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Large ongoing measles outbreak in a religious community in the Netherlands since May 2013

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Despite vaccination coverage over 95%, a measles outbreak started in May 2013 in the Netherlands. As of 28 August, there were 1,226 reported cases, including 82 hospitalisations. It is anticipated that the outbreak will continue. Most cases were orthodox Protestants (n=1,087/1,186; 91.7%) and unvaccinated (n=1,174/1,217; 96.5%). A unique outbreak control intervention was implemented: a personal invitation for measles-mumps-rubella (MMR) vaccination was sent for all children aged 6–14 months living in municipalities with MMR vaccination coverage below 90%.

Outbreak description

The first two measles cases in this outbreak that were reported, occurred in an orthodox Protestant school in the Netherlands and were reported on 27 May 2013. As of 28 August, a total of 1,226 measles cases (incidence 73.1 per 1 million) who acquired infection in the Netherlands have been reported by 19 Municipal Health Services (Figure 1). The case with the earliest date of onset of exanthema in this outbreak had not travelled abroad and the source of infection remains unknown.

Case definition

The routine measles case definition is based on the presence of clinical measles symptoms (fever and maculopapular rash and cough, coryza or conjunctivitis) in combination with laboratory confirmation or an epidemiological link (contact in the previous three weeks) to a laboratory-confirmed case.

Laboratory confirmation is based on either measles-specific IgM serology for venous- or fingerstick-blood samples or specific detection of measles virus RNA by polymerase chain reaction (PCR) in throat swabs, oral fluid or urine specimens.

Of the 1,226 cases, 176 (14.4%) had complications including encephalitis (1 case), pneumonia (90 cases) and otitis media (66 cases) and 82 (6.7%) were admitted to hospital. (For a case description of the encephalitis case (in Dutch), see [1].) There were no deaths.

The median age of cases was 10 years (range: 0-54). Most cases were 4–12 years of age (n=717; 58.5%), while 200 (16.3%) were aged 13–15 years (Figure 2). Nearly all cases were unvaccinated (1,174; 96.5% of 1,217 with known vaccination status), 39 cases (3.2%) were vaccinated with one dose of a measles-containing vaccine and four cases (0.3%) were vaccinated with two doses.

Most cases were orthodox Protestant (1,087; 91.7% of 1,186 cases with information). Reasons for being unvaccinated were: 1,072 (93.6% of 1,145 cases with information) orthodox Protestantism, 3 (0.3%) anthroposophical, 30 (2.6%) parents' or own critical attitude towards vaccination, and 40 (3.5%) other. Most cases (719; 58.6%) occurred in municipalities with MMR vaccination coverage below 90% (Figure 3). Of the cases occurring in high-vaccination coverage (≥90%) areas, the majority (425; 86.4% of 492 cases with information) was also orthodox Protestant.

