MOLECULAR ANALYSIS OF A MEASLES VIRUS ISOLATE FROM BRAZIL: A CASE ORIGINATING IN JAPAN

M.I. OLIVEIRA[1*], S.P. CURTI[1], C.A. FIGUEIREDO[1], A.M.A. SARDINHA[1], M.A.M. SALLUM[2], E.L. DURIGON[3]

[1]Instituto Adolfo Lutz, São Paulo, Brasil; [2]Faculdade de Saúde Pública, Universidade de São Paulo, São Paulo, Brasil; [3]Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil

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Summary. – This study reports on molecular analysis of a Measles virus (MV) isolate from a patient who was infected in Japan but showed symptoms after arriving to Brazil. This patient had typical clinical measles infection symptoms: fever, rash, cough and coryza. After isolating the virus in B95a cells, a fragment of the nucleoprotein (N) gene was amplified by reverse transcription–polymerase chain reaction (RT-PCR) and subjected to direct nucleotide sequencing. The sequence data showed that the MV isolate of concern is of the D5 genotype.

Key words: Measles virus; nucleotide sequence; molecular epidemiology; nucleoprotein; Brazil

Introduction

The efforts of each individual country and global efforts through the Expanded Program on Immunization have substantially reduced measles morbility and mortality throughout the world. Despite this success, measles remains endemic in many areas of the world, occurring in developed, developing and underdeveloped countries that are not able to run an adequate immunization program.

The Pan American Health Organization has reported a low number of measles cases in the Americas in 2000–2001, reflecting the overall success of measles control programs in the Western Hemisphere (CDC, 2001). The case-surveillance data including molecular epidemiological information is constantly challenged in the Americas by measles viruses originating from other regions of the world in which the virus circulation is endemic (CDC, 1998; Hanses *et al*., 2000). It is possible to monitor changes in the MV genotypes circulating in a particular region, when both standard epidemiological and case surveillance data are analyzed in conjunction, because this information, when analyzed in conjunction with standard epidemiological data, has helped to document the interruption of transmission of endemic measles (Rota *et al*., 2003).

Molecular epidemiology plays an important role in the worldwide effort to characterize MV genotypes, especially the wild type (wt) one, which seems to be prevalent in more or less confined geographic regions (Rima *et al*., 1995; Bellini *et al*., 1998). Several studies on genetic characterization of wt MV have been conducted using the coding regions of the hemagglutinin and N genes, which display up to 8% variability at the nucleotide level. One of the most variable part of the MV genome is the 450-nucleotide long region, which codes for the C-terminus of the N gene. In this region the nucleotide variability reaches up to 12% among wt viruses (Xu *et al*., 1998).

During the resurgence of measles in the state of São Paulo, Brazil, in 1997, the sequence of the MV isolate as reference (AF495863), obtained during the outbreak has indicated the presence of the D6 genotype (Oliveira *et al*., 2002), which is prevalent in Europe. Prior to 1997, the D5 genotype has caused sporadic cases occurring in the state of Bahia in 1996 (Siqueira *et al*., 2001), while the C2 genotype was involved in a small outbreak in the state of Santa Catarina in 1996.

*Corresponding author. E-mail: olive40@uol.com.br; fax: +5521-30883753.

Abbreviations: CPE = cytopathic effect; EDM = Edmonston; hLRT = hierarchical likelihood ratio test; IgM = immunoglobulin M; ML = maximum likelihood; MV = Measles virus; MVi = MV isolate from cell culture; MVs = MV isolate from clinical material; N = nucleoprotein; PBMCs = periferal blood mononuclear cells; RT-PCR = reverse transcription–polymerase chain reaction; wt = wild type

The virological surveillance has demonstrated that both the genotypes D3 and D5 have been co-circulating in Japan for the last 10 years (Katayama *et al*., 1997; Sakata *et al*., 2000). Moreover, the genotypes D8, C1, and H1 were associated with several measles cases imported from Japan (Rota *et al*., 2003). These data have shown that in Japan several MV genotypes have been both circulating or co-circulating.

The main objective of the present study was to determine the genotype of the MV isolated from a patient, who was infected in Japan and developed the disease in the state of São Paulo, Brazil. Molecular characterization of viruses and standard epidemiological surveillance data are powerful tools for both the confirmation of the source of virus infection and the evaluation of the effectiveness of measles control programs. Consequently, both these approaches have been adopted by the measles surveillance program of the state of São Paulo, Brazil.

