MEASLES: A MOLECULAR APPROACH FOR SURVEILLANCE

INTRODUCTION

For at least 5,000 years, measles has been part of the human conditions and responsible for severe epidemics throughout the world. Measles incidence varies cyclically for a given community depending on the size of population and its immune status, as an increase in measles incidence results in a reduced number of susceptible people and consequently the chance of infection person-to-person declines. The number of susceptible people will rise again in the population due to births and/or migration until it reaches threshold density culminating in new measles transmission in the community. Mass vaccination acts preventing the spread of measles through individual immunization preventing the virus circulation. Since measles vaccine was introduced in the middle of 1960’s, most developed and many developing countries have reduced measles morbidity and mortality. When in 1977, the smallpox, one of the mankind’s great plagues, was effectively eradicated, it raised the hope that measles among other virus diseases could also be eradicated. The technical feasibility of measles eradication is due to the absence of non-human reservoir, the antigenic stability of the virus, the existence of a very effective vaccine and the availability of fairly sensitive and specific diagnostic tools. These elements allow the early interruption of transmission chain, in suitable surveillance systems. Such characteristics led to an international consensus at the World Summit for Children in 1990, implementation of a worldwide program as a major step towards the global measles eradication in the long term (Clements & Cutts 1995).

Measles disease.

Measles virus (MV) is highly contagious and its transmission is efficient through direct exposure to an infected individual by respiratory droplets. Measles is most infectious during the prodrome, and

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it has an incubation period of 10 to 14 days, from exposure until the onset of symptoms. It is estimated that 76% of household exposure of susceptible persons lead to measles. The clinical disease (prodromal period) is characterized by fever over 38°C, cough and/or conjunctivitis and/or coryza, and 2-4 days later, this period is followed by the appearance of a characteristic red rash (erythematous maculopapular eruption) that appears first on the face, and behind the ears and then spreads in a centrifugal fashion to the trunk and extremities. Communicability period continues for 4-5 days after rash onset. The eruption onset coincides with the appearance of the immune response. Rash begins to fade within 3 or 4 days, and in severe cases, desquamation may occur. Other constitutional symptoms and signs, such as anorexia, diarrhea and generalized lymphadenopathy may also be present. In uncomplicated measles, clinical recovery begins soon after the appearance of the rash and is accompanied by lifelong immunity to reinfection. Most complications associated with measles infection are pneumonia, otitis media, post infection encephalitis (1/1000), subacute sclerosing panencephalitis (SSPE) (1/1000,000) and death (Griffin 2001, WHO 2000).

Laboratory diagnosis.
In areas with low incidence of measles, the clinical diagnosis is often complicated due to the sporadic nature of the disease and the occurrence of other rash-causing illness. The differential diagnosis of measles clinical picture includes other clinically similar diseases such as rubella, dengue fever, parvovirus B19, coxsackie, and herpes virus 6 viruses, therefore, confirmation must be made by laboratory. Measles infection is diagnosed serologically detecting IgM or demonstrating a significant rise (fourfold increase) in IgG between paired acute (first specimen) and convalescent (second specimen) sera. However measles virus can also be detected from clinical samples by using cell culture or molecular techniques.

Sero logical assay.
Measles infection is confirmed by the presence of measles-specific IgM or low avidity IgG in serum or saliva. Antibody measure by ELISA may require only one blood sample for case confirmation and several capture IgM ELISA kits are commercially available. IgM capture tests for measles are often positive on the day of rash onset; however, in the first 72 hours after rash 20% of tests for IgM may give false negative, and the tests are more sensitive between day 4 and 28 after rash onset. Measles-specific IgG antibodies peaks approximately 2 weeks after rash onset and gradually increases in avidity and subsequently declines, but IgG antibodies are detectable for years after infection (Griffin 2001, WHO 2000).

