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Note from the editors: MERS-CoV – the quest for the reservoir continues

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Two papers in this last *Eurosurveillance* issue of 2013, one by Reusken et al. [1] and one by Hemida et al. [2], look into the potential animal reservoir for the Middle East Respiratory Syndrome (MERS) coronavirus (CoV). This virus, which emerged in 2012 and was reported for the first time in September, has caused 163 cases and 71 deaths as of 2 December 2013 [3]. However, many questions remain on its origin, reservoir and transmission patterns [4].

The two papers investigate the seroprevalence of antibodies against MERS-CoV and MERS-like CoV in a similar set of domestic livestock, namely camels, cattle, goats, sheep and chicken, in two different geographic hotspots in Jordan and Saudi Arabia, respectively, where the largest described clusters of MERS have occurred to date. The papers complement each other and support the authors' earlier findings that dromedary camels could be a potential reservoir for MERS-CoV [5,6]. The results presented now are compelling evidence that in the studied regions high proportions of dromedary camels are exposed to a MERS-CoV or MERS-like CoV already in their first year of life. Hemida at al. conclude that camels could be infected early in life, and Reusken et al. additionally raise the possibility that the serological reactivity early in life could be due to maternal antibodies.

While the presented studies confirm the potential role of dromedary camels as MERS-CoV reservoir, they do not support a similar role of other common domestic livestock in the affected regions in the Middle East. Neither of the two studies detected antibodies in chicken, cattle or goats. Although most tests in sheep were negative, one particular assay gave positive results in a few animals, and the authors stress that this needs further investigation.

While the papers in today's issue provide further insight into the possible animal reservoir, the primary source of MERS-CoV infections remains unclear and the link to humans needs to be elucidated further as exposure to animals has only been documented for a limited number of human MERS cases. We look forward to seeing more studies in the near future that will shed light on the as yet unknown characteristics of this disease that raised much attention among infectious disease experts in 2013.

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Autochthonous case of dengue in France, October 2013

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In October 2013, autochthonous dengue fever was diagnosed in a laboratory technician in Bouches-du-Rhône, southern France, a department colonised by *Aedes albopictus* since 2010. After ruling out occupational contamination, we identified the likely chain of local vector-borne transmission from which the autochthonous case arose. Though limited, this second occurrence of autochthonous dengue transmission in France highlights that efforts should be continued to rapidly detect dengue virus introduction and prevent its further dissemination in France.

In October 2013, the French National Reference Laboratory for arboviruses (NRL) – hosted at the Institut de Recherche Biomedicale des Armées, Marseille – reported an autochthonous case of dengue fever to the Regional Health Authority of Provence-Alpes-Côte d'Azur. The case lived in the department of Bouches-du-Rhône, France. The national and regional health authorities initiated a multidisciplinary investigation to determine the source of infection of the case and the extent of possible dissemination of dengue virus (DENV).

Case report

On 11 October 2013, a female laboratory technician in her early fifties, residing and working in the area of Aix-en-Provence, Bouches-du-Rhône department, developed sudden fever with incapacitating myalgia, predominantly in her legs. She had not left the department in the 15 days before onset of symptoms. Four days later, she developed a rash on her legs and consulted her general practitioner, who prescribed symptomatic treatment of fever and aches. As the symptoms persisted, she was taken to a hospital's emergency department on two days later. On admission to hospital, she was normotensive, with a body temperature of 38.4 °C. Laboratory analyses showed a normal white blood cell (7,200/mL; norm: 4,000–10,000 /mL) and platelet count (197,000/mL; norm: 150,000–450,000/mL) and an elevated C-reactive protein level (145 mg/L; norm: ‹7.5 mg/L). As her condition had improved, despite the rash having expanded to her arms and back, she was discharged after 24 hours, with a diagnosis of 'probable viral infection'. She consulted a dermatologist three days later, who suspected an arboviral infection and sent blood samples to the NRL.

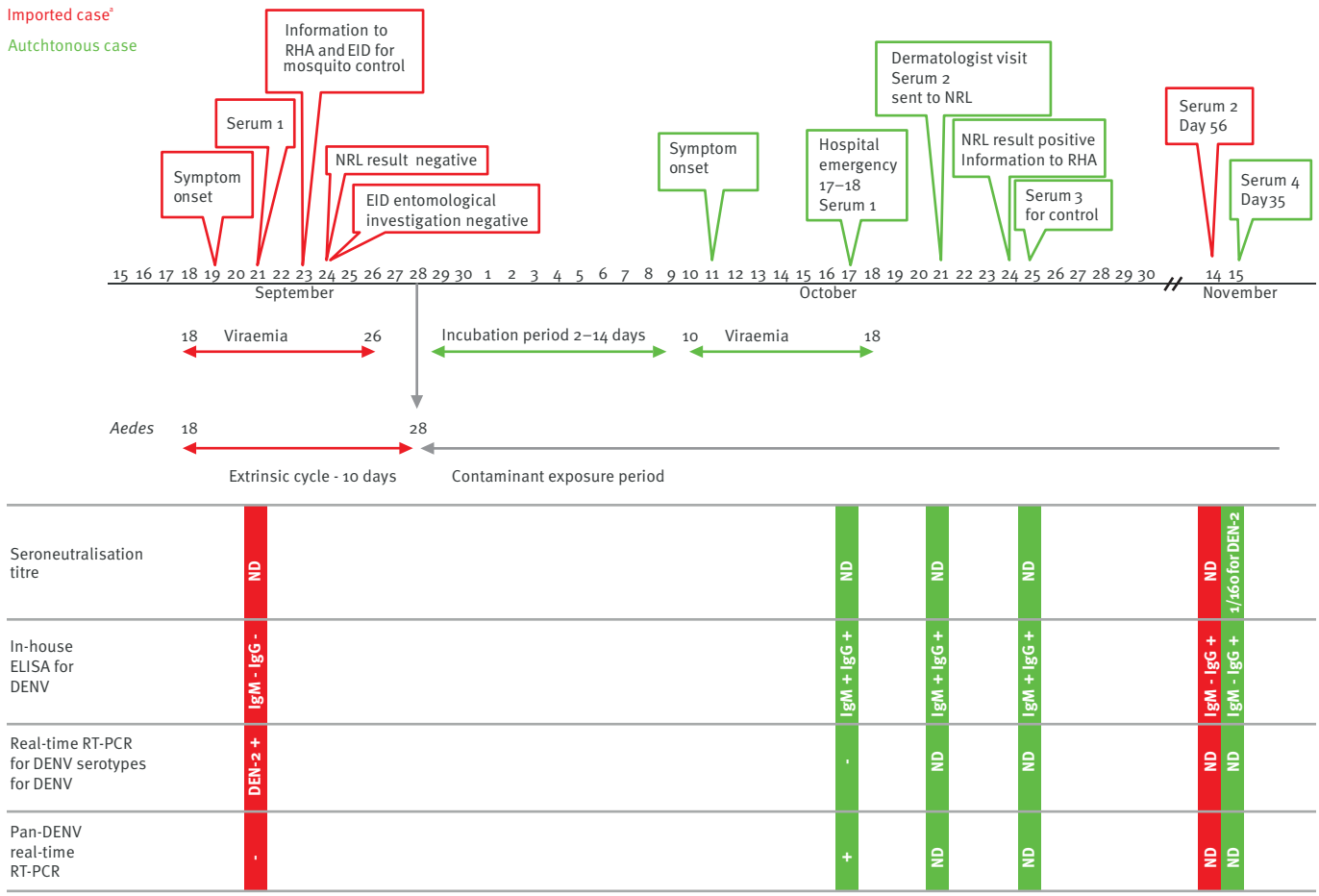
A panel of sera obtained during the acute and convalescent phases (days 6, 10, 14 and 35 after symptom onset) was investigated using in-house real-time reverse transcription polymerase chain reaction (RT-PCR) and serological assays (in-house IgM antibody capture (MAC)-enyzme-linked immunosorbent assay (ELISA) and indirect IgG ELISA) for DENV and West Nile, chikungunya and Toscana viruses (Table). For the first serum sampled on day 6, the real-time RT-PCR for DENV was positive, with a high cycle threshold (Ct) value (indicating a very low viral load), serotyping by real-time RT-PCR was negative, DENV non-structural protein 1 (NS1) detection by rapid diagnostic test (SD Bioline) was negative, but IgM and IgG antibodies against DENV antigens were detected). IgM and IgG antibodies against DENV were also detected in the next two serum samples (on days 10 and 14). For the last serum specimen, sampled on day 35, only DENV-specific IgG antibodies were detected: the specificity of these antibodies was determined by seroneutralisation against DENV serotypes 1 to 4 (DEN1–4) and West Nile virus [1]. For West Nile virus, DEN1, DEN3 and DEN4, the 90% neutralisation titre was ‹1/20. A 90% neutralisation titre of 1/160 against DEN-2 was highly suggestive of an infection of this patient by DEN-2.

Background

Infection with DENV – a member of the family *Flaviviridae*, genus Flavivirus – leading to dengue haemorrhagic fever and shock syndrome, is responsible for substantial morbidity and mortality in populations living in the tropics and among travellers to these

Figure 1

Timeline of epidemiological features and laboratory results of sera from autochthonous and imported case of dengue, Bouches-du-Rhône, France, September–November 2013



DENV: dengue virus; EID: Entente Interdépartementale pour la Démoustification du littoral Méditerranéen; ELISA: enzyme-linked immunosorbent assay; ND: not done; NLR: National Reference Laboratory for arboviruses; RHA: regional health agency ; RT-PCR: reverse transcription polymerase chain reaction.

[a] Initially reported as a suspected case.

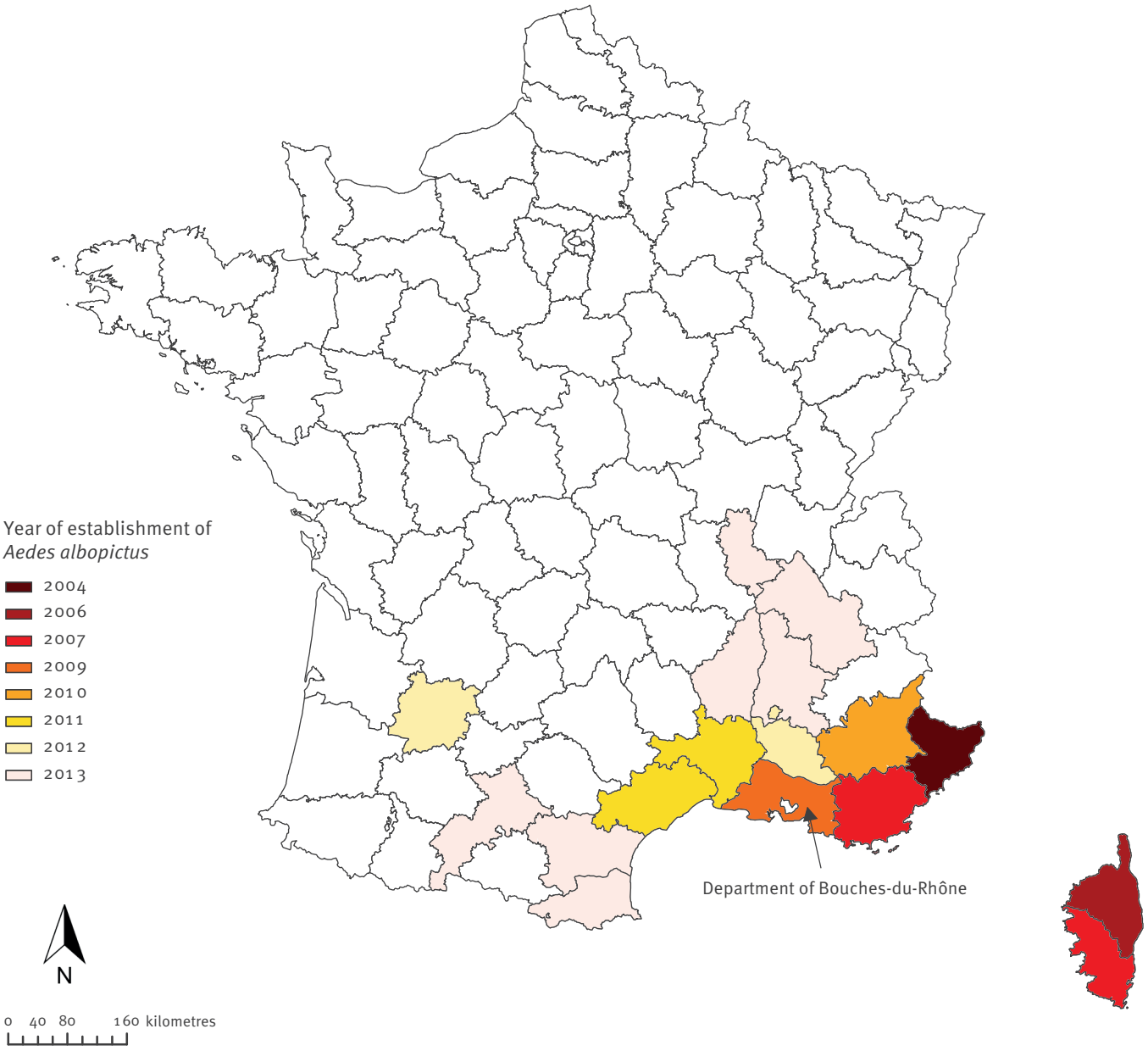
regions [2,3]. The global burden of dengue ranges from an estimated 50–100 million DENV infections worldwide every year according to the World Health Organization [3] to a recent estimate of 390 million DENV infections per year [4]. The virus is considered an emerging threat to Europe because of its recent detection in southern Europe due to the continuous spread of *Aedes (Stegomyia) albopictus (Skuse)*, an invasive mosquito species and well-known vector of chikungunya virus and DENV [5,6]. Public health concern has been heightened since limited foci of local transmission of DENV were reported in September 2010 in Nice, southern France, and Croatia and more recently in 2012 in Madeira, Portugal (where the vector was *Ae. aegypti*) [7-9].

Ae. albopictus was introduced in southern France in 2004, near the Italian border [10]. Since then, it has continuously spread eastwards and northwards and has to date colonised 17 departments (administrative districts) including Bouches-du-Rhône since 2009 (Figure 2) [10,11]. Prevention of the introduction of DENV and chikungunya virus in the departments where *Ae. albopictus* is established falls under a national preparedness and response plan created in 2006 [12]. Implemented each year during the vector activity period, from 1 May to 30 November, the plan is based on enhanced surveillance aiming at the early detection of imported dengue and chikungunya cases.

In mainland France, dengue has been a mandatorily notifiable disease since April 2006 [13]: a case should be reported immediately after laboratory confirmation [12]. In addition, in the areas and period of vector activity, physicians and laboratories are asked to immediately notify suspected imported cases to the local health authorities and to send blood samples directly to the NRL for laboratory confirmation. These actions

Figure 2

Departments colonised by *Aedes albopictus*, France, 2004–2013



Source: IGN-GéoFLA, 1999; French Institute for Public Health Surveillance (Institut de Veille Sanitaire, InVS), 2013.

guide entomological investigations followed by vector control measures when appropriate.

The investigation

As the case could have been exposed to blood during her laboratory work, two hypotheses on the source of infection and mode of DENV transmission were explored: occupational transmission through accidental exposure to blood from a viraemic patient or local vector-borne transmission.

Occupational transmission

Considering an incubation period of 2 to 14 days, in agreement with earlier reports of occupational DENV infection [14-16], we defined the period of likely exposure as 27 September to 8 October. The autochthonous case carried out on average 60 venous punctures a day from patients. She had no recollection of any direct blood exposure during this period. However, she reported not wearing gloves while collecting blood and presented skin excoriations on her fingers. Among patients sampled in her workplace, none had blood taken for dengue fever suspected by a physician. Nevertheless, we screened for DENV all patients sampled in the laboratory during the case's likely period of infection who presented with symptoms of or a history compatible with dengue. The criteria for screening were fever without diagnosis, reported travel in an

area where dengue was reported, leucopenia, thrombocytopenia, hepatic cytolysis, negative viral serology and negative rapid or blood smear tests for malaria. The NRL conducted DENV real-time RT-PCR and serology on the 15 blood samples still available from the 18 patients who met the selection criteria. All tested negative.

Vector-borne transmission

The patient reported no mosquito bites but remembered a sudden pricking sensation compatible with a mosquito bite on the evening of 3 October (eight days before symptom onset), when she was near her workplace in Bouches-du-Rhône. She reported no recent contact with travellers returning from an area with current epidemics or endemic for dengue.

A review of the surveillance database did not identify any case of imported dengue confirmed by the NRL in Bouches-du-Rhône since 1 August 2013. However, one suspected case had been notified in a neighbouring department in a woman who developed fever and a rash on 19 September, five days after returning from the Caribbean island of Guadeloupe, where a dengue outbreak was ongoing [17]. Sera collected on day 2 of her illness tested negative for DENV, West Nile, chikungunya and Saint Louis encephalitis viruses by our in-house real-time RT-PCR and serological assays. An entomological investigation of her residential area and places visited had been carried out on 24 September, before the negative test results were available. Among the places visited, the woman mentioned a short visit, the day before symptom onset, close to (less than 200 metres from) the workplace of the autochthonous case. Although an ovitrap placed nearby this workplace had been found colonised with 43 eggs of *Ae. albopictus* in late September 2013, no evidence of mosquito activity was found during an investigation on 23 September, hence no vector control measures were implemented at that time.

After the detection of the autochthonous case, we retested the serum sample of the suspected imported case by sero-specific real-time RT-PCR for DENV and by rapid diagnostic test for NS1 detection. An additional serum sample was collected on day 56 for serology testing. The NS1 test and the pan-DENV real-time RT-PCR were negative, the real-time RT-PCR for DEN-2 was positive with a high Ct value. In the later serum sample (day 56), only IgG antibodies against DENV were detected. These laboratory findings confirmed an infection with DEN-2 for this patient returning from Guadeloupe (considered the index case).

Control measures

Under the hypothesis of local vector-borne transmission, two places were chosen for identifying primary or secondary cases of DENV infection and for conducting immediate control measures: the autochthonous case's home, where she stayed while viraemic, and her place of work close to which eggs of *Ae. albopictus* had been detected in September.

The local health authorities and vector control operators jointly carried out the following activities in an area of 200 metres around the autochthonous case's home and workplace: door-to-door case finding; any mosquito breeding sites treated by mechanical destruction or larvicide treatment sites; and adulticide sprayings. Physicians and laboratories in the area were asked to report any patients with symptoms compatible with DENV infection since 1 August, including sudden onset of fever (>38.5 °C) and at least one pain symptom, including headache, arthralgia, myalgia, lower back pain or retro-orbital pain. Two suspected cases were identified. Neither tested positive for DENV by real-time RT-PCR or serology.