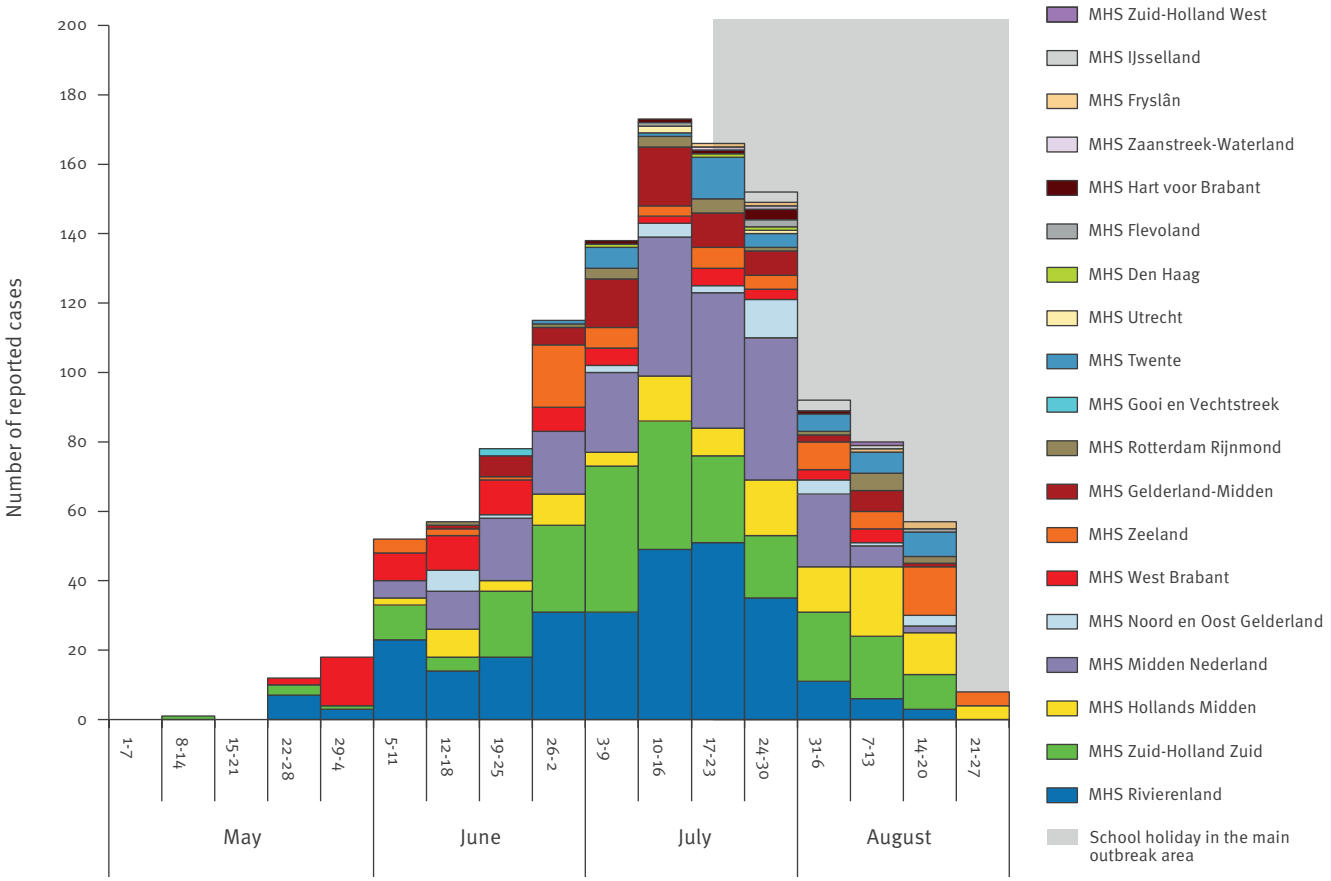
Of the 1,226 reported cases, 10 were healthcare workers who probably acquired the infection at their place of work. Nine were unvaccinated and one was vaccinated with two doses of measles-containing vaccine. Nosocomial transmission to patients has not been reported.

Laboratory confirmation and genotyping

Laboratory confirmation was obtained in 363 of 1,226 cases (29.6%); the other cases were notified on the basis of an epidemiological link with a laboratory-confirmed measles case. The vast majority of laboratory-confirmed cases are confirmed using PCR testing of oral fluid specimens from cases who were captured through exanthema surveillance, which had been implemented since 2003.

Figure 1

Reported measles cases by week of onset of exanthema and Municipal Health Service region, the Netherlands, 1 May–28 August 2013 (n=1,199)[a]

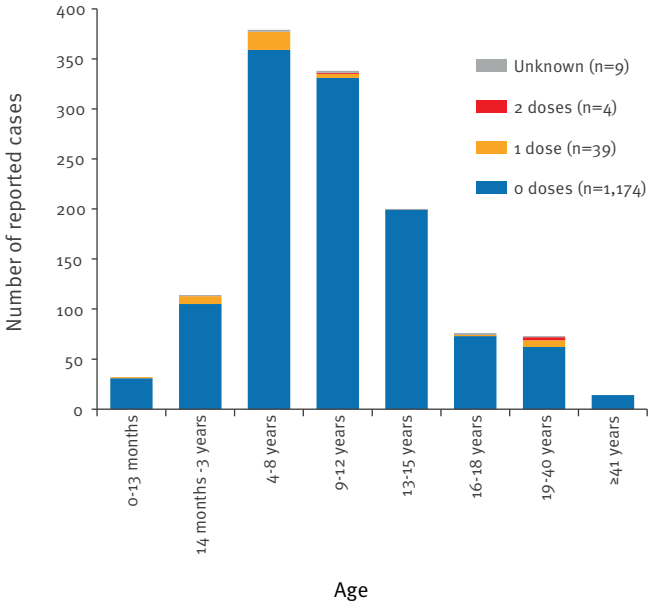


MHS: Municipal Health Service.

