vaccination of mice with *Neospora caninum*: Response to oral challenge with *Toxoplasma gondii* oocysts. *Journal of Parasitology* 84, 311–15, 1998

Lindsay DS, Dubey JP, Duncan RB. Confirmation that the dog is a definitive host for *Neospora caninum*. *Veterinary Parasitology* 82, 327–33, 1999a

Lindsay DS, Lenz SD, Blagburn BL, Brake DA. Characterization of temperature-sensitive strains of *Neospora caninum* in mice. *Journal of Parasitology* 85, 64–7, 1999b

Mainar-Jaime RC, Berzal-Herranz B, Arias P, Rojo-Vazquez FA. Epidemiological pattern and risk factors associated with bovine viral-diarrhoea virus (BVDV) infection in a non-vaccinated dairy-cattle population from the Asturias region of Spain. *Preventive Veterinary Medicine* 52, 63–73, 2001

McAllister MM, Huffman EM, Hietala SK, Conrad PA, Anderson ML, Salman MD. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. *Journal of Veterinary Diagnostic Investigation* 8, 355–7, 1996

McAllister MM, Dubey JP, Lindsay DS, Jolley WR, Wills RA, McGuire AM. Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* 28, 1473–8, 1998

McAllister MM, Björkman C, Anderson-Sprecher R, Rogers DG. Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. *Journal of the American Veterinary Medical Association* 217, 881–7, 2000

Moen AR, Wouda W, Mul MF, Graat EAM, vanWerven T. Increased risk of abortion following *Neospora caninum* abortion outbreaks: A retrospective and prospective cohort study in four dairy herds. *Theriogenology* 49, 1301–9, 1998

Nishikawa Y, Ikeda H, Fukumoto S, Xuan X, Nagasawa H, Otsuka H, Mikami T. Immunization of dogs with a canine herpesvirus vector expressing *Neospora*

caninum surface protein, NcSRS2. *International Journal for Parasitology* 30, 1167–71, 2000

Nishikawa Y, Inoue N, Xuan X, Nagasawa H, Igarashi I, Fujisaki K, Otsuka H, Mikami T. Protective efficacy of vaccination by recombinant vaccinia virus against *Neospora caninum* infection. *Vaccine* 19, 1381–90, 2001

Paré J, Thurmond MC, Hietala SK. Congenital *Neospora* infection in dairy cattle. *Veterinary Record* 134, 531–2, 1994

Paré J, Thurmond MC, Hietala SK. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. *Canadian Journal of Veterinary Research* 60, 133–9, 1996

Paré J, Fecteau G, Fortin M, Marsolais G. Seroepidemiologic study of *Neospora caninum* in dairy herds. *Journal of the American Veterinary Medical Association* 213, 1595–8, 1998

Pfeiffer DU, Wichtel JJ, Reichel MP, Williamson NB, Teague WR, Thornton RN. Investigations into the epidemiology of *Neospora caninum* infection in dairy cattle in New Zealand. *Proceedings of the 15th Annual Seminar of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*. Pp 279–92, 1998

Pfeiffer DU, Williamson NB, Reichel MP. Long-term serological monitoring as a tool for epidemiological investigation of *Neospora caninum* infection in a New Zealand dairy herd. *Proceedings of the IX Symposium of the International Society of Veterinary Epidemiology and Economics*. Pp 524, 2000

Raghupathy R. Th-1 type immunity is incompatible with successful pregnancy. *Immunology Today* 18, 478–82, 1997

Reichel MP, Pfeiffer DU. An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle. *Veterinary Parasitology* (in press), 2002

Ruiz A, Frenkel JK. *Toxoplasma gondii* in Costa Rican cats. *American Journal of Tropical Medicine and Hygiene* 19, 1150–60, 1980

Schares G, Peters M, Wurm R, Barwald A, Conraths FJ. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Veterinary Parasitology* 80, 87–98, 1998

Schares G, Conraths FJ, Reichel MP. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. *International Journal for Parasitology* 29, 1669–76, 1999

Thornton RN, Thompson EJ, Dubey JP. Neospora abortion in New Zealand cattle. *New Zealand Veterinary Journal* 39, 129–33, 1991

Thornton RN, Gajadhar A, Evans J. *Neospora* abortion epidemic in a dairy herd. *New Zealand Veterinary Journal* 42, 190–1, 1994

Thurmond M, Hietala S. Strategies to control *Neospora* infection in cattle. *Bovine Practitioner* 29, 60–3, 1995

Thurmond MC, Hietala SK. Culling associated with *Neospora caninum* infection in dairy cows. *American Journal of Veterinary Research* 57, 1559–62, 1996

Thurmond MC, Hietala SK. Effect of *Neospora caninum* infection on milk production in first-lactation dairy cows. *Journal of the American Veterinary Medical Association* 210, 672, 1997

Trees AJ, Davison HC, Innes EA, Wastling JM. Towards evaluating the economic impact of bovine neosporosis. *International Journal for Parasitology* 29, 1195–1200, 1999

Trees AJ, McAllister MM, Guy CS, Smith R, Williams DJL. Oral infection of pregnant cows with *Neospora caninum* oocysts. In: Buxton D, Innes E, Maley S, Wright S, Rae A, Bartley P, Curran C, Noble S (eds). *Neospora 2001. A Symposium On All Aspects of Bovine Neosporosis*. P 25. Moredun Research Institute, Edinburgh, 2001

Uggla A, Stenlund S, Holmdahl OJM, Jakubek EB, Thebo P, Kindahl H, Björkman C. Oral *Neospora caninum* inoculation of neonatal calves. *International Journal for Parasitology* 28, 1467–72, 1998

Wallach M. The importance of transmission-blocking immunity in the control of infections by apicomplexan parasites. *International Journal for Parasitology* 27, 1159–67, 1997

Williams DJ, Guy CS, McGarry JW, Guy F, Tasker L, Smith RF, MacEachern K, Cripps PJ, Kelly DF, Trees AJ. *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. *Parasitology* 121, 347–58, 2000

Woods LW, Anderson ML, Swift PK, Sverlow KW. Systemic neosporosis in a California black-tailed deer (*Odocoileus hemionus columbianus*). *Journal of Veterinary Diagnostic Investigation* 6, 508–10, 1994

Wouda W, Moen AR, Visser IJR, Knapen Fv, Van Knapen F. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. *Journal of Veterinary Diagnostic Investigation* 9, 180–5, 1997

Wouda W, Moen AR, Schukken YH. Abortion risk in progeny of cows after a *Neospora caninum* epidemic. *Theriogenology* 49, 1311–16, 1998

Wouda W, Dijkstra T, Kramer AMH, van Maanen C, Brinkhof JMA. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. *International Journal for Parasitology* 29, 1677–82, 1999

Wren G. Turning a *Neospora*-infected herd around. *Bovine Veterinarian* May/June, 22–30, 2000

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2. CONCLUSIONS

Four separate papers established or validated techniques for the diagnosis of three animal pathogens of importance to the New Zealand primary producers (Kittelberger and Reichel 1998, Reichel *et al.* 1998a, Reichel *et al.* 1999a, Reichel *et al.* 1999b) and demonstrated the author's aptitude in establishing serological tools in the field.