Discussion

This second report of autochthonous dengue in mainland France follows a cluster of two locally acquired cases in Alpes-Maritimes in 2010 [7]. Because our patient was a laboratory technician who daily collected blood specimens, we not only explored vector-borne local transmission of DENV but also thoroughly investigated potential occupational transmission. The latter hypothesis appears unlikely since, unlike the situation for other occupational dengue cases [14-16], our investigation pinpointed neither a viraemic or infected patient sampled nor any accidental exposure to blood at the laboratory during the likely exposure period of the case.

Several findings are in favour of vector-borne transmission in the Bouches-du-Rhône department. Firstly, our retrospective laboratory confirmation of an imported case of dengue, who had visited the immediate vicinity of the autochthonous case's workplace, while potentially viraemic (one day before symptom onset). Secondly, the 22-day delay between symptom onset of the imported and autochthonous case, which is compatible with the intrinsic (1–14 days) and extrinsic (10 days) incubation period for DENV [18]. Thirdly, the presence of *Ae. albopictus* eggs in the ovitraps in September, indicating the presence of the potential vector. Finally, laboratory confirmation of DENV infection of the same serotype, DEN-2, in both the autochthonous case and the case imported from Guadeloupe. In August to October 2013, DEN-2 was not the prevailing circulating serotype in Guadeloupe, but remained frequent [17].

The virological data presented in this paper on the two human cases of DENV infection do not follow the classical and average kinetics of viraemia and antibody response. The individual host response is known to be variable regarding the viral load in blood, the duration of viraemia and the duration of IgM detection and is also dependent on the DENV responsible for the infection [19,20]. Unfortunately, although viral RNA was detected in the acute phase sample of the autochthonous case,

we were unable to serotype and sequence the amplified product due to a very low viral load. Further comparison of the virus isolates by sequencing is therefore impossible. Two blind passages on Vero and C6/36 cell lines will be carried out to try to isolate the virus from the acute phase sera. NS1 was not detected for these two cases. However, the detection of NS1 is generally less sensitive than viral genome detection by real-time RT-PCR [20,21].

This local transmission of dengue highlights once again that mainland France is subject to overspill of dengue outbreaks, particularly from the French Antilles in the Caribbean. The implemented investigations and control measures were derived from a national plan against dengue and chikungunya that provides a framework for rapid review and exchange of information between epidemiological, entomological, laboratory and medical experts and decision-makers.

The autochthonous case was diagnosed only after a third and specialised medical consultation. We need therefore to further raise the awareness of physicians and laboratories regarding diagnosis of dengue in international travellers and the possibility of autochthonous transmission in areas where *Ae. albopictus* is established. Similarly, we should not discontinue our efforts to inform travellers to areas affected by dengue about individual protection against mosquito bites and early symptoms of dengue.

No further case could be related to this local transmission cycle of dengue in Bouches-du-Rhône. Although precautionary mosquito control was applied, this could very well have been a self-limiting viral dissemination since it occurred shortly before the end of the vector activity period in late November.

Conclusion

Although limited, this autochthonous transmission of DENV in southern France is a clear reminder that local transmission can be triggered in Europe by the introduction of the virus in areas colonised by *Ae. albopictus*, as it occurred already in 2010 in Nice. The French preparedness and response plan, in operation since 2006, proved pivotal to detect and control this threat.

Reducing the risk of local DENV dissemination to zero appears an elusive goal in the context of the continuous spread of Ae albopictus. Coordinated enhanced surveillance and response are therefore the backbone of the prevention of the occurrence of autochthonous cases and the containment of possible outbreaks. Such a plan requires, however, multidisciplinary expertise and resources and should be adapted wisely and regularly to ensure its sustainability and efficiency. In addition, innovative vector control methods and further elucidation of the dynamics of DENV transmission in non-endemic areas are needed to keep Europe safe from dengue.

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Conflict of interest

None declared.

Authors' contributions

All authors contributed to the writing of this manuscript and approved the final version. Elodie Marchand, Caroline Six, Harold Noel, Veronique Vaillant, Marie-Claire Paty drafted the manuscript and contributed to the epidemiological investigation. Caroline Six, Harold Noel designed the protocol for the rapid survey. Thibaut Bergmann, Nicolas Roux, Jeanne Rizzi contributed to the epidemiological investigation. Elisabeth Lafont and Valerie Busso conducted interviews and took part in the clinical management of the patients. Joël Deniau contributed to the epidemiological investigation and managed the national database for enhanced surveillance of dengue and chikungunya. Caroline Six coordinated the investigation at the regional level. Christine Prat, Olivier Flusin, Isabelle Leparc-Goffart were central in the laboratory investigation. Marie-Claire Paty coordinated the investigation at the national level.

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Cluster of Legionnaires' disease cases caused by *Legionella longbeachae* serogroup 1, Scotland, August to September 2013

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We report six confirmed cases of Legionnaires' disease in Scotland caused by *Legionella longbeachae* serogroup 1, identified over a four-week period in August–September 2013. All cases required admission to hospital intensive care facilities. All cases were amateur gardeners with frequent exposure to horticultural growing media throughout their incubation period. *L. longbeachae* was identified in five samples of growing media linked to five cases. Product tracing did not identify a common product or manufacturer.

We describe a cluster of Legionnaires' disease cases caused by *Legionella longbeachae* identified in Scotland between August and September 2013. This was an unprecedented cluster due to the number of cases identified in such a short time period and the geographical proximity of the cases. A national Incident Management Team (IMT) investigated this cluster, focussing on epidemiological, clinical microbiology and environmental issues.

Scotland, with a population of 5.2 million inhabitants, usually records 20–45 cases of Legionnaires' disease per year, of which around 60% are travel-related [1]. Since 2008, a small but increasing number of cases of Legionnaires' disease caused by *L. longbeachae* have been identified in Scotland. This amounts to 18 cases since 2008, with eight cases in 2013, as of 18 November [2, unpublished data for 2013]. This increase has not been mirrored across the rest of the United Kingdom (UK) and Europe. In 2012, a national IMT investigated the 10 cases detected between 2008 and 2012. The investigation focussed on: case ascertainment; case characteristics; growing media production; and a discussion of whether additional public health action was warranted [2]. It identified that the most likely reason for case ascertainment in Scotland but not the rest of the UK, was the testing protocol in use, driven by an active Scottish *Legionella* Reference Laboratory (SHLMPRL) that routinely used a *Legionella* species PCR test and *L. longbeachae* specific serology tests. These tests were not routinely used in the rest of the UK. No differences in production of horticultural growing media in Scotland and the rest of the UK were identified [2].

Investigation of the cluster

Incident management

Following identification of two confirmed (culture positive) cases of *L. longbeachae* and an additional suspected third case (*Legionella* species PCR positive), NHS Lothian (one of the health boards in Scotland) called a Problem Assessment Group on 6 September 2013. Upon confirmation of the third case (by culture), identification of a fourth suspected case and another confirmed case in neighbouring NHS Tayside, a national IMT meeting was held on 12 September 2013. Leadership of the investigation was passed to Health Protection Scotland (HPS), the national organisation for health protection in Scotland.

Information about presentation of illness, details of testing and background information on *L. longbeachae* was provided to local medical services in NHS Lothian and Tayside, including general practice, emergency care, respiratory wards and to the national health helpline (NHS24), following the first IMT meeting. All health boards in Scotland received public health alerts highlighting the situation. Other UK countries also received these alerts and the European Centre for Disease Prevention and Control (ECDC) was informed.

There was significant press interest in this cluster of cases. Reactive media statements were prepared after every IMT meeting. Following press reporting of this

Table 1

Case definitions for Legionnaires' disease cases caused by *Legionella longbeachae* serogroup 1, Scotland, August–September 2013

Confirmed case	• clinical or radiological evidence of community-acquired pneumonia with disease onset on or after 1 August 2013 AND • evidence of having been exposed in Scotland to horticultural growing media (including composted material produced locally or domestically) in the 14 days prior to the onset of symptoms AND • isolation of *Legionella longbeachae* from respiratory secretions
Probable case	• clinical or radiological evidence of community-acquired pneumonia with disease onset on or after 1 August 2013 AND • evidence of having been exposed in Scotland to horticultural growing media (including composted material produced locally or domestically) in the 14 days prior to the onset of symptoms AND • detection of *Legionella* species specific nucleic acid in respiratory secretions (accompanied by a negative urinary antigen test), or a detected rise in *L. longbeachae* serum antibody levels of at least fourfold, or a single high titre of *L. longbeachae* serum antibody
Possible case	• clinical or radiological evidence of community-acquired pneumonia with disease onset on or after 1 August 2013 AND • evidence of having been exposed in Scotland to horticultural growing media (including composted material produced locally or domestically) in the 14 days prior to the onset of symptoms AND • no current microbiological evidence as to the causal agent

cluster, representatives from growing media retailers approached HPS for advice they could provide to customers to reduce risk of illness. Growing media retailers have had increased awareness of Legionnaires' disease from press reporting of cases in Scotland since 2008. A statement was developed for the retail industry describing the low risk of infection and highlighting general gardening hygiene.

Epidemiological investigation

Case definitions are detailed in Table 1. These were adapted from the ECDC case definitions [3] with additional details of time, place and exposure. A 'possible' category was introduced to include those who met clinical and epidemiological criteria whilst test results were awaited. In reality, this 'possible' category was not used as cases were only notified to public health teams following microbiological analysis establishing them as 'confirmed' or 'probable' cases. Cases had a date of onset between 11 August and 10 September (Table 2). Cases were confirmed as *L. longbeachae* infections on average 12 days (range 7–16 days) after the initial diagnosis of community-acquired pneumonia. Due to the relative rarity of this infection, clinicians may not consider Legionnaires' disease until some time into the diagnostic process. This is particularly likely where there is a negative *Legionella* urinary antigen test result, which is often used by clinicians to exclude Legionnaires' disease. Cases were within 130 km of each other, in two neighbouring health board regions.

All confirmed cases were interviewed by nurses in the health board's health protection team, using a standard questionnaire. This sought details on clinical presentation and testing, travel history, recent hospitalisation, possible water aerosol exposures, possible horticultural growing media exposure, gardening activities. In the first instance, partners and relatives were interviewed, as the cases themselves were too unwell to be interviewed.

All cases had severe community-acquired pneumonia and were all admitted to intensive care facilities. They remained in hospital for an average of 22 days (variation 11–43 days). The cases comprised of three females and three males with a mean age of 70 years (range 55–84 years). Five out of six cases had health problems contributing to underlying immunosuppression. Five out of six cases were active or ex-tobacco smokers. All cases had lived at home throughout their exposure period of 14 days and had not undertaken any activities outside their usual activity pattern. Five out of six cases were keen amateur gardeners who had regular exposure to horticultural growing media during the incubation period of their illness. One case did not describe any clear exposure to growing media. No other relevant exposures were identified for these cases.

In addition to the six confirmed cases of Legionnaires' disease there was one case of probable Legionnaires' disease in a keen gardener with frequent exposure to growing media during the incubation period of their illness. This patient had a moderately high titre of 1:128 to *L. longbeachae* which reverted to negative on follow up testing; no acute serum sample or sputum was available for testing. This case was clinically less severe than the other cases and was managed in the community. The patient was younger than the other cases and did not have any underlying morbidity. This case was detected some time after the six confirmed cases, but had an estimated date of onset within the four-week period between August and September. This case was diagnosed retrospectively, following treatment for pneumonia and it is likely that detection was

Table 2

Summary of clinical microbiological testing for the six cases of Legionnaires' disease caused by *Legionella longbeachae* serogroup 1, Scotland, August–September 2013

Case	Date of onset of illness (2013)	Urinary antigen test	PCR		Culture	Serology (*Legionella longbeachae* specific antibody response)	Organism
			Legionella pneumophila	*Legionella* species			
1	11 August	negative	negative	positive	positive	four-fold rise	*Legionella longbeachae* serogroup 1
2	15 August	negative	negative	positive	positive	four-fold rise	*Legionella longbeachae* serogroup 1
3	24 August	negative	negative	positive	positive	four-fold rise	*Legionella longbeachae* serogroup 1
4	27 August	negative	negative	positive	positive	single high titre	*Legionella longbeachae* serogroup 1
5	28 August	negative	negative	–	positive	–	*Legionella longbeachae* serogroup 1
6	10 September	negative	negative	positive	positive	single moderate titre	*Legionella longbeachae* serogroup 1

–: test not performed

due to information about this cluster being circulated to general practice physicians.

Clinical microbiological findings

Cases 1–4 were identified by the local clinical diagnostic laboratory in NHS Lothian, which had implemented testing of severe community-acquired pneumonia lower respiratory tract samples by both L. pneumophila and *Legionella* species PCR in 2010. *Legionella* species PCR positive samples were referred to SHLMPRL for confirmation and culture. All isolates were *L. longbeachae* serogroup 1. Cases 5 and 6 were identified in NHS Tayside shortly after the first Lothian cases. Sputum/bronchial alveolar lavage samples were cultured in the local diagnostic laboratory and *Legionella* colonies were isolated. These isolates were identified as *L. longbeachae* serogroup 1. This local diagnostic laboratory did not routinely use a *Legionella* species PCR test. All patient isolates were genotyped by amplified fragment length polymorphism (AFLP) at SHLMPRL. Testing results are summarised in Table 2.

Environmental investigation

The environmental investigation focussed on three main areas:

1. identifying specific gardening activities and exposures which may be considered as a high risk;
2. establishing sources through microbiological testing;
3. tracing supply chain and manufacture of potential sources.

There was no common theme in gardening activities and no particular single gardening activity or garden exposure was common amongst the cases, other than use of recently purchased shop-bought growing media. Samples of any remaining growing media were taken for microbiological testing.

In five out of six cases, the use of growing media was investigated. Shop-bought growing media was of a range of brands bought in different premises. All had been stored at home inside, either in the cases' house or greenhouse/polytunnel/garden shed/garage. Using the barcodes on the bags of growing media, batch number and manufacturer details were obtained. There was no common manufacturing site; manufacturing sites were located in England, Scotland and Northern Ireland. Of those growing media produced at the same manufacturing site, there was no common batch number. In addition, there was no common supplier of composted material to these manufacturing sites. All of these growing media contained composted green material and four out of five contained peat.

Microbiological testing of bagged growing media and other garden samples obtained from the cases' homes, detected *L. longbeachae* serogroup 1 in five out of 11 samples tested, resulting in positive growing media samples linked to five cases. In all cases, the same AFLP DNA profile was found in the patient isolate and the implicated growing media isolate. Each patient and growing media isolate was identified as one of three circulating AFLP types regularly identified in Scotland.

The genetic similarity or diversity of all the strains is currently being analysed by whole genome sequencing. Given the match in organism and exposure during the incubation period, it is highly likely that these growing media were the source of infection for these cases. Further work is ongoing looking at further genetic analysis and comparison of the *L. longbeachae* clinical and environmental isolates, using whole genome sequencing.

Discussion

Detection of *L. longbeachae* infection is unusual in Europe with 43 cases reported to the European Surveillance System (Tessy) between 2005 and 2012 (personal communication, Encarna Gimenez, September 2013). Diagnosis of Legionnaires' disease in the European Union relies heavily on urinary antigen testing that does not detect *L. longbeachae*. Culture has always been the gold standard for the definitive diagnosis of Legionnaires' disease [3]. However, *Legionella* culture requires specific laboratory media and expertise; it may miss cases that would be detected by PCR [4] and results may not be available in a timely manner to allow effective clinical and public health action. Detection of the four Lothian cases relied on the local diagnostic service using a *Legionella* species PCR test, a test which, to our knowledge, no other diagnostic laboratory in the UK uses. In addition to culture and PCR, a four-fold change in titre to a *L. longbeachae* specific antibody was seen in the three cases from whom sufficient samples were taken. Serological diagnosis of Legionnaires' disease for serogroups and species other than L. pneumophila serogroup 1 has never been fully validated because of the rarity of these infections. However, in cases of severe Legionnaires' disease, a *Legionella* species-specific positive PCR and a greater than four-fold rise in titre to a particular *Legionella* species, have helped to verify the causative organism. These data support the growing suspicion that *L. longbeachae* infection is under-ascertained in Scotland and probably across Europe. Following detection of this cluster, Public Health England has implemented a *Legionella* species PCR test in the National Reference Laboratory for a trial period. Assessment of this should take into account the possible seasonal nature of *L. longbeachae* infections, coinciding with seasonal use of growing media. It is also recommended that national surveillance units consider ways of raising awareness amongst frontline clinical staff, to consider the diagnosis of Legionnaires' disease (including non-pneumophila species infections) in those with community-acquired pneumonia.

L. longbeachae infection accounts for approximately half of all cases of Legionnaires' disease in Australia [5] and New Zealand [6], where growing media is peat-free and a major component is composted green material such as pine woodchip and bark. Predominantly peat-based growing media is used in Europe and this is likely to be a low risk for *L. longbeachae* contamination. In the UK, there is political pressure and legislation in place to reduce the volume of peat used in growing media to preserve peat stocks [7]. As peat is phased out, the volume of composted green material in growing media will increase. It is therefore likely that we will see increasing numbers of cases of Legionnaires' disease caused by *L. longbeachae* infection, providing that we can detect them.

No single source of contaminated growing media could be identified in this cluster, which supports research findings that *L. longbeachae* is ubiquitous in soils and growing media [8,9]. Growing media manufactured in the UK for retail is required to meet manufacturing standards (PAS 100 [10]) which includes some microbiological testing, but not testing for *Legionella* bacteria. As in many other infections, a combination of infectious dose, mode of infection and host susceptibility is likely to influence outcome. It is not clear whether clinical infection follows inhalation of a particular infectious dose of bacteria, or at which stage in the manufacturing and/or retail process a bacterial load might be reached which poses a risk to susceptible individuals. It is possible that higher concentrations of *Legionella* bacteria develop during storage of growing media prior to use. All of the cases in this cluster with exposure to growing media had stored bags of growing media in their home or in sheds or other enclosed spaces. It is possible that the unusually warm summer in Scotland in 2013 caused a rise in temperature of the growing media during the day whilst being protected from cool nights indoors, providing opportunity for the *Legionella* to grow and resulting in a higher than usual concentration of these organisms in the product.

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Conflict of interest

None declared.

Authors' contributions

All authors contributed to the writing of this manuscript and approved the final version.

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Middle East Respiratory Syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013

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Between June and September 2013, sera from 11 dromedary camels, 150 goats, 126 sheep and 91 cows were collected in Jordan, where the first human Middle-East respiratory syndrome (MERS) cluster appeared in 2012. All sera were tested for MERS-coronavirus (MERS-CoV) specific antibodies by protein microarray with confirmation by virus neutralisation. Neutralising antibodies were found in all camel sera while sera from goats and cattle tested negative. Although six sheep sera reacted with MERS-CoV antigen, neutralising antibodies were not detected.

In the period between June and September 2013, sera from 11 dromedary camels, 150 goats, 126 sheep and 91 cows were collected predominantly in the al Zarqa governorate, Jordan, where the first human Middle-East respiratory syndrome (MERS) cluster appeared in April 2012 [1]. All sera were tested for the presence of MERS-coronavirus (MERS-CoV) specific antibodies by protein microarray with confirmation by virus neutralisation. Neutralising antibodies to MERS-CoV were found in all sera from dromedary camels while the sera from goats and cattle tested negative. Although six of 126 sheep sera reacted with the MERS-CoV antigen, neutralising antibodies were not detected. The reactivity of sheep sera from this region observed in the microarray warrants further study.