[a] Information on date of exanthema onset was missing for 27 cases.

Figure 2

Reported measles cases by age group and measles vaccination status, the Netherlands, 1 May–28 August 2013 (n=1,226)



The cases that have been genotyped (n=150) were all genotype D8 and all had the same sequence type (MVi/DenHaag.NLD/8.13/1, WHO/MEANS ID 32423). As of January 2013, genotype D8 has been reported for the majority of measles cases within the World Health Organization (WHO) European Region and the Dutch sequence is identical to what is currently referred to as the Taunton sequence-type of D8 (K. Brown, Public Health England, personal communication, 5 April 2013 and M. Mulders, World Health Organization, personal communication 12 June 2013).

Background

A single dose of monovalent measles vaccine was included in the Dutch national immunisation programme in 1976 for children aged 14 months. Since 1987, children have been offered vaccination against measles, mumps and rubella in a two-dose schedule, at 14 months and nine years of age. Vaccination coverage is generally high in the Netherlands. In 2012, the MMR coverage was 96% for the first dose and 93% for the second dose (birth cohorts 2010 and 2002, respectively). However, vaccination uptake is low in some specific groups, for religious reasons (orthodox Protestantism), anthroposophic reasons, and in those

with a critical attitude towards vaccination. While the last two groups are spread throughout the Netherlands, orthodox Protestants are a close-knit community of 250,000 persons, mostly living in an area that stretches from the south-west to the north-east of the country, the so-called Bible belt. Vaccination coverage in general among orthodox Protestants was assessed in 2006-2008 as about 60% [2]. Predestination is an important theme in their beliefs: refusal of vaccination is based on the idea that people should not interfere with divine providence [3]. Since they intensively share educational, social, cultural and religious activities, they do not benefit from herd immunity that protects unvaccinated individuals living elsewhere in the Netherlands [4].

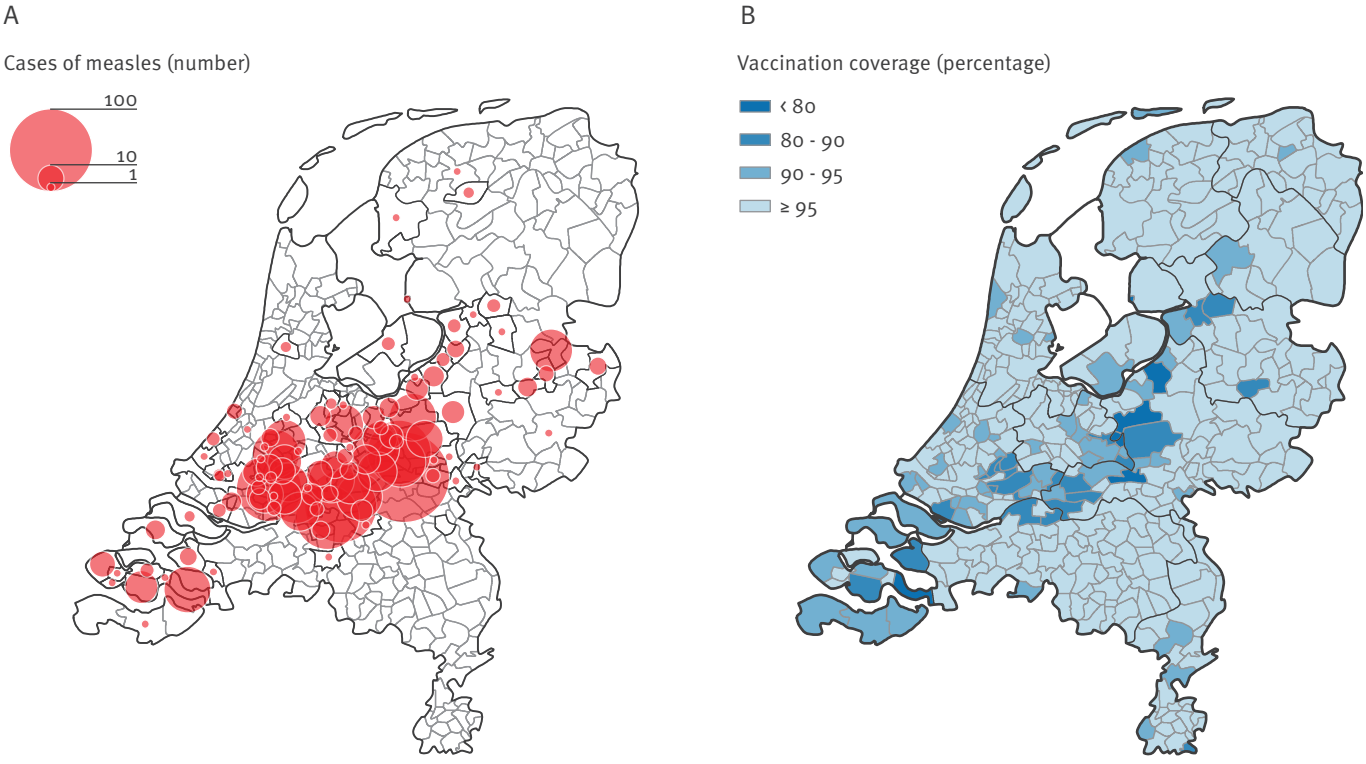
Measles has been a notifiable disease in the Netherlands since 1976. Since introduction of measles vaccination, outbreaks among unvaccinated individuals occurred every four to seven years, e.g. a small outbreak among anthroposophists occurred in 2008 [5, 6]. The most recent large outbreak in the Bible belt occurred in 1999–2000, with more than 3,200 reported cases, 3 deaths and an estimated 150 hospitalisations [7, 8]. In the Bible belt, there are 29 municipalities with a vaccination coverage for the first dose of MMR of less than 90%, in which approximately 5% of the Dutch population lives [9]. As measles is a highly contagious disease, in these 29 municipalities, all non-immune individuals – orthodox Protestant as well as others – are considered to be at risk of contracting measles during an outbreak.

