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European survey on laboratory preparedness, response and diagnostic capacity for Crimean-Congo haemorrhagic fever, 2012

M D Fernandez-García (mdfernandez@isciii.es)[1,2,3], A Negredo[1], A Papa[4], O Donoso-Mantke[5,6], M Niedrig[5], H Zeller[3], A Tenorio[1], L Franco[1], the ENIVD members[7]

1. National Microbiology Centre, Instituto de Salud Carlos III, Madrid, Spain
2. European Public Health Microbiology Training Programme (EUPHEM)
3. European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
4. Department of Microbiology, National Reference Centre for Arboviruses, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece
5. Zentrum für Biologische Gefahren und Spezielle Pathogene (ZBS-1), Robert Koch Institute, Berlin, Germany
6. Present address: Gesellschaft fuer Biotechnologische Diagnostik mbH, Berlin, Germany
7. The ENIVD members are listed at the end of the article

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Crimean-Congo haemorrhagic fever (CCHF) is an infectious viral disease that has (re-)emerged in the last decade in south-eastern Europe, and there is a risk for further geographical expansion to western Europe. Here we report the results of a survey covering 28 countries, conducted in 2012 among the member laboratories of the European Network for Diagnostics of 'Imported' Viral Diseases (ENIVD) to assess laboratory preparedness and response capacities for CCHF. The answers of 31 laboratories of the European region regarding CCHF case definition, training necessity, biosafety, quality assurance and diagnostic tests are presented. In addition, we identified the lack of a Regional Reference Expert Laboratory in or near endemic areas. Moreover, a comprehensive review of the biosafety level suitable to the reality of endemic areas is needed. These issues are challenges that should be addressed by European public health authorities. However, all respondent laboratories have suitable diagnostic capacities for the current situation.

Introduction

Crimean-Congo haemorrhagic fever (CCHF) is a zoonotic viral disease caused by the tick-borne CCHF virus (CCHFV), which is classified into the genus *Nairovirus* within the *Bunyaviridae* family. In humans, the disease is highly pathogenic and life-threatening as it can cause severe illness with prominent haemorrhages reaching case fatality rates of up to 50%. In nature, CCHFV usually circulates between asymptomatic animals and ticks in an enzootic cycle. Humans may become infected through the bite of a tick, mainly of the *Hyalomma* genus, through direct contact with blood or tissues from viraemic livestock or through direct contact with the blood or secretions of a viraemic patient [1]. Thus, risk groups include individuals with outdoor activities, mainly those who have occupational contact with animals, as well as healthcare workers in hospital settings (nosocomial hazard). Because of the potential for epidemics and nosocomial outbreaks, high fatality ratio, limitations for treatment and the lack of safe vaccine, CCHF is a disease listed for immediate notification to public health authorities as it constitutes a major threat to public health. Therefore, CCHFV is considered a high-risk pathogenic organism and classified as a biosafety level (BSL) 4 containment agent.

The disease is endemic in wide areas of Africa, the Middle East, central and south-western Asia and the south-eastern European region. More particularly, some Balkan countries (e.g. Albania, Bulgaria, Greece and Kosovo under UN Security Council Resolution 1244) are endemic zones for CCHF [2]. During the last decade, CCHF re-emerged in Albania, Greece, Kosovo under UN Security Council Resolution 1244 and countries bordering the Black sea: Georgia, south-western Russia Turkey, and Ukraine. In Greece, the detection of the non-pathogenic strain AP92 in ticks in 1975 was followed by the notification of the first human CCHF case in June 2008 [3]. However, the vast majority of CCHF cases have been recorded in Turkey (since 2002) and the south-western regions of Russia (since 1999), with expanding outbreaks and increasing numbers of associated fatalities [2]. In northern and south-western Europe, no human cases have been reported except for imported ones in France [4], Germany [5] and the United Kingdom [6]. Limited serological evidence in humans has been reported in parts of Hungary and Portugal [7,8].

In Europe, the tick vector most commonly associated with CCHFV is *Hyalomma marginatum*, which is present in southern Europe and has sporadically been detected in southern Germany, the Netherlands and the United Kingdom following expansion of its geographical range associated with movement of migrant breeding birds [9-12]. The spreading of the vector represents a risk factor for introduction of the virus from endemic to unaffected areas of Europe, increasing the occurrence of CCHF [13]. However, virological evidence has never been addressed in western Europe until 2010, when a study conducted in Spain detected for the first time CCHFV in populations of *H. lusitanicum* collected from indigenous deer [14]. Moreover, the recent discovery of antibodies against CCHFV in livestock in Romania, with prevalence values similar to those observed in other regions where the disease is endemic, suggests an extension of the circulation zone of CCHFV in Europe [15].

In 2008, after the first case in Greece was detected, the European Centre for Disease Prevention and Control (ECDC) organised an expert consultation on CCHF to identify preparedness interventions in Europe [13]. In 2011, under the initiative of the European Network for Diagnostics of 'Imported' Viral Diseases (ENIVD; www.enivd.org), a multicenter study of CCHF diagnostic tests and an external quality assessment

Table 1

ENIVD survey on Crimean-Congo haemorrhagic fever, responding laboratories, by country, 2012 (n=31)

Participating countries	Participating laboratories	Acts as NRL	WHOCC
Austria	Medical University of Vienna, Vienna	No	No
Belgium	Institute of Tropical Medicine, Antwerpen	Yes	No
Bulgaria	National Centre for Infectious and Parasitic Diseases, Sofia	Yes	No
Croatia	University Hospital for Infectious Diseases, Zagreb	No	No
Czech Republic	Institute of Public Health, Ostrava	No	No
Estonia	National Institute for Health Development/Health Board, Tallinn	No	No
Former Yugoslav Republic of Macedonia	Institute of Health Protection of the FYROM	No	No
France	1. Institut Pasteur, Lyon	Yes	Yes
	2. Aix-Marseille University and AP-HM Public Hospitals, Marseille	No	No
Germany	1. Bernhard-Nocht Institut, Hamburg	Yes	Yes
	2. Institut für Mikrobiologie der Bundeswehr, Munich	No	No
Greece	Aristotle University, Thessaloniki	Yes	No (discontinued since 20/ Oct/2008)[a]
Italy	National Institute for Infectious Diseases "L.Spallanzani", Rome	Yes	Yes
Kosovo under UN Security Council Resolution 1244	National Institute of Public Health of Kosovo, Pristina	Yes	No
Latvia	Infectology Center of Latvia, Riga	Yes	No
Lithuania	National Public Health Surveillance Laboratory, Vilnius	Yes	No
Malta	Mater Dei Hospital, Valletta	No	No
The Netherlands	Erasmus University Hospital, Rotterdam	Yes	Yes
Norway	Norwegian Institute of Public Health, Oslo	Yes	No
Portugal	National Institute of Health, Águas de Moura	Yes	No
Romania	National Institute of Public Health, Bucharest	Yes	No
Russia	Central Research Institute of Epidemiology, Moscow	Yes	No
Serbia	Torlak Institute of Virology, Belgrade	Yes	No
Slovakia	Institute of Virology, Slovak Academy of Sciences, Bratislava	No	No
Slovenia	University of Ljubljana, Ljubljana	Yes	No (discontinued since 1/ Sep/2008)[a]
Spain	Instituto de Salud Carlos III, Madrid	Yes	No
Sweden	Swedish Institute for Infectious Disease Control, Karolinska Institute Stockholm, Solna	Yes	No
Switzerland	University Hospitals of Geneva, Geneva	Yes	No
Turkey	Refik Saydam Hygiene Institute, Ankara	Yes	No
United Kingdom	1. Public Health England, Colindale	No	No
	2. Public Health England, Porton Down	Yes	Yes

ENIVD: European Network for Diagnostics of 'Imported' Viral Diseases; NRL: National Reference Laboratory; WHOCC: World Health Organization Collaborating Center (http://apps.who.int/whocc/) for Viral Haemorrhagic Fevers from the EURO region.

[a] Discontinued means that the institution is no longer a WHOCC.

(EQA) for CCHF molecular diagnosis were carried out to monitor and compare the performance of the different techniques applied for diagnosis of CCHF [16,17]. The current situation with continous high transmission in Turkey and south-western Russia, new imported cases in the European Union (EU), detection of the virus for the first time in the western Mediterranean region, and new evidence of seroprevalence in animals, make necessary a new assessment on preparedness and laboratory capacities for CCHF in the European region. Here, we describe the results of a questionnaire survey conducted in 2012 to assess the laboratory preparedness and response capacities for CCHF diagnosis in the European region.

Methods

To gather information on CCHF diagnostics, preparedness and response capacities in Europe, a questionnaire was developed and sent electronically in January 2012 to laboratory contact points in the ENIVD database, covering 28 Member States of the EU as well as nine non-EU countries, Russia, Norway, Switzerland, Bosnia and Herzegovina, Serbia, Kosovo under UN Security Council Resolution 1244, Albania, the Former Yugoslavia Republic of Macedonia and Turkey. All completed questionnaires were received by April 2012. The first part of the questionnaire assessed preparedness and response capacities, while the second part was designed to collect information on diagnostic capacities and quality assurance. Questions on the following topics were included in the questionnaire: CCHF case definition, training necessity, biosafety assurance, diagnostic tests and quality assurance. The list of respondents is shown in Table 1. Respondents were National Reference Laboratories (NRL) for Arbovirus and Viral Haemorrhagic Fever (VHF) and/or World Health Organization Collaborating Centers (WHOCC). An NRL was defined as a laboratory involved in reception/management of suspected samples of CCHF, either for diagnostic and reference activities or for shipment abroad in case of lack diagnostic capacity

