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ORIGINAL RESEARCH

Transfusion Medicine

TRANSFUSION

The incidence of donor white blood cell survival (transfusion-associated microchimerism) in Australian pediatric patients

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Abstract

Introduction: Donor leucocyte survival following red blood cell (RBC) transfusion, known as transfusion-associated microchimerism (TAM), can occur in some patients. In Australia, despite the introduction of leucocyte filtration (leucodepletion) during RBC manufacture, TAM has been detected in adult trauma patients. However, the incidence of TAM in Australian pediatric patients has not been analyzed.

Methods: Patients aged 0–16 years were recruited across two cohorts. Retrospective participants had RBC transfusion between January 1, 2002 and November 15, 2017 and prospective participants received RBC transfusion between December 1, 2016 and November 25, 2020. Twelve bi-allelic insertion/deletion (InDel) polymorphisms were used to detect microchimerism amplification patterns using real-time PCR (RT-PCR) and droplet digital PCR (ddPCR).

Abbreviations: ALL, acute lymphoblastic leukemia; CI, confidence interval; ddPCR, droplet digital PCR; gDNA, genomic DNA; InDel, insertion deletion polymorphisms; NBMS, National Blood Management System; RBC, red blood cell; RT-PCR, real-time PCR; TAM, transfusion-associated microchimerism.

Results: Of the retrospective cohort ($n = 40$), six patients showed amplification of InDel sequences indicating potential microchimerism. For three patients, minor InDel sequences were detected using RT-PCR only, two patients had minor InDel amplification using ddPCR only, and one patient had minor InDel amplification that was confirmed using both techniques. Amplification of minor sequences occurred in three patients who had received a bone marrow transplant in addition to RBC transfusion. In the prospective cohort ($n = 25$), no InDel amplification indicating potential microchimerism was detected using RT-PCR.

Discussion: Cell-based therapies had been administered in three patients where microchimerism amplification patterns were detected. Three patients have microchimerism that may be attributed to RBC transfusion. In prospective patients, who received leucodepleted and gamma-irradiated RBC units, no potential microchimerism amplification were detected. ddPCR may be a suitable technique for TAM analysis but requires further evaluation.

KEYWORDS

droplet digital PCR, leucodepletion, microchimerism, pediatrics, red blood cell, transfusion

1 | INTRODUCTION

It has been speculated that some adverse patient outcomes associated with red blood cell (RBC) transfusion can result from the presence of donor white blood cells (leucocytes) that remain within RBC units following manufacture.[1–3] One long-term phenomenon that has been described following RBC transfusion is the survival of small numbers of blood donor derived leucocytes within the blood transfusion recipient.[4–6] This phenomenon is termed transfusion-associated microchimerism (TAM), where "microchimerism" describes the presence of small numbers of genetically distinct cells. Generally, several days following transfusion, blood donor cells are identified by the recipients immune system and destroyed.[7] However, in cases where TAM is identified, blood donor derived leucocytes can endure for many years and appear to engraft and proliferate accounting for up to 5% of the recipients' hematopoietic cells.[8–17] The incidence of TAM may be more common in patients who have substantial perturbation of the immune system or require significant volumes of RBC transfusion.[9,11,13,14] Conversely, TAM has been detected in some recipients' following the transfusion of only a single RBC unit.[8,11,13,14] The molecular pathway involved with the engraftment of blood donor cells is currently unknown, although it has been shown that pro-inflammatory cytokines are slightly elevated in patients who require RBC transfusion.[16]

Current understanding of the long-term clinical outcomes for individuals detected to have TAM is limited. Chimeric cells have been detected in tissues of a woman with systemic lupus erythematosus and tumor invasiveness has recently been shown to be correlated with the presence of chimeric cells.[18,19] Furthermore, studies into the naturally occurring phenomenon known as fetal-maternal microchimerism have indicated some connections between chronic immune and nonimmune related outcomes.[20–24]

To reduce the number of blood donor leucocytes within RBC units prior to transfusion, a filtration step known as leucodepletion can be performed. In Australia, leucodepletion was introduced for all RBC units manufactured from October 2008. However, leucodepletion is not universally performed by all blood providers globally due to the associated financial burden. Furthermore, despite the use of leucodepletion, manufactured RBC units may contain up to 1 million leucocytes and remain within component specifications.