Control measures

An outbreak management team was convened on 17 June 2013 to provide scientific advice on control measures. The team defined infants between 6 and 14 months of age living in municipalities with MMR vaccination coverage below 90% as the main risk group for developing measles complications. This age group is at relatively high risk since most mothers are currently vaccinated against measles, which leads to lower levels of maternal antibodies than does natural infection [10]. The team advised that children in this age group should be targeted for an additional (for children aged 6–12 months) or early (for children aged 12–14 months) MMR vaccination. Parents of children in this age group living in municipalities with vaccination coverage below 90% received a personal invitation by post through the routine vaccination programme register. The Netherlands has a very complete national vaccination registration, which allows direct targeting of additional vaccination to risk groups [11].

Figure 3

Reported measles cases by municipality, 1 May–28 August 2013 (panel A, n=1,226) and vaccination coverage of first MMR vaccine dose by municipality[a] for birth cohort 2010 at the age of two years (panel B, n=184,230), the Netherlands



MMR: measles-mumps-rubella.

[a] There are 30 municipalities with MMR-1 vaccination coverage below 90%, of which 29 are within the 'Bible belt'. The other municipality is Vaals, in the far south-east of the Netherlands. A considerable number of the infants living in Vaals receive their vaccinations in Germany and are therefore not registered in the Dutch vaccination registration, which explains the low vaccination coverage (84.3%).

Although previous research and practical experience have shown low acceptance of catch- up vaccination among orthodox Protestants during outbreaks [4], the team advised offering MMR vaccination to all unvaccinated orthodox Protestant children from six months to 19 years of age, even if they were living in municipalities with high vaccination coverage. The aim was to provide individual protection and increase vaccine coverage. As a person's religion is not registered, this offer was publicised through media focusing on the orthodox Protestant community.

In addition, all unvaccinated individuals aged 14 months up to 19 years were invited for catch-up vaccination through the general media.

Post-exposure guidelines [12] recommend vaccination of contacts of a case of measles when they are unprotected and aged six months or older. For younger infants who have had contact with a measles case passive immunisation with immunoglobulin, or MMR vaccination when aged four months or older, could be considered, depending on the time since exposure and the measles immune status of the mother.

National recommendations to reduce the risk of measles in healthcare workers were recently finalised [13]. These suggest that healthcare workers born after 1965 should actively check their vaccination or measles infection status and complete their MMR vaccination schedule if needed. Healthcare workers born before 1965 and those vaccinated twice are considered immune. All hospitals in the Netherlands have been approached and encouraged to comply with these recommendations. The effects of the control measures will be evaluated.

