Molecular characterization of measles viruses isolated in Victoria, Australia, between 1973 and 1998

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Molecular epidemiology studies have made significant contributions to the control of measles virus infection through the identification of source and transmission pathways of the virus. These studies allow observation of changes in measles virus genotypes over time in a particular geographical location, clarification of epidemiological links during measles outbreaks, separation of indigenous strains from newly imported strains and distinction between vaccine- and wild-type virus-associated illness. A total of 35 wild-type measles viruses identified in Victoria, Australia, between 1973 and 1998 were characterized by nucleic acid sequence analysis of the nucleoprotein gene and, in some cases, the haemagglutinin gene. Relatedness between the viruses was studied and genotypes were assigned using a classification scheme recently proposed by the World Health Organization. Five recognized genotypes (C2, D1, D4, D5 and H) and one previously undescribed genotype, which we propose to be D7, were identified. Successive replacement of measles virus genetic lineages occurred in Victoria, with no evidence of temporal overlap, during this 25 year period. This pattern of circulation is likely to represent serial importation of wild-type measles virus strains from overseas foci of measles virus infections.

Introduction

Despite its status as a disease preventable by vaccination, measles virus (MV) infects up to 30 million individuals and causes nearly one million deaths each year, mostly in Africa and south-east Asia (WHO, 1999). Eradication of MV has been set as a target by the WHO Expanded Program on Immunization and considerable progress has been made towards this objective, particularly in the Americas (WHO, 1999), although constant attention to the implementation of appropriate elimination strategies remains a priority in this region.

MV is serologically monotypic and vaccines derived from the 1954 Edmonston isolate provide protection against all MVs presently circulating (Rota et al., 1994). However, genetic heterogeneity exists among MVs (Bellini & Rota, 1998) and this provides a basis for the study of their molecular epidemiology. A unified nomenclature for MV strains and a definition of their genotypes has recently been proposed (WHO, 1998). Under this scheme, sequencing of the MV nucleoprotein (N) and haemagglutinin (H) genes is used to assign MVs to one of eight clades, designated A to H. At present, clades B, C and D are further subdivided into genotypes B1 and B2, C1 and C2, and D1 to D6, respectively. Clades are used to indicate the relationship between the various genotypes. The genotype designations are the operational taxonomic units. Reference sequences for each recognized genotype have been designated (WHO, 1998). Since the publication of the WHO nomenclature guidelines, two new genotypes, B3 (Hanse et al., 1999) and G2 (de Swart et al., 2000), have been proposed.

Because of a lack of sufficient studies and the complexity of the virus at the molecular level, an understanding of MV molecular epidemiology is currently incomplete. Many MV genotypes show clear geographical associations, while others appear to be more widely distributed (Rima et al., 1995; Bellini & Rota, 1998; Santibanez et al., 1999). For example, isolates from central and western Africa have mostly been members of clade B, while recent isolates from the Republic of South Africa belong to genotypes A, D2 and D4 (Kreis et al., 1997; Hanses et al., 1999). A particularly complex pattern of genotypes is evident in the USA and the UK, possibly as a result of more
widespread molecular strain surveillance and the frequency of international travel to these countries (Rota et al., 1996, 1998; Jin et al., 1997; Bellini & Rota, 1998).

It remains unclear how many MV genotypes circulate in regions without effective control through vaccination. However, experience gained in the USA suggests that genetic diversity of MV might be relatively restricted under conditions of low herd immunity. The introduction of a two-dose vaccination schedule and aggressive vaccination programs, such as those conducted by the Pan American Health Organization, are two possible reasons for the interruption of the widespread occurrence of the single MV lineage present between 1988 and 1992 in the USA (Rota et al., 1996; Bellini & Rota, 1998). Subsequent MV outbreaks in the USA have been attributable to multiple genetic lineages imported from overseas (Rota et al., 1996, 1998). In contrast, a recent study of Nigerian and Ghanaian MV isolates revealed the co-circulation of two distinct viruses, suggesting that the number of susceptible individuals in these areas was sufficient to support the circulation of multiple chains of transmission. This may be a characteristic pattern in communities with low vaccination rates (Hanses et al., 1999). A similar picture was also observed during an outbreak in the People’s Republic of China, where two distinct genotypes circulated in four of its provinces during 1993 and 1994 (Xu et al., 1998). Similarly, in Russia and central Europe considerable genotypic intermixing occurs, with evidence of the appearance of a number of distinct genotypes in several countries during the late 1980s and 1990s (Santibanez et al., 1999).

