Laboratory methods supporting measles surveillance in Queensland, Australia, 2010–2017

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Abstract

Purpose. Australia was officially recognised as having eliminated endemic measles transmission in 2014. Maintaining laboratory support for surveillance of vaccine-preventable diseases, such as measles, is an essential component of reaching and maintaining transmission-free status.

Methodology. Real-time and conventional PCR-based tools were used to detect, differentiate from measles vaccine virus (MeVV), and sequence fragments of measles viruses (MeV) identified from specimens collected in Queensland. Specimens were mostly from travellers who had visited or returned to Queensland from international or interstate sites or been in contact with a case from either group.

Results. Between 2010 and 2017, 13,678 specimens were tested in our laboratory using real-time RT-PCR (RT-rPCR), identifying 533 positives. Most specimens were swabs (70.98%) and urines (25.56%). A MeVV RT-rPCR was used on request and identified 154 instances of MeVV. MeV-positive extracts were genotyped as required. Genotypes identified among sequenced specimens included B3, D4, D8, D9, G3, and H1 as well as members of clade A as expected from the detection of MeV among virus introductions due to global travel and vaccination.

Conclusion. We describe the workflow employed and results from our laboratory between 2010 and 2017 for the sensitive detection of MeV infection, supporting high-quality surveillance to ensure the maintenance of Australia’s measles-free status.

INTRODUCTION

The species *Measles morbillivirus* is an enveloped, single-stranded, non-segmented, negative-sense RNA member of the genus *Morbillivirus*, family *Paramyxoviridae* [1]. Detection of measles virus (MeV) RNA by real-time RT-PCR (RT-rPCR) is a highly specific and sensitive rapid diagnostic tool to identify infection [2–5].

Despite a safe and effective vaccine being available for over 50 years, MeV remains a serious public health threat worldwide. There are hurdles in the path to global measles elimination and the eventual eradication of the MeV. In developing countries, a lack of political and financial support and areas of conflict give rise to low vaccine coverage through a limitation of access to adequate healthcare. In developed countries with little or no transmission, vaccine hesitancy and mistrust among parts of the population can cause gaps in immunity and, when concentrated in specific geographic locations, can result in local transmission and even sizable outbreaks after measles is introduced via infected travellers [6]. Most measles cases in Australia are due to direct importation or contact with travel-related cases [7]. Despite large gains in vaccination coverage resulting from the control initiatives, the global coverage for measles and rubella containing vaccines has plateaued at 85% for the past ten years [8].

Following MeV detection in the laboratory using RT-rPCR, genetic characterisation of MeV genotype primarily relies on sequencing a 450 nucleotide (nt) carboxyl-terminal region of the nucleocapsid (N450) protein and secondarily, on sequencing the entire coding region of the hemagglutinin
genes, to produce internationally agreed-upon measles genotype assignments [9–11].

There is only a single serotype of MeV, but analysis of MeV genetic variation has identified eight MeV distinct clades (A–H) and 24 subtypes, referred to as genotypes [10–12]. Not all genotypes are active. The World Health Organization (WHO) has declared six genotypes, not detected for at least 15 years, as officially inactive and there are a further five genotypes with no documented cases since 2006 [13]. During 2016 to 2018 the number of genotypes decreased from six to four (B3, D4, D8 and H1) and diversity of viruses also declined [14].

RT-PCR can also be designed to identify the presence of a vaccine-derived measles virus (MeVV). This approach is useful to distinguish between wild-type MeV infection and MeVV in individuals who received MeV-containing vaccine as post-exposure prophylaxis following exposure to wild-type virus. It has also supported the investigation of post-vaccination illness [15]. However, the methods we describe below cannot discriminate between the presence of MeVV and wild-type MeV when occurring as a co-infection.

We describe a workflow that was used for the molecular diagnostic detection and characterisation of MeV in patients, particularly in Queensland, that has proven useful for supporting ongoing efforts to maintain endemic measles-free status in Australia between 2010 and 2017 [7].