Detection of virus – Culture.
Measles virus could be cultured from hep-arinized blood, nasopharyngeal aspirates, throat swabs and urine. Clinical specimens should ideally be obtained within 7 days of rash onset. A marmoset B-cell line B95-8 or B95a cells are the most sensitive lineages to isolate the virus. In MV cell culture the virus causes a distinct cytopathic effect (CPE) with the formation of multinucleated syncytia (giant cells), containing numerous nuclei of fused cells. Measles virus isolation is recommended with the purpose of genomic analysis for comparing with others strains from different locations and years, providing information of its origin and transmission history, identifying either new virus strains or existing viruses due to present circumcision (WHO 2000).

Reverse Transcription Polymerase Chain Reaction (RT- PCR).
PCR technology and sequencing methods has enable investigators to obtain genetic information that has expanded the understanding of genetic variation of measles virus. Nucleotide sequence data can be obtained sequencing PCR products derived from RNA extracted from virus isolated on cells culture or directly from clinical specimens as nasopharyngeal aspirate, throat swab, peripheral blood lymphocytes, saliva or urine collected as soon as possible after rash onset, within seven days (WHO 2000).

Measles control.
Prevention and control of measles infection consist of immunization followed by a significant reduction in the incidence of and mortality from measles. During the 1980s, remarkable progress was made worldwide in measles control through increasing routine measles vaccination. Measles vaccination coverage with one dose of measles vaccine among infants remained about 80% over the
past decade. However, coverage varied widely between countries. Despite the increasing and sustaining coverage in the American, Eastern Mediterranean and European regions, measles is still responsible for 31 million cases and 770000 deaths annually among children under 5 years-old worldwide. Mortality has taken place in the world’s poorest countries resulting from a combination of factors such as crowding, undernourishment and low vaccination coverage, being the latter the most important cause of high measles morbidity and mortality. WHO regions with most measles deaths are Africa (452,000), Southeast Asia (220,000), and the Eastern Mediterranean (81,000) (Murray et al. 2001, WHO 2001a, 2002a, de Quadros et al. 2003).

The World Health Organization (WHO) has encouraged progress towards measles control in the world, and the improvement of surveillance and widespread use of measles vaccine in many developed countries has resulted in greater reduction of confirmed cases. In all countries and regions that have implemented aggressive measles controls or elimination strategies (e.g., the Americas and southern Africa), measles cases and deaths due to the disease have been reduced. In some cases the transmission of the measles virus has been interrupted (de Quadros et al. 2003, Munyoro et al. 2003).

In the American Region, the Pan American Health Organization PAHO has been working towards measles elimination and, in 2001, ongoing transmission of measles occurred in some countries in South America, Dominican Republic and Haiti, and during 2001-2002 in Venezuela and Colombia. In 2001, the Dominican Republic and Haiti interrupted indigenous measles transmission successfully, and no indigenous measles has been reported from Argentina, Bolivia or Brazil since 2000 (WHO 2002b, CDC 2002).

In Brazil, the transmission was interrupted due to vaccination strategies, intensive efforts to reach high routine vaccination coverage during 1999-2000, and measles epidemiological and laboratorial surveillance. Since 2001 measles vaccine coverage has remained about 91% by one-year-old. Besides high vaccine coverage, the measles eradication task force group a key component of intensified measles elimination activities, was implemented in 1999. One surveillance technician was assigned to each state of the country to assist the State Secretariats of Health in strengthening surveillance through the following strategies: weekly negative notification; timely and complete investigation of cases and outbreaks, with rapid implementation of control measures and vaccination of high-risk groups (Prevots et al. 2003).