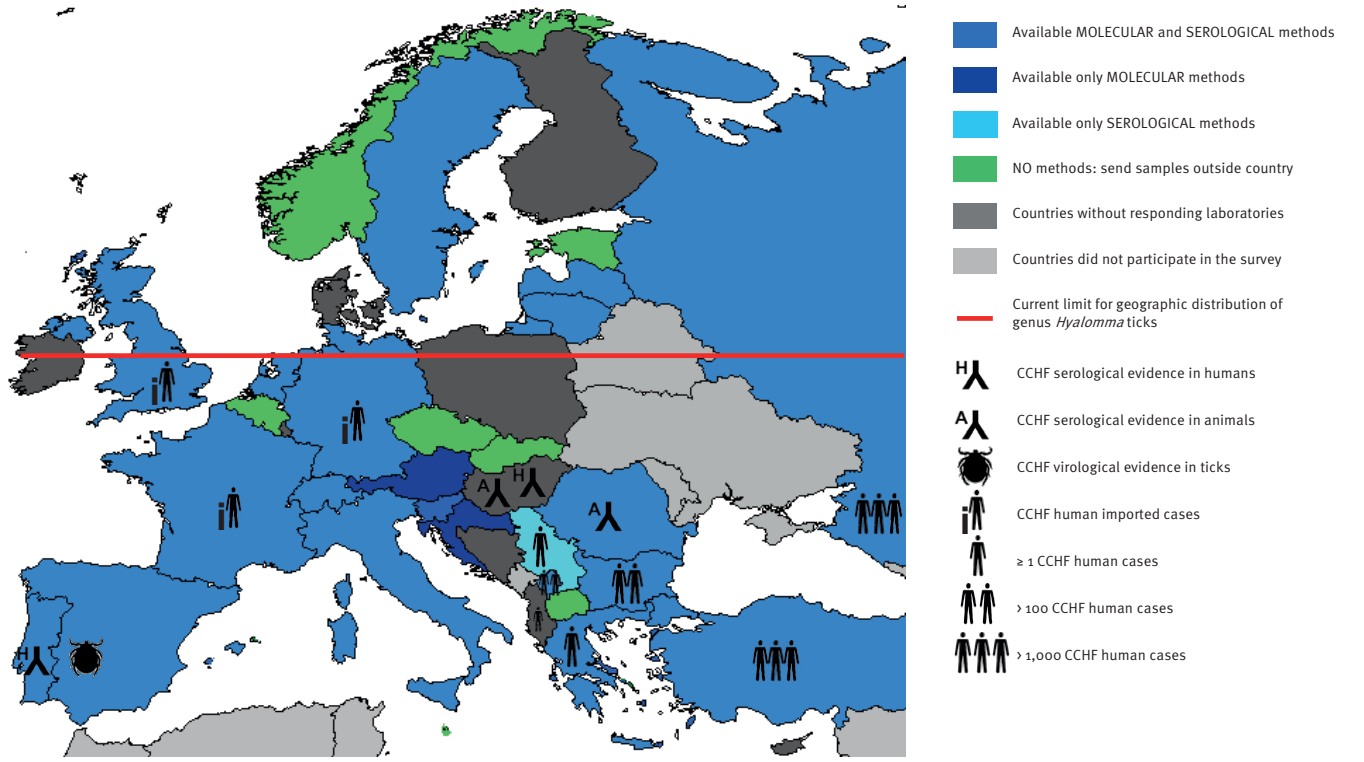
Results

Participation

Sixty-eight laboratories from 37 countries (28 EU Members States and nine countries outside the EU), were contacted for this survey. Thirty-one laboratories from 28 countries returned their answer, except Albania, Bosnia and Herzegovina, Cyprus, Denmark,

Figure 1

Diagnostic capacities and occurrence of Crimean-Congo haemorrhagic fever in Europe since 2000



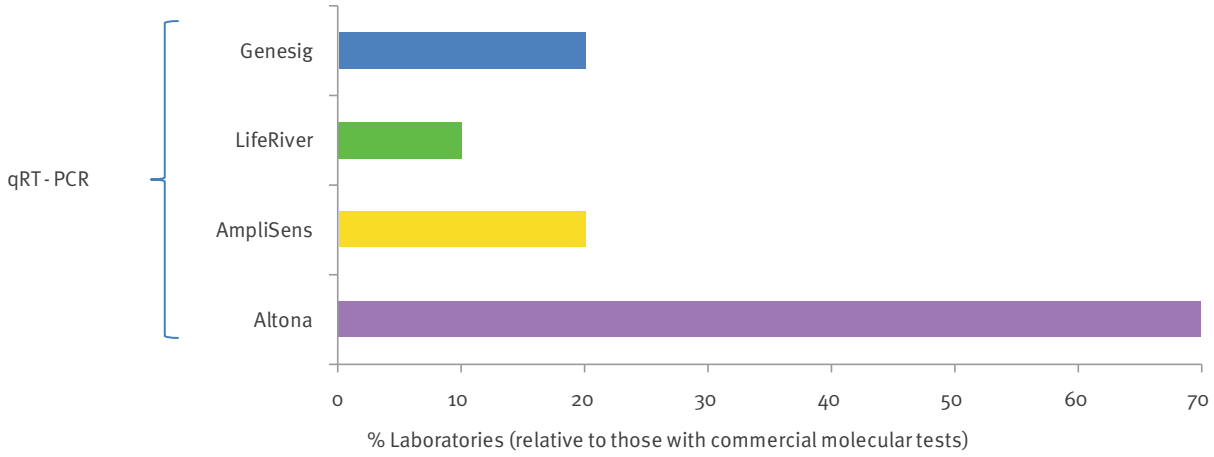
CCHF: Crimean-Congo haemorrhagic fever; ECDC: European Centre for Disease Prevention and Control; WHO: World Health Organization.

Colour code indicates diagnostic capacities as assessed in the present survey. Human silhouettes indicate occurrence of CCHF in humans according to the WHO database (http://data.euro.who.int/cisid), the ECDC consultation [13] and the Public Health England database (http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/CCHF/EpidemiologicalData/cchfoutbreaks/). Tick silhouettes indicate virological evidence of CCHF in ticks in those countries where no human cases have been reported. Antibody silhouettes indicate serological evidence of CCHF in humans or animals in countries where no human cases have been reported.

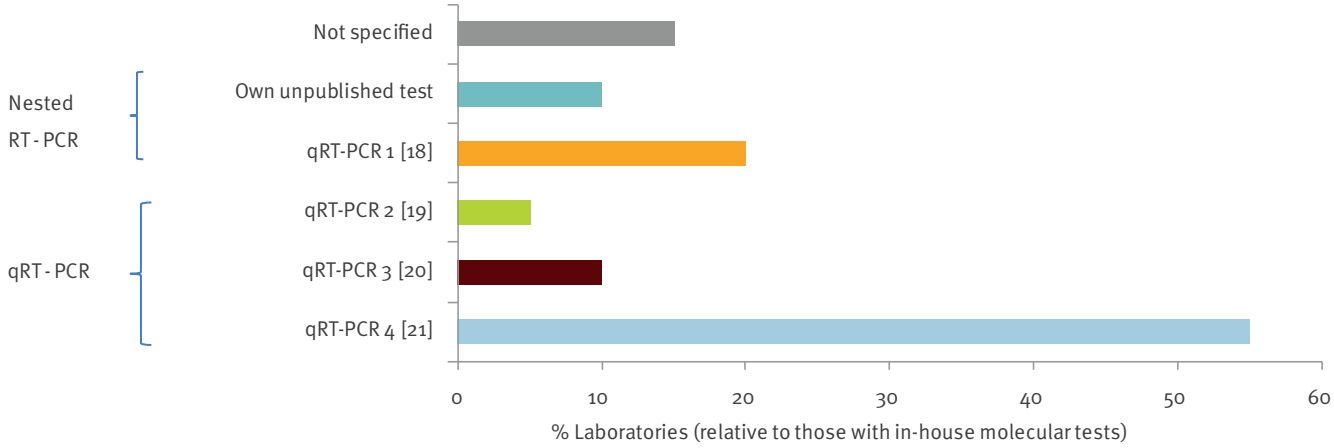
Figure 2

Application of Crimean-Congo haemorrhagic fever molecular diagnostic methods, ENIVD survey, 2012 (n=23 laboratories)

A. Percentage of countries using commercial molecular tests



B. Percentage of countries using in-house molecular tests



- Kosovo under UN Security Council Resolution 1244 , Romania
- Latvia
- Lithuania, Russia
- Germany (Hamburg, Munich), Greece, Kosovo under UN Security Council Resolution 1244, Portugal, Slovenia, Turkey
- France Lyon, Germany Munich, Romania
- The Netherlands, Russia, Spain
- Bulgaria, Croatia, Greece, Slovenia
- Slovenia
- Greece, Italy
- Austria, France Marseille, Germany Hamburg, Greece, Kosovo under UN Security Council Resolution 1244Sweden, United Kingdom Colindale, United Kingdom Porton Down

ENIVD: European Network for Diagnostics of 'Imported' Viral Diseases; qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction; RT-PCR: reverse transcription polymerase chain reaction.

Finland, Hungary, Ireland, Luxembourg and Poland, corresponding to a participation of 76% of the countries and 45% of the laboratories. Of all laboratories that participated in the survey, 21 acted as a NRL for VHF and five act as a WHOCC.

Preparedness and response

All respondent laboratories declared that CCHF was a notifiable disease in their countries and that they followed the generic case definition for VHFs, while six countries (Bulgaria, Greece, Germany, Turkey, Russia and Spain) had their own case definition for CCHF (Table 2).

Most laboratories (25/31) stated that they had trained staff authorised to handle CCHF samples and that there was trained staff in their countries skilled in assessing VHF cases/outbreaks; 19 laboratories emphasised a need for further training, not only for laboratory workers, but also for medical and nursing staff. Half of the 24 laboratories with CCHF diagnostic capacity stated their availability to offer training services for CCHF diagnosis to other laboratories in and outside their countries.

Of all responding laboratories, 20 had standardised procedures for specimen collection and storage of CCHF infected material, and 25 for processing and shipping suspected CCHF specimens for confirmation diagnosis in other laboratories.

Diagnostic capacities

Of the 31 laboratories that participated in the survey, 24 declared to have set up diagnostic capacities to detect CCHF infection. The remaining seven laboratories in countries where CCHF diagnostic capacities has not yet been established, declared that they were sending samples to reference laboratories or WHOCCs outside their countries (Figure 1).

Among the 24 laboratories with diagnostic capacities, all except the laboratory in Serbia had CCHF molecular tests based on either quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) or nested RT-PCR. Information on the type of commercial or in-house protocol used was requested from the participants. Among the 23 laboratories which performed CCHF molecular diagnosis, 20 used an in-house method, 11 used commercial assays and eight combined both in-house and commercial approaches (Figure 2).

The serological diagnosis of CCHFV infection is based on the detection of specific IgM and IgG antibodies against recombinant nucleoprotein as the predominant available antigen, either in an enzyme-linked immunosorbent assay (ELISA) or in an indirect immunofluorescence assay (IFA). Most laboratories (22/24) with diagnostic capacities had available at least one serological technique, ELISA or IFA. Respondents were also asked about the availability of specific in-house or commercial (Vector-Best, Novosibirsk, Russia) ELISA tests for CCHF as well as in-house or commercial (EuroImmun, Luebeck, Germany) IFA (Table 2).

Eleven of 21 countries declared doing research to improve in-house molecular methods, and six of the 21 declared investigating also new serological in-house methods.

Regarding quality assurance, this survey revealed that 19 of the 23 laboratories with molecular diagnostic methods participated in the EQA on CCHF molecular diagnosis organised by ENIVD in 2011 [16], while only four participated in the exercise organised by the Quality Assurance exercise and Networking on the Detection of Highly Infectious Pathogens (QUANDHIP) project (www.quandhip.info) (Table 3).

Biosafety

The 24 laboratories with diagnostic capacities informed about the inactivation process before handling specimens for diagnostic purpose. Among them, six laboratories inactivated specimens under BSL4, 12 in BSL3 and five in BSL2 conditions (Figure 3). Of 11 laboratories performing viral isolation and propagation, six did so in BSL4 facilities and five in lower-grade BSL facilities.

