Molecular epidemiology of measles virus in Italy during 2008

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Abstract
Introduction. In view of the goal of measles elimination, it is of great importance to assess the circulation of wild-type measles virus (MV). Genetic analysis is indispensable to understand the epidemiology of measles. A large measles outbreak occurred in Italy in 2008, with over 4000 cases reported to the enhanced measles surveillance system introduced in 2007, 37% of which were laboratory confirmed.
Methods. Urine and saliva samples were collected during 2008. A phylogenetic analysis of measles sequences was performed in order to understand the epidemiological situation of wild-type (MV) circulation in that period.
Result and discussion. Data showed predominant circulation of the genotype D4. Genotypes A, D8, D9 and H1 were also detected in a small number of samples, probably representing imported cases.

Key words
• measles
• epidemiology
• genotype
• virus
• infectious disease

INTRODUCTION

Globally, measles morbidity and mortality rates have dramatically declined since 1963, thanks to the availability of a safe and effective vaccine and the implementation of enhanced vaccination strategies. Nonetheless, measles remains a leading cause of childhood mortality worldwide, with an estimated 164 000 measles deaths in 2008 (a 78% reduction compared to mortality in 2000) [1, 2]. Interruption of indigenous transmission of measles virus (MV) has been reported in the Americas and in several countries [3, 4]. However, measles cases continue to be reported even in those countries with high vaccination coverage, following the importation of the virus from endemic regions.

In the World Health Organization (WHO) European Region, measles has been targeted for elimination by 2015 [5].

In Italy, the incidence of measles declined since the introduction of the combined measles-mumps-rubella (MMR) vaccine but large outbreaks continue to occur. A national elimination plan was first approved in 2003 with the aim of achieving measles elimination by 2010. Measles vaccination coverage increased from 83.9% in 2003 to 90.1% in 2008, and 90.6% in 2010. Following a period of very low incidence (≤ 1 case/100 000 inhabitants in 2005 and 2006), Italy faced a resurgence of measles starting in September 2007 [6]. A new elimination plan was approved in March 2011 targeting measles elimination by 2015 [7].

Genetic characterisation of wild-type MVs, combined with epidemiological data, helps to document transmission pathways and to illustrate the progress made towards measles elimination, by differentiating indigenous and imported viruses.

Genetic characteristics of representative wild-type MVs, identified in Italy during the year 2008, were analysed in this study.

METHODS

An enhanced surveillance system was introduced In Italy in 2007 [8], to improve timeliness, completeness of case reporting, and case investigation, including laboratory confirmation of diagnosis. According to this system, physicians are required to report all suspected measles cases to the local health authorities within 12 hours (as opposed to within 48 hours in the statutory notification system). For each suspected case the local health authorities are required to carry out an epidemiological investigation, including obtaining specimens for laboratory confirmation and genotyping, and to complete a standard measles notification form, which is then to be sent to regional health authorities. The regional authorities forward the forms immediately to the Ministry of Health and to the Infectious Diseases Epidemiology Unit of the Italian National Institute of Health (Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, ISS). Moreover, a National

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Table 1
List of the representative sequences analyzed in 2008