It still remains unclear whether the use of leucodepleted RBC units has influenced the potential for TAM since there have been conflicting findings, one study reported a reduction in the incidence of TAM while the frequency was unchanged in others.[8,11,25,26] To date, TAM studies have been mostly conducted in adult trauma patients due to the potential for large transfusion volumes, in addition to, major perturbation of the patient immune system. It is also unclear whether trauma

patients are the only affected patient group. Studies in obstetric and pediatric transfusion patients have been scarce with conflicting outcomes. Most reports from obstetric and pediatric patient groups show no incidence of TAM, most likely due to the use of leucodepleted and gamma-irradiated RBC units where gamma-irradiation further reduces the potential for viable leucocytes to be present.[25,27] However, one other recent study from Ghana showed that up to 70% of female pediatric transfusion recipients had TAM.[28] Establishing the potential long-term outcomes of RBC transfusion in pediatric transfusion recipients is of clinical importance given many patients survive to adulthood and may have the potential for enduring consequences.

Australian pediatric transfusion recipients have not been previously analyzed for any incidence of TAM. Therefore, this study aimed to use both real-time PCR (RT-PCR) and droplet digital PCR (ddPCR) assays to analyze Australian pediatric patients who received RBC unit transfusion for TAM. ddPCR was investigated for TAM detection, as this ultra-sensitive PCR technique uses a water–oil emulsion principle to partition the target samples into individual PCR droplets containing only a few copies of the template DNA. Since the potential of TAM relies on the detection of a very small number of genetically distinct blood donor cells against a background of patient cells, the analysis by ddPCR, in addition to traditional RT-PCR, is of interest. ddPCR for microchimerism detection has been demonstrated in cellular therapy and organ transplant settings but has not been evaluated to date in TAM settings.[29–31]

2 | MATERIALS AND METHODS

2.1 | Participant selection

The study was reviewed by the Hunter New England Human Research Ethics Committee and was conducted in accordance with Australia's National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research (2007). The study was led by Australian Red Cross Lifeblood (Lifeblood) with study participants recruited from John Hunter Children's Hospital (Newcastle, New South Wales, Australia) and Queensland Children's Hospital (Brisbane, Queensland, Australia).

For this study we defined pediatric patients as anyone aged 0–16 at the time their first RBC unit was administered.

For the retrospective cohort, pediatric patients transfused with at least one RBC unit between January 1, 2002 and November 15, 2017 were identified from institutional databases. Potential participants that were over the age of 18 at the time of study recruitment were directly invited via postal mail. If potential participants remained under the age of 18 at the time of study commencement, the invitation letter was addressed to the parent/s or guardian/s. Participants were included for analysis after they provided written consent and a research blood sample via the participating hospital site or a commercial pathology blood collection center.

For the prospective cohort, potential participants were identified by the lead investigator at each site. The parent/s or guardian/s were approached by the clinical team to obtain written consent. Patients were eligible to participate in the study if they had been transfused with at least two RBC units upon admission and were only enrolled once the parent/s or guardian/s had provided the study team with written consent. If the patient required multiple transfusions, a study sample was taken alongside any clinical routine sampling requirements. Participants were only included for final analysis if a follow-up sample, at least 12 months following cessation of transfusion was provided. The first patient was enrolled on December 1, 2016 with the last participant enrolled on November 25, 2020.

Wherever it was possible to ascertain, any female participants who were pregnant or had previously been pregnant were excluded to reduce the possibility of detecting naturally occurring foetal-maternal microchimerism.

2.2 | Medical record and blood donation analysis

For both the retrospective and prospective cohorts, participant medical records were analyzed by clinical staff at each hospital site to provide relevant study information and data on the administered RBC units to the researchers.

The age of blood for each transfused unit was calculated from the date of collection from the blood donor. The date of collection was determined from blood donation records stored on the National Blood Management System (NBMS) which is administered by Lifeblood. Only donation records from the January 1, 2008 were reliably available; therefore, patients transfused before 2008 may not have the RBC unit age at the time of transfusion calculated.

The volume of blood administered to each participant was unavailable for this study. Lifeblood recommends 10–20 mL/kg as a standard pediatric RBC transfusion dose unless there is active major bleeding. In Australia, pediatric RBC units are manufactured by splitting one standard 180–380-mL RBC unit into 3–4 pediatric units

with a total volume of 60 ± 4 mL (range 25–100 mL). For this study, the transfusion of either one pediatric unit or one standard volume RBC unit is recorded as the transfusion of one total RBC unit.

In Australia, universal leucodepletion of RBC units (both pediatric and adult) was introduced from October 1, 2008; therefore, potential participants who had received blood transfusion up to 6 weeks after this date may have received a mixture of leucodepleted and non-leucodepleted RBC units. Patients may also have received some RBC units that were gamma-irradiated throughout the study period. Gamma-irradiation of the RBC unit may have been specifically requested from Lifeblood by hospital sites, and this requirement would have been recorded as a transformation in the NBMS records. However, for some RBC transfusions, the hospital transfusion laboratory may have performed gamma-irradiation onsite, which may not have been captured in the NBMS records. Therefore, the patient transfusion records provided to the study team may not adequately capture the irradiation status of all the transfused RBC units.

