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Applicability of Oral Fluid Collected onto Filter Paper for Detection and Genetic Characterization of Measles Virus Strains

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Expansion of measles molecular surveillance to developing countries where measles is endemic will help facilitate measles control. Limited infrastructure in these areas is a barrier to referral of specimens suitable for measles virus (MV) genotyping. In this study, we demonstrate that oral fluid dried onto filter paper can be used for the detection and characterization of MV strains. Using this approach, an MV-positive sample by reverse transcriptase PCR could be obtained from 67% of serologically confirmed acute measles cases. Mimicking certain environmental conditions and duration of transportation established that MV RNA remained detectable and suitable for nucleic acid sequencing in oral fluid spots for at least 1 week. In the context of a measles outbreak in a remote region of the world where infrastructure is poor, oral fluid samples dried onto filter paper and sent to a specialized laboratory for testing will aid in the identification and characterization of the causative MV strain.

Vaccination programs have controlled measles infection in many parts of the world, and the interruption of measles virus (MV) transmission has been demonstrated in some countries including the United States (1) and Australia (4). Despite these achievements, measles continues to be a major childhood disease, causing nearly 800,000 fatalities annually (22), and this has prompted the World Health Organization to undertake a phased approach to its elimination in other geographical locations.

Laboratory diagnosis of measles infection is achieved primarily through the demonstration of MV-specific antibodies. The introduction of PCR into diagnostic laboratories has provided an alternative method to virus isolation for detecting MV strains and has facilitated their characterization. In the MV genome, genetic variability exists in the nucleoprotein (N) and hemagglutinin (H) genes, with the greatest degree occurring in the carboxy-terminal end of the expressed N gene (1). This variability can be utilized in molecular typing of wild-type MV strains. A standard genotyping system and nomenclature for characterization of MVs have been established by the World Health Organization and are updated annually (24). Currently, eight clades, designated A to H, have been identified and incorporate 22 genotypes (24).

When coupled with epidemiologic techniques, molecular characterization has proven to be a powerful adjunct to measles control by providing the means to identify the infected source and subsequent transmission pathways of the virus. In addition, it facilitates assessment of the effectiveness of measles vaccination programs by detecting temporal changes in MV genotypes within a particular geographical region and distinguishing indigenous from imported transmission.

As nucleic acid techniques are used by more virology laboratories worldwide, the number of newly identified genotypes and the rate at which they have been found have increased (24). This suggests that our understanding of the extent of MV genetic heterogeneity is incomplete. To obtain a better understanding of the global picture of MV genotypes, the expansion of measles surveillance to remote areas of the world that lack the infrastructure for appropriate collection, processing, storage, and shipment of specimens for virologic testing is important.

MV initially infects and replicates in the respiratory epithelium. The virus extends to local lymph nodes where it is amplified, resulting in viremia. A few days before the onset of rash, virus can be cultured from the mucous membranes of the nasopharynx, conjunctivae, and mouth of an infected individual, suggesting that the respiratory tract is the site of virus release and that oral fluid is a rich source of the virus (19). Accordingly, oral fluid (saliva) has been used successfully for the detection of MV-specific antibodies (2, 10) and also for the detection of MVs by reverse transcriptase (RT)-PCR and their subsequent genetic characterization (12, 13, 18, 19). A major advantage of oral fluid dried onto filter paper is that it provides a format for specimen transport that is simple, convenient, and inexpensive. In addition, it offers the potential for use as a single specimen for measles serological confirmation and MV genotyping.

The utility of dried blood spots on filter paper has also been established for the detection of MV-specific antibodies (5, 11, 17) and the detection of MV RNA by RT-PCR (6, 14). However, we have shown that blood for the detection of MV RNA by RT-PCR is a less optimal specimen type than samples collected from the respiratory tract, where MV RNA remains detectable for a period of up to 2 weeks after rash onset (21). Consequently, we compared the suitability of oral fluid samples dried onto filter paper with that of nose/throat swabs (NTS) for simultaneous detection and genetic characterization of MV strains in laboratory-confirmed measles cases, including an assessment of the effects of heat, humidity, and transport time.

