Detection of *Chlamydia trachomatis* mRNA using digital PCR as a more accurate marker of viable organism

Running title: Detecting viable *Chlamydia trachomatis* infections

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Introduction

Chlamydia trachomatis (CT) is the most commonly reported bacterial sexually transmitted infection (STI) in developed nations [1]. In 2015 the World Health Organisation (WHO) estimated the global prevalence of CT to be 4.2% among 15 to 49-year-old men and women [2]. CT is asymptomatic in over 80% of cases among women, and in about 10% of cases, it can lead to pelvic inflammatory disease (PID), which increases the risk of ectopic pregnancy or infertility [3-6].

Spontaneous resolution of chlamydia has been described in the literature with reports of up to 44% of infections clearing between diagnosis and treatment [7-10]. However, the key limitation of these reports is that they all used DNA or rRNA based amplification tests to diagnose CT and these assays do not differentiate between viable and non-viable DNA. Given the sensitivity of NAAT assays and the fact that they do not require viable organism to diagnose an infection, it is possible that a percentage of these initial diagnoses represented non-viable DNA elements rather than viable infectious particles, thereby over-estimating spontaneous resolution.

To overcome potential miss-classification bias, the detection of mRNA in addition to DNA could allow the differentiation between viable infection and non-viable DNA. In this study, we modified a previously published CT mRNA detection (*omp2)* method from reverse transcriptase qPCR (RTqPCR) to digital PCR (dPCR), (allowing for direct quantification of the target molecule using microfluidic generated droplets and capillary flow fluorescence detection) [11] and aimed to evaluate it using a sample of CT DNA PCR positive women to differentiate between viable and non-viable DNA. Previous studies have shown that dPCR has better sensitivity with as much as a 50-fold improvement on current real time PCR assays and provides quantitative bacterial load data on every sample [12]. We then assessed dPCR for detection of chlamydial mRNA and DNA versus DNA detection alone.

Methods

This study included two components:

1) In vitro control experiment

This experiment aimed to establish the longevity of CT DNA post antibiotic exposure and to assess the ability for dPCR to detect mRNA only. A single isolate of *C. trachomatis* genovar D was used to infect McCoy B cells at a multiplicity of infection (MOI) of 1.0. The infected cells were then treated with 0.250 µg/ml azithromycin (twice the MIC for this isolate), 4 h post infection and wells harvested at 46 h, 96 h and 144 h into RNAlater™

stabilization solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Also included was an infected and un-infected cell line with no azithromycin added, to act as positive and negative controls, respectively. The RTqPCR crossing threshold was then plotted over the azithromycin treatment in hours post infection and was then plotted as a line graph using Microsoft Excel 2013.

2) Evaluation of mRNA assay in a cohort of women diagnosed with chlamydia

Specimens from a sample of CT DNA positive women participating in the Australian Chlamydia Treatment Study (ACTS) were included in this study. ACTS was a cohort study that aimed to estimate chlamydia treatment failure in women treated with one gram azithromycin following diagnosis with urogenital CT [1]. To be eligible for ACTS, women were diagnosed with chlamydia using the Cobas® 4800 CT/NG test (Roche Molecular Diagnostics) according to the manufacturer's instructions; women were treated with one gram azithromycin and followed for up to 56 days, with weekly specimen collection for chlamydia load and genovar detection using real time PCR [1]. A total of 306 women were recruited and 36 tested chlamydia positive on or after day 28 of follow up. Specimens were available from 29 of these 36 women for this study and included self-collected vaginal swabs obtained at the time of recruitment (within the clinic) immediately prior to treatment with one gram azithromycin (visit 1). Follow up specimens were available from 26 women providing self-collected vaginal swabs obtained approximately seven days after treatment in the clinic (visit 2).

The vaginal swabs were swirled immediately in RNAlater™ stabilization solution and stored at -80°C. Samples stored in RNAlater™ stabilization solution were extracted and then half of the eluted total nucleic acid (TNA) was treated with DNase.

DNA extraction

All samples had 200µl aliquot extracted using the automated system, MagNA Pure 96 (Roche Applied Science, Germany), according to the manufacturer's instructions using the MagNA Pure 96 DNA and Viral NA Small Volume Kit. The TNA was then eluted in a final volume of 100µl in MagNA Pure 96 elution buffer. In addition, with samples from the clinical study, to assess sample and TNA extraction and sample adequacy, an aliquot was amplified by real time PCR for the human β-globin gene [13-15] (The McCoy B cells were excluded from this test).