METHODS
Specimens
Specimens for RT-PCR testing were provided to this reference laboratory by hospitals and private laboratories from around the northeastern Australian State of Queensland. This testing, in response to clinical need, occurred as part of the laboratory's role in supporting public health efforts to identify cases of measles. As such, specimens were obtained by clinical request for measles testing. Whilst clinical information was not available with test requests, it is anticipated most cases would have a febrile-rash illness. Detected strains were characterised by analysing the N450 region as required. Throughout seven years key reaction mixes were procured from the same companies with only batches changing. Oligonucleotide sequence remained constant but batches were replaced as they were consumed and each batch was compared to the previous using the same reaction mix and the results logged. These changes did not affect the process or require re-analysis of analytical sensitivity, specificity or limit of detection.

Nucleic acid extraction
Upon receipt of specimens into the laboratory, viral RNA was extracted using the Qiagen EZ1 Mini kit v 2.0 (Qiagen, Hilden, Germany).

Real-time RT-rPCR screening and controls
Two RT-rPCRs were in use during the past decade to detect wild-type MeV and to discriminate MeVV from wild-type MeV (Table 1, Fig. 1). The primer, probes, and cycling conditions of the MeV F gene assay [16] and the MeVV RT-rPCR assay [17] are described online.

The MeV RT-rPCR (Fig. 1) was used as a screen for the detection of any *Measles morbillivirus*. The assay included a final primer concentration of 0.3 µM and probe concentration of 0.15 µM. The MeVV RT-rPCR was used as needed to discriminate circulating MeV genotypes from genotype A, which is not circulating at large but is used in MeV vaccines. This assay included primers and probe at a final concentration of 0.3 µM. However, sequencing was the preferred option to confirm the presence of MeVV.

### Table 1. Oligonucleotides used to detect, differentiate, and characterise MeV

| Assay (target) | Primer or probe | 5’–3’ nucleotide sequence | Genomic positions¹ |
|----------------|-----------------|---------------------------|-------------------|
| MeV RT-rPCR (F gene) [16] | Measles MGB FP | GCTCAAATTGCAGATACATACAGAAA | 6066–6094 |
| | Measles MGB RP | GCAGATATGGGTCCCGTAA | 6137–6118 |
| | Probe Measles Fusion Probe | FAM-CTTCGTATTAGGGCC-MGBNFQ | |
| MeVV RT-rPCR (M gene) [17] | Measles F 4729 Vac | AAACCCCCAGCAATTGGAA | 4729–4747 |
| | Measles R 4795 Vac | GGTCACCTCGGTCGCTTGT | 4813–4795 |
| | Measles Probe 4757 | FAM-CCCTCTCTCACCACACAGMGBNFQ | 4757–4773 |
| Genotyping RT-PCR (N gene) | MVF1 | TACCCCTGTCAGGAGCTAATG | 1092–1115 |
| | MVB1 | AACAATGATGGAGGATGACGTG | 1736–1716 |
| | MVF2 | GATGAGGAGGCTACAGTCG | 1208–1228 |

FAM=FAM (Fluorescein); MGBNFQ=minor groove binder nonfluorescent quencher; RT-rPCR=reverse transcription real-time PCR; RT-PCR=PCR-conventional reverse-transcriptase polymerase chain reaction; ¹-numbers refer to nucleotide positions on the genome of the Edmonston MeV genotype A virus, GenBank accession AF266288.
Synthetic primer and probe controls were developed using a method previously described [18, 19]. These were used for both RT-rPCR assays, alongside no-template controls (NTC) and negative extraction controls. A threshold cycle (C_T) of ≤40 was used to indicate detection of MeV RNA whereas C_T values >40 were used to define a negative result.

**Conventional nested RT-PCR and sequence confirmation**

Confirmation of selected RT-rPCR positive specimens used a semi-nested conventional RT-PCR (N-gene RT-PCR) to generate a 528 basepair (bp) fragment. Analysis was confined to the 450nt sequence within this which encodes the carboxy-terminus of the nucleoprotein gene (N450) [20]. First-round RT-PCR amplification was performed after adding 5µl of RNA extract to 15µl SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) mixes. A 30 min reverse transcription incubation at 55 °C and a 2 min inactivation at 94 °C preceded 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 68 °C for 60 s, generating a 644 bp fragment. A second round of amplification was performed by transferring 5 µl of 1 : 100 diluted Round 1 product into 15 µl Fast Cycling PCR Mix (QIAGEN, Hilden, Germany) reactions (528 bp fragment). A 5 min denaturation at 96 °C preceded 40 cycles of 96 °C for 5 s, 60 °C for 5 s, and 68 °C for 18 s, with a final 72 °C incubation for 60 s.