The above body of research as it refers to *N caninum* infection and abortions describes the *status quo* of knowledge about the parasite in New Zealand succinctly and comprehensively. An initial paper established base-line data for the sero-prevalence of *N caninum* infection in various canid populations around the world, including Australia (Barber *et al.* 1997a). After the establishment and validation of serological tools under New Zealand conditions (Reichel and Drake 1996, Reichel and Pfeiffer 2002), subsequent sero-prevalence studies in that country established the extent of infection in dog and cattle populations (Reichel 1998, Tennent-Brown *et al.* 2000). A clinical case in a dog was also described with the use of state-of-the-art diagnostic tools and techniques (Reichel *et al.* 1998b). Subsequent case studies established techniques for the investigation of abortion outbreaks and established the advantages of sero-epidemiological approaches (Cox *et al.* 1998) versus individual cow diagnosis. A further case-study provided data regarding the possible mode of transmission of the parasite (Schares *et al.* 1999) and another long-term patterns of serological status in a large New Zealand dairy herd that had experienced a severe abortion epidemic and the beginning of the observational period (Pfeiffer *et al.* 2002).

Subsequent to the above work, two reviews were published, discussing the disease in Australasia and aspects of its serological diagnosis (Atkinson *et al.* 2000a, Reichel

2000) and finally one that suggested control options for the parasite, given the current state of knowledge (Reichel and Ellis 2002).

Future work in New Zealand should be directed at establishing the epidemiological association and inter-action between dogs and cattle on dairy farms. This has repeatedly been reported overseas (Sawada *et al.* 1998, Wouda *et al.* 1999, Dijkstra *et al.* 2002a, Dijkstra *et al.* 2002b) but it needs to be demonstrated in the New Zealand context. Further more, the exact steps that lead to *N caninum*-induced abortion epidemics need to be investigated. While experimentally *N caninum* infected dogs shed only low numbers of oocysts, recent work demonstrates that this may be affected by the origin of the infective material. Tissue cysts derived from a murine system appear to be less effective in inducing oocyst production than infective tissues of bovine origin (Gondim *et al.* 2002).

This includes also investigating the role of concurrent infection with potentially immunosuppressive animal pathogens, such as bovine pestivirus, which has been demonstrated by some to increase the risk of abortion 3-fold in already *N caninum*-infected cattle (Björkman *et al.* 2000, Pfeiffer *et al.* 2000). Bovine pestivirus infection is extremely prevalent in NZ dairy cattle with approximately 58% of cattle having serum antibodies (Littlejohns and Horner 1990) and efficacious vaccines for its control exist (Brownlie *et al.* 1995). Controlling bovine pestivirus might aid in preventing abortion epidemics or reduce their incidence.

3. CONFERENCE CONTRIBUTIONS

1. deLisle, GW and Reichel, MP (1995)

   The New Zealand JD situation. Proc. Johne's disease Diagnosis Standardisation Workshop, 51-56, CSIRO, Melbourne

2. Reichel, MP and Pomroy, WE (1996)

   Neospora spp. and bovine abortions. In: Proc. Cattle session. 2nd PanPacific Veterinary Conference, Christchurch. Veterinary Continuing Education, Massey University, New Zealand, Publication No. 171, 21-27.

3. Reichel, MP; Cox, BT and Griffiths, L (1997)

   Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study

   International Conference for Protozoology, Sydney, 21-25 July 1997

4. Hayes, DP; Pfeiffer, DU; Morris, RS; Burton, LJ; Reichel, MP and Hoggard, GK (1997)

   Factors associated with bovine leukaemia virus infection in seasonally calved dairy herds in New Zealand. Proceedings of 8th Symposium of the International Society for Veterinary Epidemiology and Economics, Paris, France, 8-11 July 1997. Special issue of Epidemiologie et Santé animale 31-32, 11.C.14.

5. Hayes, DP; Hoggard, GK; Reichel, MP; Burton, LJ; Pfeiffer, DU and Morris, RS (1997)

   The suitability of milk samples from a national milk analysis centre for bovine leukaemia virus surveillance. Proceedings of 8th Symposium of the International Society for Veterinary Epidemiology and Economics, Paris, France, 8-11 July 1997. Special issue of Epidemiologie et Santé animale 31-32, 07.B.32.

6. West, DM; Stafford, KJ; Sargison, ND and Reichel, MP (1998)

Brucella ovis infection in deer - current and future implications in sheep. In: Proc. 28th Seminar Sheep & Beef Cattle Veterinarians NZVA, Dunedin, **180**, 149-56

7. West, DM; Stafford, KJ; Sargison, ND and Reichel MP (1998)

Brucella ovis infection in deer - current and future implications in sheep. In: Proc Deer Course for Veterinarians. **15**, 93-100

8. Reichel, MP; deLisle, GW; Johns, AH and Cousins, D (1998)

Comparison of bacterial culture and serology for the diagnosis of Johne's disease in cattle. Proc. of OIE/WHO International Congress on Anthrax, Brucellosis, CBPP, Clostridial and Mycobacterial Diseases. Berg-en-Dal, Kruger National Park, South Africa, 9-15 August 1998, 352-357.

9. Reichel, MP; Kittelberger, R and deLisle, GW (1998)

Immunoblot analyses of M. paratuberculosis antigens and immune responses in cattle. Proc. of OIE/WHO International Congress on Anthrax, Brucellosis, CBPP, Clostridial and Mycobacterial Diseases. Berg-en-Dal. Kruger National Park, South Africa, 9-15 August 1998, 358-362.

