

Transmission and molecular characterisation of wild measles virus in Romania, 2008 to 2012

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Molecular characterisation of measles virus is a powerful tool for tracing transmission. Genotyping may prove the absence of endemic circulation of measles virus, i.e. transmission for more than 12 months, which is one of the criteria for verifying elimination of the disease. We have genetically characterised measles viruses detected in Romania from 2008 to 2012, focusing on the recent outbreaks from 2010 to 2012 that affected mainly groups with limited access to healthcare and schools. The findings emphasise the importance of genotyping during the different phases of an outbreak. A total of 8,170 cases were notified, and 5,093 (62%) of the 7,559 possible cases were serologically confirmed. RT-PCR was performed for 104 samples: from the 101 positive samples obtained from sporadic measles cases or clusters from different counties, 73 were genotyped. Sporadic measles cases associated with D₄ and D₅ viruses were observed from 2008 to 2009. Genotype D₄-Manchester was predominant in 2011 and 2012. In addition, the related variant D₄-Maramures and MVs/Limoges.FRA/17.10[D₄] and a few D₄-Hamburg strains were detected. The detection of several distinct MV-D₄ genotypes suggests multiple virus importations to Romania. The outbreak associated with D₄ genotype is the second largest outbreak in Romania in less than 10 years.

Introduction

Measles is a highly contagious respiratory viral disease characterised by the appearance of fever and a rash and that can be very serious or even fatal. Measles remains one of the leading causes of mortality in young children although a safe and cost-effective vaccine has been available for decades [1]. Although improvements have been made to control measles in Europe, large-scale outbreaks have recently still been observed [2-7]. The World Health Organization (WHO) was forced to postpone a number of times the target date for measles elimination from the European region, most recently to 2015 [8].

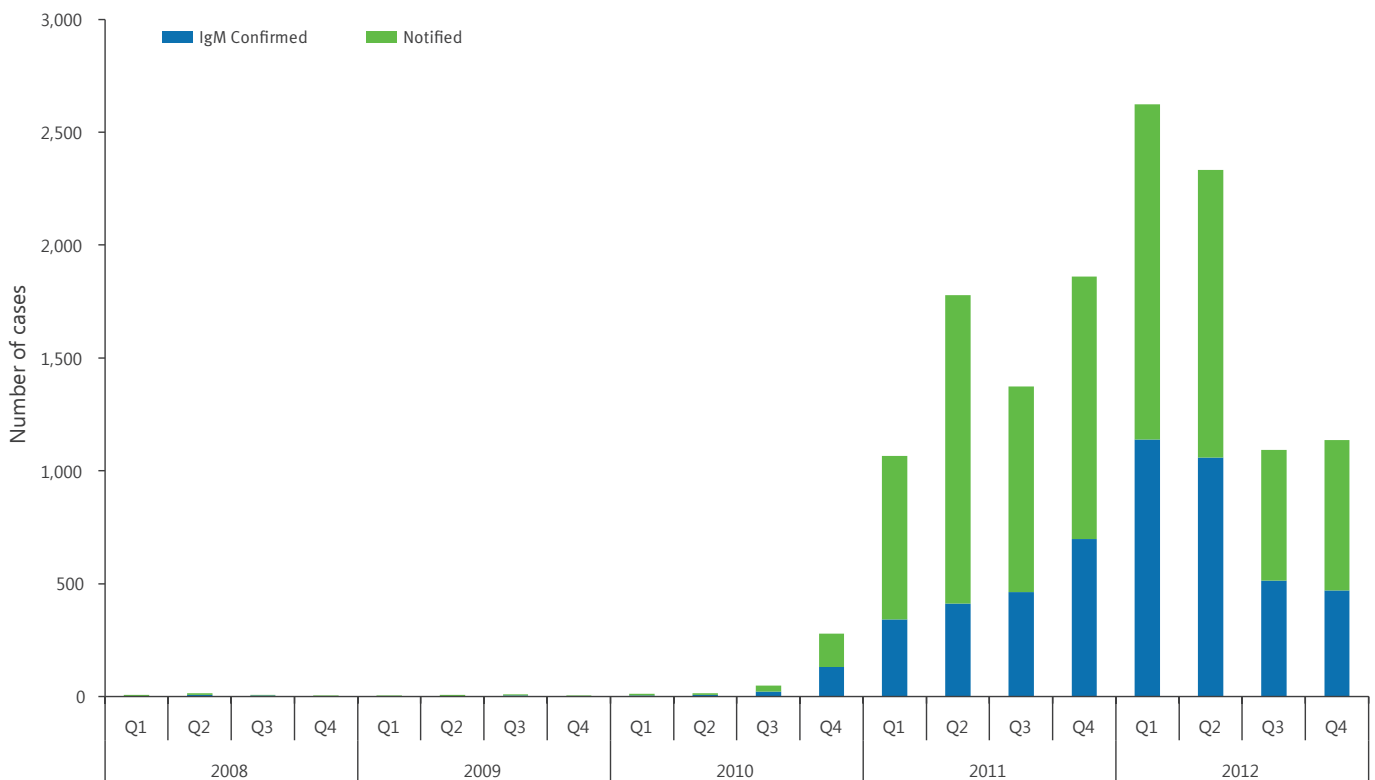
Measles has been a statutorily notifiable disease in Romania since 1978, and medical practitioners must report all clinically possible measles cases to the regional public health authorities. The first monovalent measles-containing vaccine was introduced in 1979 into the Romanian vaccination schedule for children aged nine to 11 months. The combined measles-mumps-rubella (MMR) vaccine replaced the monovalent measles vaccine in 2004 and was recommended as a first dose for children aged 12 to 15 months. The second MMR vaccine dose has been recommended since October 2005 for children aged six to seven years. In the period from 2000 to 2010, the coverage for the first dose of measles vaccine was estimated at 95–98% [9]. In 2011, measles vaccination coverage for the first dose of MMR vaccine was estimated at 84% for children aged 12 months and 93.2% for those aged 18 months [10].

