Genetic analysis of Asian measles virus strains – new endemic genotype in Nepal

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Abstract

In many parts of Asia measles virus (MV) continues to be endemic. However, little is known about the genetic characteristics of viruses circulating on this continent. This study reports the molecular epidemiological analysis based on the entire nucleocapsid (N) and hemagglutinin (H) genes of the first isolates from Nepal and Taiwan, as well as of recent MV strains from India, Indonesia, and China. Four isolates collected in various regions in Nepal during 1999 belonged to a new genotype, tentatively called D8. Another Nepalese isolate and one from India belonged to genotype D4. The diversity of the Nepalese strains indicated that measles continues to be endemic in this country. The isolate from Taiwan grouped with D3 viruses and one Chinese strain isolated in The Netherlands was assigned to the previously described clade H, known to be endemic in Mainland China. Molecular characterization emerges as an important tool for monitoring virus endemicity and vaccination efforts. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Measles virus; Genotype distribution; Molecular epidemiology; Asia

Measles is a highly contagious disease and one of the leading causes of childhood morbidity and mortality. Considerable efforts are currently undertaken by an increasing number of countries to eliminate measles from their territories. As a result of these intensified efforts, the interruption of
transmission of indigenous measles virus (MV) was declared for the first time in 1993 in the US (Watson et al., 1998). Molecular characterization of measles strains demonstrated that the previously endemic virus had ceased to circulate (Rota et al., 1996). In some parts of Europe, however, measles remains endemic as the virus can circulate within the population without interruption, but occasional outbreaks in other parts of Europe have been linked to importation events from other endemic areas in Africa (Kreis et al., 1997; Hanses et al., 1999; Truong et al., 1999) and Asia (Jin et al., 1998; Xu et al., 1998). Several regions in Asia are particularly affected: Bangladesh had a dramatic increase in the reported measles incidence by 445% between 1990 and 1997, perhaps as a result of improved reporting (CDC, 1999). Other high risk regions in Asia include the border areas of the Democratic People’s Republic of Korea and the urban centers in India and Nepal. In Nepal, the number of reported measles cases increased steadily since 1990 (CDC, 1999).

Molecular epidemiology plays an important role in the world-wide effort to control measles. Genotypic characterization of wild-type (wt) MVs has focused on the nucleoprotein (N) and hemagglutinin (H) genes, which contain the highest sequence variability within the genome (WHO, 1998). Several studies have shown that distinct lineages of wt MVs seem to be prevalent in more or less confined geographic regions (Rima et al., 1995; Bellini and Rota, 1998).

While virus isolates have been reported from a number of countries in Europe (Rima et al., 1995; Jin et al., 1997; Santibanez et al., 1999), the USA (Rota et al., 1996, 1998), and Africa (Kreis et al., 1997; Outlaw et al., 1997; Hanses et al., 1999; Truong et al., 1999), most of the available MV sequences from Asia were derived from strains isolated in Japan (Katayama et al., 1997; Yamaguchi, 1997; Rota et al., 1998) and China (Jin et al., 1998; Xu et al., 1998).

In this study, we report the first sequences from viruses isolated in Nepal and Taiwan, as well as some new isolates imported from India, Indonesia, and China (Table 1). Phylogenetic analysis

Table 1

<table>
<thead>
<tr>
<th>MV isolate (Mvi)</th>
<th>Country of isolation</th>
<th>Country of origin</th>
<th>Genotype</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
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<tr>
<td>Taiphe.TWN/94</td>
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<td>Taiwan</td>
<td>D3</td>
<td>AJ250060 AJ250068</td>
</tr>
<tr>
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<td>India</td>
<td>D4</td>
<td>AJ250067 AF193513</td>
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<tr>
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<td>Nepal</td>
<td>D4</td>
<td>AJ250065 AJ250073</td>
</tr>
<tr>
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<td>Nepal</td>
<td>Nepal</td>
<td>D8</td>
<td>AJ250061 AJ250069</td>
</tr>
<tr>
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<td>Nepal</td>
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<td>AJ250062 AJ250070</td>
</tr>
<tr>
<td>Hetauda.NEP/2.99</td>
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<td>D8</td>
<td>AJ250063 AJ250071</td>
</tr>
<tr>
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<td>Nepal</td>
<td>D8</td>
<td>AJ250064 AJ250072</td>
</tr>
<tr>
<td>Amsterdam.NET/48.97</td>
<td>Netherlands</td>
<td>Indonesia</td>
<td>G2</td>
<td>AF171231 AF171232</td>
</tr>
<tr>
<td>Amsterdam.NET/27.97</td>
<td>Netherlands</td>
<td>China</td>
<td>H1</td>
<td>AJ250066 AF193512</td>
</tr>
</tbody>
</table>