Discussion

This survey has been carried out in 28 countries of the European region, including 10 countries where human cases are frequently or sporadically reported, or where there has been evidence of CCHFV circulation in animals or ticks. The presence of potential CCHFV vectors in other European countries may extend the current geographical distribution of the disease. In addition, imported cases in travellers have been reported in the EU. Hence, early recognition of the suspected CCHF cases is critical, in order to initiate the proper treatment of the patient and to apply control measures for containment of the disease. Some authors argue that Europe needs to implement a harmonised case definition for CCHF in order to enhance notifications and to estimate the diseases burden and epidemiological trends in various areas and countries [2]. The survey revealed that all responding countries used the generic case definition of VHFs to identify and notify CCHF cases. However, this survey has some limitations since not all responding countries clearly specified the source and reference of the generic or specific case definitions.

Networking and training are key factors in ensuring a rapid and effective response to CCHF. The survey revealed that the majority of countries belong to at least one network apart from ENIVD that could assure support, management, training in the diagnosis of CCHF cases, expert consultation, exchange of experiences and protocols, and scientific support if needed. Considering that some respondents did not have procedures in place for specimen collection, processing or

Table 2

Application of Crimean-Congo haemorrhagic fever serological diagnostic methods, ENIVD survey, 2012 (n=22 respondents)

Serological diagnostic method	Countries	Proportion of countries (relative to those with CCHF serological tests)
Commercial assay	Bulgaria, Latvia, Lithuania, the Netherlands, Portugal, Romania, Spain	37%
In-house assay	France, Serbia, Sweden, Switzerland, United Kingdom	26%
Commercial and in-house assay	Germany, Greece, Kosovo under UN Security Council Resolution 1244, Italy, Russia, Slovenia, Turkey	42%

CCHF: Crimean-Congo haemorrhagic fever; ELISA: enzyme-linked immunosorbent assay; ENIVD: European Network for Diagnostics of 'Imported' Viral Diseases; IFA: indirect immunofluorescence assay..

transporting, the networks could also play a key role in closing this gap. The networks could also foster training via organising international workshops on CCHF diagnosis and biosafety.

Laboratory techniques are the cornerstone of CCHF diagnosis, essential for effective surveillance, management of individual patients and outbreak prevention. In 2008, the multidisciplinary consultation of CCHF experts organised by ECDC showed that according to ENIVD, 15 of 27 countries performed CCHF diagnostics [13].The current survey launched in 2012 indicated an increase to 21 of 28 countries performing CCHF diagnostics. Our results show a strong increase in the diagnostic capacity for CCHF from 2008 to the present, possibly due to the nomination of CCHF as a priority disease for the EU. However, as shown in Table 1, two WHOCC next to endemic areas (Greece and Slovenia), lost their status as reference centres for VHF. This issue has to be taken in consideration when a new reference centre in Europe will be designated in the future.

Currently, the routine laboratory diagnosis of CCHF is based mainly on the detection of the viral genome and specific IgM and IgG. Most surveyed laboratories with diagnostic capacities (21/24) followed international recommendations of combining molecular and serological methods for CCHF diagnosis [1,28]. This shows that most of the surveyed laboratories have essential diagnostic tools for CCHF diagnosis in place.

Molecular assays offer a rapid, sensitive and specific diagnosis of CCHF during the viraemic phase of infection up to day 16 of illness [29]. The vast majority of surveyed countries (20/21) have molecular tests available, and most of them participated in CCHF EQAs. It is highly recommended that not only endemic countries, but also neighbouring countries that lack the capacity for molecular assays try to implement them.

Of the existing molecular methods for CCHF diagnosis, the majority of respondents (18/20) used a qRT-PCR, combined or not with nested PCR, while the remaining two countries used a nested RT-PCR only. Moreover, in a recent molecular EQA, it is reported that nested RT-PCR performs considerably less well compared with qRT-PCRs [16]. Therefore, it is recommended that countries performing only nested RT-PCR implement capacities for a quantitative assay because qRT-PCRs offer advantages when over nested RT-PCR such as lower contamination rate, higher sensitivity and specificity, and better time-effectiveness. A factor that may limit the use of molecular diagnostic methods is the fact that sensitivity may be affected by the high diversity of CCHF genomes. For instance, it has been found that sensitivity of molecular methods was associated with the patients' country of origin [17]. A combination of commercial and in-house RT-PCR assays will probably ensure the detection of CCHFV strains despite their diversity. However, the survey reveals that 20 of 23 laboratories use in-house RT-PCR but only eight combine it with a commercial test.

Although serological methods may cover a broader spectrum of strains due to cross-reactivity, attention must be also paid to antigenic variation among CCHFV strains which may affect their sensitivity. However, combinations of ELISA and IFA, commercial or in-house, may increase the sensitivity of detection. A recent evaluation of two commercial kits (VectorBest ELISAs and Euroinmune IFA, both for IgM and IgG) revealed that efficient and well characterised serological assays and protocols are available for CCHF diagnosis [17]. Our survey reveals that all countries using the commercial ELISA also had available commercial IFAs and that half of them combined them with an in-house ELISAs that may compensate a potential lower sensitivity caused by antigenic diversity. We advise that each country assure that their methods are optimised for strains circulating in their area, or that they use an adapted method for CCHFV genotypes circulating in their country.

In addition, to assure that diagnostic methods perform with optimal accuracy, an increased effort is needed to establish EQA studies on a regular basis. In 2011, an international EQA for the molecular detection of CCHF was launched [16]. The majority of countries with areas endemic for or at risk of CCHF surveyed in our study

Table 3

Laboratory preparedness and response capacities for Crimean-Congo haemorrhagic fever diagnosis in the European region, ENIVD survey, 2012 (n=28 countries)

Countries	Preparedness and response					Diagnostic methods	
	Case definition		Networks	EQA		Diagnostic techniques	BSL
	Generic VHFf	Specific CCHF		ENIVD	QUANDHIP		
Austria	Yes	No	ENIVD	Yes	No	PCR	BSL2+
Belgium	NA	No	ENIVD	No	No	Referral	
Bulgaria	NA	Yes[a]	ENIVD, EpiSouth, CCH-FEVER	Yes	No	PCR, ELISA, IFA, VI	BSL2
Croatia	Yes	Yes[a]	ENIVD	No	No	PCR	BSL3
Czech Republic	NA	No	ENIVD	No	No	Referral	
Estonia	Yes	No	ENIVD	No	No	Referral	
Former Yugoslav Republic of Macedonia	NA	Yes[b]	ENIVD, EpiSouth	No	No	Referral	
France	NA	No	ENIVD, EpiSouth, Euronet-P4	Yes (Lyon)	No	PCR, ELISA, VI	BSL4
Germany	NA	Yes	ENIVD, Euronet-P4	Yes (Hamburg)	Yes	PCR, ELISA, IFA, VI	BSL4
Greece	NA	Yes[c]	ENIVD, EpiSouth, Arbo-Zoo-net, CCH-FEVER	Yes	No	PCR, ELISA, IFA, VI	BSL3
Italy	NA	No	ENIVD, EpiSouth, Euronet-P4	Yes	Yes	PCR, IFA, VI	BSL4
Kosovo under UN Security Council Resolution 1244	NA	Yes	ENIVD, EpiSouth	No	No	PCR, ELISA, IFA	BSL2
Latvia	NA	Yes	ENIVD	Yes	No	PCR, IFA	BSL3
Lithuania	NA	No	ENIVD	No	No	PCR, IFA, VI	BSL3
Malta	Yes (ECDC)	No	ENIVD, EpiSouth	No	No	Referral	
The Netherlands	Yes	No	ENIVD	Yes	No	PCR, IFA	BSL3
Norway	Yes	No	ENIVD	No	No	Referral	
Portugal	NA	No	ENIVD	Yes	No	PCR, ELISA, IFA	BSL3
Romania	Yes	No	ENIVD, EpiSouth	Yes	No	PCR, IFA	BSL2
Russia	No	No[d]	ENIVD	Yes	No	PCR, ELISA, IFA	BSL3
Serbia	Yes	Yes[a]	ENIVD, EpiSouth	No	No	IFA, VI	BSL2
Slovakia	No	No	ENIVD	No	No	Referral	
Slovenia	Yes	No	ENIVD, CCH-FEVER, Arbo-Zoo-Net	Yes	No	PCR, ELISA, IFA, VI	BSL3+
Spain	Yes	Yes[e]	ENIVD, EpiSouth	Yes	No	PCR, IFA	BSL3
Sweden	Yes	No	ENIVD, CCH-FEVER, Euronet-P4, Arbo-Zoo-Net	Yes	Yes	PCR, IFA, VI	BSL4
Switzerland	Yes	Yes	ENIVD	Yes	No	PCR, ELISA	BSL4
Turkey	NA	Yes	ENIVD, EpiSouth, CCH-FEVER	Yes	No	PCR, ELISA, IFA	BSL3
United Kingdom	Yes	No	ENIVD, Euronet-P4	Yes (Porton Down)	Yes	PCR, ELISA, IFA, VI	BSL4

Arbo-Zoo-Net: Network for Capacity Building for the Control of Emerging Viral Vector Borne Zoonotic Diseases; BSL: biosafety level; CCH-FEVER: Crimean Congo Haemorrhagic Fever Network; ECDC: European Centre for Disease Prevention and Control; ELISA: enzyme-linked immunosorbent assay; ENIVD: European Network for Diagnostics of 'Imported' Viral Diseases; EpiSouth: Network for Communicable Disease Control in Southern Europe and Mediterranean Countries; Euronet-P4: European Network of Biosafety-Level-4 laboratories; EQA: external quality assessment; IFA: indirect immunofluorescence assay; NA: not available; PCR: polymerase chain reaction; QUANDHIP: Quality Assurance exercise and Networking on the Detection of Highly Infectious Pathogens project; VHF: viral haemorrhagic fever; VI: viral isolation.

[a] [22,23].

[b] National guides in preparation.

[c] [24].

[d] Not formal case definition [25].

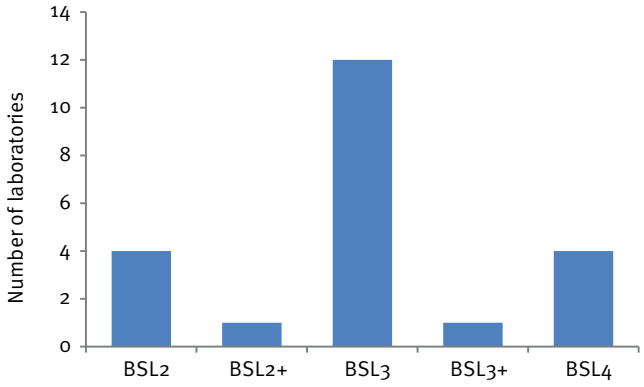
[e] [26]

[f] EU case definition for VHF [27].