10. Kittelberger, R; Reichel, MP; Penrose, ME; Meynell RM and deLisle GW (1998)

Identification of immuno-dominant antigens in ruminants infected with *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis)*. Microbes and Molecules '98. Joint meeting of the NZ Society for Microbiology and the NZ Society for Biochemistry and Molecular Biology. 30. November - 3. December 1998, Solway Park, Masterton, New Zealand. Paper 59.

11. Reichel, MP (1999)

Neosporosis in cattle. In: Proc. 29th Seminar Sheep & Beef Cattle Veterinarians NZVA, Hastings, Veterinary Continuing Education, Palmerston North, Publication No. 189, 55-60

12. Williamson, NB; Pomroy, WE; Pfeiffer, D and Reichel, MP (1999)

Vertical transmission of *Neospora caninum* in a large dairy herd

NZ Society for Parasitology, **28**, Palmerston North, New Zealand

13. Reichel, MP; Pomroy, WE; Pfeiffer, D; Williamson, NB; Conraths, FJ and Schares, G (1999)

Transmission patterns of *Neospora caninum* in New Zealand dairy herds.

Proc. ASP Annual meeting, Rockhampton, Abstract C 59, September 1999.

14. Schares, G; Reichel, MP and Conraths, FJ (1999)

Bovine neosporosis: comparison of serological methods using outbreak sera. Proceedings Annual Workshop COST 820, Vaccines against animal coccidioses, November 11-14 1999, Interlaken, Switzerland

15. Reichel, MP; Kittelberger, R, Meynell, M and Gwózdz, JM (1999)

Analysis of the antibody response by immunoblot and ELISA in sheep infected with *Mycobacterium paratuberculosis* Proc 6th Int. Colloq. Paratuberculosis, Melbourne February 1999, 604-615

16. West, DM; Stafford, KJ; Fenwick, SG; Wilson PR; Ridler AL and Reichel MP (1999)

Brucella ovis in deer - research results and research needs.

A deer course for veterinarians. Volume 16. Palmerston North: Deer Branch of the New Zealand Veterinary Association. p 123-6.

17. Pfeiffer, DU; Williamson, NB and Reichel, MP (2000)

Long-term serological monitoring as a tool for epidemiological investigation of *Neospora caninum* infection in a New Zealand dairy herd

IX Symposium, ISVEE, Breckenridge, Colorado, Abstract 524

18. Williamson, NB; Antony, A; Pfeiffer, DU; Pomroy, WE; Reichel, MP and Walton, JK (2000)

Recent Research Into *Neospora caninum* In New Zealand

XXI World Buiatrics Conference, Puenta del Este, Uruguay

19. Williamson, NB; Pomroy, WE; Walton, JK and Reichel, MP (2001)

Vertical Transmission of *Neospora* in a New Zealand Dairy Herd

WAAVP, Stresa, 26-30 August

20. Reichel, MP and Ellis, JT (2001)

A review of neosporosis in Australasia

Neospora 2001 Symposium, Edinburgh, 13-16 September

4. BIBLIOGRAPHY

1. **Anderson ML, Palmer CW, Thurmond MC, Picanso JP, Blanchard PC, Breitmeyer RE, Layton AW, McAllister M, Daft B, Kinde H, Read DH, Dubey JP, Conrad PA, Barr BC**. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. *Journal of the American Veterinary Medical Association* 207,1206-1210,1995

2. **Anonymous.** Principles of validation of diagnostic assays for infectious diseases. *Manual of Standards for Diagnostic Tests and Vaccines*. Paris: Office International des Epizooties, 1996:8-15. vol 3).

3. **Atkinson R, Harper PA, Reichel MP, Ellis JT**. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. *Parasitology Today* 16,110-4,2000a

4. **Atkinson RA, Cook RW, Reddacliff LA, Rothwell J, Broady KW, Harper P, Ellis JT**. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a dairy cattle herd. *Australian Veterinary Journal* 78,262-6,2000b

5. **Bailey K**. Naturally acquired *Brucella ovis* infection in a deer. *Surveillance* 24,1997

6. **Barber JS, Gasser RB, Ellis J, Reichel MP, McMillan D, Trees AJ**. Prevalence of antibodies to *Neospora caninum* in different canid populations. *Journal of Parasitology* 83,1056-1058,1997a

7. **Barber JS, Trees AJ**. Clinical aspects of 27 cases of neosporosis in dogs. *Veterinary Record* 139,439-443,1996

8. **Barber JS, Trees AJ, Johnson AM**. Naturally occurring vertical transmission of *Neospora caninum* in dogs. *10th International Congress of Protozoology* 28,57-64,1998

9. **Barber JS, vanHam L, Polis I, Trees AJ**. Seroprevalence of antibodies to *Neospora caninum* in Belgian dogs. *Journal of Small Animal Practice* 38,15-16,1997b

10. **Barr BC, Anderson ML, Sverlow KW, Conrad PA**. Diagnosis of bovine fetal Neospora infection with an indirect fluorescent antibody test. *Veterinary Record* 137,611-613,1995

11. **Baszler TV, Adams S, Vander-Schalie J, Mathison BA, Kostovic M**. Validation of a Commercially Available Monoclonal Antibody-Based Competitive-Inhibition Enzyme-Linked Immunosorbent Assay for Detection of Serum Antibodies to *Neospora caninum* in Cattle. *Journal of Clinical Microbiology* 39,3851-7,2001

12. **Beier D, Siakkou H**. A comparison of serological tests for the diagnosis of enzootic bovine leukosis and eradication of infection from a large herd. *Tierarztliche Umschau* 49,356-360,1994

13. **Bergeron N, Fecteau G, Paré J, Martineau R, Villeneuve A**. Vertical and horizontal transmission of *Neospora caninum* in dairy herds in Quebec. *Cananadian Veterinary Journal* 41,464-7,2000

14. **Bjerkås I, Mohn SF, Presthus J**. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Zeitschrift für Parasitenkunde* 70,271-274,1984

15. **Björkman C, Alenius S, Manuelsson U, Uggla A**. *Neospora caninum* and bovine virus diarrhoea virus infections in Swedish dairy cows in relation to abortion. *The Veterinary Journal* 159,201-6,2000

16. **Björkman C, Holmdahl OJM, Uggla A**. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. *Veterinary Parasitology* 68,251-260,1997

17. **Björkman C, Johansson O, Stenlund S, Holmdahl OJM, Uggla A**. *Neospora* species infection in a herd of dairy cattle. *Journal of the American Veterinary Medical Association* 208,1441,1996