Romania experienced a measles epidemic that started in December 2004 and lasted until early 2007 [11]. More than 9,000 cases were detected mostly in non-immunised patients belonging to the Roma ethnic group. The outbreak was caused by the strain MVs/Bucharest.ROM/48.04/2[D₄] and variants divergent by two nucleotides or less were detected during the period 2004 to 2006 [11]. Closely related strains were detected from 2005 to 2007 in Bosnia and Herzegovina, Germany, Italy, Portugal, Serbia, Spain and Switzerland [11-12]: outbreaks associated with MVs/Bucharest.ROM/48.04/2[D₄] occurred in 2005 in Germany (223 cases in Hesse, MVs/Frankfurt.DEU/03.05[D₄]) [13] and from August 2006 to February 2007 in Spain (over 200 cases in Catalonia, MVs/Barcelona.SPA/41.06/1[D₄]) [11].

D₄ measles viruses are endemic in India, South-East Asia and in South Africa [14]. Outbreaks associated with this genotype have been reported since 2007 from all continents. In Europe, many distinct variants descend from D₄-Enfield (MV/Enfield.GBR/14/07) which became endemic in the United Kingdom (UK) in

FIGURE 1

Quarterly distribution of notified and confirmed measles cases, Romania, 2008–12 (n=8,170)



2007 [15–17]. The variant D4-Hamburg initiated a transmission chain of 25,000 cases that was detected in Europe for a period of more than two years, 2009 to 2011 [18]. In 2010, the D4 genotype became predominant in Europe [2].

Genetic characterisation of measles viruses constitutes an important part of laboratory surveillance. Molecular epidemiology confirms the transmission pathway of measles virus, thereby complementing classical epidemiology. Moreover, interruption of endemic measles virus transmission (i.e. circulation of a certain variant for more than 12 months) is an important criterion for verification of measles virus elimination in Europe. Therefore, it is necessary to distinguish between endemic and imported viruses using molecular methods [16].

This study describes the genetic characterisation of measles viruses detected in Romania from 2008 to 2012, focusing on the recent outbreaks that occurred in the country between 2010 and 2012 that affected mainly groups with limited access to healthcare facilities and schools. This study underlines the importance of measles genotyping during the different phases of an outbreak.

Methods

Patients and specimens collection

According to the national strategy of measles surveillance, approved by Romanian MOH, a measles case is defined as a person with fever and maculopapular rash and at least one of the following symptoms: cough, coryza, or conjunctivitis. Possible cases are persons who met the clinical case definition with no epidemiological link to a laboratory-confirmed case. Confirmed cases are either laboratory-confirmed (by detecting measles IgM antibodies in serum samples, virus isolation, a significant rise in measles antibody levels, or measles PCR detection in all possible cases) or confirmed by the presence of measles case symptomatology and an epidemiological link to a laboratory-confirmed case.

At national level, notifications of measles cases are collected and analysed by the National Centre for Communicable Diseases Surveillance and Control in Bucharest, Romania. Specimens are sent for confirmation to the National Reference Laboratory for Measles and Rubella in Cantacuzino Institute, Bucharest, Romania.

TABLE

Molecular characterisation of wildtype measles viruses and relationship of genotypes and epidemiological activity, Romania, 2008–12

Detection period	Measles virus strain	Measles virus genotype/ variant	Imported from	Epidemiological remarks
March 2008	MVs/Dolj.ROU/13/08/1	D4-Enfield	Italy	1 sporadic case in Dolj
April 2008	MVs/Bucharest.ROU/20.08/	MVs/Lucerne.SWI/46.06[D5]	Greece	1 sporadic case in Bucharest
August 2009	MVs/Arad.ROU/35.09/1	D4-Hamburg	Ireland	Small cluster (5 cases) in Arad,
2010: 8 districts affected				
February–March	MVs/Tulcea.ROU/08.10/	D4-Manchester	France	Small clusters (5 cases) in Tulcea
April–May	MVs/Timis.ROU/18.10/1	D4-Hamburg	Unknown	2 cases in Timis (siblings)
June	MVs/Neamt.ROU/26.10/	MVs/Limoges.FRA/17.10/[D4]	Unknown	Small cluster (3 cases) in Neamt; 1 case in Timis
August	MVs/Neamt.ROU/34.10/	MVs/Limoges.FRA/17.10/[D4]	Unknown	Nosocomial outbreak (28 cases, 1 death) in Neamt
September	MVs/Vaslui.ROU/39.10/	MVs/Paris.FRA/40.10[G3]	France	1 sporadic case in Vaslui,
October 2010– September 2011	MVs/Maramures.ROU/03.11	D4-Maramures	Unknown	615 cases in Maramures
2011: 39 of 41 districts plus Bucharest affected				
March	MVs/Buzau.ROU/12.11/	D4	Turkey	2 sporadic cases in Buzau
April	MVs/Calarasi.ROU/16.11/	B3	Spain	1 sporadic case in Calarasi
October	MVs/Tulcea.ROU/41.11/	B3	Unknown	1 sporadic case in Tulcea
November	MVs/Buzau.ROU/46.11/	D8	Italy	1 case in Buzau
February–November	MVs/Ialomita.ROU/06.11/ MVs/Bucuresti.ROU/44.1/1	D4-Manchester	Not imported	Galati (25 cases), Sibiu (17), Caras-Severin (132), Arges (2), Constanta (190), Giurgiu (20), Bucuresti (49), Ialomita (30)
2012: All districts affected				
January–June	MVs/Ifov.ROU/01.12/ MVs/Ifov.ROU/23.12/	D4-Manchester	Indigenous	Ifov (50 cases), Iasi (383), and Ialomita (92)
April	MVs/Brasov.ROU/14.12/	D4-Maramures	Indigenous	62 cases in Brasov
June–July	MVs/Gorj.ROU/26.12/ MVs/Olt.ROU/28.12/	D8 - Frankfurt Main	Unknown	2 sporadic cases in Gorj and neighbouring Olt district
August	MVs/Brasov.ROU/35.12/	D4- Maramures	Indigenous	16 cases in Brasov
September	MVs/Arges.ROU/36.12/ MVs/Olt.ROU/37.12/	D4-Manchester	Indigenous	1 sporadic case in Olt and outbreak in Arges (72),
	MVs/Prahova.ROU/37.12/	D8 - Frankfurt Main	Unknown	1 sporadic case in Prahova,
October	MVs/Suceava.ROU/41.12/	D8 - Frankfurt Main	Not imported	5 sporadic cases (1 death) in different parts of the county
	MVs/Suceava.ROU/41.12/3	D4- Manchester	Indigenous	1 sporadic case in Suceava
	MVs/Brasov.ROU/44.12/ MVs/Dolj.ROU/43.12/	D4- Manchester	Indigenous	2 sporadic cases in Brasov and Dolj
November	MVs/Calarasi.ROU/47.12/	D4- Manchester	Indigenous	1 sporadic case in Calarasi,

Laboratory analysis

Serology

Serum samples were tested for measles-specific IgM using Enzygnost Anti-Measles-Virus/IgM according to the manufacturer's instructions (Siemens Healthcare Diagnostics Products GmbH).