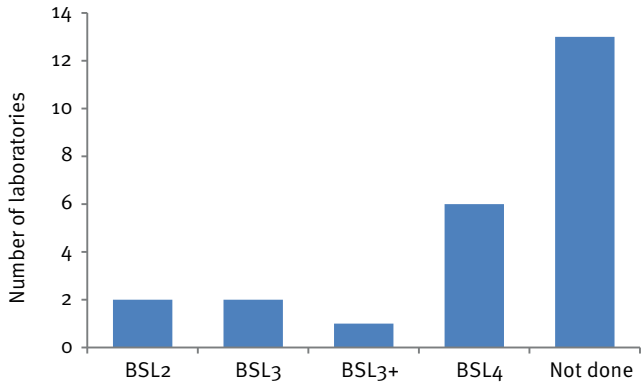
Figure 3

Biosafety levels for laboratories with Crimean-Congo haemorrhagic fever diagnostic capacities, ENIVD survey, 2012 (n=24)

A. For virus inactivation



B. For virus propagation



also participated in this EQA, in which 53 datasets were received from 44 laboratories worldwide, mostly European. Twenty of the datasets (38%) met the criteria with optimal performance.

The most definite way of CCHF diagnosis is detection of viral RNA combined with detection of IgM antibodies. Virus isolation as a diagnostic tool is rarely applied because high biocontainment laboratories (BSL4) are required. None of the European BSL4 laboratories are situated in CCHF areas, and among 11 laboratories performing viral propagation, five reported that they do not work in BSL4 facilities. Three of these five laboratories were in CCHF endemic countries.

In conclusion, the main priority issues to be addresses by European health authorities are: (i) establishing rapid and reliable protocols for CCHF laboratory diagnosis together with guidelines on storage, processing and transportation of samples, (ii) nominating a Regional Reference Expert Laboratory or a WHOCC in or near the endemic areas, and (iii) a comprehensive review of the BSL facilities suited to the reality in the endemic areas, their capacities and capabilities.

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Conflict of interest

None declared.

Authors' contributions

MDFG, AN, AT and LF conceived and designed the study; MDFG, OD, HZ, AN and LF designed the questionnaires; LF, MN and OD coordinated the collection of data through the ENIVD network; MDFG, AN and LF were involved in data management and analyses; AT, HZ, OD, MN and AP contributed with data analysis; MDFG and LF drafted the manuscript; all co-authors reviewed and assisted in the editing of the final version of the manuscript.

Members of the European Network for Diagnostics of 'Imported' Viral Diseases (ENIVD)

Austria: Stephan Aberle at Medical University of Vienna, Department of Virology, Vienna. Belgium: Marjan Van Esbroeck at Institute of Tropical Medicine, Central Laboratory for Clinical Biology, Antwerpen. Bulgaria: Iva Christova at National Centre for Infectious and Parasitic Diseases, National Reference Laboratory on tick-borne diseases, Sofia. Croatia: Alemka Markotic and Ivan-Christian Kurolt at University Hospital for Infectious Diseases, Research Department Zagreb. Czech Republic: Hana Zelena at Institute of Public Health, National Reference Laboratory for Arboviruses, Ostrava. Estonia: Irina Golovljova at National Institute for Health Development (NIHD)/Health Board. France: Delphine Pannetier at Institut Pasteur, French National Reference Center for Viral Haemorrhagic Fevers, Lyon and Remi Charrel at Aix-Marseille University, Unité Emergence des Phatologies Virales, Marseille. Germany: Jonas Schmidt-Chanasit at Bernhard-Nocht Institut, Hamburg and Roman Wölfel at Institut für Mikrobiologie der Bundeswehr, München. Greece: Anna Papa at Aristotle University, Thessaloniki, National Reference Laboratory for Arboviruses. Italy: Maria Rosaria Capobianchi at National Institute for Infectious Diseases "L.Spallanzani", Laboratory of Virology, Rome. Kosovo under UN Security Council Resolution 1244: Xhevat Jakupi at National Institute of Public Health of Kosovo, Department of Microbiology. Latvia: Jelena Storozenko at Infectology Center of Latvia, National Microbiology Reference Laboratory. Lithuania: Algirdas Griskevicius at National Public Health Surveillance Laboratory, Molecular Biology Testing Subdivision. Former Yugoslav Republic of Macedonia: Golubinka Bosevska at Institute of Health Protection of RM, Laboratory for virology and molecular diagnosis. Malta: Clive Muscat at Mater Dei Hospital, Virology Laboratory. The Netherlands: Martin Schutten at Erasmus University Hospital Rotterdam, Department of Virology. Norway: Susanne Dudman at Norwegian Institute of Public Health, Department of Virology, Oslo. Portugal: Maria Joao Alves at National Institute of Health, Centre for Vectors and Infectious Diseases Research, Águas de Moura. Romania: Cornelia Ceianu at National Institute of Public Health, National Center for Surveillance and Control of Communicable Diseases, Lab For Vector Borne Infections and Medical Entomology, Bucharest. Russia: Alexander Platonov at Central Research Institute of Epidemiology, Laboratory for zoonoses, Moscow. Serbia: Bojana Bozovic at Torlak Institute of Virology, Vaccines and Sera, National Reference Laboratory for Arboviruses. Slovakia: Boris Klempa at Institute of virology,

Slovak Academy of Sciences, Department of Virus Ecology. Slovenia: Tatjana Avsic at University of Ljubljana, Laboratory for diagnosis of zoonoses. Spain: Antonio Tenorio at Instituto de Salud Carlos III, Laboratory of Arbovirus and Imported Viral Diseases, Majadahonda, Madrid. Sweden: Ake Lundkvist at Swedish Institute for Infectious Disease Control, Karolinska Institute Stockholm, Solna. Switzerland: Pascal Cherpillod at University Hospitals of Geneva, Laboratory of Virology. Turkey: Gulay Korukluoglu at Refik Saydam Hygiene Institute, Department of Virology, Ankara. United Kingdom: David Brown at Virus reference department, Public Health England, Colindale and Tim Brooks at Special Pathogens Reference Unit. Public Health England, Porton Down.

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Rapid spread of the novel respiratory syncytial virus A ON1 genotype, central Italy, 2011 to 2013

A Pierangeli (alessandra.pierangeli@uniroma1.it)[1], D Trotta[2], C Scagnolari[1], M L Ferreri[2], A Nicolai[3], F Midulla[3], K Marinelli[2], G Antonelli[1], P Bagnarelli[2]

1. Virology Laboratory, Department of Molecular Medicine, Sapienza University, Rome, Italy
2. Department of Biomedical Sciences and Public Health, Virology Unit, Università Politecnica Marche, Ancona, Italy
3. Department of Paediatrics, Sapienza University, Rome, Italy

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Respiratory infections positive for human respiratory syncytial virus (RSV) subtype A were characterised in children admitted to hospitals in Rome and Ancona (Italy) over the last three epidemic seasons. Different strains of the novel RSV-A genotype ON1, first identified in Ontario (Canada) in December 2010, were detected for the first time in Italy in the following 2011/12 epidemic season. They bear an insertion of 24 amino acids in the G glycoprotein as well as amino acid changes likely to change antigenicity. By early 2013, ON1 strains had spread so efficiently that they had nearly replaced other RSV-A strains. Notably, the RSV peak in the 2012/13 epidemic season occurred earlier and, compared with the previous two seasons, influenza-like illnesses diagnoses were more frequent in younger children; bronchiolitis cases had a less severe clinical course. Nonetheless, the ON1-associated intensive care unit admission rate was similar, if not greater, than that attributable to other RSV-A strains. Improving RSV surveillance would allow timely understanding of the epidemiological and clinicopathological features of the novel RSV-A genotype.

Introduction

Human respiratory syncytial virus (RSV) is a major cause of lower respiratory-tract infection (especially bronchiolitis) in infants, but also affects immunocompromised and elderly patients [1]. In a small proportion of infected infants, severe RSV disease is characterised by wheezing, hyperinflation, atelectasis, increased mucus secretion, tachypnoea, retraction and consolidation. RSV, a *Pneumovirus* of the *Paramyxoviridae* family, has an enveloped, non-segmented, single-stranded, negative-sense RNA genome of approximately 15,000 nt, coding for 11 genes. It has been further divided into subtypes A and B [2] based on reactions with monoclonal antibodies against the glycoproteins G and F. The attachment glycoprotein G is a transmembrane glycoprotein with an extracellular domain that consists of two hypervariable regions separated by a central conserved region spanning amino acids 151–190, comprising the receptor binding site [3]. Although the amino acid sequence of the hypervariable region can vary widely between subgroups and even among isolates, its amino acid content is reminiscent of mucin proteins [3]. These mucin-like domains present different N- and O-linked glycosylation sites which may affect antigenicity [3]. Indeed, G is the major antigenic protein of RSV; besides exhibiting marked genetic variability in the amino acid sequence, position and number of glycosylation sites, it may also differ in length due to insertions and mutations in premature stop codons [3]. The main genetic change detected to date is a 60 nt insertion in G (generating a 20 aa insertion in the C-terminal domain) in RSV-B strain BA [4].

Based on the genetic diversity of glycoprotein G, RSV-A has been classified into genotypes GA1–GA7, SAA1 and, recently, NA1–NA2 [5-7], and RSV-B into GB1–GB12, SAB1–SAB3 and BA [4-6]. Most molecular epidemiological studies have analysed the G gene's hypervariable C-terminal domain; importantly, sequencing of the entire RSV genome of several circulating strains [8] confirmed that the evolutionary patterns and clades seen in the C-terminal part of the G gene reflect those of the whole genome. Phylogenetic analysis documented that several C-terminal positions are positively selected hypervariable sites that may contribute to immune escape, promoting re-infection and recurrent circulation of a genotype [9,10]. Multiple genotypes can co-circulate during successive epidemic seasons, but a new subtype, or one spreading from different countries, may replace dominant strains [7,8,11]. Genotype BA spread slowly and sequentially worldwide [12], but over several years, a divergent BA lineage replaced all RSV-B genotypes [13,14].

Aside from the epidemiological impact, novel variants may display enhanced clinical severity with increased replication in the lower respiratory tract and/or hyperresponsiveness of the airways [1,15], underlining the importance of monitoring their spread.

In Italy there is no ongoing national surveillance for circulating RSV, and only one study has investigated the genetic diversity of RSV-A in different seasons, up to 2006 [16]. In that and in other European studies [12,17], phylogenetic analysis disclosed co-circulation of genotypes GA2 and GA5 from 1998 to 2006/07; GA7 was less common.

Within the framework of an ongoing study of paediatric respiratory infections, we genotyped RSV-positive cases detected in Rome and Ancona, central Italy, in the 2011/12 epidemic season. Unexpectedly, we detected ON1, the new RSV-A variant of GA2 genotype identified in Ontario (Canada) in 2010 [18]. ON1 bears a 72 nt insertion in the G hypervariable region, corresponding to 24 aa (of which 23 are duplicated), the largest G protein genetic modification ever reported [18]. The ability of the G protein to host long inserts without impairing function is due to the relatively loose structural constraints of the mucin-like domain, which determines rapid evolutionary changes and contributes to the pathogenicity of RSV and other negative-stranded RNA viruses encoding this domain [19].