**Measles genome.**

Measles virus is an enveloped virus that belongs to the genus Morbillivirus (Family Paramyxoviridae), and its genome is a linear single stranded RNA molecule of negative polarity (figure 1), with a length of about 15900 nucleotides (nt). Six genes code for six structural proteins N, P, M, F, H, its own RNA-dependent RNA polymerase, protein L, and two nonstructural proteins C and V, expressed by the single gene P. Protein C is produced by the translational choice of the open read frame (ORF) which overlaps the N-terminal of the gene P ORF in the +1 frame, and the protein V is translated from a mRNA produced by a novel RNA editing mechanism. Each gene is flanked by untranslated 3’ and 5’ region, and the gene-end boundaries are intergenic regions that contain exactly 3 nucleotides. The genes N, P/C/V, M, F, H, and L (Figure 1) have 1683, 1648, 1462, 2368, 1953e 6639 nucleotides respectively (Horikami & Moyer 1995).

![Figure 1 - Measles virus genes organization, intergenic spaces, sequence leader (+) 3’ and trailer (−) 5’, (Horikami & Moyer 1995).](image-url)
Molecular epidemiology

Molecular methods have become important tools in standard epidemiology providing the most powerful instrument for measuring the evolution of a particular virus in nature, distinguishing differences between virus strains, characterizing strains isolated in outbreaks, allowing identification of chains of infection, and also for measuring effectiveness of measles control programs by monitoring the disappearance of specific virus strains. Application of appropriate molecular tools will aid the surveillance of infectious agents. Selection of the most appropriated sequences and the location and size of the genomic interval selected for analysis is fundamental. Sequencing the whole gene, or in some cases, small sequences should be enough to detect changes in the antigenicity associated with genetic alterations. Protein H is the major target of neutralizing and protective antibodies, but measles vaccine continues to be effective in all genotypes described in the world so far, in spite of the antigenic difference detected in the H protein among different strains. It has been suggested that mutations in critical epitopes could lead generations of vaccine mutants to escape from antibody protection (Tamin et al. 1994, Jin et al. 1998, Woelk et al. 2001). Molecular surveillance should be continued in order to detect any mutation with potential to generate escape mutants.

As measles is targeted to be eradicated, it is important to include viral surveillance in all phases of measles control. Besides, attempts should be made to obtain specimens for virus detection at first contact with a suspected case, from each transmission chain or each sampling point in endemic areas (WHO 2000).

Measles virus (MV) has long been considered an antigenically stable monotypic virus. Nevertheless, variability has been described in N and H genes by nucleotide sequence analysis and has been exploited to determine the relatedness between viruses, their transmission pathways within countries or continents, and to assist virological surveillance in order to identify the source of virus as vaccine or wild-type. The molecular data are analyzed with the epidemiological data or clinical history, in order to obtain logical conclusions.

As more laboratories have adopted molecular techniques as part of the routine surveillance, the number of newly identified genotype has increased. For molecular epidemiological purposes, the genotype designations are the operational taxonomic unit, while clades are used to indicate the genetic relationship between the various genotypes. Genetic characterizations of measles virus should be performed according to the international standards established by the WHO global laboratory network. The sequence of the 450 nucleotides that code for the COOH-terminal 150 amino acids of the N protein is the minimum amount of data required for determining the genotype of a measles virus. Complete H gene sequences should be obtained from representative strains or if a new genotype is suspected.

Since the H protein is a major target of the neutralizing antibody response, sequencing of the H genes will help to identify viruses that may have modified antigenic properties. Based on these recommendations, measles virus has been classified into eight separated clades, designated A, B, C, D, E, F, G, H and comprising 22 recognized genotypes (Rota et al. 2000, WHO 2003).

Within clades A to H, described for measles, some clades contain only one genotype and, in this case, the clade and the genotype are the same as, for example, clades A, E, and F, while others such as clade D contain multiple genotypes and are designated as the clade letter and genotype number. The genotypes E, F, G1, D1 are considered inactive, since they have not been isolated in the last 15 years (WHO 2003).