2.3 | Blood sampling

A single non-fasting blood specimen of volume ranging from 0.1–1 mL was provided in a 3-mL sodium heparin blood collection tube (catalogue number 367876, BD Biosciences, San Jose, CA). For the retrospective cohort, the samples were collected using standard phlebotomy techniques at commercial pathology collections centres.

For the prospective cohort, blood sampling was performed in conjunction with standard-of-care blood sampling regimes. The pre-transfusion study samples were taken by appointed blood collection staff, and if the consented participants subsequently had a blood transfusion, the blood collection staff obtained follow-up samples before each RBC transfusion episode. The final sample was obtained at least 12 months following the completion of RBC transfusion requirements and was collected alongside any routine clinical sampling collection that was conducted by the study site to reduce the burden for the participant and parent/s or guardian/s. Sample volumes were restricted by these parameters, especially for infant participants. Therefore, ddPCR analysis was not performed on this cohort.

Specimens were transported to Lifeblood research laboratories via commercial or Lifeblood couriers at room temperature within 36 h of collection. Nucleated cell populations were obtained from the buffy coat layer of the blood sample following centrifugation at 1000 × *g* for 10 min. The subsequent buffy coat was removed and stored at −80°C.

2.4 | PCR testing

Genomic DNA (gDNA) was purified using QIAamp DNA blood mini kit (catalogue number 51106, Qiagen, Hilden, Germany) from the thawed buffy coat aliquot. gDNA concentration was analyzed using a Nanodrop spectrophotometer (model ND-2000, Thermo Fisher scientific, Waltham, MA).

For TAM detection, previously described phenotypically silent bi-allelic insertion or deletion regions spanning across nine different chromosomes were screened.[30] The 12 insertion/deletion (InDel) bi-allelic markers are known as SO1, SO2, SO3, SO4, SO4B, SO6, SO7, SO7B, SO8, SO8B, SO9, and SO11 with the amplification primer sequences as previously published.[8,32,33] Triplicate RT-PCR single plex reactions for each marker was conducted on each participant sample using 50 ng of purified gDNA in a 20-μL reaction volume on the Rotor-Gene Q Real Time PCR system and software version 2.3.4 (Qiagen) as previously described.[8] Positive amplification was determined if amplification curves had a fluorescence change threshold greater than 30%.

It should be noted that not all 12 InDel sequences are expected to be amplified, generally 4–9 sequences amplify by cycle number 23 to indicate the predominant patient cell population. For individuals where potential microchimerism may be found, approximately 1–3 sequences may amplify later during the RT-PCR cycles around cycle number 30–32. These signals indicate the presence of "minor" genetically distinct sequences representing the presence of a small number of blood donor cells.

For the retrospective cohort, patient samples had sufficient sample available for analysis using ddPCR. The probe sequences used are as previously described and the primer sequences were the same as indicated for RT-PCR.[32,33] Since ddPCR allowed for multiplex reactions to be conducted, the probes for SO1, SO3, SO6, SO7, SO7B, and SO9 were labeled with FAM and Black Hole Quencher® 1 and the probes for SO2, SO4, SO4B, SO8, SO8B, and SO11 were labeled with HEX and Black Hole Quencher® 2 (Integrated DNA Technologies, Inc., Coralville, IA). Reaction pairs were multiplexed as follows SO1/SO2, SO3/SO4, SO6/SO4B, SO7/SO8, SO7B/SO8B, SO9/SO11.

ddPCR was performed on the Bio-Rad QX200 platform according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). Then, 20 ng of each patient's gDNA was digested using 10 units of HindIII restriction enzyme in 1× buffer (New England Biolabs, Ipswich, MA) at 37°C for 1 h with heat deactivation at 80°C for 5 min. ddPCR reactions were setup with final volume of 22 μL where each contained 11 μL ddPCR Supermix for Probes (no dUTP; Bio-Rad),

20 ng of digested gDNA, 500 nM of each primer and 200 nM of each probe. Samples were processed using the QX200 droplet generator and transferred to an Eppendorf twin. tec semi-skirted 96 well PCR plate (Eppendorf, Hamburg, Germany), sealed and amplified in a C1000 touch thermal cycler (Bio-Rad) using the following conditions: enzyme activation (95°C for 10 min), denaturation and annealing/extension cycles (95°C for 30 s, 60°C for 1 min for 40 cycles) with a ramp rate of 2°C/s. Amplified droplets were then analyzed on the QX200 droplet reader and processed using QuantaSoft software (Bio-Rad).