In this study, phylogenetic analysis of the G gene of RSV-A strains circulating in the 2010/11, 2011/12 and 2012/13 epidemic seasons demonstrated the presence of ON1 genotype in the 2011/12 winter and its rapid spread in the following year. Investigating the spread of ON1 is important to understand the extent to which genetic variability can modify the epidemic behaviour of RSV at population level. Moreover, since antigenic variation may influence clinical outcomes, we also addressed the clinical impact of genotype ON1. Analysis of case distribution and clinical patient data showed differences between infections with ON1 genotype and those with previously circulating RSV-A strains.

Methods

Patients and samples

The study involved RSV-positive respiratory samples collected in the 2010/11, 2011/12 and 2012/13 epidemic seasons from two sets of patients: children presenting to the paediatric emergency department who were admitted for respiratory conditions and then diagnosed at the virology laboratory of the teaching hospital Policlinico Umberto I-Sapienza University of Rome (RM samples), and children admitted to the paediatric department of Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona-Università Politecnica delle Marche, or to other paediatric departments of the Marche region that use the Ancona virology unit (AN samples). The two cities lie about 200 km apart in central Italy.

Informed consent was sought from the children's parents for participation in the study which had been approved by the ethics committee of the two hospitals.

Demographic and clinical data were taken from the medical files. A nine-point (0–8) clinical severity score based on (age-adjusted) respiratory rate, arterial oxygen saturation in room air, retraction and oral feeding ability were determined on admission [20].

RSV detection

Each institution used their own protocol to test upper respiratory tract samples for respiratory viruses as detailed below. Bronchoalveolar lavage (BAL) samples were taken from patients admitted to a paediatric intensive care unit (PICU) for severe respiratory conditions. In Rome, nasal washings and/or BAL were tested for 14 respiratory viruses with PCR-based tests, as described previously [20,21]. In Ancona, nasal swabs and/or BAL were first tested for RSV RNA using an in-house one-step multiplex real-time RT-PCR followed by further analyses with the Seeplex RV 15 ACE Detection Kit (Seegene, Korea) in RSV-negative samples. Information on PCR primers and probes is available from the authors on request.

RSV-A sequencing

About half of the RSV-A-positive samples were randomly selected for genomic characterisation. Amplicons (502 bp) for sequencing were obtained from RSV-A-positive samples with the A-Fseq (G gene position 481–498 of the RSV-A2 reference strain) and the F1 reverse primer targeting the fusion protein gene's 5' end [5]. Experimental details are available from the authors on request.

Phylogenetic analysis

The nucleotide sequences of a fragment of the second hypervariable region of the G gene (396 nt, corresponding to codon positions 167–298) from RSV-A isolates were determined and compared with reference strains in GenBank.

Sequences were edited using Bioedit v7.1.3 and aligned with reference sequences using CLUSTAL W. We analysed evolutionary relationships between the study sequences, and sequences recently circulating in the United States [8], Canada [18], Belgium [22], Japan [7] and Malaysia [23] together with reference strains representative of all RSV-A genotypes. The best-fit evolutionary model and parameters were selected using jModeltest v0.1.1 [24]. The general time reversible +G (GTR+G) model of nucleotide substitution was the most appropriate for the dataset. The evolutionary parameters corresponding to the best-fit model were run in MEGA5 v5.2.1 [25] to obtain the distance matrix among groups, the tree topology under a strict maximum likelihood (ML) approach, and the significance of the tree topology by bootstrapping (1,000 replicates); the p-distance among sequence clusters was calculated by pairwise comparison including transitions and transversions.

Sequences were submitted to GenBank and assigned the following accession numbers: KC858158–KC858194

and KC858195–KC858198 (AN and RM sequences; 2010/11); JX988439–JX988449, JX988453–JX988486 and JX988450–JX988452, JX988487–JX988499 (AN and RM sequences with/without the 24 aa insertion; 2011/12); KC858199–KC858245 and KC858246–KC858257 (AN and RM sequences; 2012/13).

Statistical analysis

The Mann–Whitney U test was used to compare median patient age, Fisher's exact test to analyse independent categorical variables and the unpaired t-test to compare genetic distance group means. A p value of ‹0.05 was considered significant. SPSS (v17.0) was used for data analysis.

Results

RSV-positive patients

From November 2010 to May 2013, 515 RSV-positive patients were detected: 165 in Rome (mean age: 4.8 months; median age: 2.75 months; range: 0.2–29 months) and 350 in Ancona (mean age: 12.8 months; median age: 3 months; range: 0.1–163 months). Their distribution is reported in Table 1: 180 cases in 2010/11 (83 RSV-A, 97 RSV-B), 65 cases in 2011/12 (119 RSV-A, 46 RSV-B) and 170 cases in 2012/13 (158 RSV-A, 12 RSV-B). All patients were hospitalised for respiratory conditions.

Of the 360 RSV-A positive samples, 161 were successfully sequenced and categorised in relation to the presence of a 72 nt insert in the G gene (Table 2).

RSV case distribution

Analysis by week of presentation highlighted a different case distribution in the last season both in Rome and Ancona (Figure 1). In 2010/11 and 2011/12, the earliest RSV-associated hospitalisations occurred in mid-December (slightly earlier in Rome) and peaked in January and February; in 2012/13, the cases started in late November, with a larger number in both cities occurring earlier than in the previous two seasons, and peaking in week 51 (2012) and week 1 (2013) in Rome and Ancona, respectively. In 2012/13, RSV-A accounted for 93% of all cases.

Table 1

Number of respiratory syncytial virus-positive patients diagnosed at two institutions in Ancona and Rome during three epidemic seasons, Italy, November 2010–May 2013 (n=515)

Winter seasons	RSV-A cases n=360		RSV-B cases n=155		All n=515
	Ancona	Rome	Ancona	Rome	
2010/11	53	30	73	24	**180**
2011/12	79	40	34	12	**165**
2012/13	103	55	8	4	**170**

RSV: respiratory syncytial virus.

Table 2

Sequence features of respiratory syncytial virus-A-positive strains in relation to the presence of a 72 nt insert, Italy, November 2010–May 2013 (n=161)

Winter seasons	Non-ON1[a] n=95		ON1[b] n=66		All n=161
	Ancona	Rome	Ancona	Rome	
2010/11	37	4	0	0	**41**
2011/12	34	13	11	3	**61**
2012/13	7	0	40	12	**59**

RSV: respiratory syncytial virus.

[a] RSV-A genotype without the insertion.
[b] RSV-A genotype with the insertion.

ON1 detection

Phylogenetic reconstruction of 85 unique sequences is reported in Figure 2. All AN and RM strains were derived from genotype GA2 and belonged to the recently described NA1 group [7,23]. They clustered into several distinct clades which, despite non-significant bootstrap values, reflect RSV-A variability and evolution during the three epidemic seasons. The major finding was that 51 AN and 15 RM strains grouped with the novel genotype ON1 (ON67-1210A) [18], which is characterised by a 72 nt insertion in G, resulting in 24 extra amino acids of which 23 are duplications of aa 261–283. ON1 was not detected in Ancona or Rome in the 2010/11 season; it accounted for 14 of 61 (22.9%) strains sequenced in 2011/12 and for 52 of 59 (88.1%) strains analysed in 2012/13. In that last season. it was the prevalent genotype in Ancona (40 ON1/47 RSV-A) and apparently the sole genotype in Rome (12/12).

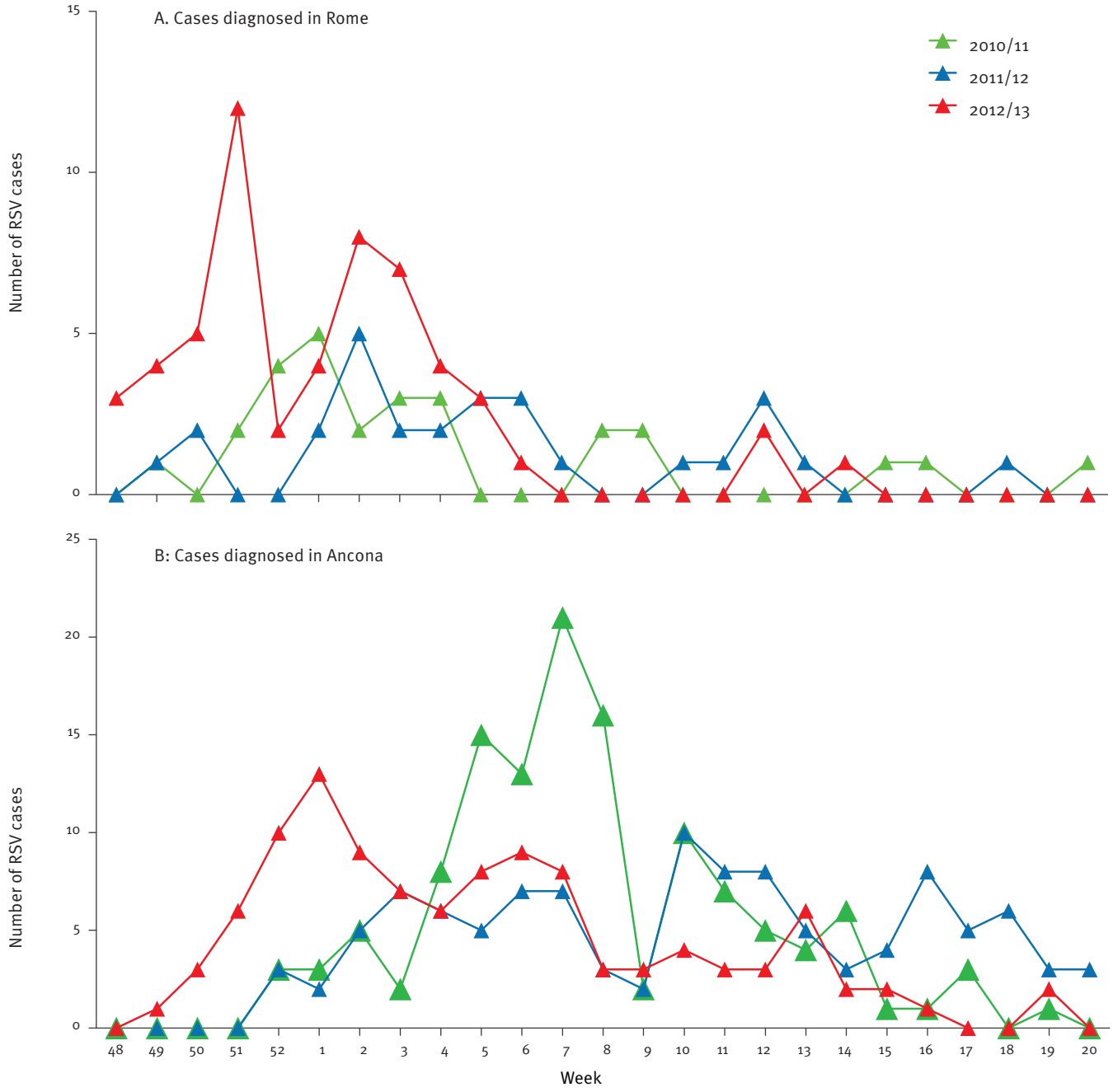
Sequence analysis

The phylogenetic tree based on G gene sequences (Figure 2) shows, next to the main branches, the amino acid substitutions identifying a subtree. At the nucleotide level, the mean p-distance among the 95 strains without the insertion (GA2; NA1 group) was greater than the one among the 66 strains bearing it (ON1), i.e. 0.022±0.016 vs 0.012±0.009 (mean±standard deviation, p‹0.0001, unpaired t-test), suggesting a more recent common origin of the ON1 strains. As expected in this highly variable region, pairwise distances were higher at the amino acid than at the nucleotide level, and were 0.043±0.030 and 0.028±0.022 among strains without and with the insert, respectively (p‹0.0001, unpaired t-test). The alignment of deduced amino acid sequences is presented in Figure 3, with grey areas representing potential N-glycosylation sites.

