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Genotyping of circulating measles strains in Italy in 2010

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Abstract

Introduction. The European Regional Office of the World Health Organization developed a strategic approach to stop the indigenous transmission of measles in its 53 Member States by 2015. In Italy, laboratory surveillance activity is implemented by the National Reference Laboratory for Measles and Rubella at the Italian National Institute of Health (Istituto Superiore di Sanità, Rome). The role of the National Reference Laboratory is to strengthen surveillance systems through rigorous case investigation and laboratory confirmation of suspected sporadic cases and outbreaks. Genetic characterization of wild-type measles virus is an essential component of the laboratory-based surveillance. This study describes the molecular characterization of measles virus strains isolated during 2010.

Methods. Dried blood spots, urine and oral fluid samples were collected from patients with a suspected measles infection. Serological tests were performed on capillary blood, and viral detection was performed on urine and oral fluid samples through molecular assay. Positive samples were sequenced and phylogenetically analysed.

Results and discussion. The phylogenetic analysis showed a co-circulation of genotypes D4 and D8, and sporadic cases associated to genotypes D9 and B3. Then, molecular epidemiology of measles cases permitted to establish that D4 and D8 were the endemic genotypes in Italy during 2010.

Key words
- measles
- molecular epidemiology
- genotype
- phylogenetic analysis

INTRODUCTION

The strategic plan of the European Regional Office of the World Health Organization (WHO) aims to halt the indigenous transmission of measles in its 53 member states by 2015 [1]. Genetic characterisation of wild-type measles virus (MV) combined with standard epidemiological methods is an essential component of the laboratory-based surveillance, performed throughout the world by the WHO Measles and Rubella Laboratory Network to reach the goal of measles and rubella elimination.

A sensitive and specific surveillance system able to promptly detect measles cases is essential to monitor the effectiveness of vaccination programs. According to the Italian reporting surveillance system, physicians are required to report all the suspected measles cases to local health authorities (LHA), which are required to carry out an epidemiological investigation, and fill a standard notification form. LHA are also required to obtain specimens from suspected measles cases, and send samples to the Regional Reference Laboratories (RRL) (if existing) or to the National Reference Laboratory (NRL) at the Italian National Institute of Health (Istituto Superiore di Sanità, Rome). RRLs confirm cases with serological and/or molecular tests, genotype and send data about sequences to the NRL.

Thus, the role of NRL is to confirm measles cases/outbreaks collected from the LHA and genotype wild-type MVs or to collect data about MV sequences provided from those regions with a RRL for molecular epidemiological purposes.

Knowledge of currently circulating MV genotype in Italy will help in monitoring the success of the measles elimination programme and will contribute to evaluate the effectiveness of future vaccination campaigns. Virologic surveillance can also help to classify suspected cases as vaccine reactions. A small proportion of measles vaccine recipients experience rash and fever 10-14 days following vaccination [2].

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Table1
List of the representative Italian measles strains analyzed in 2010