For ddPCR assay development, simulated control samples were initially tested. These included a simulated TAM positive control, that consisted of mixing 49.5 ng of gDNA from volunteer A with 0.5 ng of gDNA from volunteer B to mimic 1% of gDNA from volunteer B in a 99% background of gDNA sequences from volunteer A. A known TAM negative control was obtained from a volunteer who had never received blood transfusion, bone marrow, or stem cell transplants and had never been pregnant. No template controls were also conducted across each plate to determine background amplification. For ddPCR, predominant cell populations have very high copy number concentrations (30 copies/μL of the ddPCR reaction) and potential "minor" sequences have very low copy number amplification signals (<5 copies/μL).

3 | RESULTS

3.1 | Participant demographics

For the retrospective cohort, 234 potential participants matching the inclusion criteria were identified from the institution database (Figure 1). Of these, 40 (17.1%) provided a blood sample for the study.

For the prospective cohort, a total of 47 patients were recruited by the study sites, 5 (10.6%) had no RBC transfusion following recruitment, 5 (10.6%) patients died, and 12 (25.5%) were lost to follow-up during the study. The remaining 25 (53.2% of total recruited) patient samples were analyzed for the study (Figure 1). The number of potential participants approached for the prospective cohort arm of the study was not recorded.

Participant demographics are summarized in Table 1. For both cohorts most of the transfusion recipients were male (52.5% for the retrospective cohort and 68.0% for the prospective cohort). Prospective participants had an older median age of 9 years when they had received their first RBC transfusion compared with 5 years for the retrospective cohort. The retrospective cohort received more RBC units (median = 8) compared with the prospective participants (median = 4). The age of the RBC units at the time of transfusion for both cohorts was a median of 8 days. All of the prospective participants received leucodepleted and gamma-irradiated RBC units. This was also

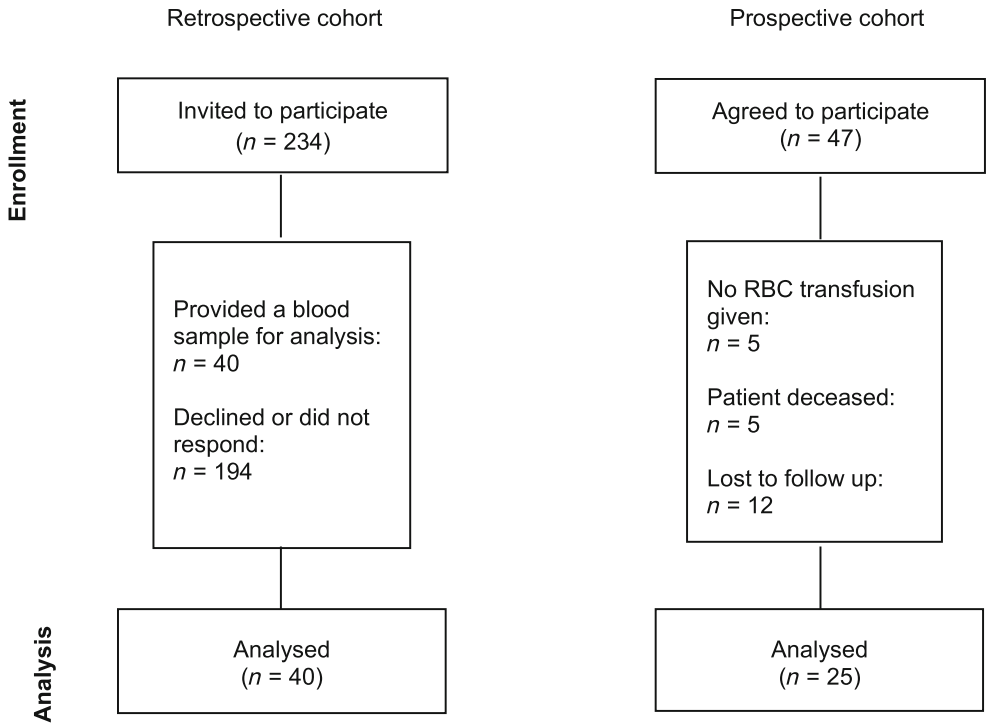

FIGURE 1 Recruitment diagram for the retrospective and prospective cohorts and final analysis numbers for each patient group. RBC, red blood cell.

TABLE 1 Participant demographics for the retrospective and prospective patient cohorts.