Initial N-gene amplicon analysis of second-round amplicon used traditional agarose gel electrophoresis in 0.5X TBE buffer, or the Qiagen QIAxcel (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For reactions producing the N450 sequence, gel electrophoresis of the remaining amplicon was followed by purification of the excised band using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Dye terminator sequencing was performed on the GeXP Genetic Analysis System (SCIEX, Framingham, MA) using the GenomeLab DTCS Quick Start Kit (SCIEX, Framingham, MA) or on the ABI3130xl Genetic Analyser (Applied Biosystems, Australia) using the Big Dye terminator cycling ready reaction kit version 3.1.

**Subgenomic sequence analysis**

Forward and reverse strands of raw sequence data were aligned with the WHO-designated MeV reference strains. Primer sequences were removed *in silico* using Sequencher software (various versions; Gene Codes Corporation). Sequence alignment for phylogenetic tree construction used Geneious version 8 and MEGA7 software, respectively [21, 22].

The phylogenetic tree was created using the Neighbour-Joining method with a bootstrap test of 500 replicates (Fig. 2). Designation of the identification of the specimens was confirmed using the MeaNS database genotyping tool [9].

**Ethical review**

The Forensic and Scientific Services Human Ethics Committee assessed this project as not requiring full HREC review. It was not recognised to be research and is an audit of practice in accordance with the definition of research, page 6, of the National Statement on Ethical Conduct in Human Research (2007) updated in 2018.

**RESULTS**

**MeV RT-rPCR (F gene) validation**

The MeV RT-rPCR could reliably and repeatedly detect 20 copies per reaction determined after titrating a quantified *in vitro*-transcript and was linear across eight log_{10} dilutions of that transcript. The assay was precise (C_T 35.0 with a standard deviation of 0.44 after amplifying 40 replicate reactions at the limit of detection).

The test detected 68 of 68 previously genotyped MeV positive specimens including examples of genotype A, B3, D2, D3, D4, D5, D8, G3, H1 and H2 MeV. The test was 100% sensitive. The specificity of the test was evaluated by testing 65 MeV-negative specimens including those known to be positive and negative for other viruses (enteroviruses, rubella virus, parvovirus) in nucleic acids extracted from a range of specimen types (tissue culture, bone marrow, nasopharyngeal aspirates, nose swabs, throat swabs). None were detected in the MeV RT-rPCR. The test was 98% specific as it detected MeV in an additional specimen from an acute parvovirus-infected patient, who was...
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MeV IgG positive but from which the N-gene RT-PCR was negative. That result was described as a false positive because it could not be confirmed.

**MeVV RT-PCR (M gene) validation**

The MeVV RT-PCR could reliably and repeatedly detect 240 copies per reaction determined after titrating a quantified *in vitro* transcript and was linear across eight log_{10} dilutions of that transcript. The assay was precise after testing of repeatability (C_{T} 33.7 with a standard deviation of 1.6 after amplifying 33 replicate reactions near the limit of detection) and reproducibility (C_{T} 33.1 with a standard deviation of 1.9 after amplifying 28 reactions near the limit of detection) on different days, performed by different operators using different reaction mix batches.

The test detected eight of eight previously genotyped MeV clade A positive specimens. The assay was 100% sensitive. In additional specificity testing, the MeVV test was evaluated on 92 MeV clade A-negative specimens which included 35 characterised MeVs (examples of genotype A, B3, D2, D3, D4, D5, D8, G3, H1 and H2) as well as examples of entirely different viral species (enteroviruses, rubella virus, parvovirus) in nucleic acids extracted from a range of specimen types (tissue culture, bone marrow, nasopharyngeal aspirates, nose swabs, and throat swabs). The assay was 100% specific.

**Summary of use between 2010–2017**

Between 2010 and 2017, we received 13,678 specimens from 9,774 people (51.67% male) for MeV RT-PCR testing.