Background

In 2012 MERS-CoV was identified in patients with severe respiratory disease in the Middle East. As of 2 December 2013, a total of 163 laboratory-confirmed cases including 70 deaths have been reported to the World Health Organization (WHO) [2]. All cases reported to date were linked to Jordan, Kuwait, Oman, Qatar, Saudi Arabia (SA) or the United Arab Emirates (UAE). Human to human transmission has been observed in healthcare and family settings [3]. Various studies indicate that the observed MERS-CoV diversity in humans results from multiple independent introductions in the human population in the Middle East [5-7] and the number of these sporadic, primary infections is still increasing [2]. The animal reservoir(s) for MERS-CoV are still unknown but serological studies demonstrated that dromedary camels in the Canary Islands, Egypt and Oman have been infected with MERS-CoV or MERS-related-CoV [8,9]. Of these countries, human cases have only been detected in Oman [2].

Data provided by the Food and Agriculture Organization of the United Nations (FAO) from 2012 show that cows, dromedary camels, goats and sheep are the main sources of meat and milk in the affected countries [4]. In addition, in Saudi Arabia, where the majority of MERS cases have been reported, roughly one sheep is sacrificed for each pilgrim or one camel for seven pilgrims in the Hajj (yearly Muslim pilgrimage to Mecca), which can amount up to the slaughter and worldwide distribution of meat of around three million Middle-Eastern sheep and camels, based on the pilgrim numbers reported for 2011 and 2012 [10]. The continued occurrence of human MERS cases, the presence of neutralising antibodies in camels and the extensive animal exposure (including animal products) of humans warrant extensive studies in livestock aimed at identifying the possible reservoir of MERS-CoV.

Figure 1

Animal sampling locations for the MERS-CoV serological study, relative to the location where MERS-CoV human cases were identified in April 2012, Jordan, June–September 2013

MERS-CoV: Middle East Respiratory Syndrome coronavirus.
A represents Al-Zarqa, the city were the first human Middle-East respiratory syndrome cases were identified in April 2012.
B-F are locations where animal sampling took place between June and September 2013.

Source: map adapted from: http://d-maps.com/carte.php?num_car=5402&lang=en

Table 1

Characteristics of animals included in the Middle East Respiratory Syndrome (MERS) coronavirus serological study, Jordan, June–September 2013 (n= 378 animals)

Animals (total numbers)	Location[a,b]	Number	Sex	Age
Dromedary camels (n=11)	E[c], outdoors	11	M	3–14 months
Sheep of Awassi breed (n=126)	E[c], indoors	20	F	› 2 years
	C, outdoors	53	F + M	All ages[d]
	D, outdoors	27	F + M	› 2 years
	B, indoors	26	F + M	All ages[d]
Cows (n=91)	C, indoors	35	F	Unknown
	F, indoors	56	F	Unknown
Goats of local breed (n=150)	D, indoors	10	F + M	Unknown
	C, unknown	91	F + M	Unknown
	B, unknown	49	F + M	Unknown

M: male; F: female.

[a] The letters B, C, D, E, and F refer to locations indicated in Figure 1.

[b] If known, it is indicated whether the animals were kept indoors or outdoors.

[c] The respective locations of the sheep and camels were 5 km apart and there was no contact between the sheep and camels.

[d] Females were older than 2 years and males were younger than 6 months.

In April 2012, an outbreak of acute respiratory illness occurred in an intensive care unit in a public hospital in Zarqa city, Zarqa governorate, Jordan. Retrospective testing identified MERS-CoV as the confirmed and probable causative agent of two and 11 patients respectively. Ten people in the outbreak were healthcare workers (HCW). The two confirmed cases, a HCW and an admitted patient, died [1]. Although epidemiological investigations identified limited nosocomial transmission, the primary source for MERS-CoV transmission to humans was not identified.

Middle East Respiratory Syndrome coronavirus serological study in livestock

Between June and September 2013, sera as well as faecal swabs from 11 dromedary camels, 150 goats, 126 sheep and 91 cows were collected predominantly in the al Zarqa governorate, Jordan (Table 1, Figure 1). All sera were tested for the presence of IgG antibodies reactive with MERS-CoV, Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), and human coronavirus OC43 (HCoV-OC43) S1 antigens exactly as described before [9,11]. HCoV-OC43 is serologically closely related to bovine coronavirus (BCoV) and used as a proxy to detect antibodies against BCoVs that are commonly circulating in ungulates [9].

All 11 dromedary camel sera and six of 126 sheep sera had antibodies against the MERS-CoV S1 antigen while there was no reactivity in goat and cow sera. Four of 11 dromedary camels, 23/91 cows, 128/150 goats and all sheep reacted with HCoV-OC43 antigen. None of the sera bound to SARS-CoV antigen (Figure 2).

For confirmation, all camel and sheep sera (n= 137) were tested in a MERS-CoV neutralisation assay, exactly as described before [9]. All camel sera had MERS-CoV neutralising antibodies with titres varying between 1:20 and 1:80, while no neutralising antibodies were detected in the sheep sera (Table 2 and data not shown). As coronavirus serology is potentially complicated due to the general circulation of BCoVs in these four livestock species (cross-reactivity needs to be excluded), a comparative plaque reduction neutralisation test (PRNT) for MERS-CoV and BCoV was performed on all camels sera and a subset of goat, sheep and cow sera, exactly as described before [9] (Table 2). All camel sera inhibited MERS-CoV plaque formation with titres varying between 1:40 and 1:80, while again no inhibition was observed with the selection of sheep sera. Four of the 11 camel sera also inhibited BCoV plaque formation with titres between 1:160 and 1:320, confirming the microarray results for these samples. BCoV neutralising titres varied between 1:40 and 1:160 for the selection of sheep sera, between 1:40 and 1:320 for the subset of goat sera and between 1:40 and 1:›1,280 for the subset of bovine sera.

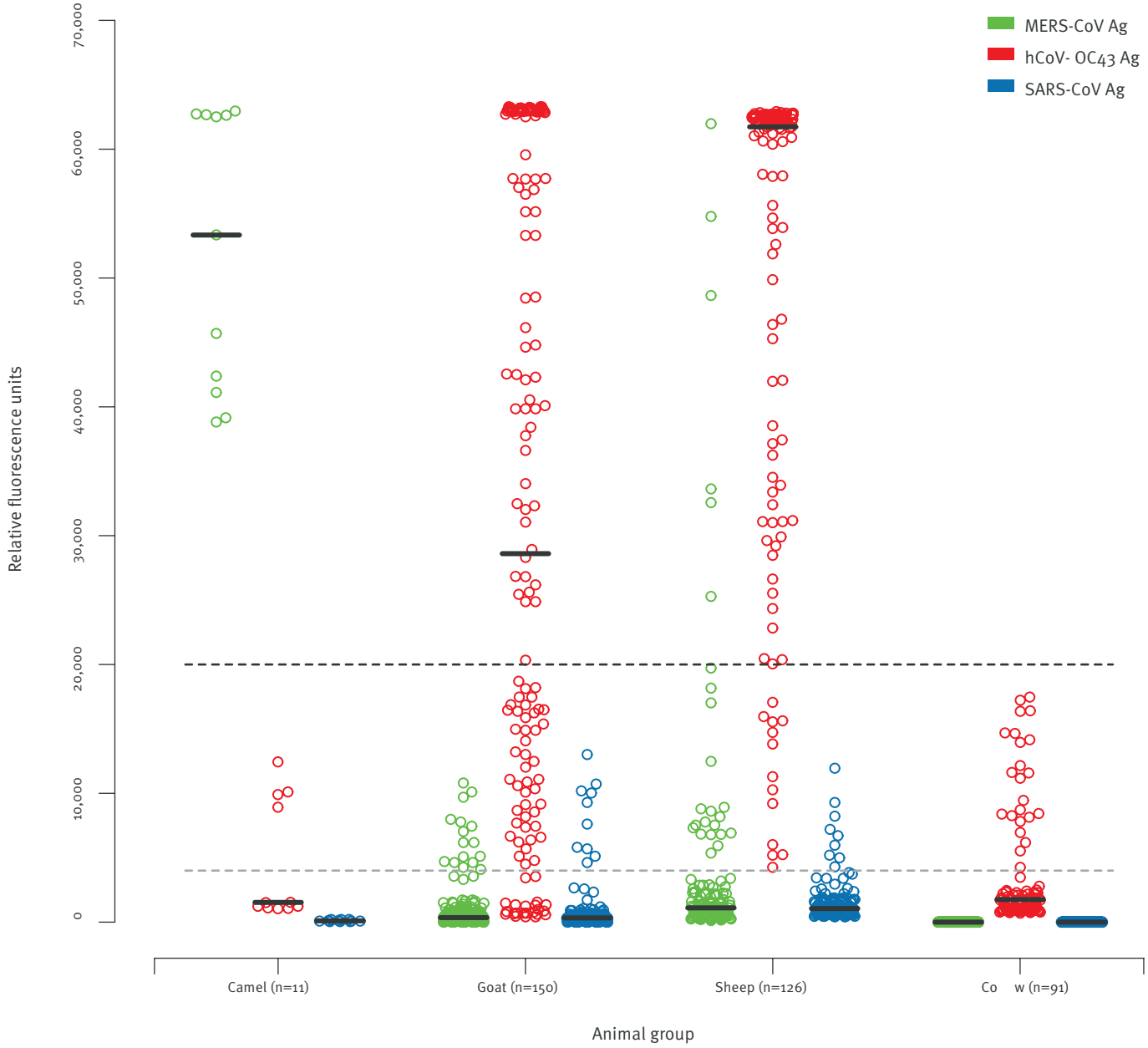
Faecal samples of camels and sheep were analysed for identification of viral sequences using pancoronavirus and specific polymerase chain reaction (PCR) methods [9]. Three BCoV sequences but no MERS-CoV or MERS-related CoV sequences were obtained from sheep rectal swabs.

Discussion

Here, we describe a serological study in various livestock species (n= 378) of economic importance in a

Figure 2

Reactivity of livestock sera (n= 378) from Jordan with three coronavirus S1 antigens



Ag: antigen; MERS-CoV: Middle East Respiratory Syndrome coronavirus; hCoV-OC43: human coronavirus OC43; SARS-CoV: Severe Acute Respiratory Syndrome coronavirus.

Column scatterplot of relative fluorescent intensities per antigen (y-axis) measured by protein microarray for dromedary camel (n=11), goat (n=150), sheep (n=126) and cow (n=91) sera from Jordan at serum dilution 1:20.

Black lines indicate medians. Dashed black line is cutoff of the assay for MERS-CoV. Dashed grey line is cutoff of the assay for HCoV-OC43.

Table 2

Results of neutralising assays for Middle East Respiratory Syndrome coronavirus and bovine coronavirus, serological study in livestock, Jordan, June–September 2013

	Number of serum samples	Positive MERS-CoV neutralisation titre[a] n (titres)	Positive BCoV neutralisation titre[b] n (titres)
Dromedary camels n= 11			
MERS-CoV antigen array signal (RFU)			
‹10,000	0	0 (NS)	0 (NS)
10,000–20,000	0	0 (NS)	0 (NS)
20,000–30,000	0	0 (NS)	0 (NS)
30,000–40,000	2	2 (1:20 to 1:40)	0 (NS)
›40,000	9	9 (1:20 to 1:80)	4 (1:160 to 1:320)
hCoV-OC43 antigen array signal (RFU)			
‹10,000	9	9 (1:20 to 1:80)	2 (1:320)
10,000–20,000	2	2 (1:80)	2 (1:160)
20,000–30,000	0	0 (NS)	0 (NS)
30,000–40,000	0	0 (NS)	0 (NS)
›40,000	0	0 (NS)	0 (NS)
Sheep n= 10			
MERS-CoV antigen array signal (RFU)			
‹10,000	4	0 (NS)	3 (1:80 to 1:160)
10,000–20,000	0	0 (NS)	0 (NS)
20,000–30,000	1	0 (NS)	1 (1:40)
30,000–40,000	2	0 (NS)	2 (1:40 to 1:160)
›40,000	3	0 (NS)	2 (1:40 to 1:80)
hCoV-OC43 antigen array signal (RFU)			
‹10,000	1	0 (NS)	0 (NS)
10,000–20,000	0	0 (NS)	0 (NS)
20,000–30,000	1	0 (NS)	0 (NS)
30,000–40,000	2	0 (NS)	2 (1:40)
›40,000	6	0 (NS)	6 (1:40 to 1:160)
Goat n= 8			
MERS-CoV antigen array signal (RFU)			
‹ 10,000	7	0 (NS)	3 (1:40 to 1:320)
10,000–20,000	1	0 (NS)	1 (1:160)
20,000–30,000	0	0 (NS)	0 (NS)
30,000– 40,000	0	0 (NS)	0 (NS)
›40,000	0	0 (NS)	0 (NS)
hCoV-OC43 antigen array signal (RFU)			
‹10,000	3	0 (NS)	0 (NS)
10,000–20,000	0	0 (NS)	0 (NS)
20,000–30,000	0	0 (NS)	0 (NS)
30,000–40,000	1	0 (NS)	0 (NS)
›40,000	4	0 (NS)	4 (1:40 to 1:320)
Bovine n= 7			
MERS-CoV antigen array signal (RFU)			
‹10,000	7	0 (NS)	7 (1:80 to 1: ›1,280)
10,000–20,000	0	0 (NS)	0 (NS)
20,000–30,000	0	0 (NS)	0 (NS)
30,000–40,000	0	0 (NS)	0 (NS)
›40,000	0	0 (NS)	0 (NS)
hCoV-OC43 antigen array signal (RFU)			
‹10,000	4	0 (NS)	4 (1:80 to 1:160)
10,000–20,000	3	0 (NS)	3 (1:160 tp 1: ›1,280)
20,000–30,000	0	0 (NS)	0 (NS)
30,000–40,000	0	0 (NS)	0 (NS)
›40,000	0	0 (NS)	0 (NS)

BCoV: bovine coronavirus; hCoV-OC43: human coronavirus OC43; MERS-CoV: Middle East Respiratory Syndrome virus; NS: not shown; PRNT: plaque reduction neutralisation test; RFU: relative fluorescence units.

[a] based on both microneutralisation with starting dilution 1:10 and PRNT with starting dilution 1:40.

[b] based on PRNT with starting dilution 1:40.

region in Jordan where a cluster of human MERS cases occurred.

No evidence for the presence of antibodies directed against MERS-CoV was found in 91 cattle and 150 goat sera. MERS-CoV neutralising antibodies were found in all 11 dromedary camel sera. Circulation of BCoV in dromedary camels is known but cross-neutralisation between MERS-CoV (a lineage C beta-coronavirus) and BCoV (a lineage A beta-coronavirus) or other CoVs, including SARS-CoV (a lineage B betacoronavirus), has been conclusively excluded in previous studies and was illustrated again in this study in the comparative PRNTs [8,9]. These observations indicate that MERS-CoV or a highly related virus circulated in dromedary camels in a region where transmission to humans occurs.

The neutralisation titres observed in the Jordan camel sera were lower than observed with sera from Oman but in the same range as those observed on the Canary Islands [9]. The dromedary camels in this study were calves, only three to 14 months of age, and these low titres might reflect the presence of waning maternal antibodies. However, maternal antibodies in dromedary camels reportedly decline rapidly two to five weeks after birth [12] and much higher antibody titres against MERS-CoV were observed in adult dromedary camels (older than four years of age) in the Middle-East region and the Horn of Africa [8,9] (and data not shown). Therefore an alternative explanation could be that the camels had just been infected and antibody titres were still rising.

Interestingly, six sheep sera reacted with MERS-CoV S1 antigen on the array while previous validation experiments using sheep sera from the Netherlands showed no reactivity [9]. This previous study included HCoV-OC43 S1-reactive and non-reactive sheep sera emphasising that there should not be any cross-reactivity between the MERS-CoV antigen and BCoV-specific antibodies due to BCoV circulation. However, none of the 126 sheep sera showed MERS-CoV neutralising activity. The applied sheep sera were highly haemolytic which may have caused some assay interferences.

Our observation strengthens our earlier study in which MERS-CoV neutralising antibodies were found in dromedary camels in Oman where human cases have been reported as well [2]. Until the virus that elicits these antibodies in camels is detected, sequenced and compared to the viruses sequenced from human patients, it remains unclear whether this livestock species is indeed infected with MERS-CoV and thus represents an immediate source for human infection. However, our observations should be used to focus virological and serological studies in livestock, especially dromedary camels and sheep, and including humans handling these animals and their products.

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Conflict of interest

None declared.

Authors' contributions

CR: coordinated the study, assisted in designing the study, analysed data, wrote manuscript. MA: sample collection, assisted in designing the study, drawing figure 1, read and revised manuscript. VSR: performed laboratory testing, analysed data, read and revised manuscript. BM: performed laboratory testing, analysed data, read and revised manuscript. AE: sample collection, read and revised manuscript. SA: sample collection, read and revised manuscript. GJG: performed laboratory testing, analysed data, read and revised manuscript. TMB: performed laboratory testing, analysed data, read and revised manuscript. IZ: performed laboratory testing, read and revised manuscript. MAM: data analysis, read and revised manuscript. BJB: produced design antigen, provided antigens, read and revised the manuscript. PJR: read and revised the manuscript. AO: read and revised the manuscript. CD: read and revised the manuscript. BH: assisted in designing the study, analysed data, read and revised manuscript. MK: assisted in designing the study, analysed data, read and revised the manuscript.

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Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013

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In Saudi Arabia, including regions of Riyadh and Al Ahsa, pseudoparticle neutralisation (ppNT) and microneutralisation (MNT) tests detected no antibodies to Middle East Respiratory Syndrome coronavirus (MERS-CoV) in sheep (n= 100), goats (n= 45), cattle (n= 50) and chickens (n= 240). Dromedary camels however, had a high prevalence of MERS-CoV antibodies. Bovine coronavirus (BCoV) infected sera from cattle had no cross-reactivity in MERS-CoV ppNT or MNT, while many dromedary camels' sera reacted to both BCoV and MERS-CoV. Some nevertheless displayed specific serologic reaction profiles to MERS-CoV.

In a seroepidemiological study of domestic livestock (sheep, goats, cattle, chicken) and dromedary camels from Saudi Arabia, we find that only dromedary camels have evidence of seropositivity to Middle East Respiratory Syndrome coronavirus (MERS-CoV), suggesting an infection with a MERS-CoV-like virus. Although some dromedary camels examined had specific serologic reaction profiles to MERS-CoV, many had sera displaying reactivity to both MERS-CoV and bovine coronavirus (BCoV) and the sera appear to have an unusually broad pattern of cross-reactivity for these related viruses. This needs to be considered when interpreting seroepidemiological data by carrying out parallel microneutralisation tests for both MERS CoV and BCoV.