RT-PCR and sequencing

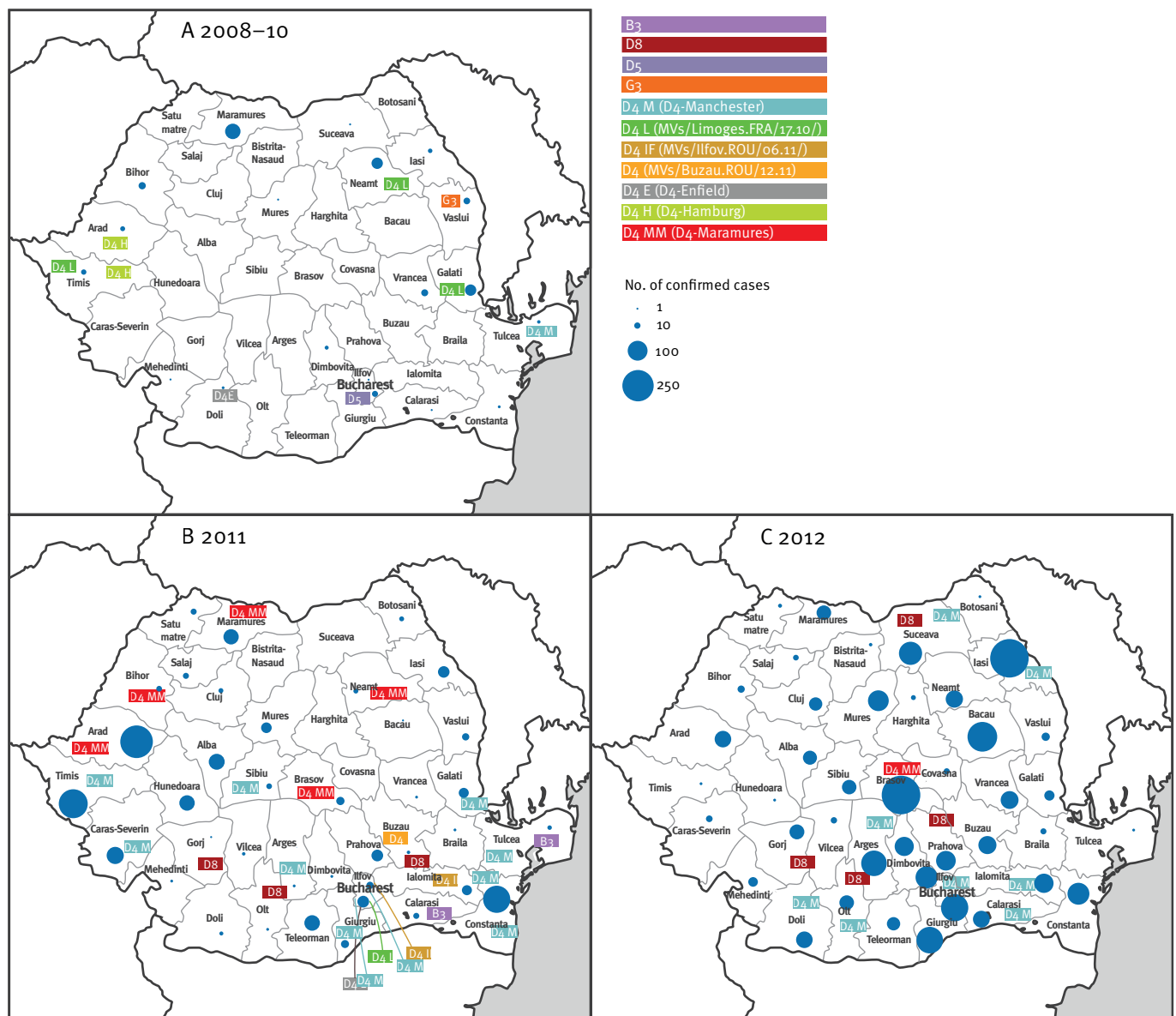
Confirmed cases were selected for genotyping from new outbreaks (index case and two or three secondary

cases). All confirmed cases with a history of travel abroad during the incubation period (7–21 days) were genotyped.

For measles virus genotyping, DNA fragments were generated by a nested RT-PCR recommended by WHO, which targeted the 450 nt region encoding the C-terminus of the nucleoprotein, as described previously [19], using the QIAGEN OneStep RT PCR Kit (QIAGEN, Hilden, Germany). Gel purification was performed with Wizard SV Gel and PCR Clean-Up System

FIGURE 2

Localisation of different measles virus genotypes and D4 variants and confirmed cases detected in Romania in 2008–12 (n=73)



For the outbreak dots that do not have a genotype assigned, genotyping was not performed. The genotype label is associated with outbreaks dots within the county's geographical boundary.

Promega (Fitchburg, Wisconsin). Sequencing was performed with the second round primers [19] using ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Foster City, California) on a four-capillary ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems Foster City, California).

Sequence analysis

Sequences were edited manually with BioEdit (version 7.0.5; North Carolina State University) and with the use of Staden Package [20]. Partial nucleocapsid gene sequences were aligned against related sequences retrieved from GenBank and MeaNS ([\[www.who-measles.org\]\(http://www.who-measles.org\)\) databases, using ClustalW \[21\] implemented in BioEdit \(version 7.1.3.0\) \[22\]. Starting with 2011, the programmes BioEdit and Gap4 have been replaced by the commercial programme Sequencer \(Gene Codes Corporation, Ann Arbor, United States\).](http://</p>
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Phylogenetic analysis was performed using MEGA (version 5) with a neighbour-joining (NJ) algorithm inferred with Tamura-Nei parameter for sequence evolution.

Results

Romania experienced a measles epidemic with continuous virus transmission from late 2004 to early 2007. In the following years 2008 and 2009, measles activity

was very low (12 cases in 2008; eight cases in 2009), all notified cases were imported by travellers coming from other European countries, leading to an incidence of less than 0.1 per 100,000 persons. The number of measles cases then increased again considerably in 2010 to 2012, reaching a total of 8,170 notified cases, of which 5,093 (62%) have been laboratory-confirmed (Figure 1).