18. **Björkman C, Lunden A, Uggla A**. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in Swedish dogs. *Acta Veterinaria Scandinavica* 35,445-447,1994

19. **Boulton JG, Gill PA, Cook RW, Fraser GC, Harper PAW, Dubey JP**. Bovine *Neospora* abortion in north-eastern New South Wales. *Australian Veterinary Journal* 72,119-120,1995

20. **Brownlie J, Clarke MC, Hooper LB, Bell GD**. Protection of the bovine fetus from bovine viral diarrhoea virus by means of a new inactivated vaccine. *Veterinary Record* 137,58-62,1995

21. **Burgess GW**. Ovine contagious epididymitis: a review. *Veterinary Microbiology* 7,551-575,1982

22. **Burton L, Allen G, Hayes D, Pfeiffer D, Morris R**. A novel approach to disease control. An industry operated programme for bovine leukaemia virus in New Zealand. 8th Symposium of the International Society for Veterinary Epidemiology and Economics. Paris: International Society for Veterinary Epidemiology and Economics:08.08.1-3 1997

23. **Chiodini RJ, Van Kruiningen HJ, Merkal RS**. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Veterinarian* 74,218-262,1984

24. **Cole RA, Lindsay DS, Blagburn BL, Dubey JP**. Vertical transmission of *Neospora caninum* in mice. *Journal of Parasitology* 81,730-732,1995

25. **Collery PM**. Neospora abortion in cattle in Ireland. *Veterinary Record* 136,595,1995

26. **Conrad PA, Sverlow K, Anderson M, Rowe J, BonDurant R, Tuter G, Breitmeyer R, Plamer C, Thurmond M, Ardans A, Dubey JP, Duhamel G, Barr B**. Detection of serum antibody response in cattle with natural or experimental Neospora infections. *Journal of Veterinary Diagnostic Investigation* 5,572-578,1993

27. **Conraths FJ, Bauer C, Becker W**. Detection of antibodies to *Neospora caninum* in cows from Hessian farms with abortions and fecundity problems. *Deutsche Tierarztliche Wochenschrift* 103,221-224,1996

28. **Cox BT, Reichel MP, Griffiths LM**. Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study. *New Zealand Veterinary Journal* 46,28-31,1998

29. **Davison HC, French NP, Trees AJ**. Herd-specific and age-specific seroprevalence of *Neospora caninum* in 14 British dairy herds. *Veterinary Record* 144,547-550,1999a

30. **Davison HC, Otter A, Trees AJ**. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *International Journal for Parasitology* 29,1683-9,1999b

31. **Dijkstra T, Barkema HW, Eysker M, Hesselink JW, Wouda W**. Natural transmission routes of *Neospora caninum* between farm dogs and cattle. *Veterinary Parasitology* 105,99-104,2002a

32. **Dijkstra T, Barkema HW, Hesselink JW, Wouda W**. Point source exposure of cattle to *Neospora caninum* consistent with periods of common housing and feeding and related to the introduction of a dog. *Veterinary Parasitology* 105,89-98,2002b

33. **Dubey JP**. Neosporosis - a newly recognized protozoal infection. *Comparative Pathology Bulletin* 24,4-6,1992

34. **Dubey JP**. Recent advances in *Neospora* and neosporosis. *Veterinary Parasitology* 84,349-367,1999

35. **Dubey JP, Barr BC, Barta JR, Bjerkas I, Bjorkman C, Blagburn BL, Bowman DD, Buxton D, Ellis JT, Gottstein B, Hemphill A, Hill DE, Howe DK, Jenkins MC, Kobayashi Y, Koudela B, Marsh AE, Mattsson JG, McAllister MM, Modry D, Omata Y, Sibley LD, Speer CA, Trees AJ, Uggla A, Upton SJ, Williams DJ, Lindsay DS**. Redescription of *Neospora caninum* and its differentiation from related coccidia. *International Journal for Parasitology* 32,929-46,2002

36. **Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A**. Newly recognized fatal protozoan disease of dogs. *Journal of the American Veterinary Medical Association* 192,1269-1285,1988

37. **Dubey JP, Hartley WJ, Lindsay DS**. Congenital *Neospora caninum* infection in a calf with spinal cord anomaly. *Journal of the American Veterinary Medical Association* 197,1043-1044,1990a

38. **Dubey JP, Hartley WJ, Lindsay DS, Topper MJ**. Fatal congenital *Neospora caninum* infection in a lamb. *Journal of Parasitology* 76,127-130,1990b

39. **Dubey JP, Jenkins MC, Adams DS, McAllister MM, AndersonSprecher R, Baszler TV, Kwok OCH, Lally NC, Björkman C, Uggla A**. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. *Journal of Parasitology* 83,1063-1069,1997

40. **Dubey JP, Leathers CW, Lindsay DS**. *Neospora caninum*-like protozoon associated with fatal myelitis in newborn calves. *Journal of Parasitology* 75,146-148,1989

41. **Dubey JP, Liddell S, Mattson D, Speert CA, Howe DK, Jenkins MC**. Characterization of the Oregon isolate of *Neospora hughesi* from a horse. *Journal of Parasitology* 87,345-53,2001

42. **Dubey JP, Lindsay DS**. Gerbils (*Meriones unguiculatus*) are highly susceptible to oral infection with *Neospora caninum* oocysts. *Parasitology Research* 86,165-8,2000

43. **Dubey JP, Lindsay DS**. *Neospora caninum* induced abortion in sheep. *Journal of Veterinary Diagnostic Investigation* 2,230-233,1990

44. **Dubey JP, Lindsay DS**. A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology* 67,1-59,1996

45. **Dubey JP, Lindsay DS**. Transplacental *Neospora caninum* infection in dogs. *American Journal of Veterinary Research* 50,1578-1579,1989

46. **Dubey JP, Metzger FL, Jr., Hattel AL, Lindsay DS, Fritz DL**. Canine cutaneous neosporosis: clinical improvement with clindamycin. *Veterinary Dermatology* 6,37-43,1995

47. **Dyer RM, Jenkins MC, Kwok OC, Douglas LW, Dubey JP**. Serologic survey of *Neospora caninum* infection in a closed dairy cattle herd in Maryland: risk of serologic reactivity by production groups. *Veterinary Parasitology* 90,171-81,2000

48. **Ellis JT**. Polymerase chain reaction approaches for the detection of *Neospora caninum* and *Toxoplasma gondii*. *International Journal for Parasitology* 28,1053-1060,1998