Chlamydia genovar and bacterial load

All references to *CT* genovar and DNA bacterial load had a 5µl aliquot of each TNA sample and control, amplified by a series of real time PCR assays targeting the ompA gene of CT for determination of chlamydial genovar as described previously [16].

TNA DNase treatment

For each sample and control, 50µl of eluted TNA was treated with a DNase enzyme to degrade the eluted DNA. Using Ambion Turbo DNA-*free*™ Kit according to the manufacturer's instructions, 0.1 volume of 10x TURBO DNase buffer and 1U of TURBO DNase was added to the TNA elution and incubated for 30min at 37°C. Following this, 0.1 volume of DNase inactivation reagent was added and incubated for 5min at room temperature. The entire volume was then pelleted by centrifugation at 10, 000 x *g* for 1.5min and the supernatant collected for testing.

Messenger ribonucleic acid (mRNA) real time PCR detection

All references to the detection of CT mRNA using the RTqPCR detection method were detected using the published method [11] with the following modifications. A 3' spacer (3SpC3) was added to probe CtP1 to stop extension on CtR1. We used the following enzymes for detection: the Roche reverse transcription enzyme for the detection of RNA targets and Bioline SensiFAST enzyme for the detection of contaminating DNA targets; both enzymes were used as per the manufactures instructions.

Messenger ribonucleic acid (mRNA) digital PCR detection

All references to the detection of CT mRNA using the dPCR detection method used an adapted version of the published RTqPCR method to Bio-Rad's dPCR system using the advanced onestep reverse transcription enzyme as per the manufactures instructions. Any samples with ≤3 positive droplets were re-tested in triplicate, if the sample returned ≥4 positive droplets combined from the triplicate testing then this sample was assessed as positive. If <4 positive droplets were detected then the sample was called negative as per manufacture protocols.

Statistical analysis

We produced scatter plots, generated lines of best fit using linear regression and calculated Pearson correlation coefficients between the DNase treated and untreated TNA results for visit 1 and 2 separately. We then examined the concordances between RTqPCR and dPCR with DNA detection (see below for detail of assays). We calculated the percentage concordance, the agreement between the two assays using Cohen's Kappa and the number of infections that would be detected using the two assays. All analyses undertaken were performed using R 3.2.4.

Ethics approval

Ethical approval for this study was granted by the Alfred Hospital Ethics Committee (HREC No. 223/12) and the Southern Eastern Sydney Local Health District Human Research Ethics Committee (Southern Sector) (HREC No. 12/143).

Results

In vitro control experiment

CT infected McCoy cells treated with azithromycin were found to be mRNA negative by 46 h post treatment. However, DNA was detected up to 6 days post treatment (Figure 1).

Evaluation of mRNA assay in clinical samples of CT positive women

Using dPCR, there was a strong correlation between mRNA and DNA ($r=0.9$, $p<0.01$) (Figure 2) in the 29 available specimens at visit 1 (pre-treatment visit). Of these 29, 23 had both mRNA and DNA detected (79.3%). For 6 specimens at visit 1, only DNA was detected (20.7%;95%CI; 78.0, 39.7)

Of the 23 women who had both mRNA and DNA detected at visit 1, 20 provided samples for visit 2. There was poor correlation between mRNA and DNA at visit 2 (7 days post-azithromycin) ($r=0.14$, $p=0.55$). Only one had both mRNA and DNA detected ([12.09%; 95%CI: 10.40, 34.58]; 11 had neither mRNA nor DNA detected and eight had only DNA detected 42.11% (95%CI: 20.25, 66.50).

Concordance and agreement of mRNA RTqPCR and dPCR with DNA:

Concordance and agreement with DNA assay results were higher for dPCR (Kappa = 48.21% concordance 72.73%) than RTqPCR (Kappa = 37.98%, concordance 65.45%). Overall, an additional four specimens were detected as CT positive (7.3%) using dPCR compared with negative for qPCR (Table 1). The Cohen's Kappa for agreement between qPCR and dPCR was 84.93% (95%CI: 70.87, 98.99) (table 2). The limit of detection for qPCR and dPCR was established at 2500 and 200 copies of mRNA per swab, respectively. All samples had beta-globin detected using quantitative qPCR.