In Australia, live attenuated measles virus vaccine was introduced and included in childhood vaccination schedules (at 1 year of age) in 1971. In 1988, the first national measles campaign was undertaken, but it failed to prevent a nationwide measles outbreak in 1993 to 1994, apparently because of less-than-optimal coverage (85%: McIntyre et al., 2000). Between 1994 and 1998, a second dose of measles–mumps–rubella (MMR) vaccine was recommended for all children aged from 10 to 16 years (Lambert et al., 2000; McIntyre et al., 2000). This age was brought forward to 4 to 5 years when a ‘catch-up’ dose was administered in 1998 to all primary school children, a strategy in line with the USA and the UK, where the transmission of indigenous MV has been interrupted.

These observations highlight the importance of identifying those MV strains circulating prior to mass vaccination campaigns. This facilitates the differentiation of subsequent outbreaks as either originating overseas or being part of continuing transmission of indigenous MV lineages, the latter suggesting failure of control measures to interrupt transmission of virus. In this study, we investigated the molecular epidemiology of MV in Victoria, Australia, between 1973 and 1998. By sequencing the N and H genes of stored MV isolates we were able to establish the identity of MV strains circulating after the commencement of a mass vaccination campaign in 1970 and prior to the ‘catch-up’ campaign in 1998.

### Methods

**Patients, specimens and virus strains.** MV sequences were obtained from specimens received between 1973 and 1998 from 35 individuals living in the state of Victoria. Specimens from which MV isolates or sequences were obtained included throat swabs, nasopharyngeal aspirates and acute phase IgM-positive sera. MV isolates were derived from a continuous monkey embryonic kidney cell line that had been inoculated with clinical material and incubated for up to 21 days at 37 °C. Supernatant from cultures showing MV-like cytopathic effects was collected and stored at −70 °C. Serum samples, obtained from whole blood that had been centrifuged at 1500 g for 10 min, were stored at 4 °C or −20 °C.

**RT–PCR.** Total RNA was extracted from the supernatant of infected cells and serum using a guanidinium isothiocyanate extraction technique (Chomczynski & Sacchi, 1987). MV RNA was reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Promega) at 42 °C for 1 h, using random hexamers as primers. Following reverse transcription, specific primers targeted to the MV Edmonston strain N gene were used to amplify a 644 bp fragment with first-round primers MVF1 (nt 985–1008, 5′ TACCCCTGCTCTGGAGCTATGCC 3′) and MVBI (nt 1629–1609, 5′ AACAAATGAGGTTAGGCG 3′) using a Taq DNA polymerase kit (QIAGEN). A second-round hemi-nested PCR was used to amplify a 528 bp fragment using primers MVF2 (nt 1101–1121, 5′ GATGGTAAAGGAGGTCAGCTGG 3′) and MVBI. The PCR cycling program consisted of denaturation for 4 min at 94 °C, followed by 40 cycles (first round) or 25 cycles (second round) of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, with final extension for 7 min at 72 °C. All products were held at 4 °C. To obtain the protein-coding sequence of MV H gene, a 2036 bp fragment was amplified using primers MVH-F (nt 7192–7211, 5′ CCTCTGGCGGAAACATATCG 3′) and MVH-R (nt 9227–9208, 5′ CAGATACCGAGGTCAATAACG 3′). The PCR cycling program consisted of denaturation for 4 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 135 s at 72 °C, with final extension for 7 min at 72 °C. All products were held at 4 °C.

**Nucleotide sequence determination.** PCR products were purified using a QIAquick PCR purification kit (QIAGEN) following the manufacturer’s instructions. Purified PCR products were sequenced in the forward and reverse directions using a cycle sequencing reaction with an ABI Prism Big Dye Terminator cycle sequencing kit (Applied Biosystems, Perkin Elmer). The carboxy-terminal 456 nt of the N gene were sequenced using the hemi-nested PCR primers MVF2 and MVB1. The protein-coding sequence of the H gene was sequenced using primers MVH-F and MVH-R and the following sequencing primers: MVH-AR (nt 7654–7635, 5′ GATCTCTGAACTGCTACTCC 3′), MVH-BF (nt 7568–7588, 5′ CACTCAGAGATTCGACTGACC 3′), MVH-AR (nt 1330–1311, 5′ GATACCGAGGTCAATAACG 3′), MVH-BF (nt 8001–8021, 5′ GATCCGGAGGTTTGAAGTAGG 3′), MVH-ER (nt 1796–1777, 5′ GAACCGTGTGTAATCAAGG 3′), MVH-FF (nt 8494–8515, 5′ GATCTGAGTCTGACGTAGAGGC 3′), MVH-GR (nt 2236–2218, 5′ CAAGCAACACAAAGTAGAGC 3′) and MVH-HF (nt 2076–2095, 5′ CAGGTTGAAACTGCTGTGG 3′). The reaction products were analysed using an ABI Prism 377 automatic DNA sequencer.