Overall our strains presented very few amino acid substitutions in the conserved central portion (up to aa 198) of the G protein, whereas, as expected, several variations with respect to the reference GA2 sequence were found in the hypervariable portion.

Figure 1

Respiratory syncytial virus cases, distribution of hospitalised children, Italy, November 2010–May 2013 (n=515)



RSV: respiratory syncytial virus.
Results are weekly data for each epidemic season.

All study sequences clearly differed from the reference GA2 genotype, as demonstrated by the R204K, L215P, S230P conserved substitutions, also found in recently circulating strains of GA2 group, i.e. the NA1, NA2 and ON1 genotypes. Importantly, amino acid positions 215 and 230 are highly variable, positively selected sites [10]. Moreover, all study strains bore the N297K substitution and all but one (17294AN, detected in 2011/12) also exhibited substitution P292S, another positively selected site [10]. Most AN and RM sequences bore the P274L substitution (a positively selected site) that had been detected in NA1 variants in Belgium, Wisconsin, Japan and Malaysia, but not in nine 2010/11 and in one 2011/12 sequences analysed in this study. The substitutions I208L and N273Y/H/D were also found. Changes at position 273 are particularly interesting as they involve the loss of a potential glycosylation site; they occurred in 18 strains from 2010/11), 23 strains from 2011/12 and six strains from 2012/13.

The N273Y substitution was also conserved in the well-defined clade containing 14 (2011/12) and 53 (2012/13) study strains with ON1 genotype (ON67-1210A). This cluster displayed the characteristic 72 nt duplication and three amino acid variations E232G, T253K and P290L, the latter a reversal mutation compared with all other study sequences. Importantly, T253K is related to the loss of another potential N-glycosylation site besides that determined by N273Y; the loss of two potential N-glycosylation sites is a major characteristic of strain ON67-1210A [18]. Moreover our ON1 strains presented several amino acid substitutions in the hypervariable portion of the G protein and in the 24 aa insert compared with the ON1 prototype (Figure 3). When amino acid sequences of the duplicated tract were compared with the homologous 23 aa portions (aa 261–283), variations from the ON1 prototype were generally found either in the insertion or in its homologous tract (Figure 3), suggesting that they arose after the insertion event. Interestingly, however, a reversion in the positively selected site 274 (L274P) was found in a single 2011/12 strain (12221AN) and in 35 of 52 (67%) 2012/13 strains (both AN and RM) in the insert or in the homologous 23 aa portion (L274P and/or LxvP), nearly always together with the YxxiH change in the insert.

Patient data and clinical diagnosis

Demographic and clinical data were available for 99 patients infected with RSV-A-positive strains sequenced in 2011/12 and 2012/13; data stratified by RSV strain are reported in Table 3.

Overall, children whose RSV strain bore the insertion were significantly younger than those infected with the other strains, they had less frequently bronchopneumonia and more frequently influenza-like illness (ILI). ON1 infection caused more, although not statistically significant (p=0.053), PICU admissions than the other RSV-A strains (Table 3).

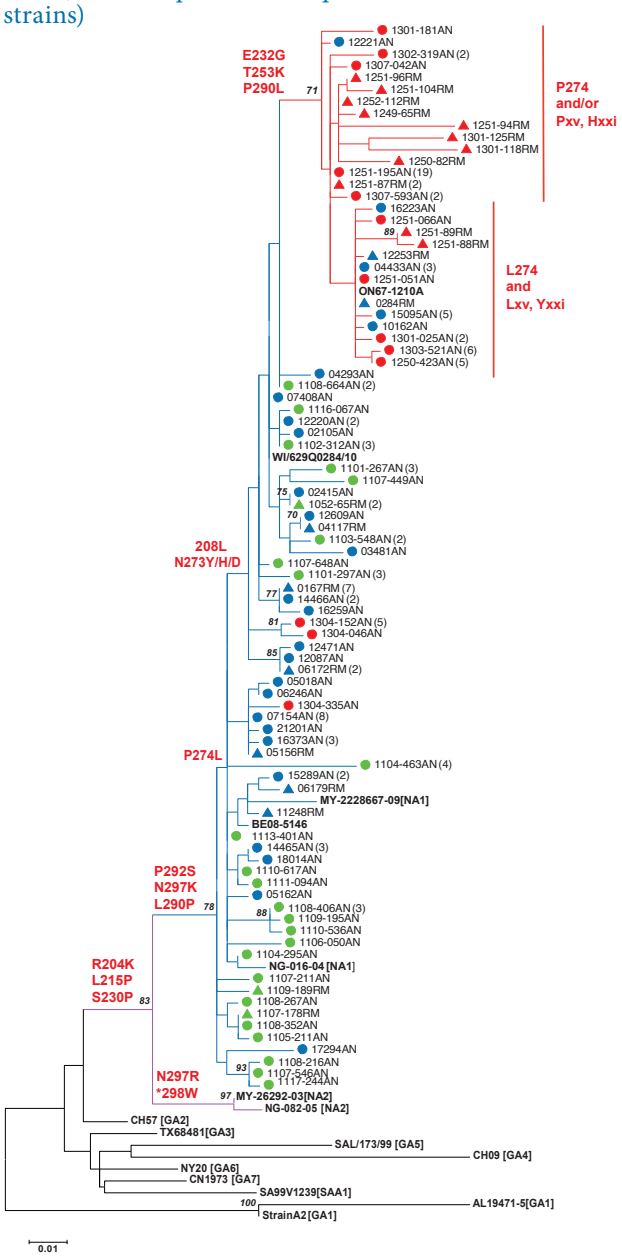
We then evaluated 52 bronchiolitis patients for whom clinical data were exhaustive (Table 3); PICU cases were excluded due to insufficient clinical information and lack of an overall assessment of risk factors for severe RSV disease. Patients positive for an RSV-A strain that bore the insertion were less likely to have an elevated respiratory rate and exhibited a lower, although not statistically significant, clinical severity score (Table 3).

Discussion

It has been estimated that RSV infects 70% of children during their first year of life and that nearly all two year-olds have been infected; in addition, more than one third of children younger than two years get infected at least twice with RSV strains not only heterologous at the subtype level (i.e. RSV-A and RSV-B), but also homologous (i.e. RSV-A and RSV-A or RSV-B and RSV-B) [1,26]. It is not entirely clear whether this is because RSV infection does not confer long-lasting protective immunity in humans, or because recurrent infections

Figure 2

Phylogenetic tree based on the second hypervariable region of the G protein gene, Italy November 2010–May 2013 (n=85 unique Italian sequences, n=16 reference strains)



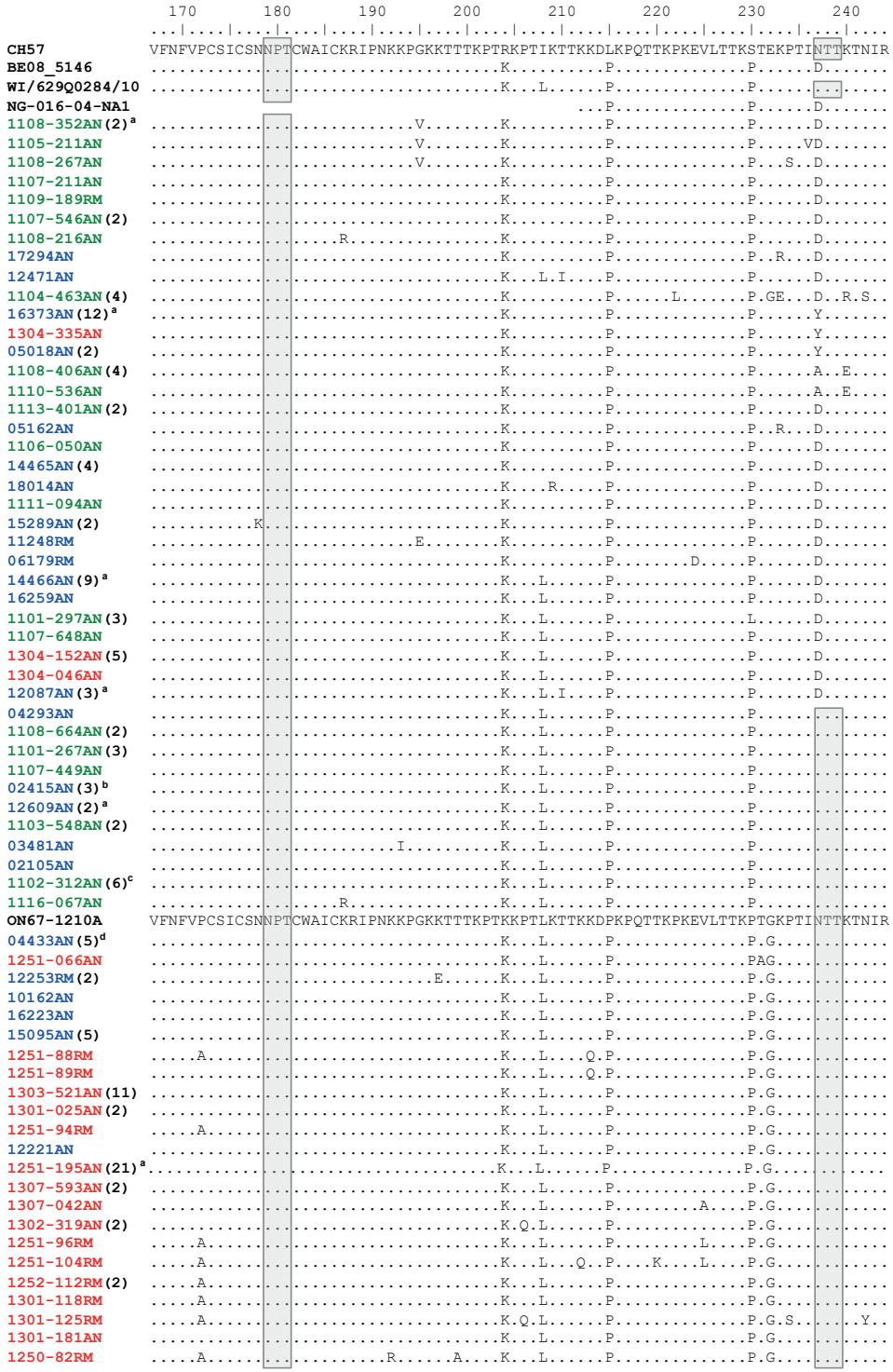
The best-fit evolutionary model and the parameters selected by the jModeltest programme were used as described in the text. The scale bar shows the proportions of nucleotide substitutions per site. Numbers at nodes are bootstrap values for 1,000 iterations; only bootstrap values of >70% are shown. Numbers in round brackets indicate the total number of strains with an identical sequence. Names in square brackets indicate the genotype of the RSV-A reference sequence.