	Retrospective cohort ($n = 40$)	Prospective cohort ($n = 25$)
Patient sex		
Male (%)	21 (52.5)	17 (68.0)
Female (%)	19 (47.5)	8 (32.0)
Median age (years) at the time of first RBC transfusion [range]	5 [0–16]	9 [0–16]
Median number of RBC units transfused [range]	8 [1–150]	4 [1–26]
Median age (days) of RBC unit at the time of transfusion[a] [range]	8 [3–28]	8 [3–19]
Manufacturing status of the RBC units transfused into each patient[a,b]		
Leucodepleted and gamma-irradiated RBC units (%)	25 (62.5)	25 (100.0)
Leucodepleted RBC units (%)	5 (12.5)	0 (0.0)
Mixture of nonirradiated and non-leucodepleted RBC units (%)	8 (20.0)	0 (0.0)
Manufacturing information unknown (%)	2 (5.0)	0 (0.0)
Reason for transfusion		
Bleeding disorders[c] (%)	5 (12.5)	0 (0.0)
Cancers[d] (%)	29 (72.5)	24 (96.0)
Other[e] (%)	6 (15.0)	1 (4.0)

Abbreviation: RBC, red blood cell.

[a]Some data may be missing due to changes in blood donor management systems.

[b]Leucodepleted units have $<1 \times 10^6$ leucocytes per unit, gamma-irradiated units are exposed to 25 Gy of gamma rays. Irradiating RBC units reduces their storage time from 42 to <14 days.

[c]Patients diagnosed with; menorrhagia, aplastic anemia, hemophilia, congenital dyserythropoietic anemia, Von Willebrand disorder, and dysfibrinogenaemia.

[d]Patients diagnosed with; acute lymphocytic leukemia (ALL), heptoblastoma, medulloblastoma, pilomyxoid astrocytoma, biphenotypic leukemia, anaplastic large cell lymphoma, Ewing's sarcoma, Hodgkin lymphoma, embryonal rhabdomyosarcoma, Wilms tumor, nephroblastomatosis, Burkitt lymphoma, mixed germ cell tumor, ectomesenchymoma, osteosarcoma, neuroblastoma, round cell sarcoma, and aplastic anemia.

[e]Patients diagnosed with; Haemolytic disease of the newborn, anemia, autoimmune haemolytic anemia, and trauma.

the case for most (62.5%) of the retrospective participants. The main indication for transfusion for both cohorts was cancer treatment-related anemia (72.5% of the retrospective participants and 96.0% of prospective participants).

3.2 | Droplet digital PCR analysis of control samples to represent microchimerism patterns

To analyze the expected ddPCR droplet outputs in relation to microchimerism, reactions were conducted using some control samples including a known TAM negative sample, a simulated TAM positive sample and no template control.

The TAM negative sample (Figure 2A) shows amplification of the SO3, SO4B, SO7B, SO8, and SO9 InDel sequences with all other tested InDel combinations remaining unamplified. The calculated gDNA concentration of the amplified InDel sequences was ≥52 copies/μL.

The simulated TAM positive sample (Figure 2B) showed amplification of the SO1, SO4B, SO7, SO8, and SO9 InDel sequences with a high signal intensity. Amplification of SO3, SO4, SO6, and SO8B InDel sequences were also detected with a reduced signal intensity, indicating that these InDel sequences were at lower concentrations. The predominant "patient" gDNA amplification signals were present with concentrations ranging from 30 to 633 copies/μL. The "minor" or "microchimeric" gDNA amplification signals were present at concentrations ranging from 0.3 to 1.4 copies/μL. Each sample used for the simulated TAM positive sample were also individually analyzed with the data shown in Figure S1. The no template control (Figure 2C) showed no amplification with any InDel primer and probe combination.

3.3 | Patient sample analysis

For the retrospective cohort, RT-PCR indicated 4 participants with amplification patterns of "minor" sequences indicating the potential for microchimerism (Table 2). All samples from the retrospective cohort then underwent confirmatory analysis using ddPCR. Of these, three patient samples did not indicate any "minor" amplification sequences using ddPCR (Table 2 and Figure 3A–C) and one patient was shown to have "minor" amplification of InDel SO1 using both RT-PCR and ddPCR (Figure 3F).

Two other patients had "minor" amplification of InDel SO1 that was detected only using ddPCR (Figure 3D,E). The concentration of InDel SO1 in these 2 patients was calculated as 0.4–0.7 copies/μL.

For the three patients, where "minor" amplification of InDel sequences by ddPCR was identified, all were diagnosed with acute lymphoblastic leukemia (ALL) (Table 2). The patient found to have microchimerism detected with both RT-PCR and ddPCR received a bone marrow transplant. For the two patients detected using ddPCR only, cellular therapy was used for one patient's