Discussion

Recently, a review of measles susceptibility of infants below the age of the first MMR vaccine dose was published [14]. This listed four European countries where early MMR vaccination (from the age of six months) was recommended during outbreaks (Greece, Italy, Romania, Spain). To our knowledge, the current vaccination campaign in the Netherlands is unique in that it is implemented using the national vaccination register, which allows a personal invitation to be sent to parents of children in the target population to have their child vaccinated. The uptake of the vaccination among the vaccine accepting population is therefore likely to be much higher than when there was only a recommendation for vaccination [15-18]. The number of MMR vaccinations administered before the age of 14 months was ten times higher in July 2013 compared with July 2012, indicating that parents adhere to the invitation. However, exact vaccination coverage is not known yet. Measles vaccination at 6–9 months of age results in suboptimal humoral immunity, which may not be completely repaired by repeated vaccination [19]. The clinical and immunological impact of the vaccination campaign will be assessed in dedicated studies.

The current outbreak was anticipated because of the large percentage of susceptible orthodox Protestant children (more than 40%) based on serological data from 2006-2007 [20]. The current percentage of susceptible individuals is estimated to be larger than prior to the 1999–2000 outbreak [21], due to the lack of natural immunity as measles virus did not circulate in this community since 2000. Therefore, we expect that the number of measles cases in the current outbreak will be higher than in the 1999–2000 outbreak, in which more than 3,200 cases were reported [7]. The current epicurve of the outbreak (Figure 1) indicates a clear decrease in the number of measles cases in the last 5–6 weeks. This is most probably due to the summer holidays, i.e. the closing of the schools, which are one of the main sources of transmission. As in the 1999–2000 outbreak, we expect the number of cases to increase again after the summer holidays. School closure or exclusion may be effective to control small local outbreaks of measles [22]. Considering that the current outbreak started in a large number of susceptibles living in a widespread area, these interventions may not be feasible or effective: it is likely that they would delay rather than stop the outbreak.

The number of reported cases in the outbreak is probably a large underestimation of the actual number of measles cases because not all patients consult a physician and not all patients seeking consultation are reported. In the 1999–2000 outbreak, it was estimated that only 9% of all measles cases were reported [7]. If we assume the same degree of under-reporting applies to the current outbreak, the actual number of cases would currently be over 13,000.

Until now, cases were mainly orthodox Protestants (92%). Based on the proportion of orthodox Protestants in the Netherlands and the vaccination coverage among these groups [23], it is estimated that only 15% of the individuals who refuse vaccination are orthodox Protestants. There is therefore a risk that the outbreak might spread to individuals who refuse vaccination because of reasons other than religion, including anthroposophists and those with a critical attitude towards vaccination, or to people who are too young or ill to be vaccinated. However, these individuals are more dispersed over the country and are therefore better protected by herd immunity. Nevertheless, it remains important to monitor the spread of the outbreak outside the orthodox Protestant community.

On 15 August 2013 the Dutch National Institute for Public Health and the Environment (RIVM) received an alert from Canadian public health authorities regarding a Dutch citizen who developed measles whilst in Canada. The strain isolated from this case was indistinguishable from the Dutch outbreak strain, consistent with his epidemiological link to two cases in the Dutch orthodox community (L. Sherrard, Public Health Agency of Canada, personal communication, 3 September 2013). Onward transmission from this Dutch

case in Canada has not been reported. For all large outbreaks of vaccine preventable diseases that occurred in the Dutch orthodox reformed community since the 1990s spread to Canada, and occasionally the US, has been documented [24]. Spread to neighbouring countries where pockets of unvaccinated people and areas with lower MMR coverage exist, such as Germany and the United Kingdom, could also occur, but there is no specific contact between the orthodox Protestant community in the Netherlands and unvaccinated people in neighbouring as is the case with Canada.

Since the 1999–2000 outbreak, the incidence of measles notifications in the Netherlands has been below the WHO European Region threshold for measles elimination (1 per 1 million population per year [25]) for all years except 2008 and 2011. However, because of the unique social and geographical clustering of religious communities with low vaccination coverage, the risk of large outbreaks remains in the Netherlands, as illustrated by the current outbreak.

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Conflict of interest

None declared.

Authors' contributions

AU, RB and MW contributed to acquisition of data. MK, ES, LM and SH analysed and interpreted the data. MK, AU and SH drafted the manuscript. ES, LM, WR, RB, MW, HM and AT critically revised the manuscript. All authors approved the final version of the manuscript.