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MATERIALS AND METHODS

Patients and specimens. Patients with clinical evidence of measles were identified by general practitioners as part of an enhanced measles surveillance strategy operating in Victoria, Australia, since 1997 (16). These patients were then visited by a registered nurse who collected specimens including nose and throat swabs and oral fluid for laboratory confirmation and genotyping. Oral fluid samples were collected using a foam swab attached to a handle (ORACOL; Malvern Medical Developments, Worcester, United Kingdom). Participants were instructed to rub the swab along the teeth/gum line for approximately 1 min, after which the swab was returned to the plastic tube with the foam swab uppermost and transported to the laboratory at 4°C, after which time oral fluid was collected into the bottom of the plastic tube by centrifugation at 3,000 × *g* for 15 min. Approximately 80 μl to 100 μl of oral fluid was applied to a filter paper disk (no. 903; Schleicher & Schuell) where it absorbed across an area of approximately 25 mm in diameter. The filter paper was allowed to dry at room temperature, transferred to a plastic resealable specimen bag, and stored at −70°C along with the original oral fluid until required for this retrospective analysis. Nose and throat swabs were pooled in 2.0 ml of viral transport medium at the time of collection, transported to the laboratory at 4°C, tested immediately by PCR, and then stored at −70°C.

A total of 39 paired oral fluid specimens and NTS from measles-specific immunoglobulin M (IgM)-positive cases identified by a commercial enzyme immunoassay (Dade Behring Enzygnost, Marburg, Germany) and collected between 1 and 14 days after rash onset in the years 2000 to 2004, inclusively, were included in the validation. As the original intention for the collection of the saliva specimens was not related to this study, some specimens were not stored optimally, including retention at 4°C for an unknown length of time, followed by storage at −20°C and being frozen and thawed on at least one occasion. Application of these specimens onto a filter paper disk was performed retrospectively.

Preparation of mock MV-positive samples on filter paper. Log dilutions from 10^{-1} to 10^{-8} of MV-infected B95 cells showing MV-specific cytopathic effects (CPE) (15) were prepared in oral fluid collected from an MV IgG and IgM antibody-negative volunteer. A volume of 100 μl of each dilution was applied to a filter paper spot and allowed to dry. Total RNA was extracted from both the diluted oral fluid and the dried filter paper spots as described below, and MV-specific RT-PCR was performed on each sample. A specimen was considered to be positive for MV RNA if a visible product of the correct size was present on an agarose gel following RT-PCR. To assess the infectivity of MV dried onto filter paper spots, a second set of dilutions (undiluted to 10^{-6}) was prepared in viral transport medium (VTM) and spotted in the same manner as described above. When dried, the spot was excised from the filter paper and soaked in VTM. A 100-μl volume of this VTM a well as 100 μl of the original dilution were transferred to separate wells of a 24-well plate containing 1 ml RF10 cell culture medium and B95 cells at 75% confluency. The plate was then incubated at 37°C in 5% CO_2 for 2 weeks or until CPE was apparent.

Extraction of RNA from filter paper. Total RNA was extracted directly from oral fluid using a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Hilden, Germany) according to the manufacturer's guidelines. Total RNA was extracted from oral fluid dried onto filter paper using the above-mentioned kit with minor modifications. Briefly, a segment corresponding to the diameter of the saliva spot was excised from the filter paper using a separate sterile scalpel for each specimen and placed in a 1.5-ml Eppendorf tube. A volume of 300 μl of the supplied binding buffer containing poly(A) RNA carrier was added, followed by 300 μl of nuclease-free water and 60 μl of proteinase K (18 mg/ml). The sample was then spiked with 20 μl of diluted bovine viral diarrhea virus to serve as an internal control for RNA extraction, reverse transcription, and amplification. The filter paper was then mulched using a sterile swab stick, thoroughly vortexed, and incubated at 72°C for 30 min. Following incubation, the specimen was spun at high speed (13,000 × *g*), and then 150 μl of isopropanol was added. The filter paper was mulched again as described above and vortexed. Supernatant devoid of filter paper was then transferred to a column, and further extraction steps were carried out according to the manufacturer's guidelines. To assess the reproducibility of the extraction procedure, a volume of 100 μl of MV RT-PCR-positive oral fluid diluted 1 in 10 in phosphate-buffered saline (PBS), pH 7.2, was applied to 20 filter paper spots which were subjected to nucleic acid extraction and subsequent RT-PCR in parallel.