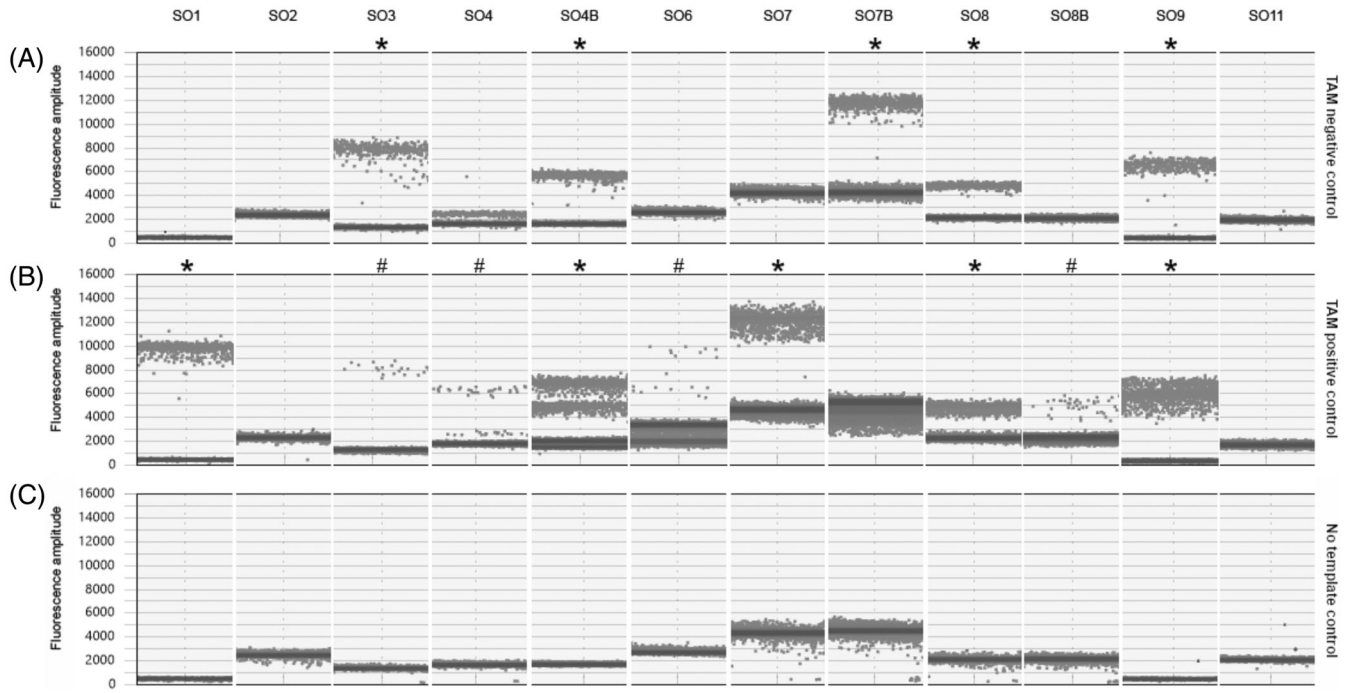

FIGURE 2 Control samples to illustrate the expected droplet digital PCR (ddPCR) outputs for transfusion-associated microchimerism (TAM) analysis. Each insertion/deletion (InDel) primer and probe combination was tested using a known TAM negative control (A), a simulated TAM positive control (B) and no template control (C). The TAM negative control (A) emulates the expected ddPCR outputs for patient samples that do not have TAM. Only amplification from predominant patient cells is expected and are labeled with "*." The sample is negative for all other primer combinations. The TAM positive control (B) emulates the expected ddPCR pattern for any patient samples with microchimerism. The dominant patient gDNA amplification was detected using InDel primer pairs labeled with "*." "Minor" gDNA amplification was detected using InDel primer pairs labeled with "#." The sample is negative for all other InDel combinations. For microchimerism, amplification of the "minor" sequences occurred less prominently as the gDNA concentration is lower than for the dominant patient gDNA. The individual samples used to make the TAM positive control were also analyzed and is shown in Figure S1. The no template control (C) indicates the background amplification of each InDel reaction. To note—every patient will have a different combination of InDel sequences that will amplify and not all primer pairs are expected to amplify even in patients with TAM. Insertion/deletion primer and probe sequences used are SO1, SO2, SO3, SO4, SO4B, SO6, SO7, SO7B, SO8, SO8B, SO9, and SO11.[8,32]

treatment. The other patient had not been exposed to any cellular therapy but is likely to have been transfused with non-leucodepleted RBC units.

For the prospective cohort, RT-PCR analysis found no patient samples with InDel amplification patterns indicating any potential microchimerism.

4 | DISCUSSION

This study investigated the potential of TAM in Australian pediatric patients following RBC transfusion across two cohorts using RT-PCR and ddPCR technology. In the retrospective cohort, 4 participants were shown to have InDel amplification patterns indicating potential microchimerism using RT-PCR but only one of these four patients had complimentary results using ddPCR. Two additional patients with microchimeric amplification patterns were identified using ddPCR only.