Materials and Methods

The patient. A seven-month-old child, born in Japan, who was not vaccinated for measles, had contact with its uncle. Both the child and its uncle were living in Japan. The latter showed first symptoms of measles infection on June 1, 2001 and was hospitalized with measles on June 3, 2001. On June 14, 2001, the child had high fever (38.9°C), which lasted 5 days. The child with its family arrived in São Paulo, Brazil, on June 16, 2001. On June 17, 2001, it showed onset of rash, which began on the face and spread throughout the body within 2 days. On June 18, 2001, the child experienced onset of cough and coryza. An epidemiological case investigation started on June 19, 2001, by collecting blood and urine samples for virus isolation. All household residents without documentation of measles vaccination or natural disease were vaccinated.

Samples. A blood sample was analyzed at Instituto Adolfo Lutz, São Paulo, the state of São Paulo, Brazil. Measles infection was also serologically confirmed as IgM-positive using the IgM ELISA Test commercial kit (Behring Diagnostics GmbH, Marburg). Periferal blood mononuclear cells (PBMCs) were purified from whole blood by using Ficoll-hypaque density gradient centrifugation. The PBMCs were washed three times with PBS. The urine was collected in a sterile container and centrifuged at 800 x g for 30 mins. The pellet was resuspended in 2 ml of Hanks' Balanced Salt Solution for virus isolation. An Epstein-Barr virus-transformed marmoset B lymphoblastoid cell line B95a (Kobune *et al*., 1990) was used to isolate MV from the urine and PBMCs. The cell cultures were infected with 0.2 ml of clinical samples and maintained in DMEM containing 0.2 mmol/l L-glutamine, 40 mg/ml gentamicin, and 2% fetal calf serum at 37°C. After 3 passages in cell line with negative results, the samples were discarded as negative for virus isolation. The cells were harvested by centrifugation when cytopathic effect (CPE) was maximal. With the urine samples no apparent CPE was observed even after 3 passages. However, with the PBMCs, CPE was observed already after 2 passages, and thus the cells were centrifuged and RNA was extracted from the cell pellet.

RT-PCR and nucleotide sequencing. RNA was extracted from the cell pellet by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski *et al*., 1987), reverse-transcribed with avian myeloblastosis virus reverse transcriptase to obtain the first-strand cDNA corresponding to the nucleotides coding for the C-terminus of N, and amplified by RT-PCR. The amplification products were sequenced using the protocol adopted by Liffick (2001) and Devereux (1984).

Phylogenetic analysis. The Bangkok THA/93 strain was included in the present study as reference of the D5 genotype, and thus for comparison with the sequence of the MV isolate of concern. A maximum likelihood (ML) analysis was carried out with the PAUP 4.0b10 (Swofford, 2003). The best model for the MV data was the ModelTest 3.06 (Posada *et al*., 1998). This program uses both a hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC) to choose among 56 available evolutionary models; when the hLRT and AIC were not in accord, the simpler model was chosen. Under the adopted model and using a Neighbor-Joining (NJ) tree as the starting tree for branch swapping, five iterative rounds of ML analysis were performed. The most likely tree identified during each of those rounds was used as the starting tree for the next search, both for calculation of updated parameter values as well as for the initiation of branch swapping. The branch-swappings were Nearest Neighbor Interchange (NNI), Subtree Pruning Regrafting (SPR), and Tree Bisection Reconnection (TBR), respectively. Bootstrapping (Felsestein, 1985) under the ML criterion utilized 100 pseudoreplicates with 10 random-taxon-addition starting trees per pseudoreplicate and TBR branch swapping. Bayesian inference of phylogeny was carried out using MrBayes 2.01 (Huelsenbeck *et al*., 2002). Since the kinds of models available in this program have limited overlap with those in the PAUP 4.0b10 (Swofford, 2003), the Bayesian analysis was conducted using the GTR (Rodríguez *et al*., 2000) plus site-specific rates model, using site partition by codon. Program default values for prior probabilities were used. The Markov Chain Monte Carlo (MCMC) was allowed to run 3 million generations and sampled every 100 generations after a burn-in of 60,000 generations.