Assessment of the impact of environmental conditions on detection of MV from dried filter paper spots. Two MV-positive oral fluid samples (sample 1 [genotype H1] and sample 2 [genotype D8]) were used to evaluate the stability of MV RNA stored on filter paper for various times at different temperatures and humidities. Additional PBS was added to both specimens to provide sufficient volume for testing (one part of sample 1 to one part of PBS; one part of sample 2 to four parts of PBS). Filter paper disks spotted with 100 μl of either of these samples were then stored at room temperature, 37°C in moist conditions of 90% humidity, 37°C in dry conditions of 22% humidity, 4°C, and −20°C at each of the time points 0, 4, 12, 24, 48, and 72 h and 1 week. RNA extracted from each of the disks was converted to cDNA as described below and then diluted in 3 log steps in nuclease-free water and tested by RT-PCR.

RT-PCR and sequencing. Following extraction of RNA from the filter paper, RT-PCR, sequencing, and phylogenetic analysis of the carboxy-terminal end of the MV N gene were performed as previously described (3). Briefly, MV RNA was reverse transcribed using random hexamers, and heminested primers were then used to amplify a 528-base-pair second-round product. The product was viewed on a 2% agarose gel containing ethidium bromide and viewed and photographed using a GelDoc 2000 (Bio-Rad, Hercules, CA). Amplification of an internal control was performed in a separate PCR described elsewhere previously (7). MV-specific PCR products were purified using a Montage PCR centrifugal filter device according to the manufacturer's guidelines (Millipore). Purified products were sequenced in the forward and reverse directions using a cycle sequencing reaction (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit; Perkin-Elmer, Applied Biosystems Division, Foster City, CA) (3). The reaction products were analyzed using an ABI 3730S capillary sequencer. Nucleotide sequences were analyzed and the amino acid sequence was determined using the SeqEd version 1.0.3 program (Applied Biosystems, Foster City, CA). Phylograms were created with PHYLIP, version 3.5c (8), using DNAdist (maximum likelihood) followed by neighbor joining. Unrooted phylograms were drawn with Treeview, version 1.5 (20).

Data analysis. Exact binomial confidence intervals and *P* values were calculated using Stata Statistical Software (release 6.0, 1999; Stata Corporation, College Station, TX).

RESULTS

Comparison of unprocessed oral fluid and oral fluid dried on filter paper for RNA extraction, detection by RT-PCR, and infectivity. The utility of unprocessed (wet) oral fluid compared to that of oral fluid spotted and dried on filter paper (dry) was evaluated for the extraction of RNA and RT-PCR for MV using measles-infected B95 cells titrated in oral fluid. The undiluted and 10^{-3} dilutions of both wet and dry preparations produced a visible product of the correct size following RT-PCR, indicating that the drying process did not reduce the ability to detect MV RNA by RT-PCR (results not shown). Of the 20 replicate filter paper spots prepared from MV-positive oral fluid, a total of 17 were positive by RT-PCR, indicating 85% extraction reproducibility of the method (results not shown). The infectivity of MV dried onto filter paper was assessed in parallel with unprocessed log dilutions of the same virus. Following 2 weeks of incubation, CPE was apparent only in the unprocessed dilutions up to 10^{-3} (results not shown). No MV-specific CPE was observed in cells inoculated with dilutions of MV-infected filter paper spots.