Sequence analysis for H and N genes has shown that the genes coding for H and/or N proteins contain up to 8% nucleotide variability in the wild-type (wt) viruses. The most variable region of measles genome is found at carboxi-terminal 456 nucleotides (nt) of the N gene that can approach 12% among wild-type viruses from different genotypes. Genetic divergence within the variable N region is between 1.7% for clade A, for clade C and D viruses reach 9.6%. This high difference is accounted for genotypes C1 and C2 that are quite distinct from each other, as D6 is distinct from the other D genotypes. Genetic divergence for clade B reaches 4.8%, clade G - 5.7%, and clade H - 5.9% (WHO 2001b, Mulders et al. 2001, Rota & Bellini 2003).

Clade A consists of live-attenuated vaccine derived from the original Edmonston strain (wtED).
isolated in 1954, and all known vaccine related to wt viruses strains. The non-Edmonston-derived vaccine contained enough genetic heterogeneity to confirm their independent origin and the vaccine strains were more related to the sequence of a low-passage observed in the original Edmonston strain. In spite of the diverse geographic origins and the different attenuation method used, the sequences for H, F, N, M genes from all vaccine strains differed no more that 0.6% at the nucleotide level (Rota et al. 1995). Four nucleotides markers in the H coding region were described, apparently linked to all members of the vaccine strains; three were synonymous substitutions (syn) C72, T129, A1149 and one non-synonymous (ns) C826 (Leu/Phe). In addition, a number of other point mutations were considered unique or rare markers of the vaccine group as the additional markers A1451 of the vaccines Zagreb, Leningrad-16, AIK-C Changchun-47 and Shanghai-191 (Christensen et al. 2002).

The number of mutations shared with respect to the consensus sequence of the genotypes of clade B is 2-9 times higher than other clades. Clade B comprises genotypes B1, B2 isolated in Cameroon in 1983 and in Gabon in 1984, respectively. There is more genetic variability within the genotype B1 and B2 viruses (up to 4.6%) than among most other genotypes.

Comparing the strains from outbreaks of 1997 in Brazil, 1998 in Argentina, and 1999 in Uruguay, to the reference sequence EUA NJ/94-1, and others belonging to genotype D6 from Central Europe, a high degree of similarity among them was observed, except for nucleotide change in the position 1508 (Siqueira et al. 2001, Canepe et al. 2000). This change resulted in one non-synonymous change at position 467 aa from Leucine to Proline in Argentina strains reported by Baumeister et al. 2000, and it could constitute a marker for virological surveillance in this continent, to document the interruption of the indigenous genotype.

Genotypes within clade H, as seen in the genotypes B, have more nucleotide substitution within the genotype than others, up to 3%. In a study in Vietnam, sequences analysis of viruses from genotype H could characterize intra-epidemic variability of measles viruses samples isolated from the same location that had identical sequences suggesting that these were linked in a single chain of transmission. In contrast, different degree of heterogeneity by genotype H2 was observed in different clinics in Vietnam within a limited geographic area; this heterogeneity is the result of multiple, co-circulate lineages of virus within a single, indigenous genotype (Lifick et al. 2001).

**Distribution of measles genotype in world**

Molecular surveillance of measles virus has been recommended as an important component of measles surveillance and epidemiological investigations to identify non-endemic genotypes and to monitor virus transmission pathways. Different genotypes are not geographically restricted, although some appear to be predominant in large areas of the world (Rima et al. 1995). In areas with endemic transmission, a limited number of genotypes has been described. In 1999, a study of Nigerian and Ghanaian MV isolates revealed the co-circulation of two distinct viruses (Muller 1999). A similar picture was also observed in China. The co-circulation of two different strains suggests that the number of susceptible individuals in these areas was enough to sustain the circulation of multiple chains of transmission, can reflect a population pattern with low levels of inherited immunity (Hanses et al. 1999, Xu et al. 1998). In areas where transmission has been interrupted, depending on migration patterns and/or frequency of international travel, a more complex pattern of genotypes may be observed and multiple genotypes may be detected resulting from serial imports of wild-type measles virus from areas where the infection was not controlled yet (Rota et al. 1998, Jin et al. 1997).