Strains	N of samples[a]	Weeks[b]	Genotype	GB Accession number
MVs/Arezzo.ITA/45.10	1	45	**D4**	*KJ650237*
MVs/Belluno.ITA/33.10	1	33	**D4**	*KJ659708*
MVs/Bologna.ITA/19.10	1	19	**D4**	KJ659709
MVs/Bologna.ITA/22.10	1	22	**D4**	*KJ659710*
MVs/Bolzano.ITA/17.10	2	17-19	**D4**	*KJ659711*
MVs/Brunico.ITA/18.10	1	18	**D4**	*KJ659712*
MVs/Bolzano.ITA/37.10	1	37	**D4**	KJ659714
MVs/Bolzano.ITA/43.10/1	13	43-49	**D4**	*KJ659713*
MVs/Catania.ITA/14.10	1	14	**D4**	*KJ659715*
MVs/Cosenza.ITA/20.10	3	20-21	**D4**	*KJ659717*
MVs/Cosenza.ITA/51.10	1	51	**D4**	KJ659716
MVs/Cuneo.ITA/14.10/1	3	14-18	**D4**	KJ659730
MVs/Cuneo.ITA/21.10/1	1	21	**D4**	*KJ670695*
MVs/Cuneo.ITA/21.10/2	1	21	**D4**	*KJ670696*
MVs/Firenze.ITA/38.10	2	38-52	**D4**	KJ659718
MVs/Foggia.ITA/17.10	4	4	**D4**	*KJ650235*
MVs/Forli.ITA/19.10	1	19	**D4**	*KJ659719*
MVs/Genova.ITA/11.10/1	1	11	**D4**	KJ659728
MVs/Genova.ITA/12.10/1	1	12	**D4**	KJ659729
MVs/Genova.ITA/26.10/1	3	26-52	**D4**	KJ659731
MVs/Genova.ITA/33.10/1	1	33	**D4**	KJ659726
MVs/Genova.ITA/52.10/3	1	52	**D4**	KJ659727
MVs/LAquila.ITA/35.10/1	2	35	**D4**	KJ659720
MVs/Lecce.ITA/21.10	2	21	**D4**	*KJ650236*
MVs/Messina.ITA/16.10/1	1	16	**D4**	*KJ659722*
MVs/Messina.ITA/16.10/2	1	16	**D4**	*KJ659721*
MVs/Napoli.ITA/24.10	1	24	**D4**	KJ659724
MVs/Napoli.ITA/30.10	3	30-43	**D4**	*KJ659723*
MVs/Napoli.ITA/41.10	1	41	**D4**	*KJ659725*
MVs/Parma.ITA/30.10	1	30	**D4**	*KJ659707*
MVs/Ravenna.ITA/16.10	2	16-17	**D4**	KJ659732
MVs/Ravenna.ITA/25.10	1	25	**D4**	KJ659734
MVs/Ravenna.ITA/27.10	2	27-29	**D4**	KJ659733
MVs/ReggioEmilia.ITA/19.10	1	19	**D4**	KJ659735
MVs/ReggioEmilia.ITA/22.10	2	21-22	**D4**	KJ659737
MVs/ReggioEmilia.ITA/29.10	1	29	**D4**	KJ659736
MVs/Roma.ITA/16.10	2	16-35	**D4**	KJ659738
MVs/Roma.ITA/36.10/1	24	17-50	**D4**	KJ659739
MVs/Rovigo.ITA/32.10	1	32	**D4**	KJ659741
MVs/Salerno.ITA/12.10	1	12	**D4**	KJ659743
MVs/Sassuolo.ITA/20.10	1	20	**D4**	KJ659742
MVs/Siracusa.ITA/07.10/1	2	7	**D4**	KJ659740
MVs/Spoleto.ITA/40.10	1	40	**D4**	KJ659744
MVs/Tivoli.ITA/38.10	1	38	**D4**	KJ659745
MVs/Tivoli.ITA/43.10	1	43	**D4**	KJ659746
MVs/Torino.ITA/22.10/1	6	14-31	**D4**	KJ659747
MVs/Torino.ITA/22.10/2	3	22-24	**D4**	KJ659748
MVs/Torino.ITA/36.10	1	36	**D4**	KJ659749
MVs/Trento.ITA/39.10	9	39-51	**D4**	KJ659751
MVs/Trento.ITA/42.10/1	1	42	**D4**	KJ659752
MVs/Treviso.ITA/43.10/1	3	43-46	**D4**	KJ659750
MVs/Venezia.ITA/32.10	1	32	**D4**	KJ659754
MVs/Verona.ITA/45.10/1	2	45	**D4**	KJ659753
MVs/Viterbo.ITA/23.10	7	23-30	**D4**	KJ659756
MVs/Viterbo.ITA/24.10	7	24-34	**D4**	KJ659755
MVs/Ugento.ITA/Riv/04/2010	3	4	**D4**	*HM173092*
MVs/Bologna.ITA/15.10	3	15-17	**D8**	*KJ573576*
MVs/Bologna.ITA/17.10/1	2	17	**D8**	*KJ573579*

(continues)

Table1(Continued)