From 2010 to 2012, a total of 104 possible cases were subjected to measles virus detection by RT-PCR (101 positive) and 73 were genotyped. The results of these investigations are summarised in the Table.

Measles cases in 2008

In March 2008, a sporadic case was notified in Dolj county (MVs/Dolj.ROU/13/08/1[D4]). This case was imported from Italy and had an identical sequence to MVs/Enfield.GBR/14.07[D4]. No secondary cases were detected. Another sporadic case was detected in Bucharest in April 2008 (MVs/Bucharest.ROU/20.08[D5]). This virus was imported from Greece and had an identical sequence to MVs/Lucerne.CHE/46.06[D5], the strain that caused a large outbreak of more than 4,400 cases in Switzerland between 2006 and 2009.

Measles cases in 2009

In August 2009, in Arad county, a small cluster of five measles cases was laboratory-confirmed by IgM. The index case was a child too young to be vaccinated returning from Ireland, who infected two other family members. One of them was hospitalised and passed the infection nosocomially to two additional cases in the paediatric ward. Genotyping a specimen from one of the secondary cases identified a measles virus (MVs/Arad.ROU/35.09/1[D4]) that was closely related to MVs/Limoges.FRA/17.10[D4] with a single nucleotide mismatch. As a response measure, the National Center for Surveillance and Control of Transmissible Diseases (CNSCBT) coordinated a supplementary vaccination campaign in the neighbouring Arad and Timis districts, targeting 1,054 unvaccinated or single-dose vaccinated children.

Measles cases in 2010

In 2010, measles activity increased to nine sporadic cases in five counties and 185 outbreak-related cases from in eight counties. Characteristic in that year was the occurrence of outbreaks in different geographic areas of the country, in the east (Neamt and Galati with 31 cases each) and in the north-west (Maramures with 95 cases) (Figure 2).

The first cluster occurred in February to March, in a Roma community from Tulcea county, totalling five cases. The index case had travelled to France shortly before. Genotyping of the strain isolated from the index case revealed MVs/Tulcea.ROU/08.10[D4], identical over the sequenced fragment to MVs/Manchester.GBR/10.09[D4] (Figure 3). Variant D4-Manchester had

circulated since 2008 in the UK and in France (MVs/Montaignu.FRA/43.08[D4], MVs/Paris.FRA/18.10[D4]).

In May 2010, two cases were confirmed in Timis county, in siblings without a recent travel history. The identical sequences found in these two cases, MVs/Timis.ROU/18.10/1[D4], differed by one nucleotide from the strain that caused the small cluster in the previous year (MVs/Arad.ROU/35.09/1[D4]), and were identical to D4-Hamburg (Figure 3).

Two outbreaks were notified in the summer of 2010 in Neamt county. The first outbreak occurred in June in a Roma community with three confirmed cases. The second outbreak started in August as a nosocomial infection in the paediatric ward of a hospital, resulting in 28 cases with one infant fatality. The sequences from both outbreaks (MVs/Neamt.ROU/26.10[D4] and MVs/Neamt.ROU/34.10[D4]) were identical to MVs/Limoges.FRA/17.10[D4] (Figure 3).

In October 2010, several outbreaks were notified in Galati, reaching 31 cases by the end of the year. The involved sequences (MVs/Galati.ROU/42.10/1[D4]) were identical to MVs/Limoges.FRA/17.10[D4], as well as the strains from Neamt. Strain MVs/Timis.ROU/50.10/1[D4], genotyped from two epidemiologically linked cases detected in December 2010 in Timis county, differed by two nucleotides.

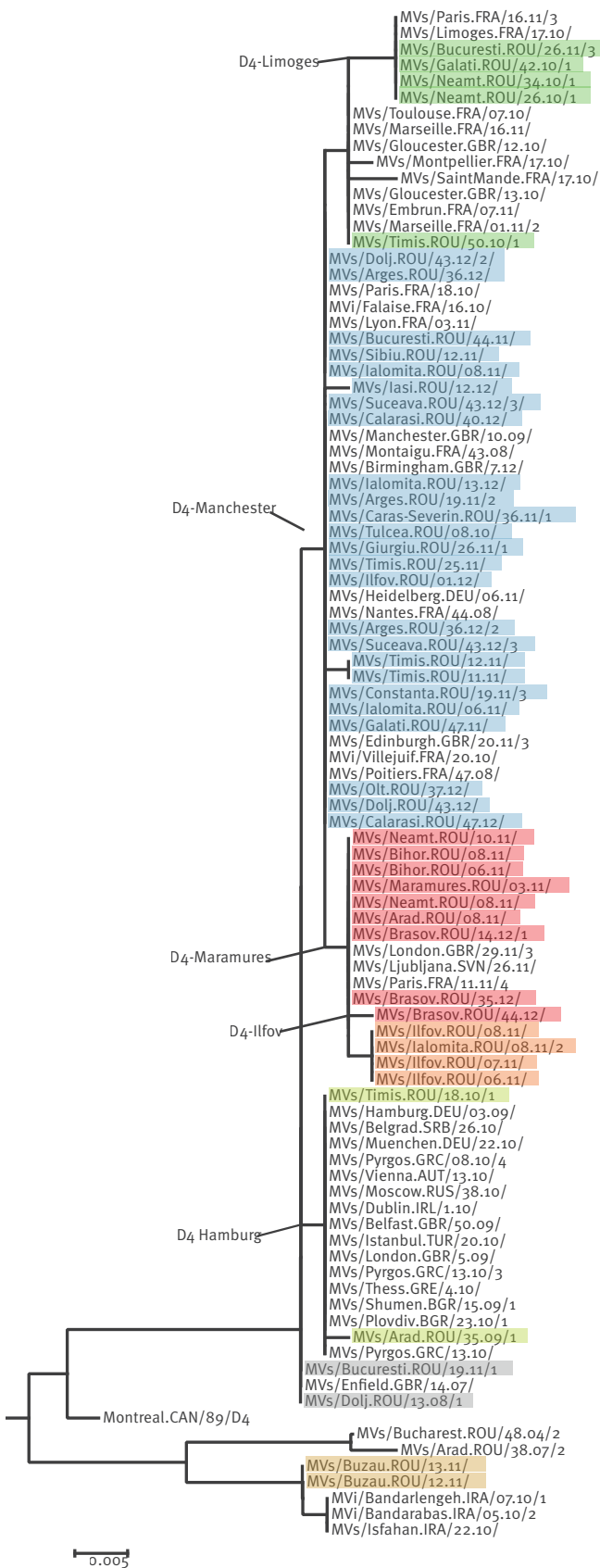
An imported case was detected in a student who travelled from Paris to Vaslui in September 2010. Sequencing revealed MVs/Vaslui.ROU.40.10[G3], identical to the strain MVs/Paris.FRA/47.10[G3] MV which circulated in France in 2010, but was also detected in Germany, the UK and Spain (Figure 4).