49. **Ellis JT, Morrison DA, Liddell S, Jenkins MC, Mohammed OB, Ryce C, Dubey JP**. The genus Hammondia is paraphyletic. *Parasitology* 118,357-362,1999

50. **Gasser RB, Edwards G, Cole RA**. Neosporosis in a dog. *Australian Veterinary Practitioner* 23,190-193,1993

51. **Gennari SM, Yai LEO, D'Áuria SNR, Cardoso SMS, Kwok OCH, Jenkins MC, Dubey JP**. Occurrence of *Neospora caninum* antibodies in sera from dogs of the city of São Paulo, Brazil. *Veterinary Parasitology* 106,177-179,2002

52. **Gondim LFP, Gao L, McAllister MM**. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and *in vitro* isolation from cattle. *Journal of Parasitology* in press,2002

53. **Gottstein B, Hentrich B, Wyss R, Thur B, Busato A, Stärk KDC, Muller N**. Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. *International Journal for Parasitology* 28,679-691,1998

54. **Greig B, Rossow KD, Collins JE, Dubey JP**. *Neospora caninum* pneumonia in an adult dog. *Journal of the American Veterinary Medical Association* 206,1000-1001,1995

55. **Greiner M, Sohr D, Göbel P**. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *Journal of Immunological Methods* 185,123-132,1995

56. **Hay WH, Shell LG, Lindsay DS, Dubey JP**. Diagnosis and treatment of *Neospora caninum* infection in a dog. *Journal of the American Veterinary Medical Association* 197,87-89,1990

57. **Hayes D, Hoggard G, Reichel M, Burton L, Pfeiffer D, Morris R**. The suitability of milk samples from a national milk analysis centre for bovine leukaemia virus surveillance. 8th Symposium of the International Society for Veterinary Epidemiology and Economics. Paris: International Society for Veterinary Epidemiology and Economics:11.C.14 1997

58. **Heydorn AO, Mehlhorn H**. *Neospora caninum* is an invalid species name: an evaluation of facts and statements. *Parasitology Research* 88,175-184,2002

59. **Hietala SK**. The options in diagnosing ruminant paratuberculosis. *Veterinary Medicine* 11,1122-1131,1992

60. **Hilbink F, Penrose M**. A comparison of two enzyme linked immunosorbent assays for enzootic bovine leucosis serology. *New Zealand Veterinary Journal* 38,80-81,1990

61. **Hilbink F, West DM, de Lisle GW, Kittelberger R, Hosie BD, Hutton J, Cooke MM, Penrose M**. Comparison of a complement fixation test, a gel diffusion test and two absorbed and unabsorbed ELISAs for the diagnosis of paratuberculosis in sheep. *Veterinary Microbiology* 41,107-116,1994

62. **Hill DE, Liddell S, Jenkins MC, Dubey JP**. Specific detection of *Neospora caninum* oocysts in fecal samples from experimentally-infected dogs using the polymerase chain reaction. *Journal of Parasitology* 87,395-8,2001

63. **Hoskins JD, Bunge MM, Dubey JP, Duncan DE**. Disseminated infection with *Neospora caninum* in a ten-year-old dog. *Cornell Veterinarian* 81,329-334,1991

64. **Jacobs RM, Song Z, Poon H, Heeney JL, Taylor JA, Jefferson B, Vernau W, Valli VEO**. Proviral detection and serology in bovine leukemia virus-exposed normal cattle and cattle with lymphoma. *Canadian Journal of Veterinary Research* 56,339-348,1992

65. **Jardine JE, Last RD**. The prevalence of neosporosis in aborted bovine foetuses submitted to the Allerton Regional Veterinary Laboratory. *Onderstepoort Journal of Veterinary Research* 62,207-209,1995

66. **Jark U, I. R, Franz B, Gerlach G-F, Beyerbach M, Franz B**. Development of an ELISA technique for serodiagnosis of bovine paratuberculosis. *Veterinary Microbiology* 51,189-198,1997

67. **Kittelberger R, Reichel MP**. Evaluation of electrophoretic immunoblotting for *Brucella ovis* infection in deer using ram and deer serum. *New Zealand Veterinary Journal* 46,32-34,1998

68. **Klein F, Hietala SK, Berthet H, Very P, Gradinaru D**. *Neospora caninum*: serological investigation of abortion in Normandy and Charolais cattle. *Point Veterinaire* 28,1283-1286,1997

69. **Klintevall K, Ballagi Pordany A, Naslund K, Belak S**. Bovine leukaemia virus: rapid detection of proviral DNA by nested PCR in blood and organs of experimentally infected calves. *Veterinary Microbiology* 42,191-204,1994

70. **Klintevall K, Naslund K, Svedlund G, Hajdu L, Linde N, Klingeborn B**. Evaluation of an indirect ELISA for the detection of antibodies to bovine leukaemia virus in milk and serum. *Journal of Virological Methods* 33,319-333,1991

71. **Lindsay DS**. Neosporosis: an emerging protozoal disease of horses. *Equine Vet J* 33,116-8,2001

72. **Lindsay DS, Dubey JP, Upton SJ, Ridley RK**. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. *Journal of the Helminthological Society of Washington* 57,86-88,1990

73. **Littlejohns IR, Horner G**. Incidence, epidemiology and control of bovine pestivirus infections in Australia and New Zealand. *Revue scientifique et techniques Office Internationale des Epizooties* 9,195-205,1990

74. **MacDiarmid SC**. Bovine brucellosis eradication in New Zealand. *Surveillance Wellington* 21,18-21,1994

75. **Mainar-Jaime RC, Berzal-Herranz B, Arias P, Rojo-Vazquez FA**. Epidemiological pattern and risk factors associated with bovine viral-diarrhoea virus (BVDV) infection in a non-vaccinated dairy-cattle population from the Asturias region of Spain. *Preventive Veterinary Medicine* 52,63-73,2001

76. **Mammerickx M**. Advantages and disadvantages of diagnostic tests for EBL. *13th conference of the O.I.E* African swine fever,enzootic bovine leukosis, Madrid, 27-30 September 1988. 1989, 113-127,1989

77. **Mammerickx M, Portetelle D, Burny A**. Application of an enzyme-linked immunosorbent assay (ELISA) involving monoclonal antibody for detection of BLV antibodies in individual or pooled bovine milk samples. *Zentralblatt fur Veterinarmedizin, B* 32,526-533,1985