Genotype A had been a major genotype before routine vaccination, implemented in the 1960s. In recent years, viruses belonging to genotype A have been isolated in United States, United Kingdom, South Africa, Russia, China and Argentina, indicating continued circulation for many decades, and showing several substitutions that distinguished them from vaccine virus strains (Rota et al. 1994, 1998, Outlaw & Pringle 1995, Kreys et al. 1997, Santibanez et al. 1999 Xu et al. 1998, Barrero 2001).

Clade B viruses contain genotypes B1, B2 and B3 and are endemic in the central and western parts of sub-Saharan Africa. In 1983, genotype B1 was isolated in Yaoundé (Cameroon) however isolated measles viruses circulating in Gabon was classified in genotype B2 one year later. Genotype B3 was isolated during 1990s and sequencing studies showed
that these viruses are heterogeneous and could be divided in two clusters: I and II. Representatives of genotype B3 have been circulating in Kenya, Sudan, Congo, Democratic Republic of Congo, Gambia, Cameroon, Nigeria and Ghana. The geographical distribution for the period 1993-2001 showed that B3 I occurred in Sudan Nigeria, Ghana and south of the Cameroon. As for B3 cluster II viruses, these strains seemed to be more limited and were described occurring in Western Africa; viruses of this genotype correspond to that isolated in Gambia, Nigeria and Ghana (Kouomou et al. 2002, WHO 2001b).

Genotypes C2 have often been isolated in Europe. It occurs in Czech Republic, Germany, Denmark, Luxembourg and Spain, D6 in Russia, Italy, Turkey, Germany, Poland, Luxembourg, Spain, and D7 in Germany and Spain. Genotype C2 was recently detected in Morocco suggesting that genotypes pattern present in northern Africa may be more similar to that of Europe than to the pattern seen in other parts of Africa (Alla et al. 2002, WHO 2001b).

Genotype D2 and D4 are known to circulate in southern and eastern African continent, South Africa, Zambia, Kenya, Zimbabwe, Namibia and Ethiopia however, measles are endemic in the Indian subcontinent and genotypes D4 viruses have been associated with imported measles cases from India circulating in Nepal, Pakistan and Iran. In 2000, Genotype D2 was also isolated during an outbreak, which occurred in an immigrant community with low vaccination coverage, in Ireland. Genotype D8 has been isolated in India and Nepal. Genotype D4 has also been described in Russia (Coughlan et al. 2002, WHO 2001b).

Viruses from genotype D3 were first isolated in Japan in the early 80s and viruses from D3 and D5 co-circulated in Japan during the late 1980s and early 1990s. In 1990-1991, genotype D3 was associated with resurgence of measles in the US, and in 90s D3 were also isolated in Taiwan, Philippines causing an outbreak in Micronesia during 1991 as well as. The genotype D5 viruses have recently become the predominant genotype in Japan and Thailand (Rota et al. 1996, 1998, Gurus 1998).

Besides the circulation in Europe, genotype D6 viruses were also related to strains obtained in Central and South America. Genetic analysis of Brazilian samples of measles viruses isolated during the outbreaks in 1997 could identify the circulation of genotype D6 (Siqueira et al. 2001). Another study showed the presence of D6 in São Paulo in 1996 (Oliveira et al. 2002), and in the same annual studies of sporadic cases in Bahia and Santa Catarina, detected D5 and C2, respectively (Prevots et al. 2003). During 1995 genotype D5 was also identified in cases in São Paulo (Oliveira et al. 2002). D5 and C2 viruses are circulating in Japan, Europe and Northern Africa respectively.