Name of strain[a]	No. of samples	No. of weeks[b]	Genotype	Accession numbers
MVs/Bergamo.ITA/11.08	2	6	D4	JX187540
MVs/Roma.ITA/13.08	26	15	D4	JX456608
MVs/Modena.ITA/12.08	6	17	D4	JX187541
MVs/Torino.ITA/4.08	57	31	D4	JX187542
MVs/Milano.ITA/9.08	13	23	D4	JX187543
MVs/Lodi.ITA/19.08	3	15	D4	JX187544
MVs/Brescia.ITA/25.08	1	1	D4	JX187545
MVs/Trento.ITA/10.08	2	10	D4	JX187546
MVs/Livorno.ITA/18.08	1	1	D4	JX187547
MVs/Torino.ITA/16.08	1	1	D4	JX187548
MVs/Monza.ITA/14.08	2	18	D4	JX187549
MVs/Bologna.ITA/16.08	9	11	D4	JX187550
MVs/Alghero.ITA/18.08	1	1	D4	JX187551
MVs/Ancona.ITA/27.08	1	1	D4	JX187552
MVs/Alessandria.ITA/14.08	3	13	D4	JX187553
MVs/Como.ITA/16.08	1	1	D4	JX187554
MVs/Firenze.ITA/9.08	5	11	D4	JX187555
MVs/Palermo.ITA/35.08	1	1	D4	JX187556
MVs/Parma.ITA/19.08	3	7	D4	JX187557
MVs/Lucca.ITA/9.08	1	1	D4	JX187558
MVs/Sassuolo.ITA/26.08	1	1	D4	JX187559
MVs/Perugia.ITA/28.08	1	1	D4	JX187560
MVs/Venezia.ITA/24.08	3	5	D4	JX187561
MVs/Spoleto.ITA/31.08	1	1	D4	JX187562
MVs/Vicenza.ITA/28.08	3	2	D4	JX187563
MVs/AscoliPiceno.ITA/9.08	5	21	D4	JX187564
MVs/Pisa.ITA/17.08	3	6	D4	JX187565
MVs/Lecco.ITA/13.08	2	12	D4	JX187566
MVs/Napoli.ITA/17.08	1	1	D4	JX187567
MVs/Arezzo.ITA/19.08	1	1	D4	JX187568
MVs/Pavia.ITA/8.08	1	1	D4	JX456609
MVs/Novara.ITA/13.08	3	10	D4	JX187569
MVs/Piacenza.ITA/8.08	2	7	D4	JX187570
MVs/Asti.ITA/17.08	2	1	D4	JX187571
MVs/Cuneo.ITA/9.08	9	19	D4	JX187572
MVs/Vercelli.ITA/15.08	1	1	D4	JX187573
MVs/Biella.ITA/19.08	5	4	D4	JX187574
MVs/Sasssari.ITA/14.08	2	3	D4	JX187575
MVs/Verona.ITA/13.08	4	7	D4	JX456607
MVs/Aosta.ITA/15.08	2	14	D4	JX187576
MVs/Cagliari.ITA/25.08	1	1	D4	JX187577
MVs/Milano.ITA/26.08	2	6	D4	JX187578
MVs/Venezia.ITA/3.08	2	2	D4	JX187579
MVs/Ancona.ITA/8.08	1	1	D9	JX187580
MVs/ReggioEmilia.ITA/5.08	1	1	D8	JX187581
MVs/Treviso.ITA/27.08	1	1	H1	JX187586
MVs/Parma.ITA/25.08	1	1	A	JX187582
MVs/Parma.ITA/47.08	1	1	A	JX187583
MVs/Torino.ITA/8.08	1	1	A	JX187584
MVs/Monza.ITA/27.08	1	1	A	JX187585

[a]The nomenclature indicates the geographical origin and the year in which each virus was first identified. 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.

Table 2
Measles cases reported to the enhanced measles surveillance system during 2008

Italy regions	No. cases	Regional population	Incidence per 100 000 population
Abruzzo	4	1 323 987	0.30
Basilicata	0	591 001	0.00
Calabria	0	2 007 707	0.00
Campania	8	5 811 390	0.14
Emilia-Romagna	195	4 275 802	4.56
Friuli-Venezia Giulia	20	1 222 061	1.64
Lazio	214	5 561 017	3.85
Liguria	3	1 609 822	0.19
Lombardia	584	9 642 406	6.06
Marche	31	1 553 063	2.00
Molise	0	320 838	0.00
Piemonte	2683	4 401 266	60.96
Puglia	14	4 076 546	0.34
Sardegna	55	1 665 617	3.30
Sicilia	8	5 029 683	0.16
Toscana	209	3 677 048	5.68
Trentino-Alto Adige	17	1 007 267	1.69
Umbria	6	884 450	0.68
Valle d'Aosta	8	125 979	6.35
Veneto	118	4 832 340	2.44
Total	**4177**	**59 619 290**	**7.01**

Reference Laboratory (NRL) of the ISS confirms outbreaks/cases by IgM serology and/or by viral RNA detection, and determines the measles virus genotypes for molecular epidemiological purposes.