Fig. 1. Phylogenetic tree based on the 456 nt carboxyl-terminal end of the N gene of Asian MV strains reported in this study (bold) and those available from GenBank. Furthermore, the WHO reference strains for each genotype (WHO, 1998) are shown in italics. The unrooted tree was generated using the ClustalX sequence analysis program (Thompson et al., 1997). The scale indicates 1% nt difference. Numbers at the nodes indicate bootstrap values (1000 replicates, in %). (*) indicate proposed genotypes: B3-1: MVI/Ibadan.NIE/97/1 (AJ232203); B3-2: MVI/New York.USA/94/N (L46753) D7: MVI/Vic.AUS/48.85 (AF243451; Chibo et al., 2000); D8: MVI/Manchester.UNK/94/140 (AF280803); G1: MVI/Berkeley.USA/83 (U01974); G2: MVI/Amsterdam.NET/48.97 (AF171234); H1: MVI/Hunan.CHN/93/7 (AF045212); H2: MVI/Beijing.CHN/94/1 (AF045217). (*) incomplete sequence. Country abbreviations: AUS: Australia, CAE: Cameroon, CAN: Canada, CHN: China, GAB: Gabon, GER: Germany, INO: Indonesia, JPN: Japan, NEP: Nepal, NET: The Netherlands, NIE: Nigeria, PAK: Pakistan, SOA: South Africa, SPA: Spain, THA: Thailand, TWN: Taiwan, UNK: United Kingdom, USA: United States of America.
was based on the sequence alignment of the hypervariable carboxyl-terminal region of the N gene (456 nt; Fig. 1) and on the full-length H gene (1854 nt; Fig. 2). Virus isolation, RT-PCR, heteroduplex mobility assay (HMA) pre-screening, sequencing and phylogenetic analyses were per-
Fig. 2. Phylogenetic tree based on the alignment of the entire H gene. Bootstrap percentages are given at the node of each genotype (1000 replicates). (*) Indicate proposed genotypes: B3-1: MV/IB/97 1 (AJ239133); B3-2: MV/New York/94 1 (U29285); G1: MV/Berkeley/USA/83 (AF079553); G2: MV/Amsterdam/NET/48.97 (AF171231); H1: MV/Hunan/CHN/93 7 (AF045201); H2: MV/Beijing/CHN/94 1 (AF045203). See for further details Fig. 1.
formed as described previously (Kreis and Whistler, 1997; Truong et al., 1999).

Five MVs were isolated in Nepal during February 1999. Strain MVi/Kathmandu.NEP/5.99 was isolated from the urine of a 9-yr old child with typical clinical symptoms, whereas the remaining four isolates were obtained by co-cultivation of peripheral blood leukocytes with B95a cells. Two of these MVs (MVi/Janakpur.NEP/2.99/1, and MVi/Janakpur.NEP/2.99/2) were obtained from children living in the Janakpur district located in the south-eastern part of the country; one originated from Pokhara (MVi/Phokhara.NEP/5.99), situated North-West of Kathmandu, and a further one was collected in Hetauda (MVi/Hetauda.NEP/2.99), a village near the capital Kathmandu.