Branches are colour-coded according to the deduced amino acid sequence, identifying subtrees and genotypes: red: sequences with insertion clustering with the novel ON1 genotype; blue: sequences of NA1 genotype clustering with sequences from the United States (Wisconsin) [8], Europe (Belgium) [22], Malaysia (Kuala Lampur) [23] and Japan (Niigata City) [7]; purple: sequences of NA2 genotype from Malaysia (Kuala Lampur) and Japan (Niigata City), added for clarity. Circles: strains isolated in Ancona; triangles: strains isolated in Rome. Symbol colour indicates epidemic season: green: 2010/11; blue: 2011/12; red: 2012/13.

GenBank accession numbers of RSV strains (this study) are reported in the text; reference strain accession numbers are: ON67-1210A (JN257693), WI/629-Q0284/10 (JF920053), MY-2228667-09 (JX256883), BE08-5146 (JX015499), NG-016-04 (AB470478), MY-26292-03 (JX256960), NG-082-05 (AB470479), CH57 (AF065258), TX68481 (AF233920), SAL/173/99 (AY472094), CH09 (AF065254), NY20 (AF233918), CN1973 (AF233904), SA99V1239 (AF348808), AL19471-5 (AF233902), strain A2 (M74568).

Figure 3

Alignment of deduced G protein amino acid sequence of RSV-A strains isolated in Ancona and Rome, Italy, November 2010–May 2013 (n=161)



[a] Strain observed in AN and RM.

[b] Strain observed in AN and RM, in 2010-11 and 2011/12.

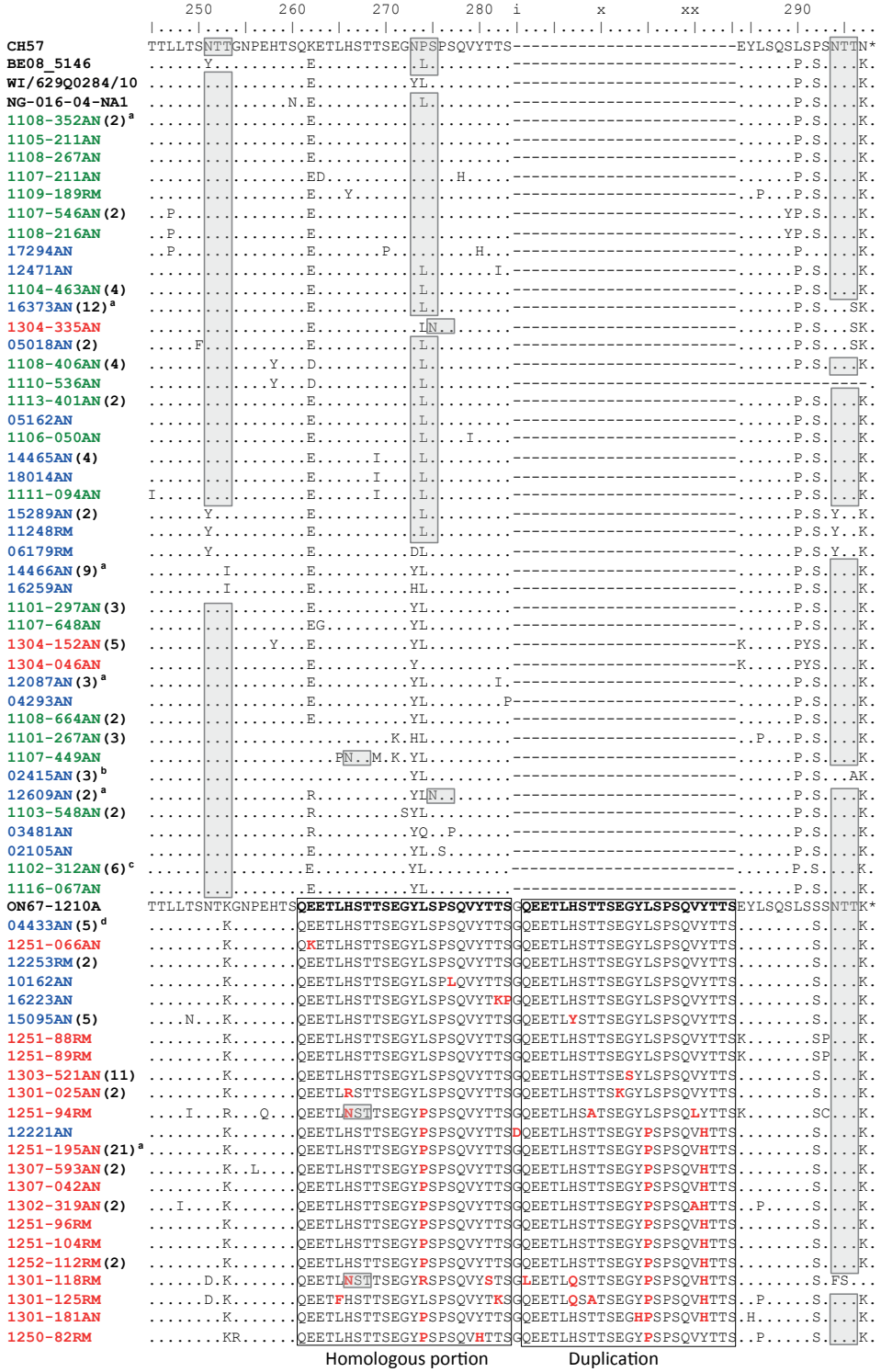
[c] Strain observed in 2010/11 and 2011/12.

[d] Strain observed in AN and RM in 2011/12 and 2012/13.

Alignments of 65 unique sequences are shown relative to the sequence of the prototype GA2 CH57 (AF065258) used as the reference strain. Reference sequences belonging to the NA1 subgroup of the GA2 genotype circulating in Europe (BE08-5146, JX015499), the United States (WI/629-Q0284/10, JF920053) and Japan (NG-016-04, AB470478) are included for clarity. Amino acid numbering from 167 to 298 refers to the G glycoprotein of the reference strains without insertion, Roman numerals indicate the position in the amino acid insertion. The ON67-1210A (ON1 genotype, JN257693) sequence with the 24 aa insertion (23 of which are duplicated) is used as the prototype for the Italian strains with the insertion. Dots indicate nucleotides identical to the CH57 strain; the sequence of the ON1 prototype is shown only for clarity. Dashes indicate the gap corresponding to the nucleotide insertions, asterisks indicate stop codons. Black boxes indicate the duplicated region (homologous portion and insertion); sequences within the box are shown independently of identities to the ON1 prototype, mutations are shown in red and bold. Grey shading indicates potential N-glycosylation sites. Colour of sequence names indicates the epidemic season: green: 2010/11; blue: 2011/12; red: 2012/13. Numbers in parentheses indicate the total number of identical strains.

Figure 3

Alignment of deduced G protein amino acid sequence of RSV-A strains isolated in Ancona and Rome, Italy, November 2010–May 2013 (n=161)



[a] Strain observed in AN and RM.

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even with the same subtype are due to significant antigenic changes in the immunodominant proteins [1,26].

Emerging RSV variants that possess a selective advantage in terms of genetic diversity can spread to neighbouring areas, gradually replacing dominant genotypes over several years [7,11,22]. A well- studied example is a 20 aa insertion in the G protein (comparable in length and position to the insertion in ON1) that arose in RSV-B BA strains; it was first detected in 1999 in Argentina [4] and spread worldwide in the course of several epidemic seasons [12-14,22]. In apparent contrast to its epidemiological success, a BA strain was efficiently neutralised by sera from patients previously infected with non-BA RSV-B [27]. However, homologous subgroup reinfections were reported with both RSV-A and RSV-B strains [26,28].

Our findings document the presence of the novel ON1 genotype in Italy in the 2011/12 epidemic season and its rapid spread in 2012/13. All other RSV-A G-gene variants described here derive from the GA2 genotype and are genetically close to the recently characterised NA1 genotype, as are most recent strains circulating worldwide [18,23,28].

The ON1 genotype was first detected in Ontario in the winter of 2010/11 [18], then in 2011/12 season in South Africa [29], Malaysia [23] and Germany [30], with an infection rate of around 10% among hospitalised children. Compared with the near absence of ON1 infections in central Italy in 2010/11, we here report a 23% rate in 2011/12 and, remarkably, a nearly 90% rate in 2012/13, i.e. a nearly complete replacement of previously circulating RSV-A strains by the new genotype within just one year. This rapid diffusion of this strain was probably made possible by its genetic diversity in the G protein. Moreover, the ON1 study strains of the 2012/13 season displayed several amino acid substitutions compared with the ON1 prototype and with ON1 strains deposited in GenBank [18,23,30].

The variability and apparent evolution seen at the positively selected site 274 in the ON1 cluster of sequences is particularly interesting. All 2011/12 strains but one (12221AN) had a leucine in this position, like most

Table 3

Demographic and clinical data of respiratory syncytial virus-A-positive patients diagnosed during the 2011/12 and 2012/13 seasons, stratified by RSV-A genotype, Italy (n=99)

Features	RSV-A n=99[a]	Non-ON1 n=43	ON1 n=56	p value[b]
Median age, months (range)	2.8 (0.4–60)	5.0 (0.5–60)	2.5 (0.4–32)	**0.030**
Patient younger than one year, n (%)	85 (85.6)[c]	33 (76.7)	52 (92.8)	**0.039**
Male sex, n (%)	66 (66.6)	30 (69.8)	36 (64.3)	0.66
Clinical diagnosis[d]				
Bronchiolitis[e], n (%)	61 (61.9) {7}	26 (60.5) {1}	35 (62.5) {6}	0.838
Wheezing/asthma, n (%)	9 (9.1)	3 (7.0)	6 (10.7)	0.727
Bronchopneumonia, n (%)	16 (16.2) {4}	12 (27.9) {2}	4 (7.1) {2}	**0.011**
ILI[f], n (%)	13 (13.1) {1}	2 (4.6)	11 (19.6) {1}	**0.036**
PICU admission, n (%)	12 (12.1)	3 (7.0)	9 (16.1)	0.053
Bronchiolitis patients[g]	RSV-A n=52	Non-ON1 n=23	ON1 n=29	
Median age, months (range)	2.0 (0.3–9)	2.5 (0.3–8)	1.6 (0.5–9)	0.110
Male sex, n (%)	35 (67.3)	15 (65.2)	20 (69.0)	1.000
Respiratory rate >45 breaths/min, n (%)	35 (67.3)	19 (79.2)	16 (55.2)	**0.043**
O2 saturation <95%, n (%)	25 (47.2)	13 (54.2)	12 (41.4)	0.403
Retractions, n (%)	36 (69.2)	17 (73.9)	19 (65.5)	0.560
Severity score 4–8[h], n (%)	13 (25.0)	9 (39.1)	4 (13.8)	0.053

ILI: influenza-like-illness; PICU: paediatric intensive care unit; RSV: respiratory syncytial virus.