The WHO currently recognizes eight clades (designated from A to H), and within these clades, there are 23 recognized genotypes. The WHO also recommends a region of 450 nucleotides, coding for the carboxyterminal 150 amino acids of the nucleoprotein (N-450), as the minimum amount of sequence data required to assign a measles genotype [9].

Specimens. Oral-fluid specimens were obtained by the salivary swab. Saliva was collected after centrifugation at 1500 rpm for 10 minutes, and then stored at -20 °C until tested. Urine samples were collected within 7 days of rash onset. Urine sediment was obtained after centrifugation at 1500 rpm for 10 minutes, washed two times in sterile PBS, and resuspended in a final volume of 0.5-1 ml. Sediments were stored at -80 °C.

RT-PCR amplification and sequencing. Total RNA was extracted using QIAmp Viral RNA Mini Kit (Qiagen) for saliva samples and RNeasy mini Kit (Qiagen) for urine samples, as per the manufacturer's protocols. Nucleic acid was tested by RT-PCR using a hemi-nested protocol directed to a highly conserved part of the MV RNA, which is located on the N gene.

Kit SuperScript One-Step RT-PCR kit with Platinum

Taq (Invitrogen) was used for RT-PCR reaction as previous described [10].

Nucleotide sequence analysis. Both strands of amplified products were sequenced by Macrogen Inc. (Seoul, Korea), using MVF2 and MVB1 primers.

Genomic sequences of reference strains used for genetic analysis of wild-type measles viruses, were obtained from database by accession number [11, 12]. Nucleotide sequences were aligned with the CLUSTAL W (BioEdit) software [13]. Phylogenetic trees were constructed using the nucleotide Kimura-2 parameter and the neighbor-joining method. Bootstrap analyses were performed through 1000 resampling of the data sets. The neighbor-joining method [14] was implemented by using MEGA-4 [15].

The sequences had been submitted to GenBank, and the corresponding accession numbers are given in *Table 1*.

RESULTS AND DISCUSSION

Between 1 January 2008 and 31 December 2008, 4177 possible, probable and confirmed measles cases were reported to the Italian enhanced surveillance system at the Infectious Diseases Epidemiology Unit of the ISS. Cases were reported from 17 of 21 Regions, with 64% of cases (2683/4177) reported from the Piedmont Region (Northern Italy). These cases were mainly reported from

Figure 1

Geographical distribution of measles virus (MV) geno-types isolated in Italy during 2008.
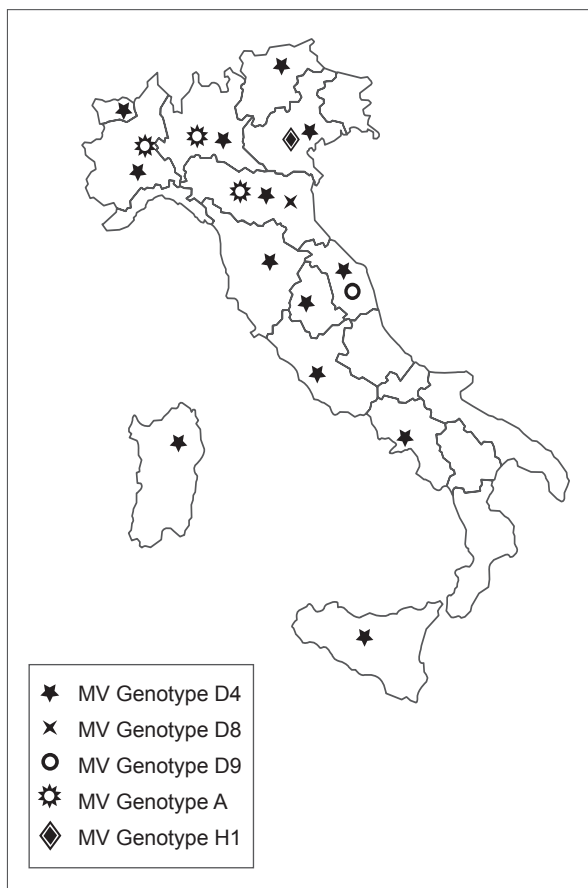
Northern and Central Italian Regions *(Table 2)*.