Middle East Respiratory Syndrome coronavirus

MERS coronavirus was recognised as a cause of severe human respiratory disease in 2012 [1]. As of 22 November 2013, 157 laboratory-confirmed, and 19 other probable cases of MERS have been reported, 69 of these being fatal [2]. Sporadic or index cases account for approximately 40% of the cases and have occurred in Jordan, Kuwait, Oman, Qatar, Saudi Arabia and United Arab Emirates. Imported cases have been reported in France, Germany, Italy, Spain, Tunisia and United Kingdom, with secondary transmission documented in some of these cases [2,3]. Human-to-human transmission has occasionally been observed, the largest cluster of such secondary transmission being reported from Al Ahsa, Saudi Arabia [4]. Phylogenetic analysis suggests considerable diversity within MERS-CoV sequences analysed to date, with two distinct clades of virus being noted [5].

Index cases of MERS appear to be zoonotic in origin, with contact with domestic livestock (e.g. camels, sheep) being reported in some of these patients [3]. But neither the proximate animal source of human infection nor the natural reservoir of the virus is known. Closely related, but not identical viruses have been found in species of insectivorous bats [6]. There is a preliminary report of a short fragment of virus genome almost identical to MERS-CoV reportedly found in Egyptian tomb bats [7].

Two recent studies from Oman and Egypt respectively, two Middle-Eastern countries adjacent to affected ones, reported high rates of MERS-CoV seroprevalence in dromedary camels [8,9]. However, there have been no seroepidemiological data of domestic livestock, camels or wildlife from affected countries, to date.

Figure

Locations where serum samples were collected for the Middle East Respiratory Syndrome (MERS) coronavirus seroepidemiological study in domestic livestock, Saudi Arabia, 2010–2013

Seroepidemiological study

Serum samples were collected from dromedary camels (n= 310), sheep (n= 100), goats (n= 45), cattle (n= 50) and chicken (n= 240) from Riyadh, Al Ahsa and other regions of Saudi Arabia (Figure).

The MERS-CoV serology methods used have been described in a previous publication [9]. The sera were heat inactivated at 56 ºC for 30 minutes and screened for antibody to MERS-CoV at a serum dilution of 1:20 in a MERS-CoV pseudoparticle neutralisation test (ppNT) [9]. Camel sera (n=56) that were seropositive at a screening dilution of 1:20 in the ppNT assay were randomly selected, representing each age group, less than one year (n=15), one to three years (n=12), four to five years (n=16) and older than five years (n=13), and titrated to end point in the MERS-CoV ppNT, MERS-CoV microneutralisation test (MNT) and bovine coronavirus (BCoV) MNT.

The MNTs were done using MERS-CoV (strain: EMC) obtained from Dr R Fouchier, Erasmus MC, Rotterdam and bovine coronavirus (ATCC BRCV-OK-0514-2). The MNTs were done on Vero cells (ATCC CCL-81) for MERS-CoV and HRT-18G cells (obtained from ATCC) for BCoV, respectively. Serial two-fold dilutions of heat-inactivated sera (56 ºC for 30 minutes) were mixed with equal volumes of 200 tissue culture infective dose $(TCID)_{50}$ of virus and incubated for one hour at 37 ºC. The virus–serum mixture was then added in quadruplicate to cell monolayers in 96-well microtitre plates. After one hour of adsorption, the virus-serum mixture

Table 1

Location and time of sampling of animals chosen for MERS-CoV ppNT and proportion of serum samples positive at 1:20 dilution, Saudi Arabia, 2010–2013 (n=745 samples)

Animals	Location (number of animals)	Collection period	Serum samples collected N	Sera positive in MERS-CoV ppNT N (%)
Dromedary camels	Al-Ahsa (120), Riyadh (190)	2012–2013	310	280 (90)
Sheep	Al-Ahsa (100)	2012–2013	100	0 (0)
Goats	Al-Ahsa (15), Taif (10), Madinah (10), Qatif (10)	2010–2012	45	0 (0)
Chicken	Al-Ahsa (120), Dammam and Alkhober (80), Abqaiq (40)	2012–2013	240	0 (0)
Cattle	Al-Ahsa (17), Taif (13), Madinah (10), Qatif (10)	2010–2013	50	0 (0)

MERS-CoV ppNT: Middle East Respiratory Syndrome pseudoparticle neutralisation test.

Riyadh province is in central Saudi Arabia; Al Ahsa, Quatif, Dammam, Alkhober and Abqaiq are in the eastern part of Saudi Arabia; Madinah and Taif are in the western part of Saudi Arabia (Figure).

was removed and 150µl of fresh culture medium was added to each well and the plates incubated at 37 ºC in 5% CO_2 in a humidified incubator. A virus back-titration was performed without immune serum to assess input virus dose.

Cytopathic effect (CPE) was read at three days post infection for MERS-CoV and four days post infection for BCoV. The highest serum dilution that completely protected the cells from CPE in half of the wells was defined as the neutralising antibody titre. Positive and negative control sera were included in each assay.

As positive controls, we used a convalescent serum from a human patient with MERS kindly provided by Dr C Drosten, Institute of Virology, University of Bonn Medical Centre, Bonn (MERS-CoV ppNT 1:160); and sera from two experimentally infected macaques (ppNT titres 1:40) and a non-infected control macaque (ppNT ‹1:20) kindly provided by Bart Haagmans, Erasmus MC as reported previously [9]. Additional positive and negative controls used were sera from dromedary camels from Egypt previously found to be seropositive (ppNT 1:320) and sero-negative (ppNT‹1:20) [9]. Antisera to BCoV from an experimentally inoculated gnotobiotic calf and guinea pig were provided by Dr. Linda Saif.

Results

Sera that were positive in the MERS-CoV ppNT test at a screening dilution of 1:20 are shown in Table 1.

A gnotobiotic calf and guinea pig B CoV antisera that had a homologous reaction titre of ≥1:1,280 and 1:80 respectively, did not cross-neutralise MERS-CoV in either the MERS-CoV ppNT or MNT. None of the sheep, goat, cattle or chicken sera had any MERS-CoV ppNT

Table 2

Stratification by age and location of dromedary camels testing positive in the MERS-CoV ppNT assay at a serum dilution of 1:20, Saudi Arabia, 2012–2013 (n=310 animals)

Age group (years)	Riyadh		Al Ahsa		Overall	
	Animals tested N	Animals testing positive N (%)	Animals tested N	Animals testing positive N (%)	Animals tested N	Animals testing positive N (%)
‹1	21	18 (86)	44	29 (66)	65	47 (72)
1–3	55	52 (94)	51	49 (96)	106	101 (95)
4–5	52	51 (98)	24	23 (96)	76	74 (97)
›5	62	57 (92)	1	1 (100)	63	58 (92)
Total	190	178 (94)	120	102 (85)	310	280 (90)

MERS-CoV ppNT: Middle East Respiratory Syndrome pseudoparticle neutralisation test.

Table 3A

Serology titres for MERS-CoV and BCoV in dromedary camel sera, Saudi Arabia, 2012–2013 (n=56)

Sera IDs	Age group in years	MERS-CoV ppNT titre	MERS-CoV MNT titre	BCoV MNT titre	Reaction profile	Region
1	<1	1:2,560	1:160	1:20	MERS-CoV specific	Riyadh
2	<1	1:320	1:20	<1:10	MERS-CoV specific	Riyadh
3	<1	≥1:5,120	1:640	1:40	MERS-CoV specific	Al-Ahsa
4	<1	1:1,280	1:320	<1:10	MERS-CoV specific	Al-Ahsa
5	<1	1:640	1:40	1:160	BCoV specific	Riyadh
6	<1	1:640	1:40	1:320	BCoV specific	Riyadh
7	<1	1:160	1:10	1:40	BCoV specific	Riyadh
8	<1	1:1,280	1:80	1:640	BCoV specific	Al-Ahsa
9	<1	<1:20	<1:10	1:160	BCoV specific	Al-Ahsa
10	<1	1:80	1:10	1:40	BCoV specific	Al-Ahsa
11	<1	1:80	1:10	<1:10	Indeterminate	Riyadh
12	<1	≥1:5,120	1:640	1:640	Indeterminate	Riyadh
13	<1	1:2,560	1:320	1:320	Indeterminate	Al-Ahsa
14	<1	1:2,560	1:320	1:320	Indeterminate	Al-Ahsa
15	<1	1:1,280	1:160	1:320	Indeterminate	Al-Ahsa
16	1–3	≥1:5,120	≥1:1,280	<1:10	MERS-CoV specific	Riyadh
17	1–3	1:160	1:20	<1:10	MERS-CoV specific	Riyadh
18	1–3	≥1:5,120	≥1:1,280	1:80	MERS-CoV specific	Riyadh
19	1–3	1:640	1:80	<1:10	MERS-CoV specific	Al-Ahsa
20	1–3	1:640	1:160	<1:10	MERS-CoV specific	Al-Ahsa
21	1–3	1:640	1:40	1:160	BCoV specific	Riyadh
22	1–3	1:20	<1:10	1:160	BCoV specific	Riyadh
23	1–3	1:2,560	1:80	>1:1,280	BCoV specific	Al-Ahsa
24	1–3	1:320	1:10	1:20	Indeterminate	Riyadh
25	1–3	1:640	1:80	1:40	Indeterminate	Riyadh
26	1–3	1:160	1:20	1:40	Indeterminate	Al-Ahsa
27	1–3	1:1,280	1:640	1:320	Indeterminate	Al-Ahsa
28	4–5	≥1:5,120	1:640	1:160	MERS-CoV specific	Riyadh
29	4–5	1:160	1:160	1:40	MERS-CoV specific	Riyadh
30	4–5	≥1:5,120	1:320	1:80	MERS-CoV specific	Riyadh
31	4–5	≥1:5,120	1:640	1:80	MERS-CoV specific	Riyadh
32	4–5	≥1:5,120	≥1:1,280	1:40	MERS-CoV specific	Riyadh
33	4–5	1:640	1:160	<1:10	MERS-CoV specific	Riyadh
34	4–5	≥1:5,120	≥1:1,280	<1:10	MERS-CoV specific	Al-Ahsa
35	4–5	1:320	1:40	1:640	BCoV specific	Riyadh
36	4–5	1:1,280	1:320	>1:1280	BCoV specific	Riyadh
37	4–5	1:2,560	1:640	>1:1280	BCoV specific	Riyadh
38	4–5	1:320	1:40	1:320	BCoV specific	Al-Ahsa
39	4–5	≥1:5,120	1:320	>1:1,280	BCoV specific	Al-Ahsa
40	4–5	1:160	1:40	1:320	BCoV specific	Al-Ahsa
41	4–5	1:1,280	1:160	1:320	Indeterminate	Riyadh
42	4–5	1:2,560	1:320	1:640	Indeterminate	Riyadh
43	4–5	1:1,280	1:160	1:320	Indeterminate	Al-Ahsa

BCoV: bovine coronavirus; ID: identity; MERS-CoV: Middle East Respiratory Syndrome coronavirus; MNT: microneutralisation test; ppNT: pseudoparticle neutralisation test.

The reaction profile was denoted as MERS-CoV specific if the antibody titre to MERS-CoV was ≥4 fold higher than BCoV; and BCoV specific if the titre to BCoV was ≥4 fold higher than for MERS-CoV. Other sera are regarded to have an indeterminate reaction profile.

Table 3B

Serology titres for MERS-CoV and BCoV in dromedary camel sera, Saudi Arabia, 2012–2013 (n=56)

Sera IDs	Age group in years	MERS-CoV ppNT titre	MERS-CoV MNT titre	BCoV MNT titre	Reaction profile	Region
44	>5	≥1:5,120	1:640	1:80	MERS-CoV specific	Riyadh
45	>5	1:1,280	1:320	1:80	MERS-CoV specific	Riyadh
46	>5	1:640	1:640	1:40	MERS-CoV specific	Riyadh
47	>5	<1:20	<1:10	1:80	BCoV specific	Riyadh
48	>5	1:80	1:80	1:320	BCoV specific	Riyadh
49	>5	≥1:5,120	1:640	>1:1280	Indeterminate	Riyadh
50	>5	1:1,280	1:320	1:160	Indeterminate	Riyadh
51	>5	1:1,280	1:640	1:320	Indeterminate	Riyadh
52	>5	≥1:5,120	1:640	>1:1,280	Indeterminate	Riyadh
53	>5	1:1,280	1:640	1:320	Indeterminate	Riyadh
54	>5	1:640	1:640	1:640	Indeterminate	Riyadh
55	>5	≥1:5,120	≥1:1,280	>1:1,280	Indeterminate	Riyadh
56	>5	1:320	1:20	1:40	Indeterminate	Riyadh

BCoV: bovine coronavirus; ID: identity; MERS-CoV: Middle East Respiratory Syndrome coronavirus; MNT: microneutralisation test; ppNT: pseudoparticle neutralisation test.

The reaction profile was denoted as MERS-CoV specific if the antibody titre to MERS-CoV was ≥4 fold higher than BCoV; and BCoV specific if the titre to BCoV was ≥4 fold higher than for MERS-CoV. Other sera are regarded to have an indeterminate reaction profile.

activity while 280 of 310 (90%) dromedary camel sera were seropositive. The age-group and location of the camels tested and their serostatus in the screening assay are shown in Table 2. While 47 of 65 (72%) of camels less than one year of age were seropositive, 233 of 245 (95%) of camels older than one year were seropositive to MERS-CoV in the ppNT test (chi-squared test, p<0.01).

A randomly selected subset of 54 ppNT seropositive sera and two ppNT negative sera were titrated by MERS-CoV ppNT, MERS-CoV MNT and BCoV MNT (Table 3). MERS-CoV ppNT titres ranged from 1:20 to ≥1:5,120 and MERS-CoV MNT titres ranged from <1:10 (in one serum only) to ≥1:1,280. Dromedary camel sera, which have ≥4 fold higher antibody MERS-CoV MNT titres compared to BCoV MNT titres are denoted as 'MERS-CoV specific' reaction patterns. Sera with comparable (within 4-fold) titres to both viruses and are denoted as 'indeterminate' reaction patterns while those that have ≥4 fold higher titres to BCoV are regarded as 'BCoV specific'.

Twelve of the 50 cattle sera were randomly selected for BCoV MNT and 11 sera were positive (Table 4).

Discussion

This study was conducted Saudi Arabia, including in central and eastern provinces from which most of the human cases of MERS-CoV hitherto had been detected; in particular, the Al Ahsa region from where the largest cluster of human-to-human transmission of the disease has occurred [4] and the Riyadh region, which appears to host the greatest genetic diversity of MERS-CoV within Saudi Arabia [5]. None of the cattle, goat, sheep or chickens had any detectable antibody to MERS-CoV. While larger numbers of such livestock in the vicinity of confirmed cases of MERS need to be examined, our data do not indicate that these species of domestic livestock are commonly infected by MERS-CoV. As expected, BCoV antibody was common in cattle with 11 of 12 cattle sera having evidence of BCoV MNT antibody, but these sera did not cross-react with MERS-CoV.

Dromedary camels from the central province of Riyadh as well as the eastern province, Al Ahsa, had comparably high levels of seropositivity to MERS-CoV by ppNT tests. Camels less than one year of age had lower seroprevalence (72%) than those older than one year of age (95%) (p <0.01). Some dromedary camels have high titres to MERS-CoV in the absence of any reactivity to BCoV confirming that these animals are being infected by a virus very different to BCoV, which could be identical or closely related to MERS-CoV [8,9]. Similarly, some animals appear to be infected by a BCoV-like virus without cross-reactivity to MERS-CoV. If we consider just the 'MERS-CoV specific' reactions, we observe that these animals are getting infected within the first year of life (Table 3).

Table 4

Serology titres for MERS-CoV and BCoV in cattle sera, Saudi Arabia, 2010–2013 (n=12)

Sera ID	MERS-CoV ppNT titre	MERS-CoV MNT titre	BCoV MNT titre
1	<1:20	<1:10	1:40
2	<1:20	<1:10	<1:10
3	<1:20	<1:10	1:640
4	<1:20	<1:10	1:160
5	<1:20	<1:10	1:80
6	<1:20	<1:10	1:40
7	<1:20	<1:10	1:160
8	<1:20	<1:10	1:40
9	<1:20	<1:10	1:40
10	<1:20	<1:10	1:640
11	<1:20	<1:10	1:160
12	<1:20	<1:10	1:160

BCoV: bovine coronavirus; ID: identity; MERS-CoV: Middle East Respiratory Syndrome coronavirus; MNT: microneutralisation test; ppNT: pseudoparticle neutralisation test.

Naturally infected cattle with BCoV titres of up to 1:640 and an experimentally infected calf with a titre of ≥1:1,280 had no cross-reaction to MERS-CoV in ppNT and MNT assays. Previously, we had shown that human sera with antibody to Severe Acute Respiratory Syndrome (SARS)-CoV failed to react with MERS-CoV, moreover SARS-CoV was not neutralised by dromedary camel sera with high antibody titres to MERS-CoV [9]. In contrast, while some dromedary camel sera had mono-specific reactions to either MERS CoV or BCoV, many sera have 'indeterminate' reaction profiles with reactivity to both MERS-CoV and BCoV (Table 3). It is possible that sequential infection of dromedary camels with different coronaviruses may lead to a broadening of the serological cross-reaction profile. Alternatively, it is possible that the unusual single chain immunoglobulins possessed by camels [10] may give broader serological cross-reactivity than is seen with other species. In any event, these findings highlight the importance of parallel titration of sera to both MERS-CoV and BCoV when interpreting seroepidemiological results.

The exact identity of this MERS-CoV-like virus can only be established by characterising this virus from dromedary camel specimens. As many of the dromedary camels sampled in this study were already seropositive to MERS-CoV, we did not attempt to detect viral RNA in the serum samples. There are (as yet unpublished) reports of detecting a MERS-CoV-like virus in camel specimens from Saudi Arabia and Qatar [2]. Assuming that MERS-CoV seropositivity in MNTs, irrespective of the infecting virus that leads to such seropositivity, correlates with resistance to, or reduced susceptibility to re-infection, these results would suggest that the search for virus in these animals should be focused on dromedary camels <1 year of age. Studies involving follow-up of herds of camels from time of calving though the first year of life with serial blood samples together with oral and rectal or fresh faecal swabs would better define the ecology of the MERS-CoV-like virus infecting these animals and provide virus for genetic characterisation. Such studies are a priority to determine whether dromedary camels are in fact a source of human MERS-CoV infection or whether they are being infected by a ubiquitous novel coronavirus closely related to MERS-CoV. As illustrated with SARS a decade ago and avian influenza A(H7N9) recently, identification of the animal source and the setting within which zoonotic transmission occurs can provide options for reducing repeated zoonotic transmissions and enhancing global public health. Finally, given the high titres of neutralising antibody to MERS CoV commonly seen in dromedary camels, serum from these animals may provide an option for passive immunotherapy of patients with MERS, from whom no specific antiviral therapy currently exists.

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Conflict of interest

None declared.