Discussion

We are the first to have developed a dPCR method to detect CT mRNA in self collected vaginal swabs and have demonstrated that mRNA becomes non-detectable within seven days following treatment with azithromycin. Using this assay, we found that approximately 20% of women who tested positive for chlamydia DNA were negative for chlamydia mRNA at visit 1 raising the question about whether these cases have spontaneously

resolved between initial diagnosis and treatment or whether the initial test result did not represent a viable infection.. We also found that if those diagnosed chlamydia DNA positive are re-tested too early following treatment, over 40% will still have DNA detected but no mRNA and are unlikely to represent viable infection. Our finding of approximately 20% mRNA negative is similar to those of Janssen and colleagues [17] who developed a viability PCR (V-PCR) assay to assess CT viability and found that 24% of vaginal swabs from women diagnosed CT PCR positive had non-viable DNA only. Unlike our assay which quantified mRNA as a marker of viable CT, their V-PCR assay consisted of propidium monoazide (PMA) treatment prior to DNA extraction followed by quantitative PCR targeting the ompA gene for the detection of CT DNA. Both mRNA and V-PCR methods rely on efficient degradation of non-cellular DNA, this is difficult to control when the following test is detecting DNA (V-PCR). However, we were able to utilize the differences between DNA and mRNA to asses this degradation in each sample, resulting in a more reliable detection of actively transcribing organisms.

When interpreting chlamydia test results, it is important to be mindful of the limitations when detecting DNA using NAAT. The in vitro study presented here showed that using NAAT targeting mRNA, viable bacteria can be detected and differentiated from non-viable DNA. We also show that within 46 h post azithromycin treatment, infected McCoy B cells at an MOI of 1.0 have been completely cleared of viable bacteria, while non-viable CT DNA is still present for up to six days. A DNA PCR test with a specificity of 99.5% in a population with a prevalence of about 4%, will lead to about 13% of test results being false positive (positive predictive value of 13%). Being able to differentiate between viable and non-viable infection could minimise unnecessary treatment and any psychosocial consequences of a positive chlamydia diagnosis. While we don't advocate using mRNA tests as an initial screening test, they may be of value as an ancillary test when there may be some concern over the accuracy of a NAAT test result, particularly in a low prevalence population.

By comparing the concordance of mRNA detection to DNA detection for both the published RTqPCR method and our modified dPCR method we found that dPCR would detect an additional 7% more cases of chlamydia than the published RTqPCR method in the detection of CT mRNA. The additional four samples positive for CT mRNA using dPCR had concentrations ranging between 205 and 2500 copies of mRNA per swab. Interestingly these four discrepant samples represented four out of the seven lowest concentrations of all detected samples. These results indicate a limit of detection for RTqPCR and dPCR as 2500 and 200 copies of mRNA per swab respectively. This was an expected result and has been reported in other studies [12] (although for different targets).

At visit 1 the there was a strong linear relationship between the DNA and mRNA (dPCR method) quantitation data that was highly significant ($p<0.001$). At visit 2 the linear relationship was lost suggesting that post antibiotic treatment there is a rapid and marked decline in detectable mRNA activity, a result which is not evident in the detection of DNA alone. This confirms earlier studies that have shown that DNA can last for several days to weeks following treatment and testing too early (within 3 weeks) following treatment with a DNA based test can lead to incorrect assumptions of treatment failure or re-infection [18].

Possible limitations of this study are that the RNAlater™ stabilization solution samples were collected and stored for up to 1 year before being tested for mRNA. Although all possible efforts were done to limit the amount of mRNA degradation we can't exclude this possibility. We also note that as the samples were self-collected swabs there could be considerable sampling variability; however previous studies have indicated this is an adequate collection method [19]. We found all samples had human beta-globin detected using a quantitative PCR demonstrating adequate sampling was performed. However, we did not perform any mRNA internal controls and therefore cannot exclude degradation of the mRNA post sample processing. Although, with our results simular those obtained by Janssen and colleagues, we are confident in our findings.

We conclude that by utilising our adapted dPCR method for the detection of CT mRNA we can accurately detect and quantify viable CT infections and discriminate this from non-viable DNA to a sensitivity of 200 copies of mRNA per swab. This study demonstrates that tests are available that can identify molecular markers of viable chlamydia DNA (specifically intra-cellular bacteria) as a marker of viable infection. Given increasing concerns about over-use of antibiotics and the potential psychosocial consequences of a positive chlamydia test result, consideration should be given to further development and use of mRNA tests as ancillary tools to detect viable chlamydia infections when there is any doubt.