Of the six patients who had microchimeric amplification patterns, five were being treated for ALL. Treatment for ALL may include cellular therapies, specifically hematopoietic stem cell transplantation. For three of the patients with microchimeric signatures cellular based therapies were confirmed and would have resulted in exposure to sources of donor cells other than RBC transfusion. One patient did not have any exposure to cellular based therapies but did potentially receive RBC transfusion using non-leucodepleted units. One patient transfused for ALL and one patient transfused for anemia, had no record of cell-based therapy administration and were transfused with leucodepleted RBC units. Therefore, 3 (7.5%) of the 40 retrospective cohort patients analyzed had microchimeric amplifications that could be attributed to RBC transfusion.

No microchimeric amplification signals were found in the prospective cohort participants using RT-PCR. All participants in this cohort received leucodepleted and gamma-irradiated RBC units.

TABLE 2 Summary of the detected minor sequences using real-time PCR and droplet digital PCR in the retrospective cohort.

Potential TAM positive patient	Sex assigned at birth	Amplification of minor sequences using real-time PCR	Amplification of minor sequences using droplet digital PCR	Number of months between study sample collection and date of the first known red blood cell transfusion	Relevant clinical treatment notes
1	Male	SO9	-	78	Patient was diagnosed with ALL but had no recorded cellular therapy
2	Male	-	SO1	172	Patient was diagnosed with ALL and received a bone marrow transplant
3	Female	-	SO1	119	Patient was diagnosed with ALL but had no recorded cellular therapy but was exposed to non-leucodepleted red blood cell units
4	Female	SO9	-	76	Red blood cell transfusion was administered to treat anemia
5	Male	SO1	SO1	100	Patient diagnosed with ALL and received a bone marrow transplant
6	Male	SO2, SO4B, SO11	-	151	Patient diagnosed with ALL and received a bone marrow transplant

Abbreviations: ALL, acute lymphoblastic leukemia; TAM, transfusion-associated microchimerism.

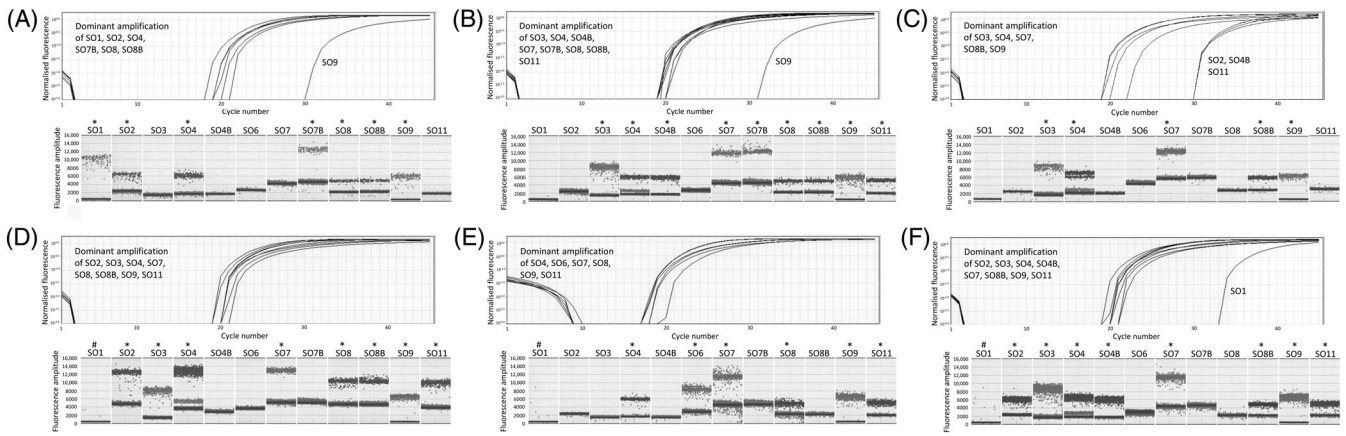

FIGURE 3 Real-time PCR (RT-PCR) and droplet digital PCR (ddPCR) outputs for retrospective cohort patient samples where potential microchimerism was detected using insertion/deletion (InDel) primer and probe sequences. RT-PCR outputs are shown as graphs and ddPCR droplet plots are shown below the RT-PCR graph. Six patients were identified to have potential microchimerism. For two patients shown in (A) and (B), RT-PCR indicated the presence of SO9 amplification after cycle number 30 but ddPCR showed copy number amplification of SO9 as higher in concentration and was therefore not present due to microchimerism. For the patient shown in (C), RT-PCR indicated the amplification of SO2, SO4B, and SO11 to occur after cycle number 30. There was no amplification of these InDel primers using ddPCR and could have been a false positive reaction. For two patients shown in (D) and (E), RT-PCR had no InDel sequences amplification after cycle number 30. However, using ddPCR, SO1 was detected at very small copy numbers. For patient shown in (F), the very low level of SO1 amplification was confirmed by both RT-PCR and ddPCR indicating the presence of microchimerism. To note—every patient will have a different combination of InDel sequences that will amplify and not all primer pairs are expected to amplify even in patients with transfusion-associated microchimerism. Insertion/deletion primer and probe sequences used are SO1, SO2, SO3, SO4, SO4B, SO6, SO7, SO7B, SO8, SO8B, SO9, and SO11.[8,32]

A major limitation of this study, particularly for the retrospective cohort, is that some patient data were incomplete, particularly if treatments were conducted across numerous health providers in Australia. Data on pertinent health interventions, such as solid organ transplantation, may not have been available. Furthermore, in

cases where cellular therapies were confirmed, cells from the donor were also not analyzed to provide comparative InDel amplification profiles.