Most specimens were swabs (70.98%; Table 2) followed by urines (25.56%), and aspirates (2.63%). Blood, cerebrospinal fluid, tissue, washes and faeces comprised a small proportion (0.83%). Of the specimens tested, 533 (71.26% swabs) from 327 people tested positive by MeV RT-PCR, mostly from those aged between 1 and 5 years of age (Table 3). The MeVV RT-PCR, which was used upon request from 2012 onwards,

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**Table 2. Specimens tested and those from which measles viruses were detected**

| Specimen     | Total tested (% of total) | MeV detected (% of MeV) | MeVV detected (% of MeVV) |
|--------------|---------------------------|-------------------------|---------------------------|
| Swab         | 9,708 (70.98)             | 341 (63.98)             | 124 (80.52)               |
| Urine        | 3,496 (25.56)             | 159 (29.83)             | 21 (13.64)                |
| Aspirate     | 360 (2.63)                | 21 (3.94)               | 8 (5.19)                  |
| Blood        | 64 (0.47)                 | 12 (2.25)               | 1 (0.65)                  |
| Cerebrospinal fluid | 37 (0.27)             | 0                       | 0                         |
| Tissue       | 9 (0.07)                  | 0                       | 0                         |
| Wash         | 3 (0.02)                  | 0                       | 0                         |
| Faeces       | 1 (0.01)                  | 0                       | 0                         |
| TOTAL        | 13,678                    | 533                     | 154                       |

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**Fig. 2.** Phylogenetic analysis of partial nucleoprotein (N)-gene sequences from 122 MeV positive clinical specimens analysed in Queensland between 2010 and 2017. The sequenced region includes the World Health Organization-recommended N450 amplicon encoding the carboxyl-terminal of the N gene. MeV sequences associated with patients are marked with red circles. MeVV sequences described recently [15] are indicated by blue triangles. WHO reference sequences are used to define genotypes (green diamonds) and clades (labelled) [13]. Sequences are labelled using GenBank accession numbers. The phylogenetic analysis used the Neighbour-Joining method in a bootstrap test (500 replicates) in MEGA7 [22, 25–27]. A higher resolution version is located at 10.6084/m9.figshare.7352489
identified 154 MeVV positive detections from 119 people (zero from 46 in 2010, zero from 30 in 2011, five from 11 in 2012, 39 from 136 in 2013, 41 from 150 in 2014, 27 from 66 in 2015, 26 from 62 in 2016, 16 from 32 in 2017). Most MeVV detections were from children aged between 1 and 4 years (94.12% of people) and the remainder occurred in those aged 10 to 39 years (5.88%).

Specimens in this study were collected in Queensland, from travellers who had returned to Queensland from overseas (the Solomon Islands, Papua New Guinea, Indonesia, India, China, Myanmar, Vietnam, the Philippines, Thailand, Pakistan), or who visited Queensland from interstate (New South Wales, our neighbouring state). Contacts of travellers and cases were also tested as required.

After accounting for multiple specimen types tested per person, 340 positive RT-PCR remained. Genotyping was not performed on every positive specimen. Some were part of clusters where the genotype in contacts was expected to be identical to that from the initial case. Some MeV positives were not subjected to sequencing as they were expected, in the absence of travel or exposure to a known case, to represent virus shed due to a recent vaccination, and thus were likely to be MeV genotype A. One hundred and twenty-two MeV viruses were characterised by nucleotide sequencing, and the N450 sequences submitted to GenBank and analysed alongside prototype genotype sequences (Fig. 2; GenBank accession MK113874, MH818212-4, MK118950, MK121378-442, MK125059, MK135495-501, MK140660, MK161510). Among the sequences were confirmed cases of genotype A, indicating detection of MeVV (blue triangles; Fig. 2) post-vaccination. Some of these were included in submissions to GenBank but their N450 sequences were unremarkable.

Apart from the clade A MeVV viruses, N450 sequencing between 2007 and 2010 identified MeV genotypes B3, D4, D8, D9, G3, and H1, like those described by other reports during similar periods [7, 10, 23]. No viruses belonging to genotype B1-2, C1-2, D1-3, D5-7, D10-11, E, F, G1-2, or H2 were identified. We did not detect any genotypes considered extinct (B1, C1, D1, E, F, G1) [13] or inactive (D2, D3, D10, G2, H3) [11].

**Conclusion**

Australia was officially recognised as having eliminated endemic measles transmission in 2014 by providing evidence of no endemic circulation of measles virus for at least three years before this [24].