Measles cases in 2011

In 2011, outbreaks expanded to 39 of the 42 counties of Romania, reaching 4,163 notified cases (45.9% laboratory-confirmed). Because the epidemic was so large, measles virus genotyping and phylogenetic analysis was restricted only to extended outbreaks and to imported cases. The majority of genotyped cases were associated with D4-Manchester and D4-Maramures variants (Figures 2 and 3). The cases were mainly seen in the north-western region of Romania, first in the Roma communities and subsequently spreading into the general population. The Salaj county was most affected (incidence: 141.9 per 100,000 population). Of the total laboratory-confirmed cases in 2011, 78% were not vaccinated. Of these, 16% were younger than 12 months and thus not eligible for vaccination. As a response measure to the growing number of measles cases, additional vaccination campaigns were implemented in 2011 that targeted children between the ages of seven months and seven years, leading to approximately 4,500 vaccinated children.

FIGURE 3

Phylogenetic tree of representative measles MV-D4 strains in relation to Mvs/Enfield.GBR/14.07/D4, Romania, 2008–12 (n=49)

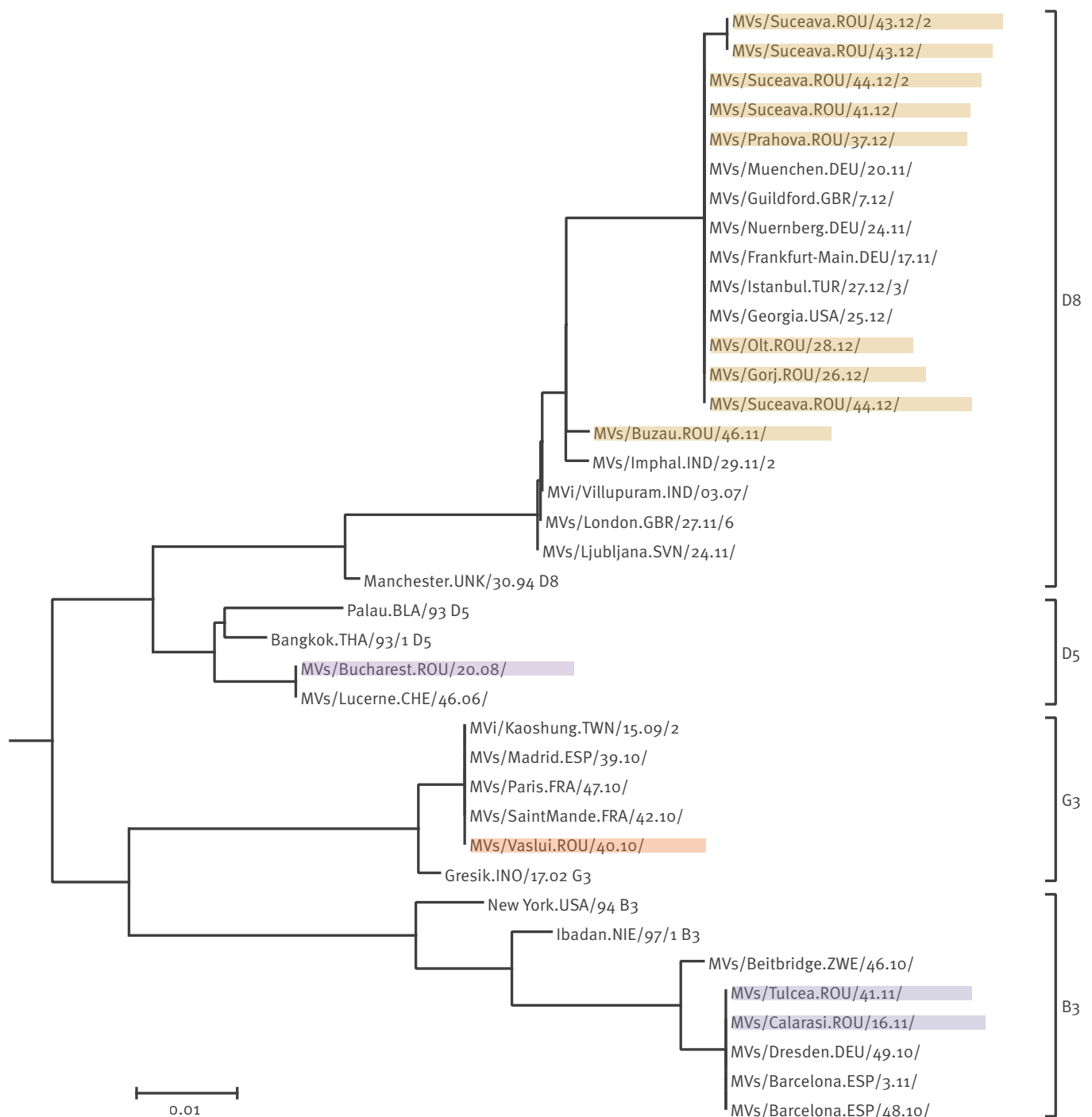


Romanian MV strains are highlighted in different colours.