Results

In this study, we report the nucleotide sequence of a part of N gene of a MV isolate from a child, who was infected in Japan but developed measles after arrival to Brazil. The infection was confirmed by measles-specific immunoglobulin M (IgM) and RT-PCR. The nucleotide sequence of C-terminus of N gene of this isolate was compared with sequences of the same region of several MV genotypes originating from different countries, deposited at the GenBank (Table 1). All the sequences were compared with each other and with the sequences from a low-passage MV EDM wt genotype A.

A fragment of 259 bp (positions 1178–1437) from the C-terminus of the N gene was obtained from the isolate MVi/São Paulo.BRA/01, deposited at the GenBank under Acc.

Table 1. Characteristics of MV strains/isolates subjected to phylogenetical analysis

Strain/isolate	Locality	Isolation year	Genotype	Acc. No.
Amsterdam.Net/3.98	Nepal, DEU	1998	D4	AF193513
Angra dos Reis/485/97	Angra dos Reis, BRA	1997	D6	AJ272480
Bangkok.THA/93/1	Bangkok,THA	1993	D5	AF079555
BRA/42.97/35175	São Paulo, BRA	1997	D6	AF495863
Edm wt	USA	1954	A	U01987
Erlangen.DEU/90 "wtf "	Erlangen, DEU	1990	C2	X84872
Funen 9/97	Funen, DEN	1997	C2	Y17025
High Wycombe/234/95	High Wycombe, UNK	1995	D6	U29302
Janakpur.NEP/2.99/1	Janakpur, NEP	1999	D8	AJ250069
Janakpur.NEP/2.99/2	Janakpur, NEP	1999	D8	AJ250070
MVi/Ámsterdam.net/27.97	China, JAP	1997	H1	AF193512
MVi/Okinawa.JPN/21.01	Okinawa, JAP	2001	D3	AB104875
MVi/OSaka C.JPN/17.97	Osaka, JAP	1997	D3	AB088149
MVi/São Paulo.BRA/01	São Paulo, BRA	2001	D5	AY425711
MVi/Sapporo.JPN/19.01	Sapporo, JAP	2001	D5	AB104876
MVi/Toquio.JPN/84/K	Toquio, JAP	1984	C1	AY 043459
MVi/Vic.AU/3.90	Victoria, AUS	1990	C2	AF243460
MVi/Vic.AU/5.91	Victoria, AUS	1991	C2	AF243462
MVi/Vic.AU/6.73	Victoria, AUS	1973	D1	AF243439
MVi/Vic.AU/9.73	Victoria, AUS	1973	D1	AF243444
MVs/Toyota C.JPN/30.01	Toyota, JAP	2001	H1	AB104874
MVs/Vic.AU/10.98	Victoria, AUS	1998	D4	AF243470
MVs/Vic.AU/27.98	Victoria, AUS	1998	D5	AF243471
MVs/Vic.AU/51.98	Victoria, AUS	1998	D5	AF243472
MVs/Vic.AU/52.98	Victoria, AUS	1998	D5	AF243473
MVs/Vic.AU/8.98	Victoria, AUS	1998	D4	AF243469
Novosibersk/97	Wroclaw, RUS	1997	D6	Y17032
Palau.BLA/93	Palau, BLA	1993	D5	L46758
Pokhara.NEP/5.99	Pokhara, NEP	1999	D4	AJ250073
Prague 243/92	Prague, CZN	1992	C2	Y17027
Sma94B	SPA	1994	D6	X84864
Stuttgart/2/93	Stuttgart, DEU	1993	D6	Y13825
Taipeh.TWN/94	Taipeh, TAW	1994	D3	AJ250068
Wroclaw/98	Wroclaw, POL	1998	D6	Y17026