The isolates from Janakpur, Hetauda and Kathmandu form a separate cluster ("Janakpur-group"), distinct from other genotypes in both the C-terminal N gene (Fig. 1), and the H gene alignment (Fig. 2). The maximum genetic variability within this cluster based on the 456 nt hypervariable region in the N gene is 3.9% (distance matrix not shown). On average, the nucleotide difference with other established D genotypes varied between 4% and 7% in this gene segment. The genetic distance with genotypes belonging to other clades is even higher (8–11%). Intra-genotypic variation in other D genotypes varied between 1.8% for D6 and 5.4% for D4. Similar values were found for other non-D genotypes. The Janakpur sequences also formed a distinct cluster in the H gene alignment, with a maximum of 1.8% nt sequence variability within this cluster and with more than 3% difference with other genotypes (Fig. 2). Tentatively, we designated this cluster as the new genotype D8. This was also supported by the high bootstrap values (Figs. 1 and 2). In the HMA analysis, the four strains belonging to the "Janakpur-group" showed similar mobility profiles among each other, but distinct when compared to profiles from other genotypes (data not shown). More importantly, however, sequences belonging to the Janakpur-group cluster closely with strain MVi/Manchester.UK/94/140 (GenBank accession number N gene: AF280803; H gene: U29285). It is possible that this strain and two other closely related strains (160 and 226; Jin et al., 1997, 1998) are linked with endemic cases in the Indian Subcontinent. Another recent isolate from Texas also clusters closely with the "Janakpur"-cluster (Dr. Paul Rota, personal communication). The maximum genetic distance based on the hypervariable domain of the N gene between the Manchester strain and the members of the Janakpur-group was 2.4%, and in comparison >4.3% with D4 genotype. The genetic variability in the H gene of the newly proposed D8 genotype is 1.9%. These findings indicate that these sequences indeed belong to the same distinct genotype.

We observed a relatively high nucleotide sequence variation among all Nepalese MV strains in the C-terminus of the N gene (6.8%), although they were isolated within a period of only 4 weeks. This was similar to the high sequence diversity of 4.6% in the N gene that we have found in Nigeria, where measles is endemic (Hanses et al., 1999). This is in contrast to the genetic variability (0.2%) during an outbreak caused by a single index case (Hanses et al., 2000). The co-circulation of viruses of different lineages indicate that measles continues to be endemic in Nepal.

Strain MVi/Amsterdam.NET/3.98 was isolated in The Netherlands in 1998 from an unvaccinated adult who developed measles shortly after returning from India. Together with the fifth Nepalese virus (MVi/Phokhara.NEP/5.99) this imported MV clustered with isolates from Karachi and the D4 reference strain MVi/Montréal.CAN/89 (Fig. 1). Although very few isolates from the Indian subcontinent have been characterized, the close relation between these Indian, Pakistani and Nepalese viruses suggest that this may be a dominant lineage in this part of the world. The close relationship of two South African strains (MVi/Johannesburg.SOA/95/73 and MVi/Johannesburg.SOA/95/78) with strains from the Indian subcontinent reflect frequent travel between both regions.

We observed typical genotype-specific mutations in the hypervariable region of the N and H
genes. The D8 genotype has four specific amino acid (aa) substitutions in the N gene (I406T, K441R, Y451N and A459L; numbering according to Mori et al. (1993), and four unique silent mutations at positions 264, 807, 1386, and 1614, and a codon change at position 1430 of the H gene. The D4 genotype had three different genotype-specific aa substitutions (T469I, P515S, R521K). The Nepalese and the Indian virus MVi/Amsterdam.NET contained an additional sixth (potential) glycosylation site at position 416 in the H gene, similar to the US (Rota et al., 1994) and Japanese viruses described earlier (Saito et al., 1992; Katayama et al., 1997). Interestingly, the MV isolate from Pokhara revealed a mutation at position 240 of the H gene, which abrogated one of the five potential glycosylation sites. While this mutation is in the neutralizing epitope (NE; aa H235–255) domain, which is involved in virus-cell fusion (Fournier et al., 1997), no mutations were found in the hemagglutinin noose epitope (HNE; aa H381–405; Ziegler et al., 1996) with any of the isolates from the Indian subcontinent. The latter two domains have been proposed as a target for a peptide based subunit vaccine that would close the window of susceptibility of infants that are insufficiently protected by maternal antibodies (Kasmi and Muller, 2001).