Unfortunately, there had been no viruses available prior to vaccination campaigns in 1992 in Brazil for molecular studies. It could have determined the indigenous genotype, and whether genotype D6 from the outbreak in Brazil had been introduced by imported cases or it could have been circulating during the non significative incidence period at low levels until the accumulation of susceptible individuals that resulted in epidemic. Soon after the outbreak in Brazil, D6 was also related to outbreaks in Argentina, Chile and Dominican Republic in 1998, Bolivia and Uruguay in 1999 and it was responsible for an outbreak in Haiti in 2000 as already observed (Baumeister et al. 2000, Cànepe et al. 2000, WHO 2001Ab). Viruses D6 have spread widely in South America; therefore, monitoring the viral genotypes pattern in this region will be important to document the circulation interruption of indigenous genotype. Recently, a new genotype, D9, has been characterized in Indonesia and in an outbreak in Venezuela, probably resulting from imported cases into this country. Genotype D9 detected in Venezuela may give an important clue to the interruption of D6 in South America.

New genotypes have been recognized as result of active virological surveillance since 2001. Genotype D9 was first isolated from an imported case into Australia from Bali. Genotype D9 viruses were also isolated in Java and Indonesia, and were responsible for an outbreak in South America, Venezuela and Colombia during 2001-2002 (Chibo et al. 2003, Rota & Bellini 2003, WHO 2003).

MV strains belonging to clade G had not been detected for 15 years, so the lineage was considered to be either extinct or inactive. However, virus belonging to clade G was isolated from an Indonesian child infected in 1997, and revealed a new genotype, now recognized as G2. This was also associated with outbreaks in Indonesia and Malaysia in 1999, suggest-
ing that this genotype has circulated in the intervening period. Another proposed genotype within clade G was isolated during 2002 in East Java, and it will be the reference strain for genotype G3. G3 viruses are circulating in East Timor (Rota & Bellini 2003, WHO 2003). The identification of new genotypes indicates that our understanding of the extent of genetic diversity in measles strains remains uncompleted.

Genotype H viruses of a highly distinct genotype was isolated in Republic of China during early 1990s. Viruses of genotypes H1 are circulating in China and Republic of Korea, while genotype H2 ones are circulating in Vietnam (WHO 2003). A distribution of genotypes endemic worldwide is described in the Table 1.

The experience from the Americas and others regions suggest that measles eradication can be achieved. This new epidemiological scenario requires new surveillance technologies, to assure early detection of imported cases and to validate routine laboratory findings, due to decrease of predictive positive values of serological tests used. In this context, the virological surveillance must be continued and expanded, so that the interruption of indigenous measles virus may be documented in countries where measles is being controlled.

Table 1 - Global distribution of wild-type measles viruses: Measles genotypes in areas with endemic transmission in the world. (Kindly supplied by Dr Paul Rota/CDC / Atlanta-USA.)

<table>
<thead>
<tr>
<th>CLADES</th>
<th>Areas with endemic transmission</th>
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<tbody>
<tr>
<td>B3</td>
<td>Congo, DR Congo, Gambia, Cameroon, Sudan, Nigeria, Ghana</td>
</tr>
<tr>
<td>C2</td>
<td>Marocco, Western Europe</td>
</tr>
<tr>
<td>D2</td>
<td>South Africa (early 90s)</td>
</tr>
<tr>
<td>D3</td>
<td>Japan, Philippines</td>
</tr>
<tr>
<td>D4</td>
<td>India, Pakistan, Ethiopia, South Africa, Russia, Kenya</td>
</tr>
<tr>
<td>D5</td>
<td>Japan, Thailand, Cambodia</td>
</tr>
<tr>
<td>D6</td>
<td>Turkey, Western Europe</td>
</tr>
<tr>
<td>D7</td>
<td>Europe, Germany, Italy</td>
</tr>
<tr>
<td>D8</td>
<td>India, Nepal</td>
</tr>
<tr>
<td>D9</td>
<td>Indonesia (West Java)</td>
</tr>
<tr>
<td>G2</td>
<td>Indonesia (West Java), Malaysia, Thailand</td>
</tr>
<tr>
<td>G3</td>
<td>East Timor, Indonesia (East Java)</td>
</tr>
<tr>
<td>H1</td>
<td>China, Korea, Mongolia, Japan</td>
</tr>
<tr>
<td>H2</td>
<td>Vietnam</td>
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REFERENCES