Authors' contributions

Maged Gomaa Hemida (mhemida@kfu.edu.sa), Malik Peiris (malik @hku.hk) designed, coordinated and supervised the study and wrote the manuscript. Ranawaka APM Perera (mahenp@hku.hk) developed the MERS-CoV and bovine coronavirus microneutralisation tests, carried out the MERS-CoV tests in BSL3 containment and the MERS pseudoparticle assays. Pei-gang Wang (pgwang@hkucc.hku.hk) developed the MERS-CoV pseudotype assay. Mohamed A Alhammadi (malhammadi@kfu.edu.sa): Specimen collection and data recording. Lewis YL Siu (ylsiu@hku.hk) and Ming-yuan Li (Imy288@hku.hk) carried out the MERS-CoV pseudoparticle assays. Leo LLM Poon (llmpoon@hku.hk): Provided advice on laboratory methods. Linda Saif (saif.2@osu.edu): Provided reagents and advised on study design. Abdelmohsen Alnaeem (aaalnaeem@kfu.edu.sa): Designed and supervised the study. All authors reviewed and commented on the manuscript.

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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].

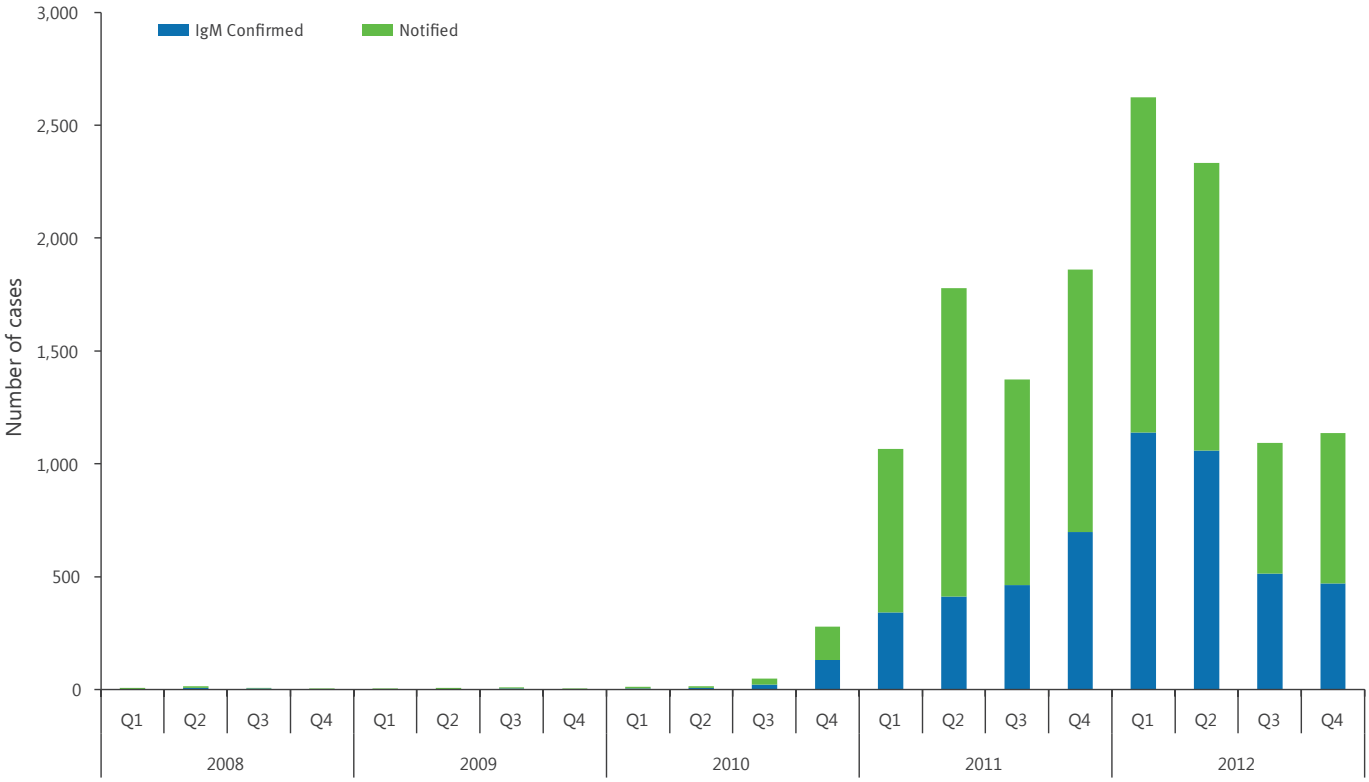
Measles has been a statutorily notifiable disease in Romania since 1978, and medical practitioners must report all clinically possible measles cases to the regional public health authorities. The first monovalent measles-containing vaccine was introduced in 1979 into the Romanian vaccination schedule 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 (MVs/Enfield.GBR/14/07) which became endemic in the United Kingdom (UK) in

Figure 1

Quaterly 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/Ilfov.ROU/01.12/ MVs/Ilfov.ROU/23.12/	D4-Manchester	Indigenous	Ilfov (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).

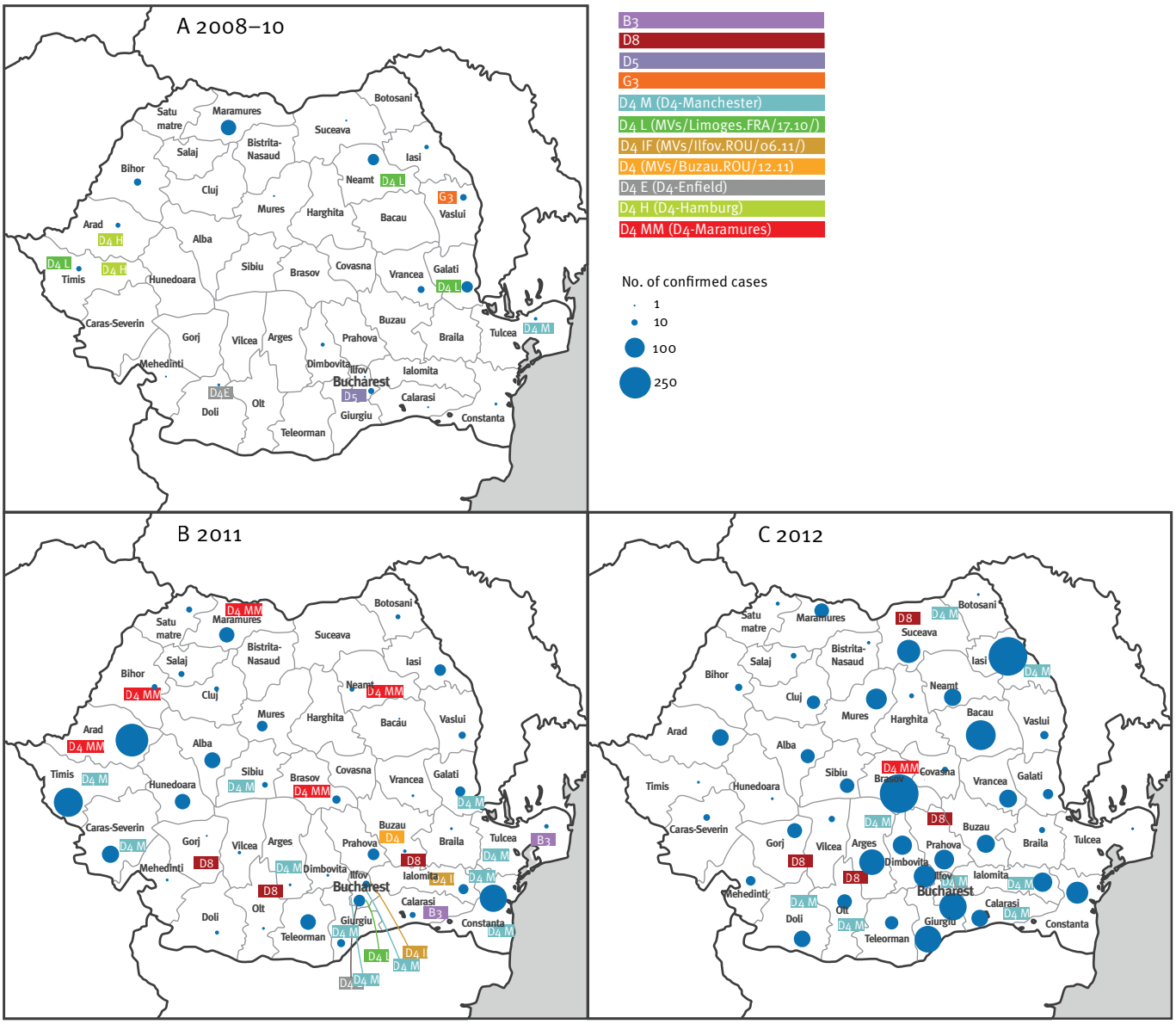
RT-PCR and sequencing

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 (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).

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/Montaigu.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.

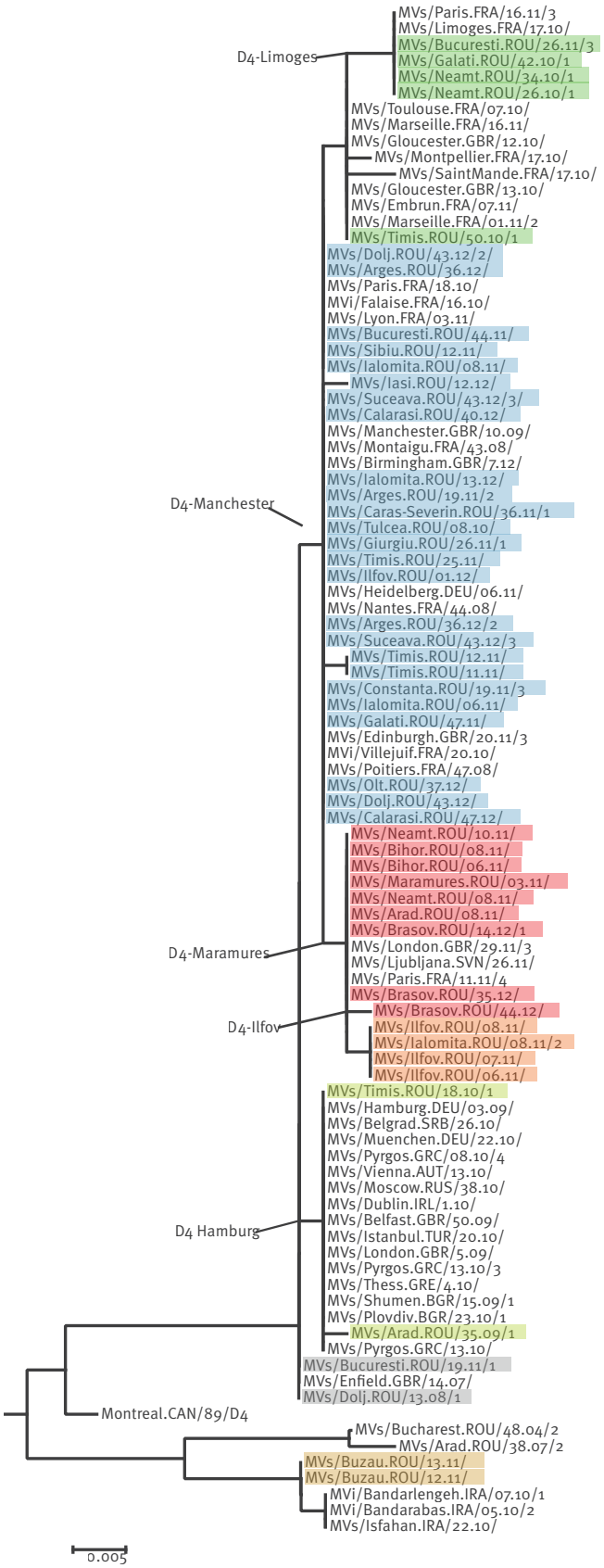
An imported case was detected in a student who travelled from Paris to Vaslui in September 2010. Sequencing revealed MVs/Vaslui.ROU.40.10[G3], identical to the strain MVs/Paris.FRA/47.10[G3] MV which circulated in France in 2010, but was also detected in Germany, the UK and Spain (Figure 4).

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)

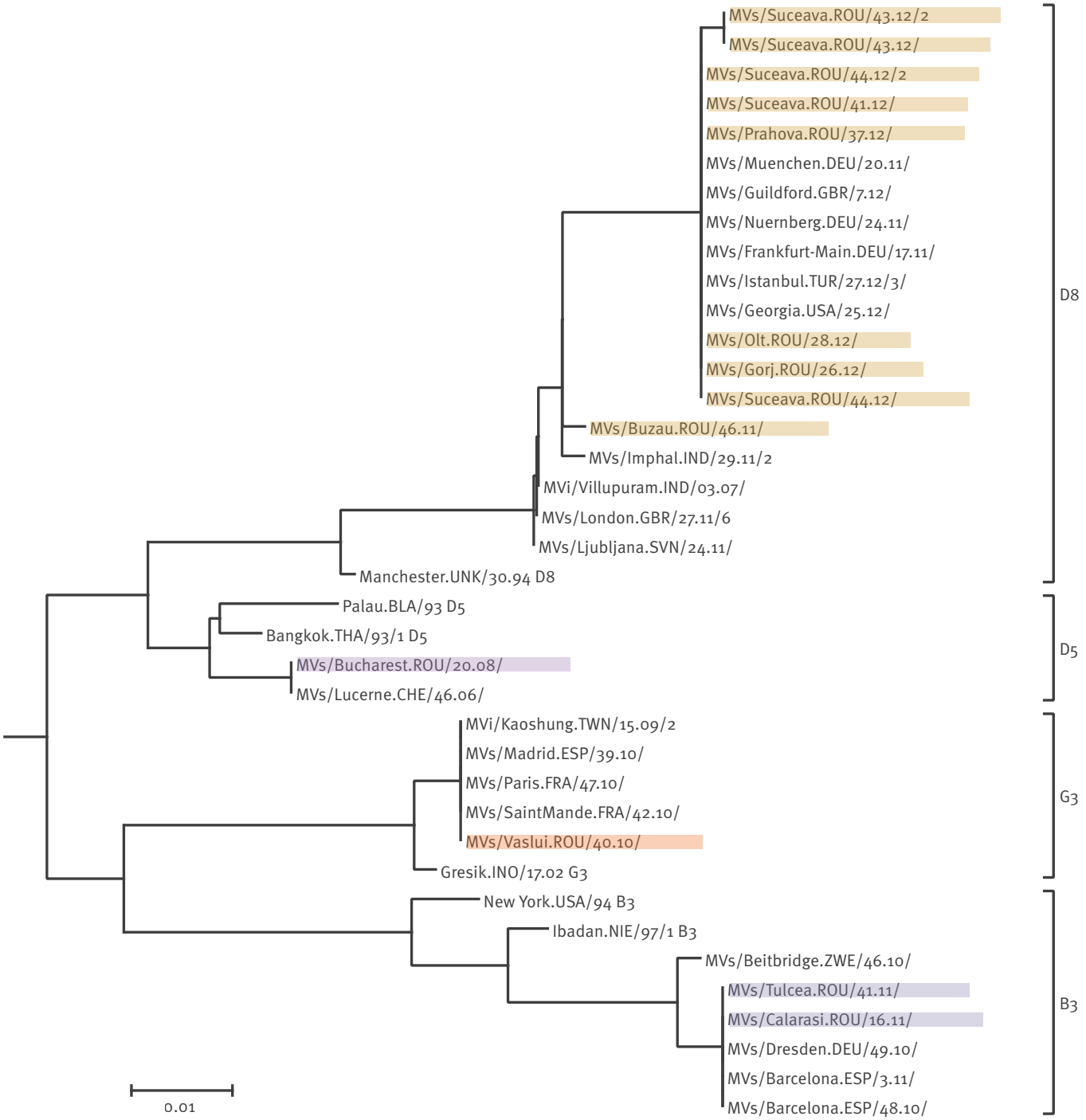


Romanian MV strains are highlighted in different colours.

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/Montaigu.FRA/43.08, GQ428173; MVs/Nantes.FRA/44.08, GQ428179; MVs/Paris.FRA/18.10, FR671444; MVs/Poitiers.FRA/47.08, GQ428172; MVs/Pyrgos.GRC/13.10/3, HM802123; MVs/Thess.GRE/4.10, HM770085; MVs/Toulouse.FRA/07.10, FR671432; MVs/Dolj.ROU/13.08/1, HQ704311; MVs/Arad.ROU/38.07/2, HQ704309; MVs/Shumen.BGR/15.09/1, HQ436103; MVs/Plovdiv.BGR/23.10/1, HQ436106; MVs/Pyrgos.GRC/13.10, HM802123; MVs/Manchester.GBR/10.09, GQ370461; MVs/Montpellier.FRA/17.10, FR671431; MVs/SaintMande.FRA/17.10, FR671435; MVi/Bandarlengeh.IRA/07.10/1, HM440228; MVi/Bandarabas.IRA/05.10/2, HM440227; MVs/Isfahan.IRA/22.10, HQ395674; MVs/Ilfov.ROU/23.12, JX847792; MVs/Brasov.ROU/35.12, NA; MVs/Arges.ROU/36.12/2, JX912278; MVs/Arges.ROU/36.12, JX912279; MVs/Olt.ROU/37.12, NA; MVs/Calarasi.ROU/40.12, NA; MVs/Suceava.ROU/43.12/3, KC172854; MVs/Dolj.ROU/43.12/2, NA; MVs/Dolj.ROU/43.12, NA; MVs/Suceava.ROU/43.12/3, NA; MVs/Brasov.ROU/44.12, NA; MVs/Calarasi.ROU/47.12, 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 month 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 Olt county, (MVs/Olt.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/Olt.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/08.10[D4]), but was apparently re-imported in early 2011 to Ialomita (MVs/Ialomita.ROU/06.11[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/08.10[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/06.11[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

Lupulescu - writing of the manuscript and interpretation of the study.

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Kinetics of serological responses in influenza A(H7N9)-infected patients correlate with clinical outcome in China, 2013

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The novel avian influenza A(H7N9) infection has recently emerged to cause severe respiratory illness in China. The objectives of this study were to define the kinetics of the antibody responses in patients with influenza A(H7N9) disease and to correlate these kinetics with clinical outcome. Serial serum samples were obtained at intervals of three to four days from 18 patients with virologically confirmed A(H7N9) disease in Shanghai. We determined the kinetics of the haemagglutination inhibition (HI) and A(H7H9) pseudotype neutralisation antibody (Nab) responses and correlated these with clinical outcomes. Most patients had robust serological responses by both HI and Nab tests. Taking into account censoring due to time of testing and death, the median time from onset of illness to Nab titre ≥1:40 was 14 days (95% confidence interval (CI): 11–18 days) in the fatal cases and 10.5 days (95% CI: 7–12) in the survivors (p=0.003). The two groups did not differ in initial Nab titres, but the rate of increase in Nab titres was significantly faster for survivors by approximately 10-fold per 15 days (p=0.007). Early and rapid induction of Nab was correlated significantly with better clinical outcome.