The isolate MVi/Amsterdam.NET/48.97 was obtained from an immuno-compromised child who became infected in Indonesia in 1997 (de Swart et al., 2000). Sequence analysis of the complete N and H genes revealed that this strain belonged to clade G, assumed to be no longer active. The Indonesian clade G virus showed 3.8% (N gene) and 3.6% (H gene) variation when compared to the two US reference isolates obtained in 1983 in the cities Boston and Berkeley, for which a new genotype G2 was proposed (de Swart et al., 2000). Rota and co-workers provided direct evidence of circulation of the G2 genotype in Malaysia and Indonesia (Rota et al., 2000). Interestingly, the three clade G MVs shared six silent nt mutations in the H gene, which have previously only been found in Chinese viruses of clade H (Xu et al., 1998).

The Taiwanese MV strain was collected in 1994 from a child living in Taipei (MVi/Taipeh.TWN/94). Based on both N and H gene sequences, this virus belonged to genotype D3 (Fig. 1). Besides Taiwan, the D3 genotype has also been found in Japan (Katayama et al., 1997; Yamaguchi, 1997), and had been imported into the US from the Philippines (Rota et al., 1998). Japanese MV strains were not included in Fig. 1 since only 300 nt of the N gene sequences were available from the GenBank database. However, when analyzing the shorter sequences the Japanese strains grouped with genotype D3 (data not shown), suggesting a Japanese, rather than a Chinese source for the Taiwanese virus.

Finally, a MV was studied that had been isolated in the Netherlands in 1997 from a patient who had most likely been infected by his girlfriend, who herself developed serologically confirmed measles shortly after travelling in China. The virus was isolated both from peripheral blood mononuclear cells and urine specimens. Both the N and H genes of the two isolates were sequenced. Apart from one silent nt change in the H gene, the urine and blood isolates were identical in sequence. The isolate MVi/Amsterdam.NET/27.97 was found to cluster with clade H viruses (Fig. 1), particularly with viruses collected in China between 1993 and 1994 (Xu et al., 1998). The nt divergence varied between 1.2–1.9% and 1.1–1.4% in the N and H genes, respectively. This observation further highlights the dominant and stable character of clade H MVs in the People’s Republic of China.

With an increasing number of reports establishing the origin and genetic characteristics of globally circulating MV strains, the picture of the geographic distribution of MV genotypes is becoming more and more complete. A WHO meeting in 1998 has attributed eight reference clades, A to H, to all known wt MVs (WHO, 1998). Some of these clades seem to be geographically distinct: clade H appears to be limited to the Asian continent, since most of these MV clade H strains were isolated in China between 1993 and 1998 (Xu et al., 1998). Furthermore, single viruses were considered to have been imported from the Philippines: genotype D5 (Bellini and Rota, 1998), and D3 (Rota et al., 1998), genotypes that also co-circulate in Japan (Katayama et al., 1997; Ya-
viruses of the USA formed genotype D3. The recent isolated for some time. The formerly indigenous D1 (Taylor et al., 1991), although D1 has not been D6 (Santibanez et al., 1999; Hanses et al., 2000) and 1997). MVs from Europe belong to genotypes C2, D4 have been found in South Africa (Kreis et al., 1996; Katayama et al., 1997; Rota et al., 1998; this paper) seem to be the most prevalent in Asia. However, also outside Asia, D viruses seem to have the widest distribution. MVs of genotypes D2 and D4 have been found in South Africa (Kreis et al., 1997). MVs from Europe belong to genotypes C2, D6 (Santibanez et al., 1999; Hanses et al., 2000) and D1 (Taylor et al., 1991), although D1 has not been isolated for some time. The formerly indigenous viruses of the USA formed genotype D3. The recent 1997–1998 measles outbreak in Argentina was attributed to D6 strains (Barrero et al., 2000). The proposed genotype D7 has been a common genotype in Australia during the late 1980s (Chibo et al., 2000). The new D8 genotype appears to be circulating not only in Nepal, but was also found in the UK (Jin et al., 1997, 1998).

Our study highlights that measles is still endemic on the Indian subcontinent, where genotypically distinct lineages co-circulate over vast geographic regions with a large reservoir of susceptible individuals. Although interruption of indigenous measles transmission has been achieved in the US, this and other reports of imported measles demonstrate that a concerted and sustained effort of all countries on all continents is required to control and eventually eradicate measles.

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References