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Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013

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We describe a novel spike pseudoparticle neutralisation assay (ppNT) for seroepidemiological studies on Middle East respiratory syndrome coronavirus (MERS-CoV) and apply this assay together with conventional microneutralisation (MN) tests to investigate 1,343 human and 625 animal sera. The sera were collected in Egypt as a region adjacent to areas where MERS has been described, and in Hong Kong, China as a control region. Sera from dromedary camels had a high prevalence of antibody reactive to MERS-CoV by MERS NT (93.6%) and MERS ppNT (98.2%) assay. The antibody titres ranged up to 1,280 and higher in MN assays and 10,240 and higher in ppNT assays. No other investigated species had any antibody reactivity to MERS-CoV. While seropositivity does not exclude the possibility of infection with a closely related virus, our data highlight the need to attempt detection of MERS-CoV or related coronaviruses in dromedary camels. The data show excellent correlation between the conventional MN assay and the novel ppNT assay. The newly developed ppNT assay does not require Biosafety Level 3 containment and is thus a relatively high-throughput assay, well suited for large-scale seroepidemiology studies which are needed to better understand the ecology and epidemiology of MERS-CoV.

Introduction

A novel lineage C beta-coroanvirus was isolated from a patient with fatal viral pneumonia in Saudi Arabia in 2012 and termed Middle East respiratory syndrome coronavirus (MERS-CoV) [1]. As of 3 September 2013, 108 human cases have been confirmed, 50 of which were fatal [2]. Locally acquired cases have been reported from Jordan, Qatar, Saudi Arabia and the United Arab Emirates, and imported index cases, sometimes with secondary local transmission, have been reported in France, Germany, Italy, Tunisia and the United Kingdom [2-4]. Clusters of cases suggestive of limited human-to-human transmission have been reported; the largest cluster of cases to date occurred at a healthcare facility in Al-Hasa, Saudi Arabia [5]. The epidemiology of the disease so far is suggestive of multiple zoonotic transmissions from an animal reservoir leading to human infection, sometimes with secondary transmission events in humans.

Phylogenetically closely related, although not identical, viruses have been found in insectivorous bats in Africa and Europe [6,7]. More recently, a very short fragment (181 bp) of the RNA-dependent RNA polymerase gene that was genetically identical to MERS-CoV has been detected in a *Taphozous perforatus* bat captured in the vicinity of the residence of a human case with MERS [8]. These findings remain to be confirmed with more definitive sequence data. Even if MERS-CoV is found in bats, the possibility of an intermediate peridomestic host remains important to explore.

Since antibody responses following coronavirus infection remain detectable for many years [9], seroepidemiology of potential animal species for MERS-CoV-specific antibody is a logical approach to identify candidate species for further investigation. A recent report suggests that MERS-CoV antibody was detected in dromedary camels in the Arabian peninsula [10]. While a number of serological tests, including ELISA assays, immunoflourescence assays and immunoassays using recombinant viral proteins have been used for detecting serological responses in infected humans [11,12], virus neutralisation is the most specific serological

test and currently considered the gold-standard. However, virus neutralisation requires the handling of live virus and requires Biosafety Level 3 containment. We have therefore developed a pseudoparticle neutralisation (ppNT) assay where the spike protein of MERS-CoV is expressed by a replication-incompetent human immunodeficiency (HIV) virus that contains a luciferase reporter gene. Similar pseudotype viruses have been used successfully in serological tests for severe acute respiratory syndrome coronavirus (SARS-CoV) and influenza viruses such as the highly pathogenic avian influenza A(H5N1) virus [13]. Pseudotyped MERS-CoV has been used to study the mechanisms of virus entry, and it has been shown that cell transduction by such particles is blocked by neutralising antibodies specific for MERS-CoV [14].

The geographical distribution of MERS-CoV in its animal reservoir is not defined. Being a Middle Eastern country with an ecology and domestic livestock practices fairly similar to some countries where human MERS infections have occurred, we reasoned that Egypt would be a relevant geographical location for seroepidemiological studies. We have used both the ppNT assay and conventional microneutralisation (MN) tests to carry out seroepidemiological surveillance in humans and livestock in Egypt. Human and animal sera collected in Hong Kong were used as controls.