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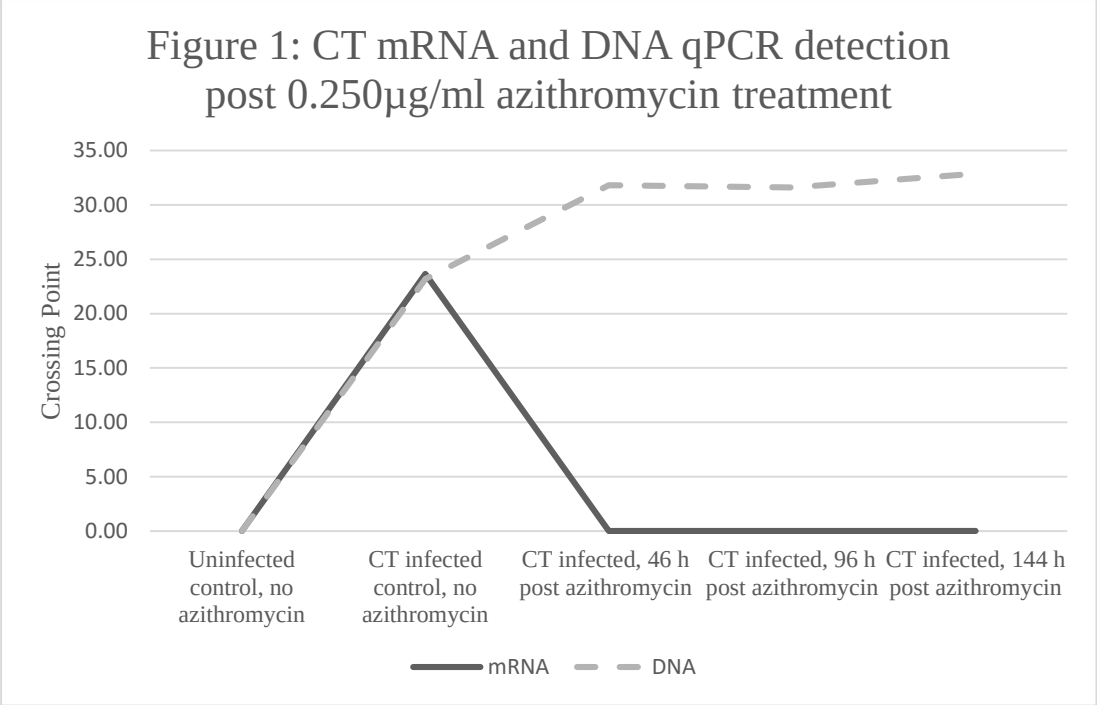

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Figure 2: Scatter plot mRNA and DNA copies per swab (Log10) Visit 1