GenBank accession numbers: MVi/Galati.ROU/20.11, JX156445; MVs/Bucuresti.ROU/19.11/1, JN615582; MVs/Timis.ROU/18.10/1, HQ704313; MVs/Arad.ROU/35.09/1, HQ704310; MVs/Tulcea.ROU/08.10, HQ704312; MVs/Timis.ROU/12.11, JN599040; MVs/Timis.ROU/11.11, JX841118; MVs/Arad.ROU/10.11/1, JN599037; MVs/Neamt.ROU/10.11, JN599039; MVs/Bihor.ROU/08.11, JN599033; MVs/Ilfov.ROU/08.11, JN599034; MVs/Ialomita.ROU/08.11/2, JN599036; MVs/Ilfov.ROU/07.11, JX841117; MVs/Ilfov.ROU/06.11, JX841116; MVs/Bihor.ROU/06.11, JX841115; MVs/Maramures.ROU/03.11, JX841109; MVs/Neamt.ROU/08.11, JX841114; MVs/Arad.ROU/08.11, JX841113; MVs/Brasov.ROU/14.12/1, JX156442; MVs/Brasov.ROU/14.12/2, JX156443; MVs/Ialomita.ROU/08.11, JN599035; MVs/Constanta.ROU/19.11/3, JN615580; MVs/Caras-Severin.ROU/36.11/1, JN873033; MVs/Giurgiu.ROU/26.11/1, JN615577; MVs/Sibiu.ROU/12.11, JN599041; MVs/Ialomita.ROU/06.11, JX841112; MVs/Neamt.ROU/26.10/1, HQ704314; MVs/Neamt.ROU/34.10/1, HQ704315; MVs/Galati.ROU/42.10/1, HQ902906; MVs/Bucuresti.ROU/26.11/3, JX841111; MVs/Timis.ROU/50.10/1, HQ902905; MVs/Arges.ROU/19.11/2, JN615581; MVs/Timis.ROU/25.11, JN615579; MVs/Bucuresti.ROU/44.11, JQ417670; MVs/Bucharest.ROU/48.04/2, AM849093; MVs/Galati.ROU/47.11, JQ417669; MVs/Iasi.ROU/12.12, JX157884; MVs/Ialomita.ROU/13.12, JX156444; MVs/Ilfov.ROU/01.12, JQ809707; MVs/Buzau.ROU/13.11, JN599042; MVs/Buzau.ROU/12.11, JX841110; Montreal.CAN/89, U01976; MVs/London.GBR/29.11/3, NA; MVs/Ljubljana.SVN/26.11, NA; MVs/Paris.FRA/11.11/4, NA; MVs/Lyon.FRA/03.11, NA; MVs/Edinburgh.GBR/20.11/3, NA; MVs/Birmingham.GBR/7.12, NA; MVs/Heidelberg.DEU/06.11, NA; MVs/Hamburg.DEU/03.09, HQ436108; MVs/Belgrad.SRB/26.10, NA; MVs/Muenchen.DEU/22.10, HQ704350; MVs/Pyrgos.GRC/08.10/4, HM366144; MVs/Vienna.AUT/13.10, HQ704298; MVs/Dublin.IRL/1.10, NA; MVs/Paris.FRA/16.11/3, NA; MVs/Marseille.FRA/01.11/2, NA; MVs/Embrun.FRA/07.11, NA; MVs/Gloucester.GBR/13.10, HM215515; MVs/Marseille.FRA/16.11, NA; MVi/Falaise.FRA/16.10, FR671445; MVi/Villejuif.FRA/20.10, FR671446; MVs/Belfast.GBR/50.09, GU479875; MVs/Enfield.GBR/14.07, EF600554; MVs/Gloucester.GBR/12.10, HM215515; MVs/Istanbul.TUR/20.10, HM579947; MVs/Limoges.FRA/17.10, FR671430; MVs/London.GBR/5.09, GU120179; MVs/Olt.ROU/37.12, NA; MVs/Dolj.ROU/43.12, NA; MVs/Constanta.ROU/19.11/3, NA; MVs/Galati.ROU/47.11, NA; MVs/Edinburgh.GBR/20.11/3, NA; MVi/Villejuif.FRA/20.10, NA; MVs/Poitiers.FRA/47.08, NA; MVs/London.GBR/5.09, NA; MVs/Olt.ROU/37.12, NA; MVs/Dolj.ROU/43.12, NA; MVs/Neamt.ROU/10.11, NA; MVs/Bihor.ROU/08.11, NA; MVs/Bihor.ROU/06.11, NA; MVs/Maramures.ROU/03.11, NA; MVs/Neamt.ROU/08.11, NA; MVs/Arad.ROU/08.11, NA; MVs/Brasov.ROU/14.12/1, NA; MVs/London.GBR/29.11/3, NA; MVs/Ljubljana.SVN/26.11, NA; MVs/Paris.FRA/11.11/4, NA; MVs/Brasov.ROU/35.12/1, NA; MVs/Brasov.ROU/44.12/1, NA; MVs/Ilfov.ROU/08.11, NA; MVs/Ialomita.ROU/08.11/2, NA; MVs/Ilfov.ROU/07.11, NA; MVs/Ilfov.ROU/06.11, NA; MVs/Timis.ROU/18.10/1, NA; MVs/Hamburg.DEU/03.09, NA; MVs/Belgrad.SRB/26.10, NA; MVs/Muenchen.DEU/22.10, NA; MVs/Pyrgos.GRC/08.10/4, NA; MVs/Vienna.AUT/13.10, NA; MVs/Moscow.RUS/38.10, NA; MVs/Dublin.IRL/1.10, NA; MVs/Belfast.GBR/50.09, NA; MVs/Istanbul.TUR/20.10, NA; MVs/London.GBR/5.09, NA; MVs/Pyrgos.GRC/13.10/3, NA; MVs/Thess.GRE/4.10, NA; MVs/Shumen.BGR/15.09/1, NA; MVs/Plodiv.BGR/23.10/1, NA; MVs/Arad.ROU/35.09/1, NA; MVs/Pyrgos.GRC/13.10, NA; MVs/Bucuresti.ROU/19.11/1, NA; MVs/Enfield.GBR/14.07, NA; MVs/Dolj.ROU/13.08/1, NA; MVs/Bucharest.ROU/48.04/2, NA; MVs/Arad.ROU/38.07/2, NA; MVs/Buzau.ROU/13.11, NA; MVs/Buzau.ROU/12.11, NA; MVi/Bandarlengeh.IRA/07.10/1, NA; MVi/Bandarabas.IRA/05.10/2, NA; MVs/Isfahan.IRA/22.10, NA.