We have described a laboratory workflow employed in Queensland, Australia, across this time point, which detected measles cases by supporting high-quality surveillance. This workflow successfully detected and genotyped MeV infections in Queensland between 2010 and 2017 and in response to clinical need. A low proportion of all specimens tested was positive, and of those, a portion was sequenced. Among the sequenced MeV viruses were a wide range of genotypes as expected in a setting where endemic measles transmission has been halted. This genetic diversity reflects MeV introductions originating from a range of international travel sources with endemic or time-limited measles transmission.

Ongoing MeV mutation can challenge molecular diagnostic assays, resulting in reduced test performance or even test failure because of mismatches between oligonucleotides and target. We recently updated our MeV RT-rPCR test to better accommodate viral evolution [15]. Continual curation, optimisation and validation of molecular detection tools are essential to support disease diagnosis. Future use of whole-genome sequencing technologies is also needed to better support contact-tracing and enhance understanding of virus change over time. The concurrence of wild-type MeV and MeVV also presents a diagnostic challenge which is currently incompletely addressed by a mix of laboratory support, good clinical history, and serology.

Most of our MeV positives were the result of an infection acquired during travel to Australia from countries with ongoing endemic measles transmission or time-limited outbreaks: either from returned Australian travellers and their

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**Table 3. Age distribution of sampled individuals**

| Age-group (years) | Total tested (% of total) | MeV detected (% of MeV) | MeVV detected (% of MeVV) |
|-------------------|--------------------------|-------------------------|--------------------------|
| <1                | 1774 (18.15)             | 7 (2.14)                | 0                        |
| 1–4               | 3645 (37.29)             | 137 (41.90)             | 112 (94.12)              |
| 5–9               | 1267 (12.96)             | 9 (2.75)                | 0                        |
| 10–14             | 507 (5.19)               | 20 (6.12)               | 1 (0.84)                 |
| 15–19             | 444 (4.54)               | 33 (10.09)              | 2 (1.68)                 |
| 20–24             | 374 (3.83)               | 32 (9.79)               | 2 (1.68)                 |
| 25–29             | 330 (3.38)               | 19 (5.81)               | 1 (0.84)                 |
| 30–34             | 321 (3.28)               | 38 (11.62)              | 0                        |
| 35–39             | 276 (2.82)               | 18 (5.50)               | 1 (0.84)                 |
| 40–44             | 223 (2.28)               | 6 (1.83)                | 0                        |
| 45–49             | 153 (1.57)               | 3 (0.92)                | 0                        |
| 50–54             | 130 (1.33)               | 1 (0.31)                | 0                        |
| 55–59             | 110 (1.13)               | 1 (0.31)                | 0                        |
| 60–64             | 78 (0.80)                | 2 (0.61)                | 0                        |
| 65–69             | 48 (0.49)                | 0 (0.00)                | 0                        |
| 70–74             | 46 (0.47)                | 0 (0.00)                | 0                        |
| 75–79             | 27 (0.28)                | 0 (0.00)                | 0                        |
| 80–84             | 14 (0.14)                | 0 (0.00)                | 0                        |
| >85               | 5 (0.05)                 | 0 (0.00)                | 0                        |
| No DOB            | 2 (0.02)                 | 1 (0.31)                | 0                        |
| TOTAL             | 9774 (100.00)            | 327 (19.38)             | 119 (69.42)              |

DOB=Date of birth.
contacts or from specimens provided for testing/confirmation directly from these countries as part of our reference laboratory service. Our findings reinforce Australia’s status as having achieved measles elimination. We did not detect any formally extinct or any non-circulating genotypes in this study despite detections from travellers arriving in Queensland from a range of points of embarkation. High-quality laboratory services are required in settings where endemic measles circulation has been halted for identification of imported virus and its transmission and to confirm continuing elimination status.

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Author contributions
JLM – investigation, analysis, manuscript review and editing. JN – investigation, analysis, manuscript review and editing. MF – investigation, manuscript review and editing. ML – investigation, manuscript review and editing. SBL – conceptualisation, manuscript review and editing, supervision. IMM – conceptualisation, planned the study, analysed data, wrote the manuscript. All authors reviewed, revised and approved the final version.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The Forensic and Scientific Services Human Ethics Committee assessed this project as not requiring full HREC review. It was not recognised to be research and is an audit of practice in accordance with the definition of research, page 6, of the National Statement on Ethical Conduct in Human Research (2007) updated in 2018.

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