The potential for TAM should be analyzed locally as outcomes between studies may not be comparable since RBC unit manufacturing procedures can be disparate. One contemporary example is pediatric transfusion recipients from Ghana where 70% of their patients were found to have TAM.[28] In the study from Ghana, whole blood units were transfused and were unlikely to be leucodepleted or irradiated. Therefore, these units would have contained high numbers of viable donor leucocyte resulting in a higher likelihood for TAM. Gamma-irradiation diminishes the potential of viable donor leucocytes following RBC unit manufacture and has been shown to reduce TAM in some studies.[25] Despite this, RBC unit irradiation cannot be routinely implemented across all transfusion settings since it significantly reduces the RBC unit storage shelf-life from 42 to 14 days and has been shown to increase RBC potassium release and storage lesions.[34–37]

Since the irradiation status was not confirmed for all of the RBC units transfused in the retrospective study participants nor was the total volume of transfusion known for either the retrospective or prospective cohorts, it is challenging to ascertain which patients received more exposure to RBCs from numerous donors, which can increase the likelihood of TAM.[4–6] Despite this limitation, the majority of participants approached for this study would have been exposed to RBC transfusion as either neonates or pediatric, Hematology or Oncology patients, and most likely would have received irradiated RBC units.

It is also challenging to analyze samples for microchimerism. ddPCR has been reported to be more sensitive than RT-PCR and has been used to analyze the potential of donor cells survival in other clinical settings, such as organ transplant.[29–31,38] One advantage of ddPCR is that multiplex analysis can be conducted and estimations of gDNA concentrations can be obtained without using calibration curves. In the patient samples where microchimeric signatures were amplified, the "minor" gDNA populations were present at <5 copies/μL and the dominant gDNA populations were present at >30 copies/μL. Therefore, any potential microchimerism detected using ddPCR appears to occur with cell populations of less than 1%, which is similar to previous reports.[11] However, this study was limited since small sample volumes meant that ddPCR was not performed for prospectively recruited patients.

Other disadvantages of ddPCR include, challenges for high throughput sample volumes, requirement for specialized equipment, higher reagent costs compared with RT-PCR, extra sample preparation, and lengthy assay time as the droplets need to be generated and "read" individually. Other aspects that require consideration when evaluating the techniques are that ddPCR has the most sensitivity and specificity when performed with probes, which may have compounded the differing results with RT-PCR. ddPCR is also subject to inhibition effects and for this study, digestion of genomic DNA was required to reduce this, and RT-PCR assays are subject to suboptimal amplification and false positives reactions especially for detecting these subtle cellular changes.[39] Further evaluation of the application of ddPCR technology for TAM settings along with more detailed evaluation of sensitivity would be of value.

The patient cohorts in this study are small; however, it should be noted that it is extremely challenging to obtain pediatric samples for transfusion outcome analysis. Few pediatric patients receive RBC transfusion, and even fewer receive significant volumes of RBC transfusion to study potential TAM outcomes. Another limitation, was not having a larger proportion of the study participants who had not been exposed to other cellular therapies, thus limiting the conclusive analysis of microchimerism from RBC transfusion exposure alone. It would also have been valuable to analyze samples from pediatric participants who have never had a blood transfusion, organ transplant or other cellular therapies to determine the incidence of background foetal-maternal microchimerism. However, this would be difficult to perform due to iatrogenic blood loss and associated ethical challenges.

Overall, this study indicates that the potential of TAM is low in pediatric patients transfused with RBC units in Australia. The current Australian practice of transfusing leucodepleted and gamma-irradiated blood products for pediatric patients appears to prevent TAM detection, especially when compared with adult trauma patients previously analyzed.[8,16,17] Furthermore, ddPCR may be a suitable confirmatory method for TAM detection that requires further evaluation. The data from this small patient cohort showed differing TAM rates between RT-PCR and ddPCR methodologies, and it remains to be determined if ddPCR specificity or sensitivity explains this difference in the context of TAM detection.

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CONFLICT OF INTEREST STATEMENT

The authors have disclosed no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data can be made available upon request to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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