Overall 37% of cases (no. = 1550) were laboratory confirmed, 45% (no. = 1860) were probable cases and 18% (no. = 767) were possible cases. Most cases were non vaccinated and almost 60% of cases were aged > 15 years. The mean age was 18 years.

In 2008, the NRL sequenced a total of 203 samples collected from 13 out of 21 Italian Regions *(Figure 1)*. The percentage of samples tested in 2008 was low and genotype information was available for 203 out of 1550, only 13%.

Genotype D4 was identified in 196 of 203 specimens *(Table 1; Figure 2)*. All D4 sequences were closely related to each other and showed overall only a single nucleotide difference, supporting the hypothesis of a common origin for the outbreak. Furthermore, these strains showed a 100% identity with those isolated in Italy in 2007, and in Europe and Asia in 2007 and 2008 (GenBank accession numbers: *JQ783000.1; FJ917754.1; EU585740.1; GQ428178.1; EU585844.1; GU371654.1)*.

The remaining 8 sequences belonged: 4 to genotype A, 1 to genotype D8, 1 to genotype D9 and 1 to genotype H1. During the year 2008, genotypes A, D8, D9 and H1 were also identified in Italy in sporadic cases, probably as result of importation *(Figure 2)*.

Genotype A was isolated in the regions of Piedmont, Emilia Romagna and Lombardy. A comparison of the Italian A MVs genotype with those deposited in

GenBank revealed a close relation with the Schwarz vaccine strain, as well as with the wild-type genotype A strain *(Figure 2)*. Further analysis will be performed to establish whether they are vaccinal cases.

The D8 genotype isolated in Emilia Romagna was found to be closely related to a MV strain detected in Canada and India in 2008 and 2007 respectively (GenBank accession numbers: *EU650202.1; EU650202.1)*.

Genotype D9, detected in the Marche Region, was closely related to the strains isolated in Europe and China in 2008 (GenBank accession numbers: GQ428185.1; EU878302.1; FJ911610.1; EU368827.1), and in Thailand in 2007 (GenBank accession numbers FJ356073.1) and was probably imported from the latter.

Genotype H1, isolated in the Veneto Region, was closely related to Chinese and Russian strains identified in 2007 (GenBank accession numbers: *EU423318.1; GU237349.1; EU597257.1)* suggesting a possible route of importation.

Both D9 and H1 had never been reported in Italy previously.

While certain genotypes, such as A, D8, D9 and H1, were identified in only a few regions, the distribution of genotype D4 was isolated from 13 Italian Regions *(Figure 1)*.

In summary, MV D4 strain was endemic in Italy during 2008 and caused a large outbreak in the Piedmont Region. Phylogenetic analysis indicates that the first case reported in the Piedmont Region was imported from the United Kingdom in 2007. This shows the importance of international efforts in controlling measles transmission in Europe [16].

Genetic characterization of wild-type MV provides a means to study the transmission pathways of the virus and is an essential component of laboratory-based surveillance. In particular, genetic data can help confirm the source of the virus, may suggest a source for unknown-source cases, and may establish links, or lack thereof, between imported cases and outbreaks.

Virological surveillance will therefore become increasingly important to document the interruption of endemic measles transmission in Italy as in the European Region.