Introduction

A novel avian influenza A(H7N9) infection emerged to cause an outbreak in the Yangtze River delta in early 2013, subsequently spreading to other provinces in China [1-4]. In the first wave of influenza A(H7N9) infections from February to July 2013, 135 patients were reported from 11 provinces and municipalities in China, leading to 45 deaths [5]. Further cases have been reported since October 2013. Genomic analysis revealed that the novel H7 haemagglutinin is genetically distinct from other historical and contemporary human influenza viruses [3,6]. Adverse clinical outcomes have been associated with co-existing medical conditions or the development of drug resistance [7,8]. Previous H7 subtype influenza virus infections in humans such as the influenza A(H7N7) outbreak in Netherlands in 2003 were poorly immunogenic and serodiagnosis and seroepidemiology were challenging [9]. We explored the kinetics of the serological responses to this novel virus in haemagglutination inhibition (HI) assays and in a recently developed H7N9 pseudotype virus particle neutralisation (Nab) test [10]. Viral pseudotypes have been previously shown to provide reliable correlation with conventional microneutralisation tests for influenza A(H5N1) serological studies [11]. We investigated correlations between serological responses and clinical outcome.

Methods

Patients and samples

In April 2013, 18 patients confirmed with influenza A(H7N9) infection by real-time PCR were hospitalised at the Shanghai Public Health Clinical Center (SHAPHC). Serum specimens were collected every three to four days following admission with two to seven serial serum samples being collected from each patient. Clinical data including patient demographic information, treatment, clinical investigations and disease progression were retrieved from the clinical notes. Written informed consent was obtained from all participants. The overall study was reviewed and approved by the Ethics Committee of SHAPHC.

Haemagglutination inhibition assay

The methods used were as previously described and used horse erythrocytes [12]. Serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken Co Ltd., Tokyo, Japan) to remove non-specific inhibitors. Stored serum samples collected in 2009 from individuals not infected with influenza were used as negative serum controls. The virus strain used was

A/Shanghai/4664T/2013 (H7N9) (GenBank accession No: KC853228.1).

Pseudovirus-based neutralisation assay

To rapidly and safely assess neutralisation activities against the 2013 influenza A(H7N9) virus which caused severe disease in humans, we developed a luciferase reporter-based Nab assay which has a non-replicative human immunodeficiency virus backbone carrying influenza A H7 and H9. We have previously demonstrated that the titres quantified by Nab assay correlated well with the titres measured by traditional HI assay, using serum samples from influenza A(H7N9)-infected patients and uninfected subjects with good correlation (Spearman r=0.88) [10]. The pseudoviruses were prepared as described in our previous report [10]. The neutralising titre of human sera was defined as the highest serum dilution that gave ≥80% inhibitory concentration (IC80) of the luciferase signal in virus-infected MDCK cells. On the basis of previous studies we had defined that IC80 and a antibody titre of 1:40 were the best discriminators between patients and non-infected controls, and we employed these to define positive Nab responses.

Statistical analyses

Non-parametric Mann–Whitney test was used to test the differences in HI or Nab titres across groups. Categorical variables were compared by using the two-tailed Fisher's exact test to account for small sample size. In addition, univariate and multivariate exact logistic regression modelling were employed to identify the association of different factors with clinical outcome and allow for small cell size. The covariates used in the multivariate model included age, sex and NAb titres (1:40 and 1:640). The results were presented using odds ratios (ORs).

We fitted accelerated failure time model assuming a Gaussian distribution to compare time from illness onset to reaching a Nab titre of 1:40 between patients who recovered versus those who died, accounting for interval censoring due to time of testing. The model was also used to identify factors associated with longer time to recovery for recovered patients. We compared the initial Nab titre and rate of increase in Nab titre, adjusted for age and sex, using a linear mixed model to account for repeated measurements, assuming a linear increasing trend by days since illness onset. For analyses based on continuous measurements, titres were first log-transformed (with base 10). We used bootstrap method with 1,000 resamples to test the difference in time from illness onset to reaching a Nab titre of 1:40 between fatal cases and survivors. All statistical tests were considered significant at the level of $p<0.05$. All data were analysed by using SPSS software (version 17.0) and R (version 3.0.1).

Results

To understand the kinetics of the human serological responses to the novel influenza A(H7N9) virus, we first determined (HI) antibody and pseudotype Nab responses in 18 influenza A(H7N9)-infected patients. HI antibody titres reached a titre of 1:40 in six of 14 patients by Day 10 of illness. By Day 18 of illness, 17 of 18 patients had antibody titres of 1:40 with titres ranging from <1:10 to 1:320, and 10 of 18 patients had titres ≥1:80. All patients had evidence of seroconversion within three weeks (Figure). We next examined Nab titres quantified by the Nab assay. Nab titres reached 1:40 in five of 14 patients by Day 10 of illness and 16 of 17 by Day 18 of illness. Thirty-seven control sera collected in 2009 had negative results in both tests. To test the reliability of assays, limited repeat testing has been done on sets of sera by both assays with good reproducibility; in addition, all the sera were tested in one large assay batch to maintain comparability.

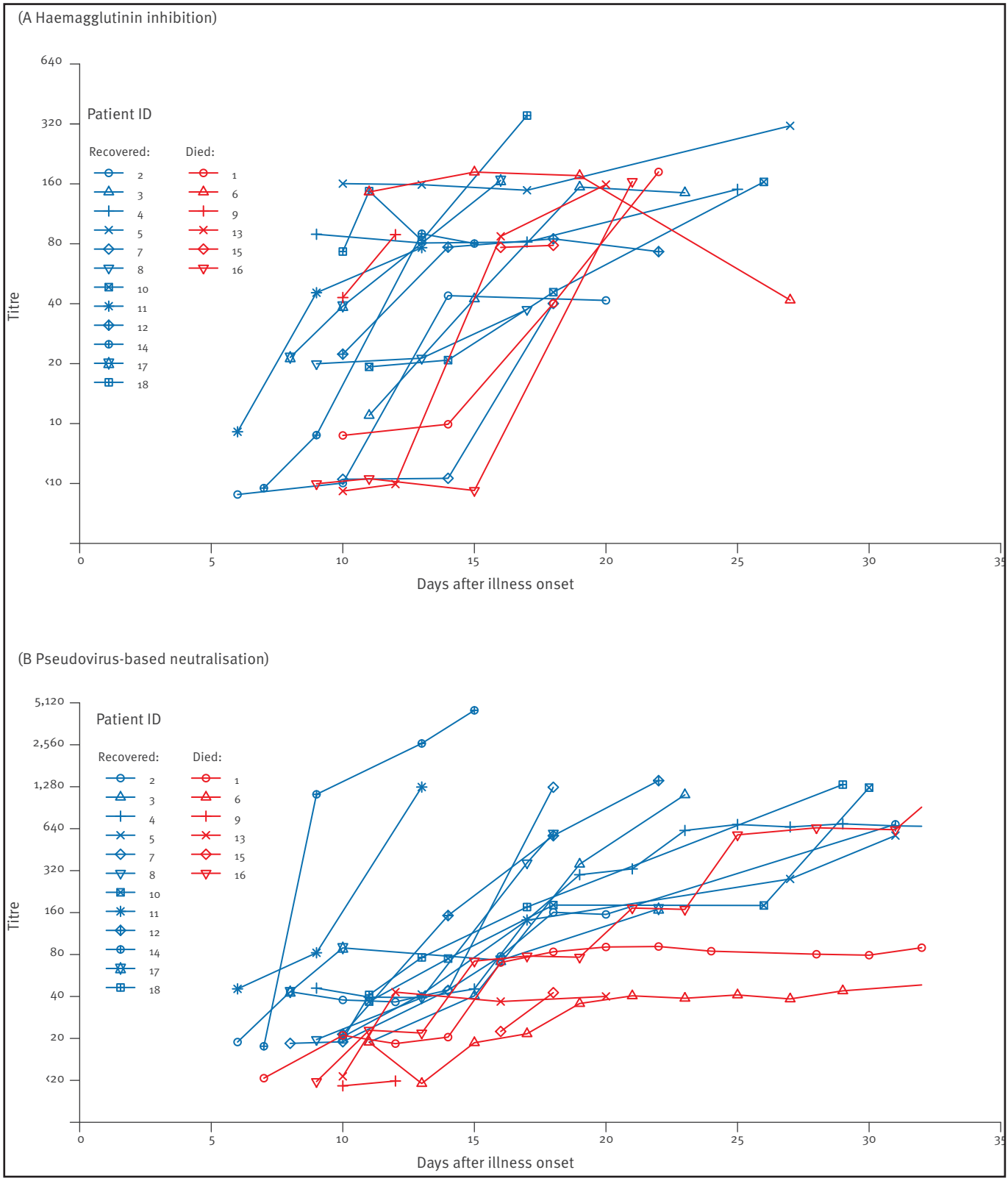
We examined the association between HI and Nab responses with clinical outcomes. The 18 patients were divided into two groups: group A were 12 patients who recovered and group B were six patients who had died (Table 1). The differences in HI titres between the two groups were not statistically significant. We next examined the development of Nab in the two patient groups, taking into account censoring due to time of testing and death. The median time from symptom onset to Nab titres reaching 1:40 was 10.5 days (95% confidence interval (CI): 7–12 days) for patients who survived and 14.0 days (95% CI: 11–18 days) for those who died. In patients who survived, it took a significantly shorter time to reach Nab titres of 1:40 for the unadjusted model ($p<0.001$) and the model adjusted for age and sex ($p<0.001$). Adjusted for age and sex, there was no significant difference ($p=0.36$) in the initial Nab titre between patients who survived and those who died. However, the Nab titre for survived patients increased significantly faster than for patients who eventually died, by approximately 10-fold per 15 days ($p=0.007$). The difference persisted when we restricted the analysis to patients with low initial Nab titres (≤1:40 on day 10, $p=0.003$).

We further examined the association between clinical outcome and sex, age, underlying medical disorders, timing for initiating antiviral treatment with oseltamivir or anti-inflammatory treatment with methylprednisolone and initial viral loads. None of these factors were significantly associated with clinical outcomes in the univariate analysis (Table 2). Nab titres reaching ≥1:640 or reaching 1:40 within 14 days were associated with survival in both univariate and multivariate analysis (Table 2). However, HI titres reaching 1:40 within 10 days were not associated with survival.

We also examined the association of these different factors with time to recovery in the survivors. Reaching Nab titres 1:640 within 14 days after illness onset was significantly associated with faster recovery ($p=0.002$) in the univariate analysis (Table 3). After adjusting for other confounding factors, it was associated with a 22% shorter recovery time, although the effect was not

Figure

Antibody responses in influenza A (H7N9) patients after illness onset, China, 2013 (n=18)



Serum samples were collected from all 18 influenza A(H7N9)-infected patients. For each patient, two to seven serial serum samples at intervals of three to four days were collected after their admission to our center. The population level kinetics of both (A) HI and (B) neutralisation antibody responses of each individual patient are shown over time. HI titres were not significantly different between survivors and fatal cases at eight to 10 days (p=0.126) and at 11 to 14 days (p=0.390) after illness onset.

Table 1

Viral loads and clinical outcomes in influenza A (H7N9)-infected patients, China, 2013 (n=18)

	Patient number	Sex	Age	Underlying medical disorders	Time between onset of symptoms and		Initial viral loads (copies/mL)	Clinical outcome
					the initiation of oseltamivir treatment (days)	first treatment with methylprednisolone (days)		Discharged after days of onset or death
Group A: recovered	11	M	53	None	5	5	$6.59x10^2$	14
	14	M	47	None	5	5	$9.30x10^3$	17
	7	M	68	Hypertension II	6	No	$1.29x10^3$	18
	8	M	65	Hypertension, urethritis	4	No	$3.49x10^2$	18
	17	F	74	Hypertension III, coronary heart disease (NYHA IV), diabetes II, cholecystitis	8	No	$5.11x10^3$	21
	12	M	74	None	8	12	$3.68x10^2$	22
	2	F	81	Arrhythmia	5	6	$3.08x10^3$	23
	3	M	67	None	5	4	$4.24x10^3$	23
	18	M	67	Diabetes II	11	9	$3.86x10^2$	24
	10	M	78	Hypertension II	11	15	$2.28x10^2$	31
	5	F	75	Hypertension II, coronary heart disease (NYHA IV), diabetes	8	9	$1.13x10^3$	33
	4	M	62	Hypertension	9	5	$5.70x10^5$	35
Group B[a]: died	16	F	79	Arrhythmia, coronary heart disease (NYHA IV), chronic bronchitis. primary biliary cirrhosis	6	6	$5.65x10^4$	Death
	1	M	56	None	3	7	$2.67x10^2$	Death
	6	M	58	Hypertension	9	8	Negative	Death
	13	M	88	Hypertension III, coronary heart disease (NYHA IV), diabetes II, chronic bronchitis	7	8	$4.06x10^4$	Death
	15	M	80	None	7	7	$5.8x10^3$	Death
	9	M	74	Coronary heart disease (NYHA III)	6	6	Negative	Death

F: female; M: male; NYHA: New York Heart Association Functional Classification.

[a] The virus isolates in patients 13 and 1 developed the Arg292Lys mutation in the neuraminidase protein, which is known to confer resistance to oseltamivir.

Patients are listed by date of discharge from our hospital.

statistically significant (p=0.108). Only age and time until oseltamivir treatment remained significant in the multivariate analysis (Table 3).

To specifically examine whether use of methyl prednisolone had an impact on antibody responses, we fitted a linear mixed model including 'initiation of methylprednisolone treatment less than eight days after disease onset' as a variable to predict trends in HI and Nab titres. Adjusted for age and sex, receiving methylprednisolone treatment within eight days did not have a significant influence on HI and Nab titres.

Discussion

The kinetics of the antibody responses in human A(H7N9) virus infections remain ill defined. Such data are important for serodiagnostic purposes as well as for the interpretation of seroepidemiological studies. Our findings indicate that influenza A(H7N9) infections are associated with generally robust HI antibody responses and, with one exception (Patient 9, a patient who died), Nab antibody responses. Therefore, serodiagnosis of hospitalised patients should not pose a major challenge, unlike human H7N7 infections in the Netherlands in 2003 [9]. It is relevant to note that

Table 2

Association of different factors with clinical outcomes in influenza A (H7N9)-infected patients, China, 2013 (n=18)

Variables	Discharge n (%)	Death n (%)	Univariate analysis		Multivariate analysis	
			OR[a] (95% CI)	p value	OR[a] (95% CI)	p value
Sex						
Male	9 (64.3)	5 (35.7)	1	–	–	–
Female	3 (75.0)	1 (25.0)	1.62 (0.096-105)	1	–	–
Age						
<75 years	9 (75.0)	3 (25.0)	1	–	–	–
≥75 years	3 (50.0)	3 (50.0)	0.344 (0.027–4.17)	0.356	–	–
Medical disorders						
No	4 (44.7)	2 (33.3)	1	–	–	–
Yes	8 (66.7)	4 (33.3)	1.00 (0.064–11.5)	1	–	–
Initiation of oseltamivir treatment						
<8 days	6 (54.5)	5 (45.5)	1	–	–	–
≥8 days	6 (85.7)	1 (14.3)	4.59 (0.346–275)	0.316	–	–
Initiation of methylprednisolone treatment						
<8 days	5 (55.6)	4 (44.4)	1	–	–	–
≥8 days	7 (77.8)	2 (22.2)	2.64 (0.253–40.5)	0.620	–	–
First viral load						
<1,000 copies/mL	5 (62.5)	3 (37.5)	1	–	–	–
≥1,000 copies/mL	7 (70.0)	3 (30.0)	1.37 (0.126–15.2)	1	–	–
Nab titre reaches 1:640						
No	1 (16.7)	5 (83.3)	1	–	1	–
Yes	11 (91.7)	1 (8.3)	35.4 (1.99–2740)	0.004	16.9 (2.19–220)	0.006
Nab titre reaches 1:40						
≤14 days	11 (84.6)	2 (15.4)	1	–	1	–
>14 days	1 (20.0)	4 (80.0)	0.0587 (0.000858–0.941)	0.022	0.0361 (0.000–0.519)	0.011
HI titre reaches 1:40[b]						
≤10 days	5 (62.5)	3 (37.5)	1	–	–	–
>10 days	7 (70.0)	3 (30.0)	1.37 (0.126–15.2)	1	–	–

–, not relevant; CI: confidence interval; HI: haemagglutination inhibition; Nab: pseudotype neutralisation antibody; OR: odds ratio.

[a] OR>1 indicates 'preferring recovery'. Multivariate analyses were performed by examining whether Nab titres reached 1:640 or 1:40 within 14 days in a model with co-factors of age and sex.

[b] Missing HI titres within 10 days were imputed conservatively in such a way that would attenuate the results toward the null hypothesis.

nearly all diagnosed H7N9-infected patients had more severe disease (namely pneumonia) compared with the outbreak in the Netherlands, where diagnosed patients had mainly mild conjunctivitis or influenza-like illness. Thus, one cannot be certain that milder or asymptomatic influenza A(H7N9) infections would manifest comparably robust serological responses. In addition, this has to be kept in mind when interpreting community-wide seroepidemiological studies. It would be interesting to have serological data from family members and contacts of our patients. Unfortunately, such a follow-up study was not conducted.

The progression of influenza A(H7N9) disease was observed to be slower than of influenza A(H5N1) disease, the median time from hospitalisation to death being 12.0 days and 5.7 days, respectively [13]. We hypothesised that the prolonged disease course of H7N9 compared with H5N1 influenza (and SARS-CoV) infection is likely to imply that there is more opportunity for the adaptive immune response to contribute to recovery and survival. Therefore, we examined the antibody responses with HI and Nab assays in influenza A(H7N9)-infected patients admitted into our clinical center. We evaluated the correlation between the kinetics of HI and Nab antibody responses and clinical

Table 3

The association of different factors with time to recovery for recovered patients

Variables	Median time to recovery (days)	Univariate analysis		Multivariate analysis	
		AF[a] (95% CI)	p value	AF[b] (95% CI)	p value
Sex					
Male	22.0	1		1	
Female	23.0	1.17 (0.831–1.64)	0.374	0.893 (0.709–1.12)	0.332
Age					
<75 years	21.0	1		1	
≥75 years	31.0	1.39 (1.03–1.86)	0.031	1.32 (1.05–1.66)	0.019
Medical disorders					
No	19.5	1		1	
Yes	23.5	1.32 (0.999–1.75)	0.051	0.994 (0.795–1.24)	0.960
Initiation of oseltamivir treatment					
<8 days	18.0	1		1	
≥8 days	27.5	1.46 (1.18–1.81)	<0.001	1.31 (1.11–1.56)	0.002
Initiation of methylprednisolone treatment					
<8 days	23.0	1		–	–
≥8 days	22.0	1.09 (0.806–1.48)	0.575	–	–
First viral load					
<1 000	22.0	1		–	–
≥1 000	23.0	1.12 (0.826–1.51)	0.476	–	–
Nab titre reaches 1:640					
>21 days or not reaching 1:640	23.5	1		–	–
15–21 days	20.0	0.878 (0.681–1.13)	0.312	0.996 (0.803-1.23)	0.968
≤14 days	15.5	0.606 (0.440–0.835)	0.002	0.784 (0.584-1.05)	0.108
Nab titre reaches 1:40					
≤14 days	22.0	1		–	–
>14 days	23.0	1.03 (0.595–1.78)	0.922	–	–
HI titre reaches 1:40[b]					
≤10 days	24.0	1		–	–
>10 days	22.0	0.88 (0.655–1.191)	0.415	–	–

–, not relevant; CI: confidence interval; HI: haemagglutination inhibition; Nab: pseudotype neutralisation antibody; AF: acceleration factor.