Comparison of NTS and wet and dry oral fluid samples from laboratory-confirmed cases for detection of MV by RT-PCR. NTS and oral fluid were available from 39 patients with serologically confirmed MV infection. Thirty-seven NTS samples, 32 unprocessed oral fluid specimens, and 26 of those oral fluid specimens dried onto filter paper were positive for MV RNA (Table 1). The sensitivities of each of these specimen types and processing methods for detection of MV relative to the serology results are shown in Table 1. The negative unprocessed oral fluid samples were also negative when processed and tested in the dry format. There was no statistically significant difference between the serology results and RT-PCR result for NTS ($P = 0.49$, Fisher's exact test). Although both wet and dry oral fluid specimens were significantly less likely to be positive for MV RNA than serology, there was no

TABLE 1. Numbers tested and sensitivity of MV RT-PCR on NTS and wet and dry oral fluid samples compared to cases confirmed by detection of MV-specific IgM

Sample type	PCR result					
	No. of IgM-positive samples	No. of positive samples	No. of negative samples	Sensitivity (%)	95% CI[a]	*P* value (vs IgM)
NTS	39	37	2	95	83–99	0.49
Oral fluid (wet)	39	32	7	82	66–92	0.012
Oral fluid (dry)	39	26	13	67	50–81	<0.001

[a] CI, confidence interval.

statistical difference between the NTS and the wet oral fluid results ($P = 0.18$; McNemar's test for paired samples with continuity correction). However, there was a statistically significant difference between NTS and dried oral fluid for detection of an MV-specific PCR product ($P < 0.01$). Overall, there was a significant decrease in sensitivity from NTS through to oral fluid dried on filter paper ($P = 0.0015$; Chi squared for trend).

Effect of time, temperature, and humidity on detection of MV on filter paper. The effect on MV RNA detection of environmental conditions likely to be experienced during transport of oral fluid spotted onto filter paper was assessed. MV RNA was detected in both tested samples following storage for 1 week at all time points and temperatures, with the exception of sample 2, stored at 37°C under humid conditions, in which MV RNA was undetectable after 48 h (Table 2). Differences in the quantity of RNA recovered from spots were observed (for example, the occasional loss of RNA from an intermediate titer and a reduced titer at 48 h in sample 2 at higher temperatures). However, RNA was always detected in undiluted samples, irrespective of the conditions tested (Table 2). The PCR products derived from sample 1 stored at 37°C under humid conditions for 1 week and from sample 2 stored at 37°C under dry conditions for 1 week were sequenced in both directions and showed no nucleotide changes compared to the original samples stored at −70°C (results not shown).

DISCUSSION

In this study, we demonstrate for the first time that oral fluid collected onto filter paper is a suitable specimen for the detection of MV RNA by RT-PCR and subsequent genotyping and phylogenetic analysis of the virus. Samples showed acceptable stability after being subjected to a variety of environmental conditions that could be experienced during transport from the field to the laboratory. Oral fluid was also shown to be a safe option for handling postcollection, as infectivity was absent once dried on filter paper. Importantly, there were no differences in the nucleotide sequences of the PCR products derived from unprocessed or dried oral fluids, indicating the stability of MV RNA when stored under harsh conditions and the overall general utility of filter paper transport of MV-positive material for subsequent genetic characterization.

Overall, MV RNA was recovered from 67% of dry oral fluid spots from patients with acute measles infection, compared to 82% of unprocessed oral fluids and 95% of NTS. A similar diminished recovery from oral fluid compared to that found with NTS was seen in a study performed during a measles outbreak in The Netherlands (23). In that study, MV RNA was detected in 93% of NTS specimens and 88% of unprocessed oral fluid samples (23). It appears from our results that the drying of oral fluid results in a further incremental reduction in recovery of MV RNA, and it is likely that the reduced efficiency of RNA extraction from filter paper disks also contributes to this. However, since measles genotyping and phylogenetic analysis need only be undertaken on a few representative samples of circulating MVs, these levels of sensitivity are acceptable. Of note, the majority of specimens we analyzed had previously been tested and stored at −20°C or −70°C and subjected to occasional rounds of freeze-thawing, procedures that are likely to have resulted in decreased MV titers.

Use of filter paper as a specimen transport matrix from which to recover MV RNA has been previously described for blood samples (6, 14). However, several groups have demonstrated that blood has some drawbacks for the detection of MV RNA. The duration of MV PCR positivity in serum is less than that in respiratory secretions and is influenced by the MV-specific IgG response (21). Similarly, a study of blood samples applied to filter paper demonstrated that the number of MV positives obtained by RT-PCR was indirectly proportional to the presence of MV-specific IgM (6).