Methods

Sera from dromedary camels (n=110), water buffaloes (n=8) and cows (n=25) were collected from two abattoirs, one located in Cairo and the second located in the Qalyubia governorate in the Nile Delta region. The dromedary camels were mostly imported from Sudan for slaughter and were five to seven years-old. Upon import, they were held on Egyptian farms for four to five months before transport to the abattoirs in open trucks. Sera from sheep (n=5) and goats (n=13) were collected from backyard animals from a village in the Nile Delta. All sera were collected in June 2013.

Human sera (n=815) were collected in 2012–13 as part of an ongoing community-based seroepidemiological study on influenza virus among healthy subjects in Cairo and the Nile Delta region. The age range of the subjects was between two and 79 years-old (median: 29 years). Fifty-eight per cent of the study subjects were female.

Sera collected in Hong Kong served as un-exposed controls. These included archived age-stratified human sera (n=528) collected in Hong Kong in 2011 and 2012, with more than 50 sera from each decade of age (range: <10 to 80 years-old). Swine sera (n=260) were collected from an abattoir in Hong Kong in 2011 and 2012. Sera (n=204) from wild northern pintails (Anas acuta) and Eurasian widgeons (*Anas penelope*) were collected in December 2010 from the Mai Po wetlands nature reserve in Hong Kong.

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, Germany), and sera from two experimentally infected macaques and a non-infected control macaque kindly provided by Bart Haagmans (Erasmus University Medical Center, Rotterdam, the Netherlands).

An acute and convalescent serum from a patient with SARS was used as a further negative control. The MN antibody titre was <10 to SARS-CoV in the acute serum, and 160 in the convalescent serum.

The study was approved by the institutional review boards of the University of Hong Kong and St Jude Children's Research Hospital and the Ethics Committee of the National Research Centre, Egypt.

Viruses and virus titration

MERS-CoV (strain EMC) virus was obtained from Dr R Fouchier (Erasmus University Medical Center, Rotterdam, the Netherlands). SARS-CoV (strain HKU-39849) was taken from the virus repository at Hong Kong University. Virus stock for MERS-CoV was prepared in Vero cell culture (ATCC CCL-81) in minimal essential medium containing 2% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin. Virus aliquots were stored at -80 °C. Virus was titrated in serial half-$\log_{10}$ dilutions (from 0.5 log to 7 log) to obtain 50% tissue culture infectious dose ($TCID_{50}$) on 96-well tissue culture plates of Vero cells. The plates were observed in a phase contrast microscope for cytopathic effect (CPE) daily for three days. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated by using the Reed Muench method and designated as one $TCID_{50}$. SARS-CoV was grown and titrated in the same manner with the exception that Vero E6 cells (ATCC CRL-1586) were used.

Microneutralisation tests

Serial two-fold dilutions of heat-inactivated sera (56 °C for 30 minutes) were made, starting with a dilution of 1:10. The serum dilutions were mixed with equal volumes of 200 $TCID_{50}$ of MERS-CoV or SARS-CoV as indicated. After 1 h of incubation at 37 °C, 35 µL of the virus–serum mixture was added in quadruplicate to Vero or Vero-E6 cell monolayers for MERS-CoV and SARS-CoV, respectively, in 96-well microtiter plates. After 1 h of adsorption, an additional 150 µL of culture medium were added to each well and the plates incubated for three more days at 37 °C in 5% CO_2 in a humidified incubator. A virus back-titration was performed without immune serum to assess input virus dose. CPE was read at three days post infection. The highest serum dilution that completely protected the cells from CPE in half of the wells was taken as the neutralising antibody titre and was estimated using the Reed-Muench method. Positive and negative control sera were included to validate the assay.

MERS-CoV spike pseudoparticle neutralisation assay

A codon-optimised spike gene was designed according to published MERS-CoV genome sequence (GenBank accession number: JX869059.1), synthesised by GeneCust (Luxembourg) and subcloned into pcDNA3.1+ vector to generate pcDNA-S. To produce HIV/MERS spike pseudoparticles, 10 µg pNL Luc E^- R^- and 10 µg pcDNA-S were co-transfected into $4x10^6$ 293T cells [13]. Supernatants of transfected cells were harvested 48 h later and quantified for HIV p24 viral protein using a p24 ELISA Kit (Cell Biolabs, San Diego, United States).