[a] AF<1 indicates reduction in time from illness onset to recovery. Multivariate analyses were performed by examining medical disorder, initiation of oseltamivir treatment after eight days and time to reach Nab titres 1:640, in a model with co-factors of age and sex.

[b] Missing HI titres within 10 days were imputed conservatively in such a way that would attenuate the results toward the null hypothesis.

disease outcome of survival or death. Strikingly, we observed that the early and rapid induction of Nab significantly correlated with the rapid recovery from illness. The two groups of patients did not differ in initial Nab antibody titres, but the rate of antibody titre increase was approximately 10-fold faster per 15 days in survivors than in fatal cases (p=0.007). In contrast, no significant association was observed between HI titres and clinical outcomes. While the HI test only detects antibodies against the receptor-binding domain of the globular head of the haemagglutinin, neutralisation could be conferred by antibodies against the stalk of the haemagglutinin as well as the neuraminidase. Therefore, it is possible that neutralisation and HI antibodies may differ in their capacity to control influenza infection, and Nab may represent a better early surrogate marker for recovery from H7N9 influenza disease.

In those who survived the infection, reaching Nab titres of ≥1:640 within 14 days of illness was associated with a trend toward faster recovery. Interestingly, while age and time to oseltamivir treatment were not significantly associated with survival in the group overall, they were associated with faster recovery in the survivors. This

difference may be related to the finding that emergence of oseltamivir resistance was associated with adverse clinical outcome in at least two of our patients with fatal outcome [8]. Another recent study reported association between faster serological responses and survival in H7N9 influenza patients, although it did not account for censoring due to time of testing and death, nor investigate the role of other confounding factors such as corticosteroid therapy or antiviral therapy [17].

Antibodies use different mechanisms to contain virus infection, including direct neutralisation of virus entry, antibody-dependent cytotoxic responses and directing presentation of virus–antibody complexes to antigen-presenting cells [14]. It is also possible that the early development of Nab antibodies and the rapid involvement of Nab antibodies in responding to influenza virus infection may alter the innate immune response and thereby result in a milder disease course. Previous reports have demonstrated that severe clinical disease in highly pathogenic avian influenza infection was usually associated with skewed innate immune responses which were characterised by the production of more pro-inflammatory cytokines and less type I interferon, whereas a mild influenza disease course was associated with a balanced type I interferon and inflammatory response [15,16]. An alternative explanation is that the antibody responses are a surrogate marker for cell-mediated immune responses.

In summary, our data demonstrate that the haemagglutinin of the 2013 A(H7N9) influenza virus is immunogenic and capable of rapidly eliciting robust HI and Nab antibody responses. The kinetics of the Nab response are correlated with clinical outcome. Cause and effect cannot yet be established because antibody responses may be a confounding factor associated with other protective determinants of clinical outcome. Convalescent plasma therapy has previously been shown to reduce mortality in patients in intensive care with pandemic H1N1 influenza [18]. Thus, our data may indicate that early passive antibody therapy may be beneficial to clinical outcome, especially in those who fail to mount rapid Nab antibody responses.

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Conflict of interest

None declared.

Authors' contributions

AZ, YH, DT, EL, YW and XL conducted the study and analysed data, ZS, YD, XZ, JZ, MB, MZ, SY, JS, ZZ, YWH, LC, CL and JW processed samples and prepared sera samples., XYZ and ZYZ coordinated the study and provided samples, MP and JX conceived and designed the study, analysed data and wrote the manuscript.

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Seroprevalence of hantavirus infections in Switzerland in 2009: difficulties in determining prevalence in a country with low endemicity

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In several European countries, diagnosis of nephropathia epidemica, a mild form of haemorrhagic fever with renal syndrome (HFRS) caused by Puumala virus infection, has increased over the past 10–20 years. In Switzerland, despite its geographical proximity to regions with epidemic outbreaks in Germany and France, infections are detected only sporadically. To estimate the actual prevalence and potential risk factors of human hantavirus infections in Switzerland, a seroepidemiological study was performed in 2009 on serum samples from 4,559 blood donors and 1,810 military personnel. Sera were screened using commercial Puumala IgG and hantavirus IgG enzyme-linked immunosorbent assays indicating a seroprevalence of 1% and 9%, respectively. Subsequently, the samples were analysed by immunofluorescence assay and immunoblot assay, showing a much lower prevalence, of 0.4% and 0.3%, respectively. Two of the serum samples achieved an 80% reduction in plaque-forming units in a neutralisation test. Statistical evaluation of questionnaires only identified an association of age (above 50 years) with hantavirus seropositivity when adjusted for sex (odds ratio: 2.36; 95% confidence interval: 1.10–5.05). This study provides baseline data (0.3–0.4%) for future monitoring of hantavirus seroprevalence in Switzerland and highlights the challenges in estimating the seroprevalence of these viruses in a country with very low endemicity.

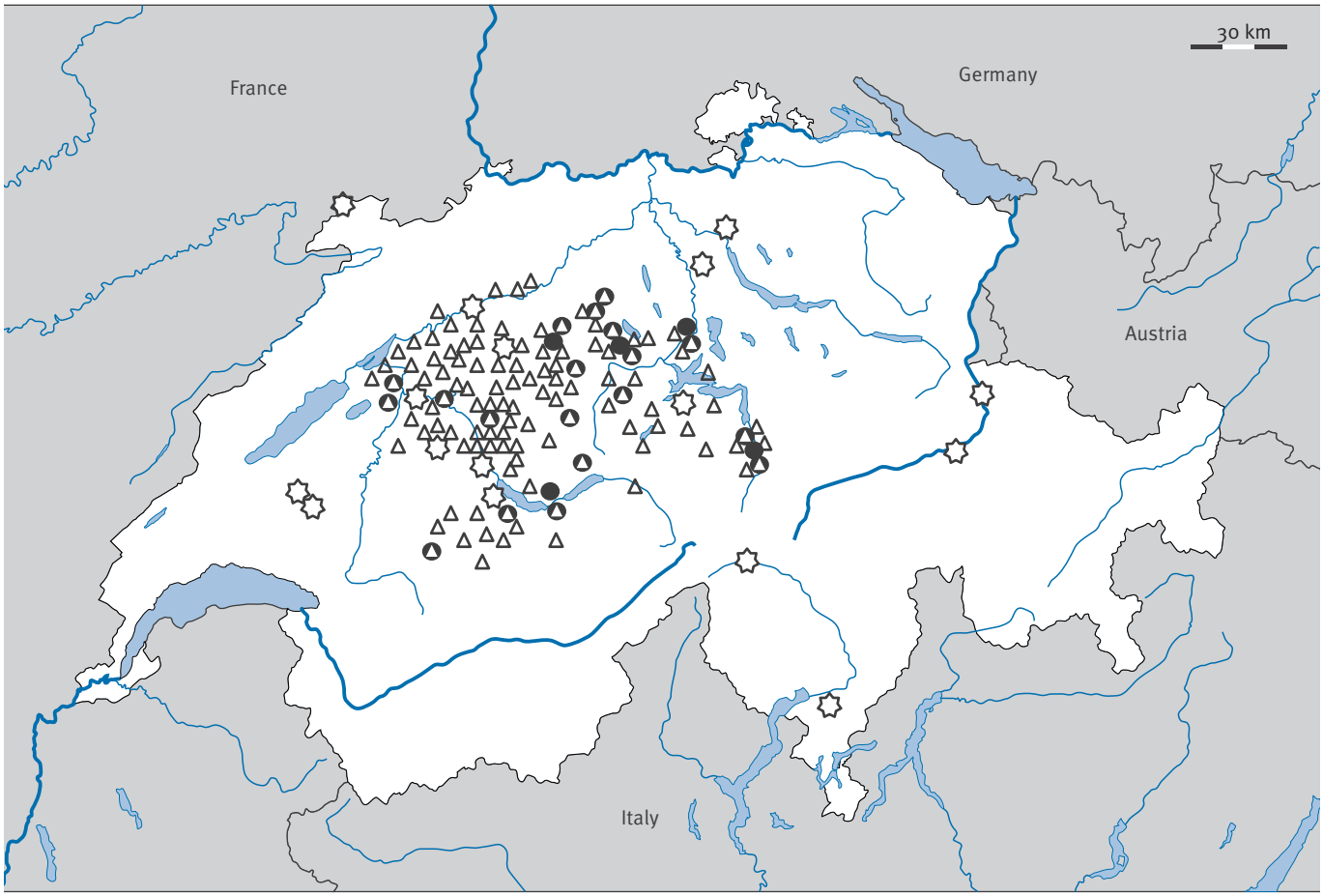
Introduction

At present, the genus *Hantavirus* includes over 20 viruses, which are mainly transmitted from rodents to humans via aerosols. Hantaviruses cause haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) and are responsible for the annual hospitalisation of 150,000 to 200,000 patients worldwide [1,2]. Hantaviruses are predominantly present in distinct regions of Asia, Europe and America. In Europe, the hantavirus species Puumala virus (PUUV), Dobrava virus (DOBV) and Seoul virus (SEOV) cause HFRS in humans. These viruses differ in their geographical distribution and course of infection. PUUV is predominantly present in the European region of Russia (7,000 cases per year), Scandinavia (1,000–3,000 cases per year) and central Europe (300–3,000 cases per year) and to a lesser extent in the eastern part of Europe (Slovenia, Slovakia, Romania, Serbia) [3-5]. PUUV causes nephropathia epidemica (NE), a mild form of HFRS, which is generally not associated with major haemorrhagic symptoms and has a low case fatality rate of approximately 0.4% [6]. In the Balkan Peninsula, DOBV causes HFRS, with a case fatality rate of 9–12% [7]. Saaremaa virus (SAAV), first isolated in Estonia and found also in Russia, Slovenia and Germany, is genetically closely related to DOBV but causes a mild form of HFRS [8,9]. Although other hantaviruses have been isolated in Germany, hantavirus infections in central Europe are generally caused by PUUV [10,11].

An increase in HFRS due to PUUV infection was observed in 2005, 2007 and 2010 in distinct regions of Belgium, Luxembourg, the Netherlands, France and Germany [4]. Some of the most affected regions were located close to the Swiss border [11,12]. While Italy, which shares a border with the south of Switzerland, reported no cases of hantavirus infection between 2005 and 2010 [4], in Austria, a country neighbouring Switzerland to the east, moderate numbers of PUUV infections were reported until 2011, with an increase in the number of

Figure 1

Location of blood sampling sites and place of residence of blood donors with positive hantavirus serology, Switzerland, 2009



Blood samples were collected from 16 military bases throughout Switzerland by a field team of the University of Zurich/ Institute of Social and Preventive Medicine, Division of Communicable Diseases and from 114 locations by mobile teams of the Blood Transfusion Service of the Swiss Red Cross Berne.

Place of residence of blood donors with positive hantavirus serology (positive enzyme-linked immunofluorescence assay screening followed by either a positive immunofluorescence assay and/or a positive or questionable immunoblot assay) is indicated. Dark-grey lines indicate borders; rivers and lakes are depicted in blue.

Map adapted from: http://d-maps.com/carte.php?num_car=2648&lang=en

human cases in 2012 in provinces bordering Slovenia [13]. Despite the proximity of Switzerland to endemic regions in Germany and France, only one case was reported here between 1988 and 2003 (Nicole Gysin, Federal Office of Public Health, personnal communication, 11 December 2013) and a few HFRS patients (between 0 and 4 cases per year) were documented in Switzerland between 2003 and 2011 [14]. However, as the majority (90–95%) of PUUV infections remain subclinical [15] and symptomatic infections may easily be overlooked, due to lack of awareness among Swiss clinicians, the actual number of hantavirus infections may be underestimated in Switzerland.

IgG antibodies produced in response to hantavirus infection persist for 20 years or more [16]. Hence, serological studies can be used to determine the proportion of a population that has been infected with hantaviruses. In European countries where infections with hantaviruses are common, the IgG seroprevalence ranges from 1% to 9% [1-3]. In Germany, the average seroprevalence was estimated at 1–2% in 1995, but

was much higher in 2005 in epidemic areas such as Baden Württemberg and Lower Bavaria (about 7%) [17-19]. A regional and smaller serological study performed in the north-eastern part of Switzerland in 2002–03 indicated that the hantavirus seroprevalence in local blood donors was in the range of 0.5%, with comparable results in selected risk groups such as forestry workers and farmers [20]. A higher seroprevalence was observed among young soldiers tested during their military service (1.9%; 2/103), but the sample size was small and the difference was not statistically significant [20].

Our study aims were threefold: firstly, to estimate the actual PUUV seroprevalence in blood donors in central Switzerland, to provide baseline data for surveillance; secondly, to determine whether there would be a statistically significant difference between the seroprevalence in army personnel and blood donors when the sample size is larger; and thirdly, to generate evidence to increase awareness and preparedness given the cyclical epidemic situations in our neighbouring countries.

We performed a sample size determination based on previous seroprevalence data for Switzerland [20] and Swiss military personnel and blood donors were selected as study populations. Since it is difficult to assess the seroprevalence in a country with low endemicity, we combined the high sensitivity of two ELISAs used for screening with the specificity provided by immunofluorescence, immunoblot and neutralisation assays to confirm the positive sera.

Methods

Study population, data collection and selection criteria

During 2009, a prospective questionnaire-based seroprevalence study was performed in Switzerland. The study protocol was approved by the relevant cantonal ethical boards. To be enrolled, adults (>18 years) had to be German-, French- or Italian-speaking Swiss residents and either soldiers of the Swiss Armed Forces during their military service or registered blood donors.

We selected 16 military bases on the basis of their location throughout Switzerland. All soldiers at the bases, who resided all over the country, were invited to participate in the study. Participation was voluntary. All soldiers were informed orally about the study, asked to give their written consent and to complete a structured questionnaire on the military base before they provided a single blood sample.

All blood donors registered with mobile teams of the Blood Transfusion Service of the Swiss Red Cross Berne received information about the study, the questionnaire and the consent form by post and were asked to take the completed forms to their next blood-donation session, if willing to participate. Samples and forms from registered blood donors were collected in 114 locations within the cantons of Berne and Lucerne and in central Switzerland (Figure 1). Sera were taken during the regular blood donation organised by the mobile teams and uncertainties concerning the questionnaire or study were clarified on site. Testing blood samples obtained by the mobile teams ensured that donors living in rural areas were included, as the teams visit villages outside urban areas.

Potential risk and confounding factors, such as place and location of residence, demographics, occupational and leisure activities, self-perceived current health status, relevant symptoms, smoking history, comorbidities and travel history in the past two years were assessed with the questionnaire.

Serological screening

All sera were screened for hantavirus-specific IgG using commercially available ELISAs. Sera were first analysed with the Hantavirus IgG DxSelect ELISA (Focus, Cypress, USA) then with Hantavirus Puumala IgG/IgM ELISA (Progen Biotechnical, Heidelberg, Germany).

Immunofluorescence assay

Serum samples that tested positive with at least one of the ELISA tests were further analysed by immunofluorescence assay (IFA) using the Euroimmun Anti-Hantavirus-IIFT Mosaic II Test (Euroimmun, Lübeck, Germany). Briefly, 1:100 diluted serum samples were added to each reaction field on biochips containing either uninfected cells or cells infected with PUUV, SAAV, DOBV, Hantaan virus (HTNV) or SEOV.

Immunoblot assay

All ELISA-positive sera were further analysed using the recomLine Bunyavirus IgG/IgM test kit (Mikrogen, Neuried, Germany). In short, serum samples diluted 1:100 were incubated on recomLine test strips containing six lines with complete nucleocapsid proteins from HTNV and PUUV, or a recombinant N-terminus of the nucleocapsid antigen from PUUV, HTNV, DOBV, SEOV or from sandfly fever virus serotype Toscana (TOSV), as well as a control band for the antibody class (IgG or IgM).

Focus reduction assay

The focus-reduction neutralisation test (FRNT) was performed as described previously [21]. An 80% reduction in the number of focus forming units (FFU) compared with the virus control was used as the criterion for virus neutralisation titres.

Sample size determination and statistical analysis

The sample size determination was based on previous data from Switzerland [20] and consisted of a two-sample comparison of proportions with a ratio of 0.5 between military personnel and blood donors (power 80%, two-sided, p=0.05). For the statistical analysis, a positive ELISA combined with either a positive IFA

Table 1

Prevalence of hantavirus antibodies in the study populations by serological tests, Switzerland, 2009 (n=6,369)

Source of sera	Number of sera tested	Number (%) of sera found positive or borderline for hantavirus antibodies			
		Hantavirus ELISA	PUUV ELISA	IFA	IBA
Blood donors	4,559	405 (8.9)	40 (0.9)	22 (0.5)	13 (0.3)
Military personnel	1,810	194 (10.7)	19 (1.0)	4 (0.2)	3 (0.2)
Total	**6,369**	**599 (9.4)**	**59 (0.9)**	**26 (0.4)**	**16 (0.3)**

ELISA: enzyme-linked immunosorbent assay; IBA: immunoblot assay; IFA: immunofluorescence assay.

result and/or a positive or questionable immunoblot assay (IBA) was classified as a positive hantavirus serology (denoting a case).

Due to the small number of cases, our analysis was restricted to the main potential risk factors. All variables were assessed univariately and by subsequent stepwise backward logistic regression using positive hantavirus infection as the outcome (STATA version 12.1).

Results

Study population

A total of 1,810 blood samples were collected from military personnel from May to December 2009. The participation rate was 49.3% (1,810/3,673), of which 1,797 (99.3%) were male and 13 (0.7%) were female. The ages of the participating personnel ranged from 18 to 56 years, with a median of 21 years.

A total of 4,559 samples were collected from blood donors in 114 locations during July to November 2009 (Figure 1). The participation rate was 48.7 % (4,559/9,359), similar to that of the military personnel; however, men (2,720; 59.7%) and women (1,743; 38.2%) were more equally represented. Data on age were unavailable for 96 (2.1%) of the donors sampled; the median age of the rest was 45 years (range: 18–65 years).

Screening by enzyme-linked immunofluorescence assay

All sera were screened for hantavirus IgG using two different ELISA test systems. When the hantavirus ELISA was used, 405/4,559 serum samples from blood donors and 194/1,810 samples from military personnel were positive, corresponding to a seroprevalence of 8.9% and 10.7%, respectively (Table 1). However, according to the results obtained with the PUUV ELISA, 40 samples from the blood donors and 19 samples from the military personnel gave positive results, corresponding to a prevalence of 0.9% in the blood donors and 1% among military personnel.