**Sequence analysis and assignment of genotype.** Nucleotide sequences were analysed with the SeqEd program, version 1.0.3. Sequence alignments were performed using Multalin (Corpet, 1988). Phylograms were created with PHYLIP, version 3.5c (Felsenstein, 1993), using DNASid (maximum likelihood) followed by neighbour-joining. Unrooted phylogenograms were drawn with Treeview, version 1.5 (Page,
Table 1. Details of the patients involved in the study and genotype of MV strains identified in Australia during 1973–1998

<table>
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<tr>
<th>No.</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Specimen type*</th>
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* Name according to the proposed nomenclature for MV strains (WHO, 1998).
† TS, Throat swab; NPA, nasopharangeal aspirate; NTS, nasal and throat swab.
‡ First accession number for D7 genotypes refers to the N gene sequence. Second accession number for D7 genotypes refers to the H gene sequence.
NA, Not available.

1996). A database of WHO-designated reference sequences for each genotype was obtained from GenBank. The assignment of each MV strain to a particular clade and genotype was based on the PHYLIP analysis. MV strains were designated according to their clade (A–H) and genotype (1–7). An MV strain of clade D genotype 1 is referred to as D1. Prototype genotypic sequences included in this analysis for comparative
purposes are indicated by block capitals. Analysis of nucleotide and amino acid sequence was carried out using Genedoc (Nicholas & Nicholas, 1997).

Results

Patients, specimens and virus strains

MV was isolated from patients ranging in age from 4 months to 29 years, with the majority being obtained from children under 6 years of age (Table 1). Most of the MV sequences analysed were derived from isolates that had been stored for several years at $-70^\circ C$. Passaging of these viruses was not undertaken prior to RT–PCR and sequencing. These wild-type MV isolates from Victoria were named according to the following scheme, MVi/Vic.AU/week.year of isolation (Table 1). MV sequences were also derived directly from the serum of five individuals (Table 1). These patients had a clinical illness consistent with MV infection and had an MV-specific IgM response in the absence of MV IgG antibody, except for one (patient no. 30), who was MV IgG antibody equivocal. Only serum specimens obtained in 1998 contained amplifiable MV sequences. These samples had been stored briefly at 4 °C before RNA extraction. MV sequences were not obtained from

![Phylogenetic analysis](image_url)

Fig. 1. Phylogenetic analysis using the maximum likelihood/neighbour-joining method of MV strains identified in Victoria, Australia (1973–1998) according to the sequence of the N gene. The nomenclature system proposed by WHO (1998) is used to indicate the geographical origin and year in which each virus was first identified. Prototype strains are shown in italic and upper case. Significant bootstrap values ($>80\%$) are indicated.
serum samples that had been stored at $-20 \, ^\circ \text{C}$ for periods of 12 months or more.

**Nucleic acid sequencing and phylogenetic analysis**

With the exception of strains circulating between 1985 and 1989, phylogenetic analysis enabled the assignment of all strains listed in Table 1 into previously identified genotypes (Fig. 1). The clustering of viruses within a particular genotype was supported by a significant bootstrap value ($> 80\%$ for 1000 replicates). Other methods of analysis, for example parsimony and distance methods (DNA and amino acid), were performed on the dataset and revealed virtually the same outcome (data not shown). The unassignable strains were closest to the Thailand ‘93 D5 genotype by nucleotide similarity and shared 445 nt of a total 456 nt in the carboxy-terminal region of the N gene (BLAST search; data not shown). However, they were phylogenetically closest to the Chicago ‘89 D3 prototype, from which they differed by 13 nt in the N gene sequence (Fig. 2). The assignment of these nine viruses to an apparently novel genotype within clade D, based on N gene analysis, was confirmed by sequence analysis of the entire H gene protein-coding region, which revealed closest similarity to the Canada ‘89 D4 prototype (BLAST search; data not shown).
shown). On the basis of these results, the nine strains were assigned to a new genotype (D7).