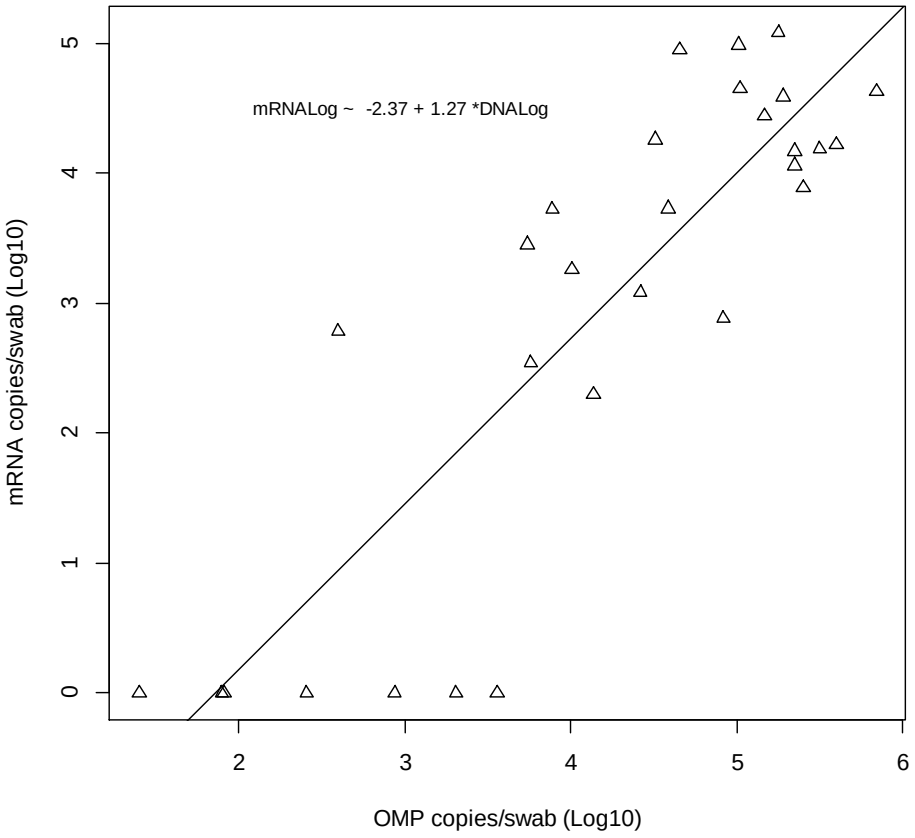

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Figure 3: Scatter plot mRNA and DNA copies per swab (Log10) Visit 2

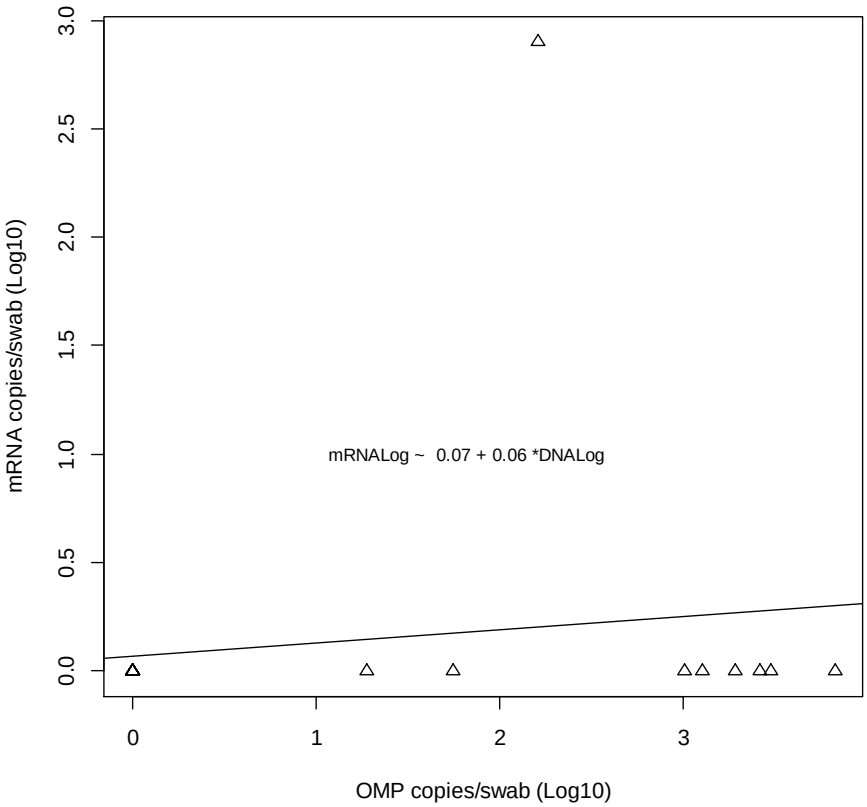

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Table 1. Concordance between RTqPCR and dPCR detection of CT mRNA with CT DNA detection*

CT result	*DNA ompA*		Total (%)	Concordance	Kappa (95% CI)
	Detected	Not Detected			
mRNA qPCR					
Detected	20	0	20 (36.4)		37.98%
Not Detected	19	16	35 (63.6)	65.45%	(20.28, 55.68)
Total (%)	39 (70.9)	16 (29.1)	55		
mRNA dPCR					

Detected	24	0	24 (43.6)		48.21%
Not Detected	15	16	31 (56.4)	72.73%	(29.09, 67.33)
Total (%)	39 (70.9)	16 (29.1)	55		

* Based on visit 1 and visit 2 data combined (55 samples)

Table 2. Cohens Kappa for agreement between RTqPCR and dPCR detection of CT mRNA*

CT mRNA result	*RTqPCR*		Total (%)	Kappa (95% CI)
	Detected	Not Detected		
dPCR				
Detected	20	4	24 (43.64)	84.93%
Not Detected	0	31	31 (56.36)	(70.87, 98.99)
Total (%)	20 (36.36)	35 (63.64)	55	

* Based on visit 1 and visit 2 data combined (55 samples)