No. AY425711. Comparing the amino acid sequence of MVi/São Paulo.BRA/01 with those of other D5 genotypes, it was observed that the similarity among them ranges from 95.1% to 100% in the C-terminus of N (MEGALIGN, DNASTAR, Inc.). ModelTest 3.06 (Posada *et al*., 1998) was used to choose among models in PAUP. The hLRT found the TrN + Γ model (Tamura *et al*., 1993), while the AIC suggested the TIM + Γ model (Transition Model with gamma distributed site-to-site rate variation). The TrN + Γ model was chosen as the simpler of the two. ML analysis under the TrN + Γ model generated a single topology with a log ML of –3702.47121(data not shown). The ML topology recovered a clade consisting of all seven sequences of the D5 genotype of MV. More important, the MVi/SãoPaulo.BRA/01 sequence clustered with the MVi/Sapporo.JPN/19.01 and Palau.BLA/93 sequences within a major clade formed by the seven sequences of the D5 genotype. ML bootstrap analysis with the TrN + Γ model provided a strong support for the D5 clade (86% bootstrap proportion) as well as for the D5 clade leading to the MVi/SãoPaulo.BRA/01, MVi/Sapporo.JPN/19.01 and Palau.BLA/93 (96% bootstrap proportion). The maximum likelihood bootstrap support for the second clade within the D5 group is moderate (64% bootstrap value). This clade consisted of the three strains isolated in Victoria, Australia and one of Bangkok, Thailand. The Bayesian 50% majority-rule consensus tree was nearly identical with the ML topology generated with the TrN + Γ model. The Bayesian analysis recovered MVi/SãoPaulo.BRA/01 within a clade consisting of the D5 genotype sequences. Furthermore, MVi/SãoPaulo.BRA/01 clustered within the clade formed by MVi/Sapporo.JPN/19.01-D5 and Palau.BLA/01-D5. The posterior probability for the clade was 1.0 and for the clade consisting of the Bangkok, Thailand, Victoria and Australia sequences was 0.99.