Of the nine prototype D7 strains identified by phylogenetic analysis, all but one (MVi/Vic.AU/29.88) shared an identical N gene nucleotide sequence. MVi/Vic.AU/29.88 diverged from the others in the group by three nucleotides, which resulted in two amino acid changes (P510S and Y518H; Fig. 3).

As a group, these nine viruses shared four nucleotides and two characteristic amino acids in the N protein, 443G and 473Q, that were not seen in any other reference prototype analysed (Fig. 3).

Analysis of the same nine strains revealed H gene divergence within the group of up to four unique nucleotide changes (in one strain MVi/Vic.AU/42.88) across the entire 1854 nucleotide sequence (data not shown). Three strains (MVi/Vic.AU/16.85, MVi/Vic.AU/48.85 and MVi/Vic.AU/52.85) shared identical sequences which differed from the other D7 strains by three nucleotides (data not shown). Of the remaining five strains, two (MVi/Vic.AU/29.88 and MVi/Vic.AU/51.86) possessed a single unique nucleotide change. All the observed mutations in the H gene were silent except for one, which resulted in an R261K amino acid change in MVi/Vic.AU/51.86. This amino acid change is unique to MVi/Vic.AU/51.86 when compared to all other MV reference prototypes. As it is unique to this single member of the D7 prototype, it is unlikely that it plays a significant role in determining the D7 genotype. As a group, however, members of the D7 genotype shared amino acids in the H protein that were not found in any other reference prototype, namely 195K, 351I, 395D and 592E.

**Temporal distribution of measles viruses**

Six genotypes circulated in Victoria during the 25 year period of study: D1, C2, H, D4, D5 and the novel D7 genotype, circulating between 1985 and 1989. Successive replacement of MV genotypes occurred during this study period without evidence of temporal overlap. All 11 MV isolates obtained between 1973 and 1981 were genotype D1, similar to MVO '74 UK prototype strain. Six isolates, obtained between 1990 and 1991, were similar to the German Fleckenstein '90 prototype strain and were therefore classified as genotype C2. A single isolate from 1993 was classified as genotype H, the prototype of which is the China '94 strain. Five viruses from early 1998 were genotype D4, of which Canada '89 is the prototype strain. The most recent three strains from late 1998 belong to genotype D5, represented by prototypes Thailand '93 and Palau '94.
Discussion

This study represents one of the most extensive descriptions of MV molecular epidemiology in a single geographical region reported to date. Multiple MV genotypic lineages were identified among Victorian MVs collected over the 25 year period between 1973 and 1998. Successive replacement of these genotypes occurred during this time, without evidence of temporal overlap. While two genotypes circulated in 1998, they did not appear to overlap in time. Our analysis shows that genotype D1 circulated in Victoria for at least 9 years. It was last detected in 1981. This is the longest circulation time of any of the MV genotypes identified in this study, and might represent the final activity of Victoria’s pre-vaccine MV. Live attenuated measles virus vaccine was introduced into Australia in 1971. Discharge diagnosis data from Fairfield Infectious Diseases Hospital, Melbourne, Victoria, reflects large biennial measles outbreaks occurring until 1971, followed by an abrupt decline over the next 10 years. Approximately 2000 measles cases were admitted to Fairfield Hospital in the 20 years after 1971, compared to 3660 cases in the preceding 10 years (Tobin & Kelly, 1999). Introduction of the vaccine also coincided with an abrupt decline in the number of complications of measles infections, such as encephalitis (Tobin & Kelly, 1999). These observations might fit with the interpretation that MV genotype D1 was Australia’s indigenous pre-vaccine strain, although in the absence of a Victorian MV isolate from this pre-vaccine period, it is not possible to confirm this.

Following the disappearance of the D1 genotype in 1981, four additional genotypes circulated for periods of up to 4 years before disappearing. A fifth genotype, first detected in 1998, is still circulating in Victoria. Comparison of this molecular epidemiological data with hospital discharge data (Tobin & Kelly, 1999) suggests that each significant burst of MV activity in Victoria since 1984 has been associated with the appearance of a distinct genetic lineage and the disappearance of the preceding lineage. One interpretation which could be drawn from this is that MV transmission associated with an indigenous strain was interrupted in Victoria because of the successful 1970 vaccine campaign, and that the MV strains identified in this study resulted from importation of new viruses from other geographical regions, most likely from overseas but possibly from other areas of Australia. For example, genotype D5, which circulated in the latter half of 1998, is known to have circulated in Japan and Thailand. Tourism between these countries and Australia is common, suggesting that importation of these strains to Victoria is a distinct possibility. The serial replacement of MV genotypes seen in our study is similar to the four abrupt genotypic replacements that have occurred since the 1960s in Spain (Rima et al., 1997). Our study extends this data by demonstrating five successive replacements of MV genotypes among a larger collection of MVs collected in real time over 25 years.