Strains	N of samples[a]	Weeks[b]	Genotype	GB Accession number
MVs/Cuneo.ITA/14.10/2	6	14-21	**D8**	*KJ625223*
MVs/Genova.ITA/11.10/2	11	11-20	**D8**	*KJ625224*
MVs/Genova.ITA/18.10/1	1	18	**D8**	*KJ625225*
MVs/Lodi.ITA/21.10	1	21	**D8**	KJ586230
MVs/Lodi.ITA/13.10	1	13	**D8**	*KJ573581*
MVs/Monopoli.ITA/ San/04/2010	1	4	**D8**	*HM173091*
MVs/Piacenza.ITA/14.10/1	2	14	**D8**	*KJ573577*
MVs/Ravenna.ITA/13.10	5	13-18	**D8**	KJ573578
MVs/Ravenna.ITA/32.10	1	32	**D8**	*KJ586229*
MVs/ReggioEmilia.ITA/21.10/1	1	21	**D8**	*KJ573580*
MVs/Torino.ITA/14.10/2	1	14	**D8**	*KJ625222*
MVs/Trento.ITA/31.10	1	31	**D8**	*KJ573575*
MVs/Bologna.ITA/24.10	1	24	**D9**	KJ573574
MVs/Genova.ITA/32.10/2	2	32-34	**B3**	*KJ573572*
MVs/Asti.ITA/21.10/1	1	21	**B3**	*KJ573573*

[a]Identical sequences collected in the same place were grouped, and a single representative sequence was submitted in GenBank; [b]period of circulation of each strain from its first isolation.

Genetic and antigenic characterization of wild-type MV identifies eight clades (A-H) subdivided into 24 genotypes (A, B1-B3, C1and C2, D1-D11, E, F, G1-G3, and H1-H2) [3, 4]. According to WHO recommendations, the 450-nt sequence encoding the C-terminal 150 amino acids of the hyper variable region of the N gene represents the minimal amount of sequence required for MV genotyping [5].

This study describes the genetic characterisation of a series of wild-type MV strains detected during the year 2010 in Italy. During this year, Italy experienced several outbreak in different regions [6].

METHODS

NRL received urine, oral fluid (OF) and dried capillary blood spots samples collected from 211 patients with suspect measles infection from different regions of Italy. Urine and OF specimens were collected from patients with suspected measles infection within the suggested time of 10 days after onset of rash, and capillary blood between 4-28 days [7]. All the specimens were treated and stored as previous described [8]. Dried blood spots were tested serologically by a specific anti-measles IgM enzyme-linked immunosorbent assay (ELISA) [9] and urine and OF specimens were tested by reverse transcriptase-polymerase chain reaction (RT-PCR).

For 11 patients only the blood sample was available, and was tested by ELISA.

Urine and OF samples were available for 164 and 36 patients, respectively. Total RNA was extracted from the urine sediment using the RNEasy Mini Kit (Qiagen) and from the OF using QiAmp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Two rounds of amplification were performed through RT-PCR and hemi-nested PCR directed on a highly conserved region 450 nucleotides long on the N gene [10] coding for the carboxy-terminal 150 amino acids of the nucleoprotein (N). Then, amplicons were analysed by electrophoresis with a 1.5% agarose gel and gel-red staining. PCR products were sequenced by Macrogen DNA Sequencing Service (http://dna.macrogen.com), with the second round primers.

Nucleotide sequences were corrected and aligned with sequences of the reference strains and with those that showed higher percentage of identity after Blast analysis, using CLUSTAL W (BioEdit) software [11].

For the phylogenetic analysis, the K2 (Kimura-2 parameter) evolutionary model was chosen as the best-fitting nucleotide-substitution model, and the tree was constructed by using MEGA version 6 [12]. Bootstrap values were obtained through 1000 resampling of data sets. Virus genotypes were named according to the new official WHO nomenclature [5, 13]. Sequences were submitted to GenBank and the corresponding accession numbers are given in *Table 1*.

RESULTS AND DISCUSSION

Molecular tests were performed on a total of 200 clinical samples – 164 urine and 36 OF samples, respectively – collected from patients with suspected measles infection in different Italian regions.

Viral genome presence was found through RT-PCR assay in 153 clinical samples, and 139/153 were sequenced. Nucleotide sequences were analysed by comparing the fragment coding for the carboxyl terminus of the nucleoprotein (450 nucleotides) with the one of the WHO reference strains. A representative set of sequences is listed in *Table 1*.

Sequence data provided from the Italian RRL of the regions of Liguria and Apulia, 36 and 10 sequences respectively, were also enclosed in the study.

The phylogenetic analysis showed that 144 MV strains belonged to the genotype D4, 37 belonged to the genotype D8, 1 to D9, and 3 to genotype B3 *(Figure 1A)*.