78. **McAllister MM**. *Neospora caninum*: its oocysts and its identity: an opinion. *Parasitology Research* 86,860,2000

79. **Mehlhorn H, Heydorn AO**. *Neospora caninum*: is it really different from *Hammondia heydorni* or is it a strain of *Toxoplasma gondii*? An opinion. *Parasitology Research* 86,169-78,2000

80. **Miller JM, Maaten MJvd, Van der Maaten MJ**. A review of methods to control bovine leukosis. *Proceedings of the United States Animal Health Association* 86,119-125,1982

81. **Milner AR, Mack WN, Coates KJ, Hill J, Gill I, Sheldrick P**. The sensitivity and specificity of a modified ELISA for the diagnosis of Johne's disease from a field trial in cattle. *Veterinary Microbiology* 25,193-198,1990

82. **Moen AR, Wouda W, Mul MF, Graat EAM, vanWerven T**. Increased risk of abortion following *Neospora caninum* abortion outbreaks: A retrospective and prospective cohort study in four dairy herds. *Theriogenology* 49,1301-1309,1998

83. **Munday BL, Dubey JP, Mason RW**. *Neospora caninum* infection in dogs. *Australian Veterinary Journal* 67,76-77,1990

84. **Obendorf DL, Murray N, Veldhuis G, Munday BL, Dubey JP**. Abortion caused by neosporosis in cattle. *Australian Veterinary Journal* 72,117-118,1995

85. **Odin M, Dubey JP**. Sudden death associated with *Neospora caninum* myocarditis in a dog. *Journal of the American Veterinary Medical Association* 203,831-833,1993

86. **Paré J, Hietala SK, Thurmond MC**. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. *Journal of Veterinary Diagnostic Investigation* 7,352-359,1995a

87. **Paré J, Hietala SK, Thurmond MC**. Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. *Journal of Veterinary Diagnostic Investigation* 7,273-275,1995b

88. **Paré J, Thurmond MC, Hietala SK**. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. *Canadian Journal of Veterinary Research* 60,133-139,1996

89. **Paré J, Thurmond MC, Hietala SK**. Congenital *Neospora* infection in dairy cattle. *Veterinary Record* 134,531-532,1994

90. **Patitucci AN, Alley MR, Jones BR, Charleston WAG**. Protozoal encephalomyelitis of dogs involving *Neospora caninum* and *Toxoplasma gondii* in New Zealand. *New Zealand Veterinary Journal* 45,231-235,1997

91. **Patitucci AN, Charleston WAG, Alley MR, O'Connor RJ, Pomroy WE**. Serological study of a dairy herd with a recent history of Neospora abortion. *New Zealand Veterinary Journal* 47,28-30,1999

92. **Pfeiffer DU, Williamson NB, Reichel MP**. Long-term Serological Monitoring as a Tool for Epidemiological Investigation of *Neospora caninum* Infection in a New Zealand Dairy Herd. IX Symposium. Breckenridge, USA: International Society Veterinary Epidemiology and Economics:524 2000

93. **Pfeiffer DU, Williamson NB, Reichel MP, Wichtel JJ, Teague WR**. A longitudinal study of *Neospora caninum* infection on a dairy farm in New Zealand. *Preventive Veterinary Medicine* 54,11-24,2002

94. **Reichel MP**. *Neospora caninum* infections in Australia and New Zealand. *Australian Veterinary Journal* 78,258-261,2000

95. **Reichel MP**. Prevalence of Neospora antibodies in New Zealand dairy cattle and dogs. *New Zealand Veterinary Journal* 46,38,1998

96. **Reichel MP, Drake JM**. The diagnosis of *Neospora* abortions in cattle. *New Zealand Veterinary Journal* 44,151-154,1996

97. **Reichel MP, Ellis JT**. Control options for *Neospora caninum* infections in cattle - current state of knowledge. *New Zealand Veterinary Journal* 50,86-92,2002

98. **Reichel MP, Kittelberger R, Penrose ME, Meynell RM, Cousins D, Ellis T, Mutharia LM, Sugden EA, Johns A, de Lisle GW**. Comparison of serological tests, immunoblot and faecal culture for the detection of *M. avium* spp. *paratuberculosis* infection in cattle and analysis of the antigens involved. *Veterinary Microbiology* 66,135-150,1999a

99. **Reichel MP, Pfeiffer DU**. An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle. *Veterinary Parasitology*,107,197-207,2002

100. **Reichel MP, Ross G, Drake J, Jowett JH**. Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams. *New Zealand Veterinary Journal* 47,1999b

101. **Reichel MP, Tham KM, Barnes S, Kittelberger R**. Evaluation of alternative methods for the detection of Bovine Leukaemia virus in cattle. *New Zealand Veterinary Journal* 46,140-146,1998a

102. **Reichel MP, Thornton RN, Morgan PL, Mills RJM, Schares G**. Neosporosis in a pup. *New Zealand Veterinary Journal* 46,106-110,1998b

103. **Reichel MP, West DM**. *Brucella ovis* accreditation in New Zealand. *Surveillance* 24,19-20,1997

104. **Ridge SE, Morgan IR, Sockett DC, Collins MT, Condron RJ, Skilbeck NW, Webber JJ**. Comparison of the Johne's absorbed EIA and the complement-fixation test for the diagnosis of Johne's disease in cattle. *American Veterinary Journal* 68,253-257,1991

105. **Ruehlmann D, Podell M, Oglesbee M, Dubey JP**. Canine neosporosis: a case report and literature review. *Journal of the American Animal Hospital Association* 31,174-183,1995

106. **Sawada M, Park CH, Kondo H, Morita T, Shimada A, Yamane I, Umemura T**. Serological survey of antibody to *Neospora caninum* in Japanese dogs. *Journal of Veterinary Medical Science* 60,853-854,1998

107. **Schares G, Conraths FJ, Reichel MP**. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. *International Journal for Parasitology* 29,1669-1676,1999

108. **Schares G, Peters M, Wurm R, Barwald A, Conraths FJ**. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Veterinary Parasitology* 80,87-98,1998

109. **Schares G, Rauser M, Sondgen P, Rehberg P, Barwald A, Dubey JP, Edelhofer R, Conraths FJ**. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. *International Journal for Parasitology* 30,1123-30,2000

110. **Sockett DC, Conrad TA, Thomas CB, Collins MT**. Evaluation of four serological tests for bovine paratuberculosis. *Journal of Clinical Microbiology* 30,1134-1139,1992