A substantial MV outbreak occurred in 1993 in most parts of Australia, but relatively little increase in MV activity was seen in Victoria (data not shown). It may be that the single clade H isolate available to us from that time represented the genotype associated with this outbreak. This clade is known to have circulated in China (Xu et al., 1998), where it was first detected in 1993. Subsequent to that time (in 1997), we have identified two New Zealand isolates as clade H viruses, obtained during a substantial MV outbreak during that year (data not shown). Two importations into the US in 1997 have also been associated with this genotype (Xu et al., 1998).

The ability to classify MV into distinct clades arises from the variability of the N gene. The nucleotide sequence of this gene has been reported to vary between MV isolates by as much as 7%, with most of this occurring in the 450 nt of the carboxy terminus, where variation can be up to 12% (WHO, 1998). Phylogenetic analysis currently defines eight MV clades, which together comprise 15 separate genotypes. At present, no criteria exist to enable the definition of a new genotype. It is expected, however, that such genotypes will be able to be assigned, albeit temporarily in some cases, to an existing clade represented by a prototype strain having a reference sequence (WHO, 1998). It has been suggested that a variation between existing genotypes of approximately 2% at the nucleotide level should be used to define a new genotype (Kreis et al., 1997). Another proposal, based on common characteristic mutations, has suggested that two sets of viruses would belong to different genotypes when at least five set-specific mutations occur in the carboxy terminus (Hanses et al., 1999). However, neither of these definitions appear to satisfactorily define or distinguish clades or genotypes in the absence of phylogenetic studies.

On the basis of phylogenetic analysis, we propose that the prototype to the novel strain we have identified be termed MV/Vic.AU/16.85 and classified as genotype D7. This new genotype differs in the N gene by 13 nt from its phylogenetically closest counterpart, the Chicago ’89 D3 genotype. Analysis of the N gene nucleotide differences between clade D genotypes reveals variation by as few as nine [between Chicago ’89 (D3) and Thailand ’93 (D5)]. A similar analysis between clades, for example Edmonston (A) and Johannesburg (D2), reveals variation in nucleotides by as few as eight in the same region. The deduced amino acid sequence of the proposed D7 genotype N gene is closest to the Thailand ’93 D5 prototype, from which it differs by six amino acids. However, it phylogenetically groups with Chicago ’89 (D3), from which it differs by seven amino acids. By comparison, some distinct genotypes [MVO ’74 UK (D1) and Johannesburg (D2)] vary by as few as four amino acids and some distinct clades [Edmonston (A) and Johannesburg (D2)] vary by as few as seven amino acids.

Comparison of the H gene of the proposed D7 genotype with both clade D genotypes and clade prototypes gave similar results to those obtained through analysis of the N
gene. MV/Vic.AU/16.85 was closest by nucleotide and amino acid analysis to the Canada '89 D4 prototype, from which it differed by 34 nt (7 aa). Comparison of the H gene of MV strains within genotypes and between clades sometimes reveals closer relationships than that existing between D7 and D4. For example, Canada '89 D4 and Palau '94 D5 differ from each other by only six amino acids. This highlights the difficulty of attempting to define new clades or genotypes on the basis of simple nucleotide or amino acid differences compared to existing reference prototype strains. Therefore, it is likely that the identification of new clades and genotypes in the future will rely considerably on phylogenetic analysis. Even then, it remains unclear as to whether a novel, distinct group identified by phylogeny will represent a truly new clade or a new genotype.

It is evident from this study that for the last 15 years, MV outbreaks in Victoria, and by inference Australia, have arisen from the importation of a variety of exotic MVs without evidence of sustained transmission of a local strain. The global distribution of MV genotypes is insufficiently understood for us to be confident of the geographical origin of these importations, and the origin of prototype strains is unlikely to help in this regard. Further studies will be necessary to increase our understanding of worldwide MV genotypic distribution and significance.

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References


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