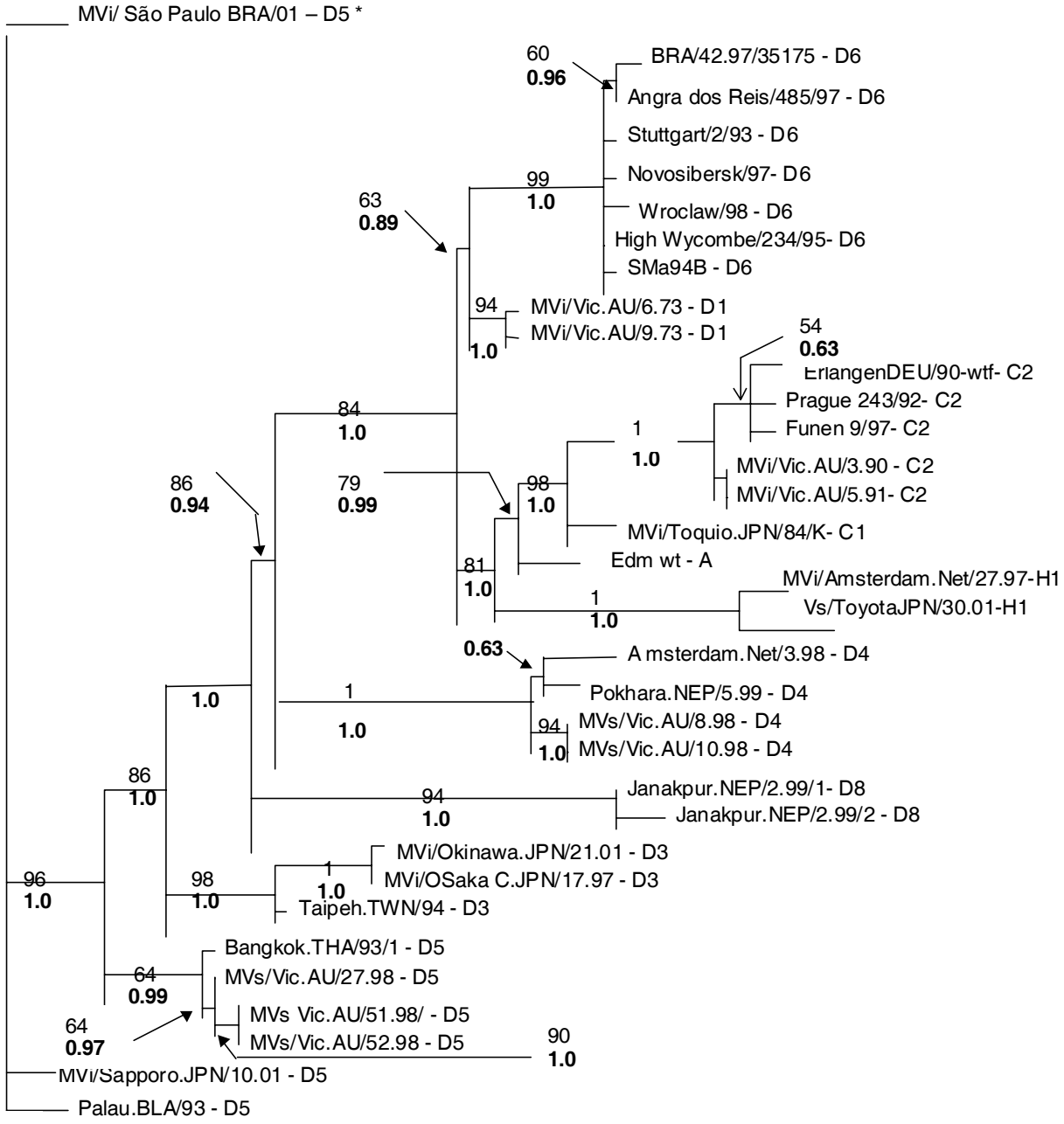

Fig. 1

The phylogenetical tree of MV strains and isolates

The majority rule 50% consensus, unrooted topology of the tree retained from the Bayesian analysis with the GTR+SS model of the N gene data set for MV. Numbers on the branches are posterior probabilities obtained from the consensus of 29,400 retained trees generated during the run of 3 million generations after a burn-in of 60,000 generations.
(*) = the proposed genotype.

Discussion

The results of the phylogenetic analysis as well as of the sequence data analysis strongly suggest that the Brazilian isolate MVi/São Paulo.BRA/01 belongs to the D5 genotype of MV. Additionally, both virological surveillance and standard epidemiological case investigation carried out by the measles control program have confirmed that the child of concern was infected in Japan and traveled to Brazil during the incubation period, arriving to São Paulo with clinical symptoms of the disease. Consequently, both the molecular analyses of the virus isolate and the epidemiological data confirmed that this isolate of D5 genotype originated from Japan. The only sample of the D5 genotype from an isolate from the state of São Paulo, Brazil from 1995 was sequenced at CDC, Atlanta, the USA. Unfortunately, the sequence has not been deposited at the GenBank.

The MVi/São Paulo.BRA/01 isolate was compared with the Bangkok THA/93 strain, which has been used in a phylogenetic analysis carried out in 1995 (Oliveira *et al*., 2002). As previously mentioned, the Brazilian isolate clustered within the clade consisting of the D5 genotype, which is the most prevalent and frequently associated with imported measles cases from Japan into other countries (Katayama *et al*., 1997; Yamaguchi, 1997; Nakayama *et al*., 2003). The D5 genotype, which has circulated in Thailand in 1993, has been used in studies focusing on the global distribution of wt genotypes of MV (Rota *et al*., 2003).

In Japan, a molecular epidemiological study has investigated which genotypes of MV circulate there. Consequently, it has been shown that the C1 genotype was circulating since 1984, while D3 from 1987 to 1999, D5 from 1990 to 1997, and D5 again in 2000. Additionally, the genotype H1, which has caused sporadic infections, became dominant in 2001, while D5 reached only a minor distribution (Takahashi *et al*., 2000; Zhou *et al*., 2003).

In Brazil, molecular epidemiological studies have detected sporadic-case of measles caused by the genotypes D5 and D6 in 1995 in São Paulo (Oliveira *et al*., 2002), D5 in 1996 in Bahia (Siqueira *et al*., 2001), and D6 in a large outbreak in 1997 in São Paulo. These reports have suggested that the genotypes, which are circulating in Brazil are similar to those occurring in Europe. Consequently, the virus strains responsible for the epidemics may have been imported from Europe (CDC, 1998). In the 1997 outbreak, the D6 genotype started an epidemic in the state of São Paulo and then spread out to several states causing a large outbreak (Barrero *et al*., 2000; Cánepa *et al*., 2000; Oliveira *et al*., 2002; CDC, 2002).

In the future, molecular epidemiological studies will help to monitor the pathways of transmission of MV throughout the world, and will enable the public health surveillance services to follow up the elimination of the transmission of indigenous MV as a result of the continuous vaccination program.

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