According to the epidemiological data available, these results demonstrated the circulation of the genotype D4 during 2010 in the regions of Piedmont, Trentino Alto Adige, Veneto, Emilia Romagna, Liguria, Tuscany, Umbria, Latium, Abruzzo, Campania, Apulia, Calabria and Sicily *(Figure 1B)*. After BLAST analysis, the strain

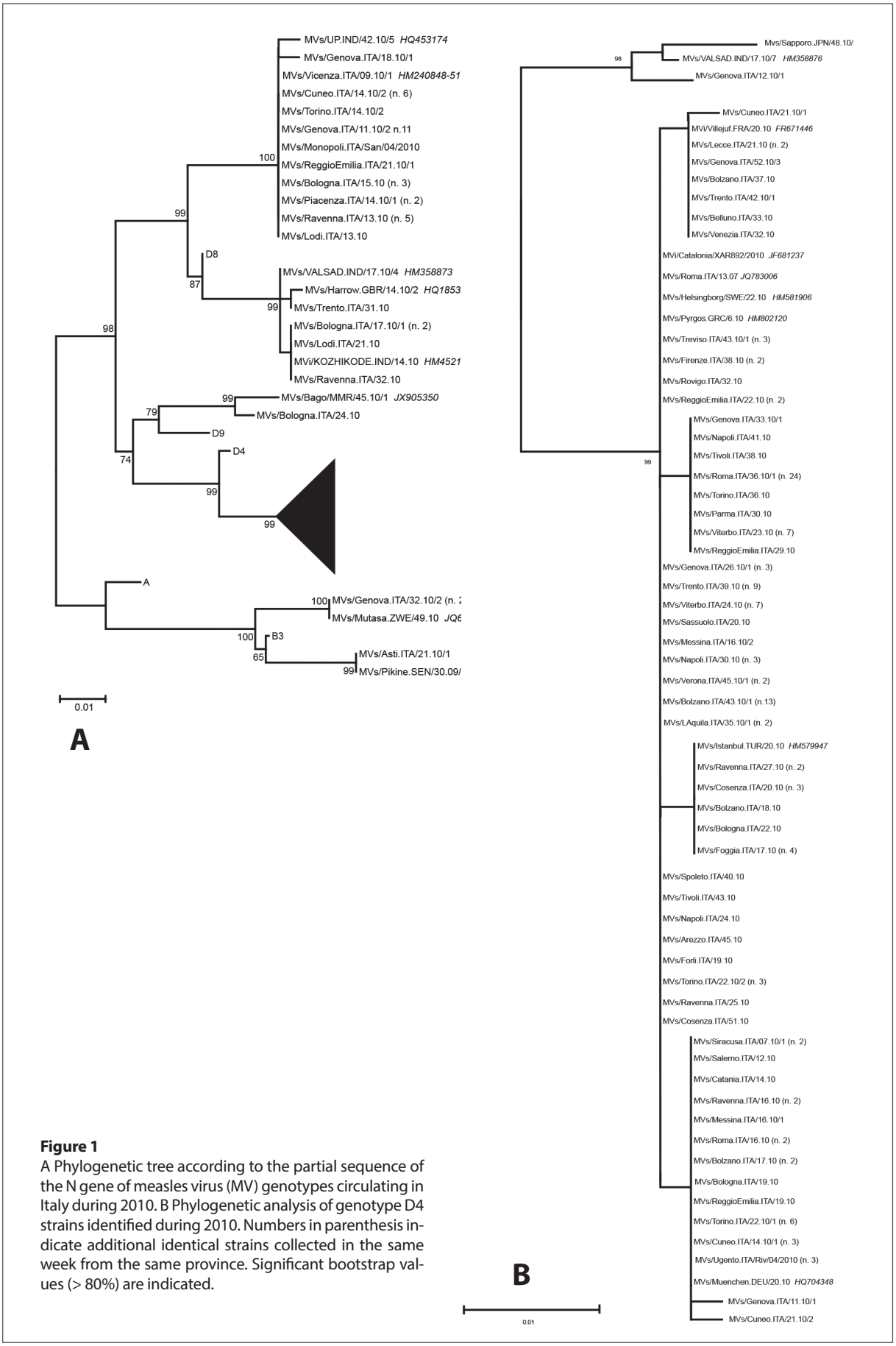

Figure 1
A Phylogenetic tree according to the partial sequence of the N gene of measles virus (MV) genotypes circulating in Italy during 2010. B Phylogenetic analysis of genotype D4 strains identified during 2010. Numbers in parenthesis indicate additional identical strains collected in the same week from the same province. Significant bootstrap values (> 80%) are indicated.