[a] Number of patients for whom data were available.

[b] Mann-Whitney test for the difference in median age; Fisher's exact test for analysis of independent categorical variables. Bold indicates a significant result.

[c] In parentheses the proportion of cases with the condition among the total cases per group.

[d] Clinical diagnosis on admission; number of PICU admissions in curly brackets.

[e] Bronchiolitis was defined as a history of upper respiratory tract infection followed by acute onset of respiratory distress with cough, tachypnoea, retraction and diffuse crackles on auscultation in infants within one year [20].

[f] ILI was defined according to the Italian Health Ministry as fever (temperature ≥38 °C), with at least one of the following symptoms: headache, asthenia, myalgia, and at least one of the following respiratory symptoms: cough, rhinitis, acute pharyngitis.

[g] Clinical data were available for 52 of 61 patients admitted for bronchiolitis (excluding PICU cases).

[h] Clinical severity score (0–8) including subscores for respiratory rate (<45 breaths/min=0, 45–60 breaths/min=1, >60 breaths/min=2); arterial oxygen saturation in room air (>95%=0, 95–90%=1, <90%=2); presence of retractions (none=0, present=1, present+nasal flare=2), and feeding ability (normal=0, reduced=1, intravenous=2) [20].

strains circulating then and in the previous winter as well as the ON1 prototype. The reversal mutation involving a proline at site 274, first detected in the 12221AN strain in the homologous portion and in the insert (LxvP), together with the YxxiH substitution, seemed to confer an evolutionary advantage, being found in most (31/52 sequences; 60%) 2012/13 strains. Notably, this variant was circulating during the 2011/12 winter as a minority strain also in Germany [30] and Japan (GenBank: AB698559). These variations may have arisen from independent evolutionary reversal events affecting the amino acid at site 274 and its homologous position in the insert, as may happen in those sites following the so called 'flip-flop' phylogenetic pattern (i.e. a frequently reversible amino acid replacement) [10]. Alternatively, the duplication event may have occurred independently in a strain already bearing the L274P substitution, generating the same substitution in its homologous position in the insert, followed by a second independent mutation at insert position xxi. ON1 strains with 274P and/or xvP in the homologous and insert portion spread widely in the population, accounting for 67% of 2012/13 strains.

Overall, a variety of genetic changes could be responsible for the spread of ON1 strains, conferring low cross-protection by pre-existing antibodies to RSV-A strains previously circulating in Italy: the 24 aa insertion, the loss of a further potential glycosylation site due to the T253K substitution, and even other amino acid changes. Unfortunately, we were not able to culture ON1 clinical isolates and could therefore not assess the antigenic properties of these strains.

Nonetheless, the ability to replace circulating RSV-A strains and the fact that the peak of RSV infections occurred earlier in 2012/13 than in the previous two seasons, could be the consequence of broad antigenic diversity. Another possible consequence of antigenic diversity could be that newborns infected with a novel genotype would be less protected by maternal antibodies against previously circulating RSV-A strains. This would be consistent with our finding that hospitalised patients with ON1 were younger than those with other RSV strains, as was also seen in a German report for July 2010 to June 2012 [30]. In contrast, a Japanese study reported that patients infected with a novel GA2 variant, NA2, had a greater mean age than those infected with the previously circulating GA5 genotype [7]. The difference could be related to viral characteristics or, more probably, to the study population, since the Japanese study described cases reported to the national surveillance by sentinel paediatric clinics, whereas all our samples were from hospitalised children; we could therefore not assess the impact of the novel genotype at the population level.

Besides the epidemiological impact, significant genetic variation in circulating strains may involve different pathogenicity and virulence. Several publications have recently documented that different patient isolates are able to induce variable pathogenesis in a mouse model [31] and in cell culture [32]. Moreover, novel substitutions and deletions were identified in RSV strains from clinical samples of severely ill patients [15].

In this study we analysed demographic and clinical data from about 100 RSV cases and from 52 well-characterised bronchiolitis patients, and found differences between infections with ON1 vs GA2 strains. ON1 patients were significantly younger than those infected with the other RSV-A strains (p=0.03); they were more frequently affected with less severe clinical conditions; bronchiolitis severity was lower, as documented by the significantly better respiratory condition (p=0.043) and the considerably lower severity score (p=0.053). A milder clinical course among children infected with genotype ON1 compared to infections with other RSV infections was recently reported in Cyprus [33]. On the other hand, PICU admissions were more numerous for ON1 than for the other RSV-A strains (p=0.053), as also reported in the German study [30]. Given these contrasting observations, the novel genotype does not seem to possess special determinants of severity compared with previously circulating RSV-A strains, but its sudden diffusion due to genetic differences could have increased the epidemic peak, the number of hospitalised patients and consequently PICU admissions.

Undoubtedly, it is of interest to investigate viral and host pathogenic factors during both severe and mild RSV infection, but the challenge is to characterise RSV strains and clinical conditions from a sufficiently large number of patients to assess associations. In several European countries, weekly reports of influenza virus characterisation also contain RSV detection in ILI cases reported by sentinel primary care physicians, issued by the European Influenza Surveillance Network (EISN). In Italy, RSV testing of ILI samples was performed in the framework of the EISN RSV Task Group up to year 2007 [34].

The Task Group's report recommended improving RSV surveillance using molecular techniques yet to be standardised and setting up a sentinel system of representative hospitals to determine the burden of RSV illness and define its epidemiology [34]. Research projects would now be well timed to monitor the diffusion of the novel ON1 genotype and of the other RSV strains in the general population and to determine their hospitalisation rate and clinical impact. This knowledge could also help include the more virulent strains in vaccines.

Acknowledgements

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Conflict of interest

None declared.

Authors' contributions

AP and PB designed the study, had primary responsibility for analysis of the results and wrote the paper; DT conducted research in Ancona and performed phylogenetic analysis; CS conducted research in Rome; MLF and KM performed virological diagnosis and collected patient data in Ancona; AN and FM collected respiratory samples and patient data in Rome; GA analysed the results and contributed to writing the paper.

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Letter to the Editor: Cutaneous diphtheria in a migrant from an endemic country in east Africa, Austria May 2014

S Huhulescu[1], S Hirk[1], V Zeinzinger[1], P Hasenberger[1], H Skvara[2], R Müllegger[2], F Allerberger (Franz.Allerberger@ages.at)[1], A Indra[1]

1. Austrian Agency for Health and Food Safety (AGES), Institute for Hygiene and medical Microbiology, Diphtheria-Reference Laboratory, Vienna, Austria
2. Landesklinikum Wiener Neustadt, Department of Dermatology, Wiener Neustadt, Austria

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To the editor:

In their recent article, Both et al. pointed out that the unavailability of diphtheria antitoxin (DAT) constitutes a risk for patients presenting with diphtheria across Europe and may hamper diphtheria diagnostics [1].

In Austria, DAT is also no longer available since 2011. However, 21 years after the last documented Austrian case of diphtheria due to toxigenic *Corynebacterium diphtheriae*, an east African teenager was diagnosed with cutaneous diphtheria in May 2014. He had been hospitalised on 25 April, after arriving in Austria via Italy, for secondary infected skin wounds with impetigo appearance mainly on extremities and treated with intravenous ampicillin/sulbactam (3 g i.e. 2 g ampicillin/1 g sulbactam every 8 hours for 7 days). On admission, he had a total white blood cell count of 13.7 x 10^9 /L (norm: 3.8-9.8 x 10^9 /L), neutrophils 10.33 x 10^9 /L (norm: 1.5-7.0 x 10^9 /L), and a C-reactive protein of 2.73 mg/dL (norm: < 0.5 mg/dL). The wound swab taken from a leg ulcer on 25 April yielded *C. diphtheriae*, *Staphylococcus aureus* and *Streptococcus dysgalactiae equisimilis* (Lancefield group C). Microbiological diagnosis was hampered by delays in specimen transport and reporting of results; the Diphtheria-Reference Laboratory received the isolate on 19 May.

The World Health Organization (WHO) Global Reference Centre for Diphtheria and Streptococcal Infections at Public Health England (PHE), London, United Kingdom, confirmed the isolate as toxigenic *C. diphtheriae* biovar *mitis*. Minimum inhibitory concentration for benzylpenicillin was 0.25 mg/L determined by Epsilometer (E) test on a blood agar plate. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) have no species specific breakpoints for *C. diphtheriae*, but the strain can be categorised as resistant to benzylpenicillin according the EUCAST recommendations for *Corynebacterium* sp.-related breakpoints [2]. Penicillin-resistance is not unusual among tropical *C. diphtheriae* strains, rendering benzylpenicillin ineffective for treatment [3].

Both et al.'s statement, that supply and access to DAT is insufficient in Europe, has been confirmed by our experience in Austria. We were unable to procure DAT for a patient with toxin-producing *C. ulcerans* infection in May 2013 and are still without any stock of DAT. The interim guidelines of PHE require that antitoxin should be given if ulcers in cases of cutaneous diphtheria are larger than 2 cm^2, as was the case in our patient [4].

It has long been recognised that *C. diphtheriae* can cause clinical skin infections characterised by chronic non-healing ulcers with a dirty greyish membrane and often superinfected by *Staphylococcus aureus* and haemolytic streptococci [5]. Skin carriage of *C. diphtheriae* can act as a silent reservoir for the organism, and it has been found that person-to-person spread from infected skin sites is even more efficient than from the respiratory tract in causing classical respiratory diphtheria [6]. The carriage of *tox*-positive lysogenic *C. diphtheriae* also poses a risk that non-toxigenic strains, which are regularly found in Austrian residents, could become lysogenised by introduction of such a beta-phage-bearing strain.

In our case, when the Diphtheria Reference Laboratory alerted the treating clinicians and public health authorities on 23 May about the diagnosis of toxigenic *C. diphtheriae*, the patient had already left the hospital on their own initiative and could not be contacted hereafter. We would like to point out that travel and migration to and from countries where diphtheria is still endemic may pose a risk for re-emergence of the disease and therefore public health authorities are well advised to ensure availability of DAT as a WHO essential medicine.

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Conflict of interest

None declared.

Authors' contributions

SH, SH, AF and AI wrote the draft manuscript. VZ and PH performed bacteriological work. HS and RM provided clinical data. All authors corrected and approved the final version.

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