FIGURE 4

Phylogenetic tree of sporadic cases infected with measles virus genotypes G3, B3, D8 and D5 detected in Romania, 2008–12 (n=13), in relation to reference strains



Romanian MV strains are highlighted in different colours

GenBank accession numbers: MVs/Tulcea.ROU/41.11, JX847793; MVs/Calarasi.ROU/16.11, JN615583; MVs/Buzau.ROU/46.11, JQ417668; New York.USA/94, L46753; Ibadan.NIE/97/1, AJ232203; Manchester.UNK/30.94, AF280803; MVs/Gorj.ROU/26.12, JX497760; MVs/Vaslui.ROU/39.10, JX497759; Palau.BLA/93, L46758; Bangkok.THA/93/1, AF079555; MVs/Imphal.IND/29.11/2, JQ687144; MVs/SaintMande.FRA/42.10, FR848083; MVs/Paris.FRA/47.10, FR848086; MVs/SaintMande.FRA/44.10, FR848084; MVs/Madrid.ESP/39.10, HQ712117; MVs/Bucharest.ROU/20.08, NA; MVs/Ljubljana.SVN/24.11, NA; MVs/London.GBR/27.11/6, NA; MVs/Lucerne.CHE/46.06, NA; Gresik.INO/17.02, AY184217; MVs/Barcelona.ESP/48.10, JF681232; MVs/Barcelona.ESP/3.11, JF681246; MVs/Dresden.DEU/49.10, NA; MVs/Istanbul.TUR/27.12/3, NA; MVs/Frankfurt-Main.DEU/17.11, NA; MVs/Nuernberg.DEU/24.11, NA; MVs/Muenchen.DEU/20.11, NA; MVs/Guildford.GBR/7.12, NA; MVi/Villupuram.IND/03.07, FJ765078; MVs/Georgia.USA/25.12, JX402879; MVs/Olt.ROU/28.12, JX497758; MVs/Beitbridge.ZWE/46.10, NA; MVs/Prahova.ROU/37.12, NA; MVs/Suceava.ROU/41.12, JX982115; MVs/Suceava.ROU/43.12/2, KC172853; MVs/Suceava.ROU/43.12, KC179763; MVs/Suceava.ROU/44.12/2, NA; MVs/Suceava.ROU/44.12, NA.

Several outbreaks leading to 520 cases started in Maramures county in October 2010 and continued until September 2011. Genotyped cases from these outbreaks revealed a new D4 variant MVs/Maramures.ROU/03.11[D4], which differed by one nucleotide from D4-Manchester. D4-Maramures was also detected in 2011 in several other counties: Arad, Neamt, Bihor (MVs/Arad.ROU/10.11/1[D4], MVs/Arad.ROU/10.11/1/2[D4], MVs/Neamt.ROU/10.11[D4], and MVs/Bihor.ROU/06.11[D4]) as well as in 2012 in Brasov county (MVs/Brasov.ROU/14.12/1[D4] and MVs/Brasov.ROU/44.12/1[D4]). The new variant D4-Maramures was exported in 2011 to Slovenia (MVs/Ljubljana.SVN/26.11[D4]), France (MVs/Paris.FRA/11.11/4[D4]), the UK (MVs/London.GBR/29.11/3[D4]) and the United States (US) (MVs/Florida.USA/28.11/1) (Figure 3).

Several strains diverging by a single nucleotide from variant D4-Maramures were detected in Ilfov county: MVs/Ilfov.ROU/06.11[D4], MVs/Ilfov.ROU/07.11[D4] and MVs/Ilfov.ROU/08.11[D4] as well as in Ialomita county (MVs/Ialomita.ROU/08.11/2[D4]). The transmission of this divergent strain was interrupted in February 2011.

Besides the epidemic caused by D4-Manchester and D4-Maramures, we identified new imported cases: a single sporadic case with measles genotype B3 was detected in April 2011 in Calarasi county. This case (MVs/Calarasi.ROU/16.11[B3]) was a teenager who had travelled to Spain. Phylogenetic analysis of this case revealed 100% identity with MVs/Barcelona.ESP/48.10[B3] detected in 2010 to 2011 in Spain (Granada, Balearic Islands and Barcelona) (Figure 4). This import apparently did not spread further. However, another case with an identical sequence (MVs/Tulcea.ROU/41.11[B3]) was detected 25 weeks later in the neighbouring Tulcea county.

Two sporadic D4 cases were identified in March 2011 in Buzau county. The index case (MVs/Buzau.ROU/12.11[D4]) had travelled to Turkey, and an isolate from a secondary case (MVs/Buzau.ROU/13.11[D4]) revealed a single nucleotide exchange compared to strains circulating in 2010 in Iran (MVi/Bandarlengeh.IRA/07.10/1[D4]) (Figure 4).

Measles virus MV-D8 (MVs/Buzau.ROU/46.11[D8]) was also detected in November 2011 in Buzau county in a person with no recent travel history. The index case of this measles virus importation was not identified, and no secondary cases were detected. Phylogenetic analysis of this case revealed close relation with the variant D8-Frankfurt Main (Figure 4).

Measles cases in 2012

In 2012, measles activity remained at comparable intensity to the previous year, reaching 4,006 cases (79.3% laboratory-confirmed) by the end of year, but the geographical distribution shifted to the south-eastern region of Romania. Of all laboratory-confirmed cases in 2012, 84.5% were unvaccinated (26.7% too young

for vaccination). The most affected age group were children younger than one year (incidence: 219/100.000) and those between one and four years of age (incidence: 78.6/100.000). The majority of these cases were associated with D4-Manchester variant (Figures 2 and 3). According to the national strategy of measles cluster control, vaccination of children aged between seven months and seven years continued in 2012, but the total number of vaccinations is not available.

Two sporadic measles cases infected with MV-D8 with identical sequences were detected in June in Gorj county (MVs/Gorj.ROU/26.12[D8]) and in July in the neighbouring Ilt county, (MVs/Ilt.ROU/28.12[D8]). These two strains belonged to the variant D8-Frankfurt-Main (MVs/Frankfurt Main.DEU/17.11[D8]) that was detected in Germany between February and June (MVs/Muenchen.DEU/20.11[D8] and MVs/Nuernberg.DEU/24.11[D8]), the UK (MVs/Guildford.GBR/7.12[D8]), the US (MVs/Georgia.USA/25.12/[D8]) and Turkey (MVs/Istanbul.TUR/27.12/3[D8]), in 2011 to 2012 (Figure 4).