MVs/Genova.ITA/12.10/1 showed 100% identity with the one mVs/Grosseto.ITA/a301 [14] and 99% identity with those isolated in India and Japan in 2010 (GenBank accession Nos: HM358876, AB605257). Except for the sequence MVs/Genova.ITA/12.10/1, all the others sequences belonging to the genotype D4 were found closely related to each other, showing a percentage of identity higher than 99% with a maximum difference of 3 nucleotides. These strains showed a close relationship with those circulating in France, Spain, Sweden, Greece, Turkey and Germany in the same year (GenBank accession Nos: FR671446, JF681237, HM581906, HM802120, HM579947, HQ704348) and with those isolated in Italy in the biennium 2006-2007 [15].

Sequences from 37 cases (collected from week 4th to week 31st of the year 2010 from the regions of Piedmont, Liguria, Trentino Alto Adige, Emilia Romagna and Apulia) were found to belong to the genotype D8. The phylogenetic tree shows these strains belonging to two distinct clades *(Figure 1A)*. Sequences in the first clade show 100% identity with Italian ones identified by the RRL of Veneto (GenBank accession Nos: HM240848-HM240851) and 99% with those isolated in India in the same year (GenBank accession No: HQ453174). Sequences in the second clade show 99% of identity with those isolated in India (GenBank accession Nos: HM358873, HM452160).

One case associated to genotype D9 was detected in the region of Emilia Romagna. It was related to the MV strains circulating in Myanmar in 2010 (GenBank accession no: JX905350).

Moreover, NRL's analysis showed 3 sporadic cases of genotype B3 from the regions of Liguria and Piedmont. Sequences MVs/Genova.ITA/32.10/2 showed high identity with those circulating in South Africa in 2010 (GenBank accession no: JQ627736). The strain MVs/Asti.ITA/21.10/1 was closely related to those isolated in Senegal in 2009 (GenBank accession no: HQ896945). Genotype B3 had circulated in Italy during 2006 and 2007, but no cases were found in 2008.

CONCLUSIONS

Genetic characterization of wild-type MV provides a mean to study the transmission pathways and to find origins and routes of MV wild-type circulation. Genetic data can help to confirm the sources of virus or suggest a source for unknown-source cases as well as to establish links, or lack thereof, between various cases and outbreaks. Then, monitoring of the MV strains circulating is a necessary component of the surveillance system. Since serologic methods cannot distinguish between a vaccine-induced antibody response and antibodies derived from natural disease, molecular characterization of viral isolates provides a method to confirm whether vaccine or wild-type measles virus caused the rash and fever. Moreover, characterizing measles virus isolates is the key role to determine whether cases come from indigenous transmission or importations.

This study involved a total of 185 MV Italian strains detected during 2010. Phylogenetic results demonstrated the co-circulation of genotypes D4 and D8 during the reviewed period.

Previous results from NRL showed that genotype D4 replaced the genotype D7 during the biennium 2006-2007 [15], and was endemic in Italy during 2008 [8]. Moreover, sporadic cases associated to the genotype D8 in the years 2007 and 2008 were demonstrated. Limited data about the molecular epidemiology of MV are available for the year 2009, because of the interruption of the surveillance activity for that year. Anyway, in 2009 some cases were found to be associated to the genotype D4 (data not published).

In summary, according to NRL's analysis we can assert that D4 continued to be endemic in Italy in the reviewed period as in the rest of Europe, and the genotype D8 seems to have become endemic in Italy together with D4. In the same year cases associated with D9 and B3 genotypes were also found.

For both endemic and sporadic cases genetic correlations with strains circulation in different countries were found, suggesting a possible route of introduction. Unfortunately, no epidemiological data were available to support this hypothesis.

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Conflict of interest statement

All named authors have read and agreed to the submitted version of the manuscript, and declare there are no potential conflict of interests, or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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