Investigation of a range of environmental conditions potentially encountered during specimen transportation, including temperature and humidity over a range of time periods up to 1 week, demonstrated that MV RNA remained detectable and yielded identical nucleic acid sequence to that obtained at baseline. Previous studies have shown that cells infected in vitro with MV and then diluted in blood and spotted onto filter paper were positive for MV RNA by RT-PCR after 25 weeks of storage at room temperature (6). These results, and the ones we report here, attest to the stability of MV under suboptimal conditions sufficient to enable subsequent RNA extraction, detection, and characterization.

This study demonstrates the utility of oral fluid collected onto filter paper for the detection of MV by RT-PCR and subsequent genotyping and phylogenetic analysis. Although sensitivity is reduced somewhat compared to that of RT-PCR from NTS, it is nevertheless acceptable for undertaking molecular surveillance. One potential drawback was the need for low-speed centrifugation of the oral fluid sample. Since then, changes to the apparatus used to collect these samples has improved, and it is now possible in those areas where a centrifuge in not available to manually squeeze the sponge to remove a sufficient amount of saliva to be spotted onto a filter paper disk (David Brown, personal communication). Oral fluid spots provide a simple, noninvasive approach to specimen col-

TABLE 2. Detection of MV-specific RNA following extraction of MV RNA from oral fluid stored on filter paper at variable times and storage temperatures

Sample	Time	cDNA dilution[a]	Temp (°C)				
			Room	37 (humidified)	37 (dry)	4	−20
1	0 h	Neat	+				
		10–1	+				
		10–2	+				
		10–3	−				
	4 h	Neat	+	+	+	+	+
		10–1	+	+	+	+	+
		10–2	−	−	−	+	+
		10–3	−	−	+	−	+
	12 h	Neat	+	+	+	+	+
		10–1	+	+	+	+	+
		10–2	+	−	+	+	+
		10–3	+	−	−	−	−
	24 h	Neat	+	+	+	+	+
		10–1	+	+	+	+	+
		10–2	+	+	+	+	−
		10–3	−	−	−	+	−
	48 h	Neat	+	+	+	+	+
		10–1	−	+	+	+	+
		10–2	−	−	−	+	−
		10–3	−	−	−	−	−
	72 h	Neat	+	+	+	+	+
		10–1	+	+	+	+	−
		10–2	−	−	+	+	+
		10–3	−	−	−	−	−
	1 week	Neat	+	+	+	+	+
		10–1	+	+	+	+	+
		10–2	+	−	−	−	+
		10–3	−	−	−	−	−
2	0 h	Neat	+				
		10–1	+				
		10–2	−				
		10–3	−				
	4 h	Neat	+	+	+	+	+
		10–1	−	−	+	+	+
		10–2	−	−	−	−	−
		10–3	−	−	−	−	−
	12 h	Neat	+	+	+	+	+
		10–1	+	−	+	+	+
		10–2	−	−	−	−	−
		10–3	−	−	−	−	−
	24 h	Neat	+	+	+	+	+
		10–1	+	−	−	+	+
		10–2	−	−	−	−	−
		10–3	−	−	−	−	−
	48 h	Neat	+	+	+	+	+
		10–1	+	−	−	+	+
		10–2	−	−	−	−	+
		10–3	−	−	−	−	−
	72 h	Neat	+	−	+	+	+
		10–1	+	−	−	+	−
		10–2	−	−	−	−	−
		10–3	−	−	−	−	−
	1 week	Neat	+	−	+	+	+
		10–1	+	−	+	−	−
		10–2	−	−	−	−	−
		10–3	−	−	−	−	−

[a] "cDNA dilution" refers to log dilutions in nuclease-free water of measles virus RNA that had been reverse transcribed to cDNA following extraction.

lection and a compact, logistically straightforward, and relatively inexpensive means of specimen transport. They provide the option of a single specimen from which IgM serology and measles genotypic analysis can be attempted. Alternatively, both blood and oral fluid spots from patients could be collected together on the same card for separate analysis. Oral fluid spots provide a very practical sample type for collection in regions where the infrastructure to support measles molecular surveillance programs is lacking. Their use offers the potential to greatly expand the geographic coverage of measles molecular surveillance and to considerably enhance our understanding of measles epidemiology.

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