

# Genotyping of circulating measles strains in Italy in 2010

Melissa Baggieri<sup>(a)</sup>, Claudia Fortuna<sup>(a)</sup>, Filippo Ansaldi<sup>(b)</sup>, Maria Chironna<sup>(c)</sup>, Antonella Marchi<sup>(a)</sup>, Paola Bucci<sup>(a)</sup>, Eleonora Benedetti<sup>(a)</sup>, Martina Del Manso<sup>(d)</sup>, Silvia Declich<sup>(d)</sup>, Loredana Nicoletti<sup>(a)</sup> and Fabio Magurano<sup>(a)</sup>

<sup>(a)</sup> Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Rome, Italy

<sup>(b)</sup> Dipartimento di Scienze della Salute, Università di Genova, Genoa, Italy

<sup>(c)</sup> Dipartimento di Scienze Biomediche ed Oncologia Umana, Sezione di Igiene e Medicina Preventiva, Policlinico di Bari, Bari, Italy

<sup>(d)</sup> Centro Nazionale di Epidemiologia, Sorveglianza e Promozione della Salute, Istituto Superiore di Sanità, Rome, Italy

## 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].

**Table 1**

List of the representative Italian measles strains analyzed in 2010

Strains	N of samples <sup>a</sup>	Weeks <sup>b</sup>	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)

Table 1 (Continued)

Strains	N of samples <sup>a</sup>	Weeks <sup>b</sup>	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

<sup>a</sup>Identical sequences collected in the same place were grouped, and a single representative sequence was submitted in GenBank; <sup>b</sup>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, C1 and 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.macro-gen.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

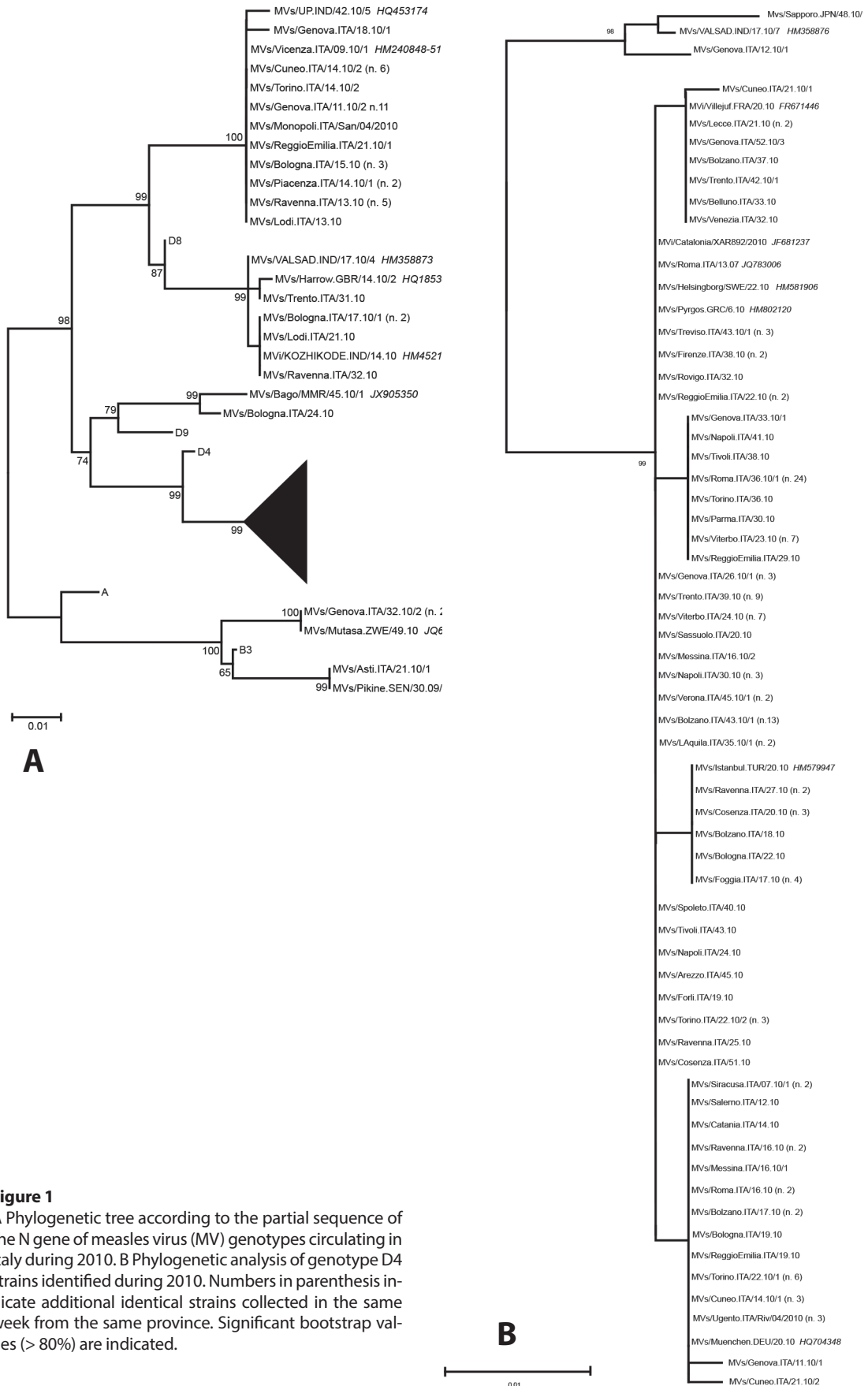
Molecular tests were performed on a total of 200 clinical samples – 164 urine and 36 OF samples, respectively – collected from patients with suspected measles infection in different Italian regions.