A fatal measles case in a young teenager (vaccinated with two doses of MMR), registered in secondary school without any underlying health problems, was recorded in October 2012 in Suceava county. MVs/Suceava.ROU/41.12[D8] also belonged to D8-Frankfurt-Main and was identical to MVs/Gorj.ROU/26.12[D8], MVs/Ilt.ROU/28.12[D8], MVs/Prahova.ROU/37.12[D8] and MVs/Suceava.ROU/44.12[D8] (Figure 4). Two cases with a single nucleotide difference were identified (MVs/Suceava.ROU/43.12/[D8]) (Figure 4). In summary, 8 MV-D8 cases were identified in 2012, five of them in Suceava.

Discussion

The 20 measles cases notified during 2008 to 2009 were attributed to MV-D4 and MV-D5 viruses imported from neighbouring countries and were not passed on to the general population. The situation changed with three outbreaks in 2010 in Tulcea and Neamt (MVs/Tulcea.ROU/08.10[D4]), (MVs/Neamt.ROU/26.10[D4]). The outbreak in Tulcea was caused by a D4-Manchester variant, whereas the viruses from Neamt and Galati (MVs/Neamt.ROU/26.10/1[D4], MVs/Galati.ROU/42.10/1[D4]) exhibited an amino acid substitution to D4-Manchester (I469L) that had previously been found only in a few French sequences deposited in GenBank (MVs/Toulouse.FRA/07.10[D4], MVs/Limoges.FRA/17.10[D4]) and in one strain from the UK (MVs/Gloucester.GBR/12.10[D4]). This finding makes prior undetected circulation of Measles Virus related to MVs/Neamt.ROU/26.10/1[D4] in early 2010 unlikely. It can be therefore assumed, that the Tulcea cluster in early 2010 and the outbreak in Neamt in mid-2010 were linked to separate importations (Figure 3). The two cases from Timis detected in December 2010 (MVs/Timis.ROU/50.10/1[D4]) had two nucleotide differences compared with sequences from the outbreak in Neamt and Galati, indicating a different source as well. The

strain MVs/Timis.ROU/50.10/1[D4] did have the substitution I469L like the sequences from the outbreak in Neamt, but shared two additional nucleotide changes with the strains circulating in France and the UK. The cases from the Timis and Neamt outbreaks may have been introduced from different sources but their genetic sequences indicate a similar origin.

The variant D4-Manchester was imported in early 2010 and caused a small outbreak in Tulcea (MVs/Tulcea.ROU/o8.10[D4]), but was apparently re-imported in early 2011 to Ialomita (MVs/Ialomita.ROU/o6.11[D4]) and was detected as late as November 2012. Genotyped viruses in 2011 to 2012 from outbreaks in a wide geographical distribution (Ialomita, Sibiu, Arges, Constanta, Timis, Giurgiu, Caras-Severin, Bucuresti, Galati, Ilfov, and Iasi, the latter with a single nucleotide mismatch) were identical to MVs/Tulcea.ROU/o8.10[D4] (Figures 2 and 3). There is no indication of continuous circulation of D4-Manchester during the period between February 2010 and February 2011; our results suggest that it was imported for the second time in early 2011. However, it is possible that some measles cases went unnoticed clinically, some time after February 2010 and before February 2011, making the time window in which D4-Manchester did not circulate shorter than indicated by our data. Variant D4-Manchester apparently co-circulated with D4-Maramures in 2011 (D4-Manchester mainly in the south-east and D4-Maramures in western and central Romania) but became predominant in 2012.

Variant D4-Maramures was widely detected in 2011 and last in October 2012 in Brasov county. Because of their phylogenetic relationship and local and temporal distribution, it can be assumed that D4-Maramures and MVs/Ilfov.ROU/o6.11[D4] represent first- and second-generation descendants of D4-Manchester. Thus, the total number of measles cases attributed to the main transmission chain had reached at least 7,300 notified cases by the end of 2012. We could demonstrate the establishment of D4-Manchester and its descendants as new endemic strains in Romania, circulating continuously for a time period of almost two years (February 2011 to November 2012). The outbreak from 2011 to 2012 associated with D4-Manchester is the second large outbreak of D4 genotype in Romania within a period of less than 10 years.

In response to the measles outbreaks, a wide range of control measures were implemented, including strengthened surveillance for timely identification and monitoring of cases and outbreaks, modified immunisation schedules, and supplementary immunisation activities (approximately 4,500 vaccinated) of the rural population. Despite these measures, more than 4,000 of the over 30,000 cases recorded in Europe in 2011 were from Romania [23]. In 2012, 3,843 of 8,230 total cases were reported by Romania to The European Surveillance System [24].

Frequent measles outbreaks in Romania could be the result of suboptimal vaccine coverage. In 2009, the coverage for the first dose of measles-containing vaccine in children aged 12 months was estimated at 85.1% (95% confidence interval (CI): 82.4–87.8), but reached the target of 95% (95% CI: 93.4–95.8) in children aged 18 months [25]. Despite this fairly high vaccination coverage in the 18 month-olds, it is of note that families not registered with a family physician are not represented in this analysis. Lately a shrinking confidence in vaccination has been observed in Romania, reflecting the progress of the anti-vaccine movement all over Europe. The ethnic group of the Roma is traditionally underserved by national health services and moreover, a part of them refuse vaccination actively. The current and previous Romanian outbreaks started in Roma communities and underline the need to develop strategies to improve their integration into the national health services. However, we want to make clear that any under-vaccinated groups or those with a low immunity rate are highly prone to measles virus infections, and represent an important factor preventing successful and sustainable elimination of measles in Romania. Our results underline the importance to strengthen immunisation programmes and to develop specific measures to address parents and their concerns regarding vaccination as well as the hard-to-reach population all over Europe.

In conclusion, a combination of epidemiological data and molecular characterisation enabled us to trace the spread of wild measles virus genotype in Romania from 2008 to 2012. Molecular surveillance of measles virus circulation in Romania will be continued to assess the effectiveness of the national measles control programme and hopefully to support the verification of measles elimination by the year 2015.

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Conflict of interest

None declared.

Authors' contributions

Gheorghe Necula - writing of the manuscript, molecular epidemiology data analysis and interpretation of the study. Mihaela Lazar - writing of the manuscript, molecular epidemiology data analysis and interpretation of the study. Aurora Stanescu - epidemiological data analysis and contributed to the revision of the draft manuscript. Adriana Pistol - epidemiological data analysis and contributed to the revision of the draft manuscript. Sabine Santibanez - interpretation of the study and contributed to the revision of the draft manuscript. Annette Mankertz - interpretation of the study and contributed to the revision of the draft manuscript. Emilia

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