Figure 2
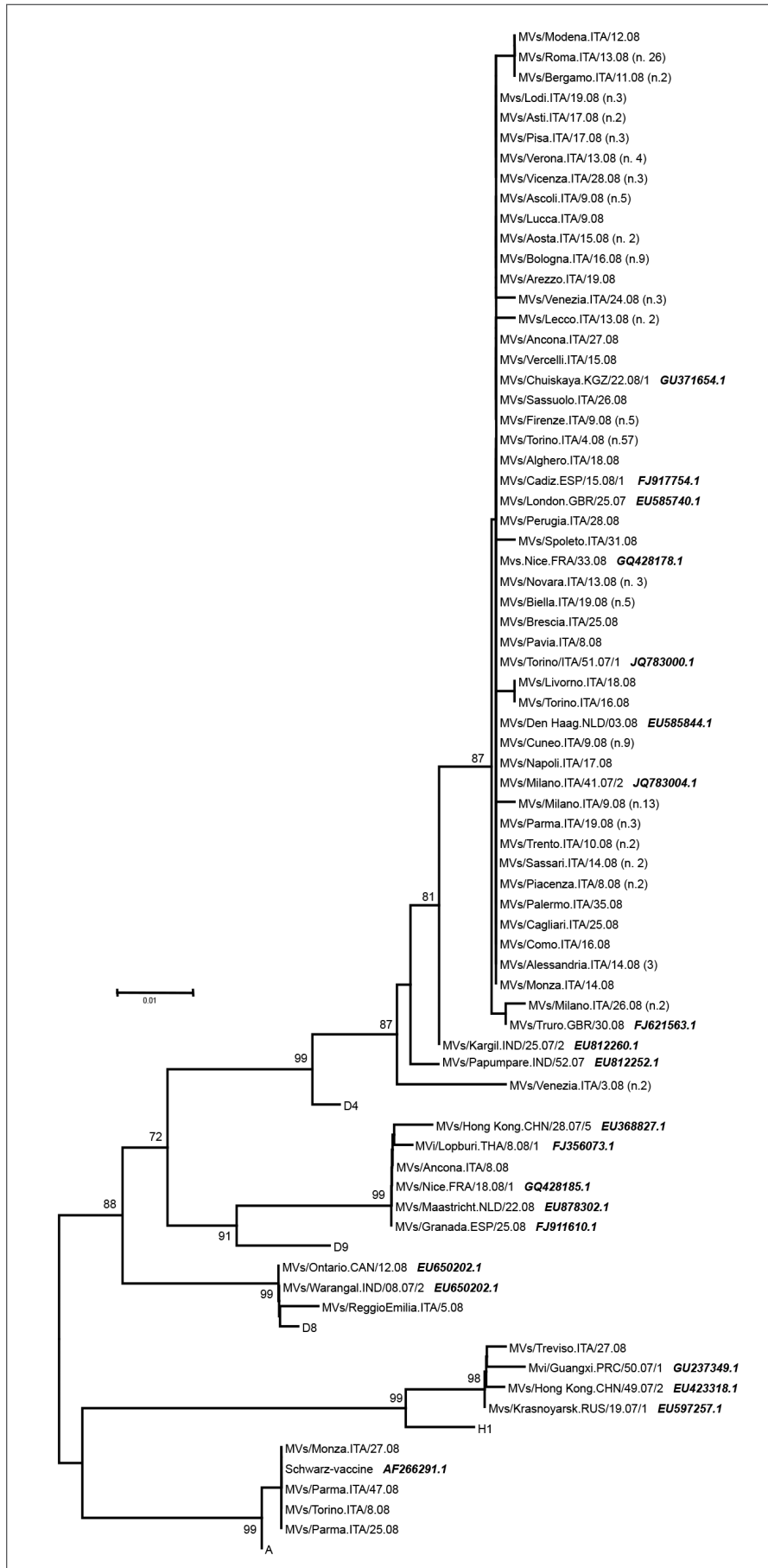Phylogenetic analysis according to the sequence of the N gene of measles virus (MV) strains identified in Italy during 2008. Numbers of identical sequence variants from the same Region are shown in parenthesis. Significant bootstrap values (> 80%) are indicated.

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