111. **Tennent-Brown B, Pomroy WE, Reichel MP, Gray P, Marshall T, Moffat P, Rogers M, Driscoll G, Reeve O, Ridler A, Ritvanen S**. Prevalence of *Neospora* antibodies in beef cattle in New Zealand. *New Zealand Veterinary Journal* 48,149-150,2000

112. **Thornton R**. Bovine abortion diagnoses in 1995. *Surveillance Wellington* 23,21-22,1996

113. **Thornton R**. Bovine abortions - laboratory diagnosis 1991. *Surveillance Wellington* 19,24,1992

114. **Thornton RN, Gajadhar A, Evans J**. *Neospora* abortion epidemic in a dairy herd. *New Zealand Veterinary Journal* 42,190-191,1994

115. **Thornton RN, Thompson EJ, Dubey JP**. Neospora abortion in New Zealand cattle. *New Zealand Veterinary Journal* 39,129-133,1991

116. **Thurmond MC, Hietala SK**. Effect of *Neospora caninum* infection on milk production in first-lactation dairy cows. *Journal of the American Veterinary Medical Association* 210,672,1997

117. **Trees AJ, Guy F, Low JC, Roberts L, Buxton D, Dubey JP**. Serological evidence implicating Neospora species as a cause of abortion in British cattle. *Veterinary Record* 134,405-407,1994

118. **Trees AJ, Guy F, Tennant BJ, Balfour AH, Dubey JP**. Prevalence of antibodies to *Neospora caninum* in a populaton of urban dogs in England. *Veterinary Record* 132,125-126,1993

119. **Williams DJL, McGarry J, Guy F, Barber J, Trees AJ**. Novel ELISA for detection of Neospora-specific antibodies in cattle. *Veterinary Record* 140,328-331,1997

120. **Woods LW, Anderson ML, Swift PK, Sverlow KW**. Systemic neosporosis in a California black-tailed deer (*Odocoileus hemionus columbianus*). *Journal of Veterinary Diagnostic Investigation* 6,508-510,1994

121. **Worthington RW, Weddell W, Penrose ME**. A comparison of three serological tests for the diagnosis of *B. ovis* infection in rams. *New Zealand Veterinary Journal* 32,58-60,1984

122. **Wouda W, Dijkstra T, Kramer AMH, van Maanen C, Brinkhof JMA**. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. *International Journal for Parasitology* 29,1677-1682,1999

123. **Wouda W, Moen AR, Schukken YH**. Abortion risk in progeny of cows after a *Neospora caninum* epidemic. *Theriogenology* 49,1311-1316,1998

5. APPENDICES

List of collaborator contributions to the jointly-authored papers:

In reference to the paper:

Comparison of serological tests, immunoblot and faecal culture for the detection of *M. avium* spp. *paratuberculosis* infection in cattle and analysis of the antigens involved.

Reichel, MP; Kittelberger, R; Penrose ME, Meynell, RM; Cousins, D; Ellis, T; Mutharia, LM; Sugden, EA; Johns, A and de Lisle, GW

Veterinary Microbiology **66**, 135-150 (1999)

This paper was the result of collaboration between a number of parties in a number of countries. MPR coordinated (and directed parts of) the project, procuring sera from Western Australia (D Cousins and T Ellis) and from infected cattle in Taranaki (A Johns). An ELISA for the serological diagnosis of Johne's disease was being validated by MPR, with technical assistance from M Penrose. GW deLisle conducted the faecal culture on infected New Zealand cattle, R Kittelberger the immunoblot analyses.

The resulting manuscript (incl. the statistical analyses of data) was largely written by MPR and submitted to the journal.

Dr R Kittelberger

Dr GW de Lisle

Mrs M E Penrose

Michael P Reichel

In reference to the paper:

Performance of an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection in rams

Reichel, MP; Ross, G; Drake Jo and Jowett, JH

New Zealand Veterinary Journal **47**, 43-46 (1999)

This paper was conceived and written by MPR with technical assistance from, mainly Gail Ross (and Jo Drake) who were working under his directions at the time as serology technicians. John Jowett performed the statistical analyses as directed by MPR.

Gail Ross

Jo Drake

Michael P Reichel

In reference to the paper:

Evaluation of electrophoretic immunoblotting for *Brucella ovis* infection in deer using ram and deer serum

Kittelberger, R and Reichel, MP

New Zealand Veterinary Journal **46**, 32-34 (1998)

This paper developed as a collaboration between the two authors from the need to adapt the established immunoblotting technique for *B ovis* infection in rams for deer. *B ovis* infection in deer was hitherto unknown in New Zealand and the serological test available for the diagnosis in rams had to be validated for deer.

The work was jointly conceived and planned, the immunoblotting work was carried out by RK. MPR added data on the serological testing of a serumbank of deer sera and had input into the writing of the resulting manuscript.

Dr R Kittelberger Michael P Reichel

In reference to the paper:

Evaluation of alternative methods for the detection of Bovine Leukaemia Virus in cattle.

Reichel, MP; Tham, KM; Barnes, S and Kittelberger, R

New Zealand Veterinary Journal **46**, 140-146 (1998)

This study was initiated by MPR, who oversaw the overall project.

ELISA-analyses were carried out on serum samples selected by MPR from New Zealand cattle and obtained from OIE reference laboratories. Kok-Mun Tham conducted the PCR-analyses of corresponding blood samples (with S Barnes assisting), Dr Kittelberger did the immunoblot analyses. MPR did the statistical analyses and wrote the main part of the resulting manuscript, incl. discussion and submitted it to the journal.

Dr K Tham

Dr R Kittelberger

Michael P Reichel

In reference to the paper:

Prevalence of serum antibodies to *Neospora caninum* in different canid populations.

Barber, JS; Gasser, RB; Ellis, J; Reichel, MP; McMillan, D and Trees, AJ

Journal of Parasitology **83**, 1056-1058 (1997)

MPR's contribution to this paper was the collection of 500 dog sera from the Falkland Islands and editorial input into the manuscript.

Dr JS Barber

Assoc. Prof RB Gasser

Assoc. Prof. J Ellis

Dr D McMillan

Prof Sandy Trees

Michael P Reichel

In reference to the paper:

Neosporosis in a pup

Reichel, MP; Thornton, RN; Morgan, PL; Mills, RJM and Schares G.