Analysis by Immunofluorescence and Immunoblot assay

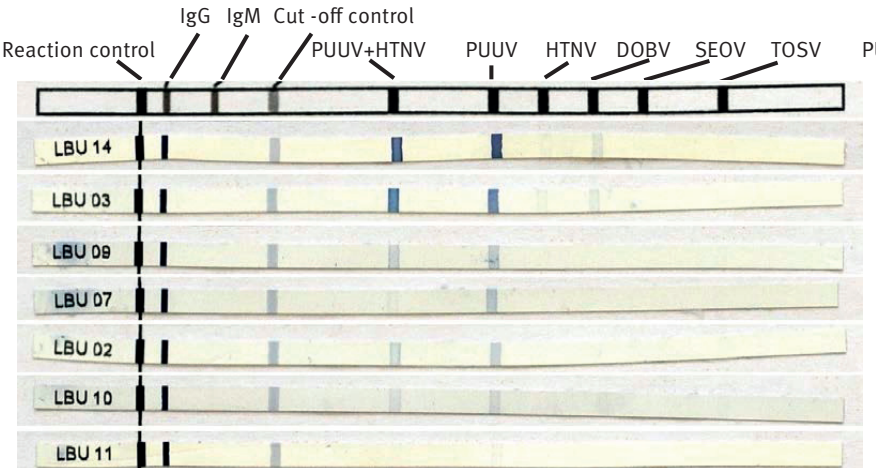
To obtain reliable seroprevalence data, following broad screening using two different ELISAs, all positive samples from both ELISAs (n=655) were further analysed by IFA and IBA. Both tests allow us to differentiate between hantaviruses causing disease in Europe and were used to confirm the ELISA results. Of the 655 serum samples tested by IFA, 25 showed positive results for PUUV; six of these sera produced a strong or very strong immunofluorescence signal and 19 sera showed a weaker but still specific PUUV-fluorescence pattern (data not shown). Cross-reactivity with other hantaviruses (HTNV, SEOV, DOBV, SAAV) was observed for several sera (n=11), but differentiation was possible in most cases (n=8) by comparing the signal intensities to the different hantaviruses. One serum showed a specific reaction for the SAAV or DOBV antigen) and two sera showed a comparable fluorescence signal for PUUV and SAAV and/or DOBV.

When analysed by IBA, even fewer (n=16) of the 655 sera tested showed positive or questionable band patterns for PUUV or other hantaviruses (illustrated in Figure 2). In most sera (11/16), the signal was specific for PUUV with only minimal cross reactivity to the antigens of other hantaviruses (Table 2). Two sera reacted specifically to DOBV, with minimal cross reactivity; another showed a strong signal for both DOBV and HTNV. Several sera (n=24/6,369) reacted against the TOSV antigen (data not shown). The agreement between the different tests was good for sera producing strong IFA and IBA results but was only around 50% when the immunofluorescence signal was weak (Table 2).

In summary, of all 655 ELISA-positive sera analysed in more detail, 30 showed clearly positive results by IFA and/or IBA. A total of 26 were positive by IFA and 16 gave positive (n=9) or questionable (n=7) band pattern in the IBA, resulting in an overall seroprevalence of the 6,369 sera tested of 0.4% (IFA) and 0.3% (IBA). The seroprevalence in the blood donors (4,559 sera) was 0.5% when confirmation was based on the IFA and 0.3% when IBA was used.

Figure 2

Immunoblot pattern of serum samples analysed to confirm IgG antibodies against hantaviruses, Switzerland, 2009



-	no band
+/-	weak band
+	band of equal intensity as cut-off
++	band of stronger intensity than cut-off

IBA: immunoblot assay; DOBV: Dobrava virus; HTNV: Hantaan virus; neg: negative; pos: positive; PUUV: Puumala virus; quest: questionable; SEOV: Seoul virus.

All sera (n=655) from blood donors and military personnel with a positive ELISA were confirmed by IBA. The immunoblots show examples of sera from blood donors rated positive, questionable and negative according to the band intensities. Serum samples were rated positive, if the PUUV+HTNV band was of stronger intensity compared with the cut-off-control (examples 4476 and 682) or if of equal intensity; at least one additional band of hantavirus serotype PUUV, HTNV, DOBV or SEOV had to be of at least equal intensity (example 2076). The result was estimated as questionable if only one of the bands was of equal intensity as the cut-off band (examples 183 and 1387) and negative, if the signal was weaker than the cut-off (example 2270) or absent (example 2798).

Confirmation by focus reduction neutralisation test (FRNT)

Of the 30 sera showing clearly positive results by IFA and/or IBA, 14 were analysed for their neutralising capacity against PUUV and HTNV. At a dilution of 1:40, only two sera (numbers 682 and 4476) could neutralise these hantaviruses (Table 2). While serum 624 was also capable of neutralising PUUV at a serum dilution of 1:80, serum 4476 was not. Both sera showed very strong positive results in the IFA and IBA assays. The other two sera with comparably strong reaction patterns for PUUV by IFA and IBA (numbers 149 and 3051) did not reach the 80% reduction level required for a positive result in the FRNT. Nevertheless, they achieved a reduction of greater than 50% in FFU, indicating that neutralising antibodies against hantaviruses might be present. Sera with only weak signals in the IFA or IBA did not lead to a significant reduction of FFU in the FRNT (data not shown).

Evaluation of risk factors for infection

To assess potential risk factors for hantavirus infection, univariate and multiple backwards logistic regression analyses were performed on positive serum samples cases (defined as having a positive ELISA test and confirmatory IFA and/or positive or questionable IBA results). No significant association of risk factors, such as recreational activities or travel to endemic countries with seropositivity was identified: only age (above 50 years) was associated with an increased risk of seropositive ELISA and IFA or IBA assay. When dichotomised into two age groups (≤50, >50 years) and controlled for sex, the odds ratio was 2.36 (95% confidence interval (CI): 1.10–5.05), which indicates an approximately 2.5-fold increased risk of hantavirus infection for people over 50 years-old. By place of residence (postal code) of 24 of the 25 blood donors with positive serum samples (information on postal code was missing for one donor), no clustering could be detected (Figure 1).

Discussion

The seroprevalence of hantavirus infections of 0.3–0.5%, estimated in blood donors from central Switzerland, is low compared with the seroprevalence observed in endemic regions of surrounding countries such as Baden-Württemberg in south-west Germany (about 2–3% [17,19]) but seems to be somewhat higher than the prevalence found in other countries where no

Table 2

Summary of serological data of hantavirus-reactive serum samples, Switzerland, 2009 (n=30)

Serum ID	Hantavirus ELISA		PUUV ELISA		IFA						IBA						FRNT	
						Signal intensity						Band intensity						
	Result	OD S/Cal	Result	OD S/Cal	Result	HTNV	PUUV	SEOV	SAAV	DOBV	Result	PUUV/HTNV	PUUV	HTNV	DOBV	SEOV	PUUV	HTNV
Blood donors																		
149	pos	4.474	eqv	1.373	pos	+	++	-	-	-	pos	++	++	-	-	-	neg	neg
183	neg	0.707	pos	1.655	pos	-	+	-	-	-	pos	+	+	-	-	-	neg	neg
682	pos	1.614	eqv	1.412	pos	+	+++	-	+	+	pos	++	++	+/-	+/-	-	1:80	1:40
977	pos	1.326	neg	0.355	neg	-	-	-	-	-	pos	++	+/-	+/-	++	-	ND	ND
990	neg	0.171	pos	2.272	pos	-	++	-	-	-	neg	-	-	-	-	-	ND	ND
1064	pos	1.742	neg	0.243	pos	+	+	+	+	+	pos	++	-	++	++	-	ND	ND
1387	pos	1.337	eqv	1.285	neg	-	-	-	-	-	quest	-	+	-	-	-	neg	neg
1474	pos	1.611	neg	0.295	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
1483	neg	0.480	pos	1.585	pos	-	+	-	-	-	quest	-	+	-	-	-	neg	neg
2076	neg	0.845	pos	1.752	pos	+	+	-	-	-	pos	+	+	-	-	-	neg	neg
2270	neg	0.743	pos	1.633	pos	+	+	-	-	-	neg	+/-	+/-	-	-	+/-	neg	neg
2404	neg	0.242	pos	1.656	pos	-	+	-	-	-	quest	+	-	-	-	-	ND	ND
2511	pos	1.847	neg	0.185	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
2551	pos	2.047	neg	0.188	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
2798	neg	0.092	pos	2.893	pos	-	+	-	-	-	neg	-	+/-	-	-	-	neg	neg
3051	pos	2.187	eqv	1.081	pos	+	++	-	-	-	pos	++	++	-	-	-	neg	neg
3115	pos	3.115	neg	0.297	pos	-	+	-	+	+	neg	-	-	-	-	-	ND	ND
3389	pos	1.205	neg	0.472	pos	-	+	-	-	-	quest	+/-	+	-	-	-	neg	neg
3529	pos	1.889	neg	0.351	pos	-	+	+	+	-	neg	-	-	-	-	-	ND	ND
3585	neg	0.245	pos	1.628	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
4162	pos	3.487	neg	0.382	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
4304	neg	0.307	pos	1.917	pos	-	+	+/-	-	+/-	neg	-	-	-	-	-	ND	ND
4320	pos	3.707	neg	0.311	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
4476	pos	3.421	pos	1.741	pos	+	+++	+	++	++	pos	++	++	+/-	+	-	1:40	1:40
4521	pos	3.024	pos	1.589	neg	unsp	unsp	unsp	unsp	unsp	quest	+/-	+	-	-	-	neg	neg
Military personnel																		
I 147	pos	1.578	neg	0.557	neg	-	-	-	-	-	pos	++	-	-	-	-	ND	ND
L55	pos	4.012	pos	1.644	pos	-	+	-	-	-	quest	+/-	+	-	-	-	neg	neg
R1	pos	3.911	neg	0.351	pos	-	-	+/-	+	+	quest	-	-	-	+	-	ND	ND
M14	neg	0.153	pos	1.807	pos	-	+	-	-	-	neg	-	-	-	-	-	ND	ND
X6	neg	0.659	pos	2.164	pos	-	+++	-	-	-	neg	+/-	+/-	-	-	-	neg	neg

Cal: calibrator; DOBV: Dobrava virus; ELISA: enzyme-linked immunosorbent assay; eqv: equivocal; FRNT: focus reduction neutralisation test; HTNV: Hantaan virus; IFA: immunofluorescence assay; ND: not done; neg: negative; OD: optical density; pos: positive; PUUV/HTNV: Puumala or Hantaan virus; PUUV: Puumala virus; ques: questionable; S: sample; SAAV: Saaremaa virus; SEOV: Seoul virus nucleocapsid protein; unsp: unspecific.

IFA

-	no signal
+/-	barely visible signal
+	weakly positive signal
++	clearly positive signal
+++	bright, positive signal

IBA

-	no band
+/-	weak band
+	band of equal intensity as cut-off
+ +	band of stronger intensity as cut-off

Listed are all samples that were found positive by ELISA screening and could be confirmed by either IFA or IBA. The OD ratio (OD of the sample divided by the OD of the calibrator for the two ELISAs and the signal intensities observed by IFA and IBA are indicated. Sera showing a positive signal by IFA or IBA were further analysed by FRNT. When positive, the serum dilution at which 80% focus reduction was achieved is indicated.

or only very few HFRS cases were reported. In Spain, seroprevalence of 0.06% was found in 2003 in more than 10,000 sera from blood donors using methods comparable to those used in our study [22], while other studies published in 2002 and 2009, based on smaller number of samples and using different methods for screening and confirmation, reported a seroprevalence of 0.31% and 2% respectively for distinct areas in Spain [23,24]. In Italy, a serological study on sera collected in 2002 in the north of the country from 488 forestry workers revealed no serum reaction to PUUV and, although a low seroprevalence for hantaviruses (0.4%) was found in bank voles [25]; no human cases of hantavirus infection were reported in Italy between 2000 and 2010 [4,26]. In Spain, only a few HFRS cases were reported during the same time period [4,26]. In Switzerland, seven cases of hantavirus infections were documented between 2003 and 2010 [14].

In 2012, eight new cases of hantavirus infections were reported to the Swiss Federal Office of Public Health (incidence: 0.1 per 100,000 population) [14]. Seven of the cases were confirmed, of whom five had been infected in an endemic region outside Switzerland (Nicole Gysin, Federal Office of Public Health, personnal communication, 10 December 2013). This is in contrast to the situation in Baden Württemberg, southwest Germany, where high numbers of infections were reported for 2012 (in weeks 1–17, n=501; incidence: 4.66 per 100,000 population) [27]. In Germany as a whole, seroprevalence of 1–2% was estimated 1995, while values of over 5% were documented more recently for highly endemic regions of Baden-Württemberg (2001) and Lower Bavaria (2009) [17-19]. Considerable differences between neighbouring regions have often been observed with hantavirus infections [18,19].

Our study highlights the difficulties arising from limited test specificity when investigating the hantavirus seroprevalence retrospectively in a population with a low incidence of infections. To ensure maximum sensitivity, we used two different ELISA kits for the screening. The hantavirus ELISA is based on a pool of recombinant nucleoprotein antigens and should detect antibodies against the most frequently detected European HFRS-causing hantaviruses. In our study, it is likely that this ELISA led to an excessively high rate of false-positive results, since very few of the 599 sera with positive results from the hantavirus ELISA could be confirmed by IFA or IBA. The reasons for this high proportion of non-specific reactions are unclear, but might be related to the recombinant antigens used or to problems associated with washing parameters of the automated ELISA system used for the study, although the assay quality controls were within the kit specifications. In other serological studies, higher serum dilutions were used for screening [24] or an increased cut-off value was proposed [28] to get around the problem of non-specific binding. The PUUV ELISA results for the same samples found that only 1% of the sera gave a positive OD ratio, indicating that this ELISA was less likely to generate false-positive results.

Of the 655 sera analysed by both ELISAs, only 30 could be confirmed by either IFA and/or IBA. Since none of the tested individuals reported symptoms that could be unequivocally attributed to a previous or current hantavirus infection, it seems impossible to determine whether they had been infected with hantaviruses or not. The FRNT, used as third method to further evaluate samples showing specific reaction to PUUV by IFA and IBA, is widely accepted as the gold standard for hantavirus serology of non-acute samples [21]. When an 80% reduction in FFU was applied as the cut-off, only two samples were positive at a serum dilution of 1:40 and two more samples achieved a 50% reduction in FFU. These four sera showed a strong signal in the IFA and IBA. Interestingly, no clear reduction in FFU was observed for any of the other sera analysed, which raises the question of whether the excellent specificity of the FRNT may be acquired at the expense of its sensitivity. For clinical infections, the presence of antibodies has been demonstrated up to 10–20 years after infection, using the FRNT [16,29]. Whether this would also be the case for subclinical infections is unknown. Furthermore, the selection of the virus strain used in the FRNT may influence the outcome. In our study, neutralisation was performed using a Russian PUUV strain (Kazan): the isolates circulating in central Europe may differ from this Russian isolate [30]. This may partially explain the fact that only sera with presumably high antibody titres led to a reduction in FFU. Interestingly, the neutralisation capacity of both sera that were positive in the neutralisation test was very similar for the two hantaviruses tested (PUUV and HTNV). This suggests that the PUUV strain used either differed substantially from the virus causing the infection and/or that some of the tested individuals might have been infected with another hantavirus species. The presence of Tula virus, for example, has been documented in Switzerland, in a 10 year-old boy bitten by a small wild rodent in 2000 as well as in rodents trapped between 2001 and 2009 [31,32].

Several laboratories in Europe use the IBA as diagnostic or confirmatory test [33,34]. This was also the method of choice in the earlier serological study performed in the north-eastern part of Switzerland [20]. This analysis of blood samples from 2002–03 revealed a seroprevalence of 0.5% among blood donors and in occupational risk groups, with a higher seroprevalence observed only in the small group of military personnel (2/103; 1.9%). When the same criterion (positive IBA) was used for confirmation, we found a slightly lower seroprevalence of 0.3% in the blood samples collected mainly in the central part of Switzerland. The rather minor difference in prevalence obtained in the two studies may result from our larger sample size, methodological differences when performing and interpreting the assay or it may reflect a truly higher prevalence in the population in the north-east of the country.

However, even a population-representative sample would scarcely allow to detect (small) foci and the high sample size needed would have been impracticable. Hence the reason we sampled a population residing in mainly rural areas, as living close to forested areas was found to be a risk factor [35].

Some recreational/leisure or work-related activities are known to be risk factors for PUUV infection [36-38]; but this was not found in our study. Travel to other endemic countries, such as in Asia or south-east Europe, was also not found to increase risk. Both findings might be explained by the low case numbers. An association of age (above 50 years) with seropositivity was found, which is consistent with observations from Sweden [39] based on seroepidemiology, but not with German findings, which were based on notification data including clinical symptoms [11]. This fact might be explained by the persistence and accumulation of hantavirus IgG antibodies in elderly persons in endemic regions, compared with those newly acquired in the course of a recent symptomatic infection within one epidemic season.

Although the large sample of blood donors was representative for the blood donor population in Switzerland, this study is limited by the fact that blood donors differ from the general population in several aspects, including health-related anthropometric and personality-related variables [40]. In addition, although the blood donors samples covered well the region of central Switzerland, we do not have data on donors in other regions of the country. Although blood donors may not represent an ideal control group for diseases related to environmentally, behaviourally or socially patterned exposures [39], due to the ease of accessibility of blood samples and personal information, blood donors are popular study populations for serological analysis. Serological data from blood donors may be used as basis value for comparative studies with population groups representing potential risk groups [20,41].

In our study, we compared the seroprevalence of blood donors residing in central Switzerland with that of young soldiers residing all over Switzerland. On the basis of prevalence data from a study performed in the north-eastern part of Switzerland in 2002–03, we investigated predominantly young soldiers as a potential risk group, but since their military service did not take place during a year of increased hantavirus activity in regions close to Switzerland, they were not at particular risk of infection [4,27,38]. Due to time-consuming preparatory work, the period of blood collection in the military personnel could not be handled flexibly and could not be postponed to the following year: in that year, increased numbers of hantavirus infections were documented in Germany [4,11]. Prevalence data from various populations, including blood donors, risk groups and symptomatic or asymptomatic volunteers from the general population, are available from several European countries, rendering them attractive for comparative analysis between different regions [18,22-24]. But, as we have shown, the methods applied to determine prevalence data need to be considered as well, when comparing different studies, since the influence of the test sensitivity and specificity on the determined seroprevalence may be substantial.

In summary, we have found a low prevalence of hantavirus infections in the study populations, but the periodic hantavirus epidemics in neighbouring countries requires attention of public health authorities and measures of preparedness including active surveillance need to be evaluated. Furthermore, at-risk populations – known from other studies to be people living in rural areas and people carrying out activities in areas and facilities infested by rodents – should be informed about potential exposure risk to hantaviruses and should be advised regarding precaution measures and symptoms.

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