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.

## Acknowledgements

The authors thank the staff at Italian Regional and Local Health Authorities for providing clinical specimens. This work was partly supported by a grant from Italian Ministry of Health-CCM 1M27 "Sorveglianza di laboratorio di malattie virali prevenibili da vaccinazioni e rinforzo del Piano di eliminazione del morbillo e della rosolia congenita".

## 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.

Received on 23 June 2014.

Accepted on 15 October 2014.

## REFERENCES

1. World Health Organization. Regional office for Europe. Resolution. Renewed commitment to measles and rubella elimination and prevention of congenital rubella syndrome by 2015 and Sustained support for polio-free status in the WHO European region. Regional Committee for Europe. Sixtieth session. Moscow: 13-16 September 2010. Available from: [http://www.euro.who.int/\\_\\_data/assets/pdf\\_file/0008/119546/RC60\\_edoc15.pdf](http://www.euro.who.int/__data/assets/pdf_file/0008/119546/RC60_edoc15.pdf).
2. Griffin DE. Measles virus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman Strauss SE (Eds). *Fields virology*. Philadelphia: Lippincott Williams, and Wilkins; 2001. p. 1401-41.
3. Rota PA, Featherstone DA, Bellini WJ. Molecular epidemiology of measles virus. *Curr Top Microbiol Immunol*



- 2009;330:129-50. Review DOI: 10.1007/978-3-540-70617-5\_7
4. Zhang Y1, Ding Z, Wang H, Li L, Pang Y, Brown KE, Xu S, Zhu Z, Rota PA, Featherstone D, Xu W. New measles virus genotype associated with outbreak, China. *Emerg Infect Dis.* 2010;16(6):943-7. DOI: 10.3201/eid1606.100089
  5. World Health Organization. Measles virus nomenclature update 2012. *Wkly Epidemiol Rec* 2012;87:73-80. Available from: <http://www.who.int/wer>.
  6. Filia A, Bella A, Rota MC, Tavilla A, Magurano F, Baggieri M, Nicoletti L, Iannazzo S, Pompa MG, Declich S. Analysis of national measles surveillance data in Italy from October 2010 to December 2011 and priorities for reaching the 2015 measles elimination goal. *Euro Surveill* 2013;18(20):1-7.
  7. WHO. *Surveillance guidelines for measles, rubella and congenital rubella syndrome in the WHO European Region update December 2012.* Available from: [www.euro.who.int/\\_\\_data/assets/pdf\\_file/0018/79020/e93035-2013.pdf?ua=1](http://www.euro.who.int/__data/assets/pdf_file/0018/79020/e93035-2013.pdf?ua=1).
  8. Magurano F, Fortuna C, Baggieri M, Filia A, Benedetti E, Bucci P, Marchi A, Nicoletti L. Molecular epidemiology of measles virus in Italy during 2008. *Ann Ist Super Sanità* 2013;49(1):50-5. DOI: 10.4415/ANN\_13\_01\_09.
  9. Novello F, Ridolfi B, Fiore L, Buttinelli G, Medda E, Favero A, Marchetti D, Gaglioppa F. Comparison of capillary blood versus venous blood samples in the assessment of immunity to measles. *J Virol Methods* 1996;61(1-2):73-7. DOI: 10.1016/0166-0934(96)02071-X
  10. Chibo D, Birch CJ, Rota PA, Catton MG. Molecular characterization of measles viruses isolated in Victoria, Australia, between 1973 and 1998. *J Gen Virol* 2000;81(Pt 10):2511-8.
  11. Hall TA: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT/. *Nucl Acids Symp* 1999;41:95-8.
  12. Tamura K, Stecher G, Peterson D, Filipksi A, and Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 2013;30:2725-9. DOI: 10.1093/molbev/mst197
  13. WHO. Expanded programme on immunization ± standardization of the nomenclature for describing the genetic characteristics of wild-type measles viruses. *Wkly Epidemiol Rep* 1998;73:265-9.
  14. Boncompagni G, Incandela L, Bechini A, Giannini D, Cellini C, Trezzi M, Atti ML Cd, Ansaldi F, Valle L, Bonanni P. Measles outbreak in Grosseto, central Italy. *Euro Surveill* 2006;3(8):11.
  15. Magurano F, Fortuna C, Marchi A, Benedetti E, Bucci P, Baggieri M, Nicoletti L. Molecular epidemiology of measles virus in Italy, 2002-2007. *Virol J* 2012;9:284. DOI: 10.1186/1743-422X-9-284