New Zealand Veterinary Journal **46**, 106-110 (1998)

MPR initiated this clinical report when the serum sample from the clinically affected pup was submitted to my laboratory. MPR co-ordinated the input from the collaborators (incl. follow-up of the serological status of dam and littermate), selected the test systems and sent tissue blocks to Dr Gereon Schares to Germany for immunohistochemistry and PCR. Dr Ron Thornton provided the pathology and histology report, Drs Morgan and Mills were the clinicians dealing with the clinical aspects of the case, incl. collection of sera.

MPR then wrote, in large parts, the manuscript for publication, incl. discussion and conclusion.

Dr Ron N. Thornton

Dr PL Morgan

Dr RJM Mills

Dr G Schares

Michael P Reichel

In reference to the paper:

Progress in the Serodiagnosis of *Neospora caninum* Infections in Cattle

Atkinson, R; Harper PAW; Reichel, MP and Ellis JT

Parasitology Today **16**, 3, 110-114 (2000)

To this review paper, MPR contributed his practical knowledge of diagnostic serology and *N caninum* serological assays. He also made further comments on various aspects of *N caninum* serodiagnosis (interpretation of results, diagnostic sensitivity/specificity) and contributed to the literature review.

R Atkinson

Dr PAW Harper

Assoc. Prof JT Ellis

Michael P Reichel

In reference to the paper:

The diagnosis of *Neospora* abortions in cattle.

Reichel, MP and Drake, JM

New Zealand Veterinary Journal **44**, 151-154 (1996)

This study was conceived by me: the Neospora-ELISA was developed by me, the sera which formed part of the serological comparison were chosen by me and the pathological information collected from the submitting diagnostic laboratories was collected by me.

I carried out all the statistical analyses and wrote the manuscript for publication.

Ms Jo Drake was working as a technician for me at the time and performed the Indirect Fluorescent antibody test (IFAT) on the sera used in the study.

Jo Drake Michael P Reichel

In reference to the paper:

An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle

Reichel,M.P. and Pfeiffer,D.U

Veterinary Parasitology **107**, 197-207 (2002)

MPRs contribution has been to develop the idea for this scientific contribution and to conduct it in conjunction with the collaborator. MPR contributed the serological testing of the serum samples and wrote major parts of the resultant publication, submitted the manuscript and guided it through the refereeing process.

Prof Dirk Pfeiffer contributed the ROC analyses and pertinent comments to the manuscript.

Date: Date:

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Prof Dirk U Pfeiffer Michael P Reichel

In reference to the paper:

Prevalence of *Neospora* antibodies in beef cattle in New Zealand

Tennent-Brown, B; Pomroy, WE; Reichel, MP; Gray, PL; Marshall, TS; Moffat, PA; Rogers, M; Driscoll, VA; Reeve, OF; Ridler, AL and Ritvanen S (2000)

New Zealand Veterinary Journal **48**, 121-122 (2000)

This publication developed as a result of long-standing collaboration with Dr WE Pomroy of Massey University on the epidemiology of *Neospora caninum*. The two of us (WEP and MPR) discussed and conceived the project, and Dr Pomroy coordinated the collection of sera and data by veterinary students.

I tested the sera for anti- *Neospora caninum* antibodies and Dr Pomroy and MPR wrote and edited the resulting paper.

Dr WE Pomroy

Anne Ridler

Michael P Reichel

In reference to the paper:

Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: A case study.

Cox, BT; Reichel, MP and Griffiths, LM

New Zealand Veterinary Journal **46**, 28-31 (1998)

In the above study my contribution has been developing the idea for this study in conjunction with the collaborators and planning the experimental design. I further contributed the serological testing of the serum samples, the coordination of the sequential bleeds and then wrote the major parts of the resultant publication (incl. statistical calculations), discussion and conclusions.

Drs Brian Cox and Lewis Griffiths initiated the study after the initial abortion outbreak; Dr Cox provided the pathology and histopathological information, Dr Griffiths obtained the epidemiological background information and the serum samples from the dairy cattle tested.

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Dr Brian T Cox

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Dr Lewis M Griffiths

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Michael P Reichel

In reference to the paper:

Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand

Schares, G; Conraths, FJ and Reichel, MP.

International Journal of Parasitology **29**, 1669-1676 (1999)

This publication developed as a result of a scholarship I received from the NZ Ministry of Research, Science and Technology which allowed me to travel to Wusterhausen in Germany.

It was my idea to collect the sera from all cattle on a farm after a *Neospora caninum* abortion outbreak occurred, including the sera of the heifers and calves grazed off the property, to determine their serological status and any association with their familial relationships. Serological testing, in IFAT and ELISA was carried out in New Zealand.

I was also at my suggestion that I took the sera with me to Germany and tested them there in Immunoblot. I also collected the epidemiological information on the abortion outbreak, and information on the familial relationship of the cattle on the property.

Discussions about the results and the conclusions took place at the end of my stay in Germany and continued via Email for the following years until completion of the manuscript.

Drs Schares and Conraths, Wusterhausen provided additional serological testing (two ELISAs) and Dr Schares completed the final manuscript and statistical analyses of data.

Dr Gereon Schares

PD Dr Franz J Conraths

Michael P Reichel

In reference to the paper:

A longitudinal study of *Neospora caninum* infection on a dairy farm in New Zealand

Pfeiffer,D.U. , Williamson,N.B., Reichel,M.P., Wichtel,J.J. and Teague,W.R.

Preventive Veterinary Medicine **54**, 11-24 (2002)

This major sero-epidemiological study in New Zealand was the result of a collaborative effort of the "Neospora"-Research group based at Massey University in New Zealand.

MPR contributed the serological testing of the serum samples, and equally contributed to the general planning of the study, the sequential bleeds and further work. MPR was then a major contributor to the writing of the resultant publication, discussion and conclusions and revision.

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Prof Dirk U Pfeiffer Prof Norm B Williamson

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Michael P Reichel Dr Jeff J Wichtel

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Dr Bill R Teague

In reference to the paper:

Control options for *Neospora caninum* infections in cattle – current state of knowledge

Reichel,M.P. and Ellis, J.T.

New Zealand Veterinary Journal **50**, 86-92 (2002)

This review and the analysis of the pertinent literature was jointly undertaken by the two authors, and the manuscript written in large parts by MPR. MPR submitted the paper and guided it through the refereeing process.

Prof John Ellis provided guidance throughout the process and valuable comments to the manuscript.

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Prof John T Ellis Michael P Reichel