For the ppNT assay, HIV/MERS pseudoparticles containing 5 ng p24 were used to infect Vero E6 cells (ATCC CRL-1586) in a single well (96-well plate format; $1x10^4$ cells/well). Infected cells were lysed in 20 µl lysis buffer and 100 µl of luciferase substrate at two days postinfection (Promega Corporation, Madison, United States). Luciferase activity was measured in a Microbeta luminometer (PerkinElmer, Waltham, United States).

For the ppNT, HIV/MERS pseudoparticles (5 ng of p24) were pre-incubated with serially diluted sera for 30 min at 4 °C and then added to cells in triplicate. Residual virus replication was assayed at two days post infection, as described above. The highest serum dilution giving a 90% reduction of luciferase activity was regarded as the ppNT antibody titre.

Results

Overall, 976 human and animal sera from Egypt and 992 human and animal sera from Hong Kong were tested by MN at a screening dilution of 1:10 and 1:20 (Table 1). None of the age-stratified human sera (n=528), swine sera (n=260) or wild bird sera (n=204) collected in Hong Kong had any neutralising activity for MERS-CoV in the MN tests. Similarly, none of the sera from humans (n=815), water buffaloes (n=8), cows (n=25), sheep (n=5) and goats (n=13) collected in Egypt were positive in the screening MN tests. In contrast, 103 of 110 sera collected in Egypt from dromedary camels neutralised MERS-CoV at the screening dilution of 1:20 or higher.

Entry of MERS pseudoparticles was shown to be inhibited by increasing concentrations of 0–20 mM NH_4Cl (data not shown), demonstrating pH dependent entry of the MERS pseudoparticles as previously reported [14]. The MERS ppNT assay was evaluated using two sera from experimentally infected macaques, one negative control serum from an uninfected macaque, a human convalescent serum from a MERS patient and five negative human control sera from Hong Kong (Figure 1).

The MERS ppNT assay was then used to screen 115 human sera from Hong Kong and 100 randomly selected human sera from Egypt which were all serologically negative for MERS-CoV. Sixteen dromedary camel sera that were positive in the MN screening assay were all found to have a high neutralising activity in the ppNT assay. In addition, five of six sera that were negative in the MN assay had a weak, but detectable, activity in the ppNT test (Table 1, Table 2, Figure 2). The camel sera that were found to be positive at a screening dilution of 1:20 in the MN test had antibody titres in the MERS NT screen ranging from 40 to 1,280 and higher, and MERS ppNT titres ranging from 640 to 10,240 and higher. One of the five MERS MN-negative sera was negative in the MERS ppNT assay, while the other four had low MERS ppNT titres ranging from 40 to 160.

Table 1

Screening results for MERS-CoV microneutralisation and MERS-CoV spike protein pseudoparticle neutralisation, human and animal samples from Egypt and Hong Kong, 2012–2013 (n=1,968)

Sera	Source of sera	MERS-CoV micro-neutralisation titre ≥1:20		MERS-CoV spike pseudotype antibody titre ≥1:20	
		Total tested	% Positive (n)	Total tested	% Positive (n)
Human[a]	Egypt	815	0 (0/815)	100	0 (0/100)
Goat[b]		13	0 (0/13)	ND	ND
Sheep[b]		5	0 (0/5)	ND	ND
Water buffalo[b]		8	0 (0/8)	ND	ND
Cow[b]		25	0 (0/25)	ND	ND
Camel[b]		110	93.6 (103/110)	110	98.2 (108/110)
Human	Hong Kong	528	0 (0/528)	115	0 (0/115)
Swine		260	0 (0/260)	ND	ND
Wild bird		204	0 (0/204)	ND	ND

MERS-CoV: Middle East respiratory syndrome coronavirus; ND: not done.

[a] Collected in 2012–13.

[b] Collected in June 2013.

Details of sera collected in Hong Kong as given in Methods.

The correlation of the MERS MN and MERS ppNT titres are shown in Figure 3 (Pearson's correlation coefficient: R=0.88). The MERS ppNT test appears to be more sensitive than the MERS MN test, and thus some of the apparently MN-negative camel sera give low titre-positive results in the MERS ppNT assay. However, none of the human sera from Egypt (n=100) or Hong Kong (n=115) had any detectable antibody in the MERS ppNT test. None of the camel sera with high antibody titres to MERS-CoV had any cross-neutralising activity to SARS-CoV (Table 2).

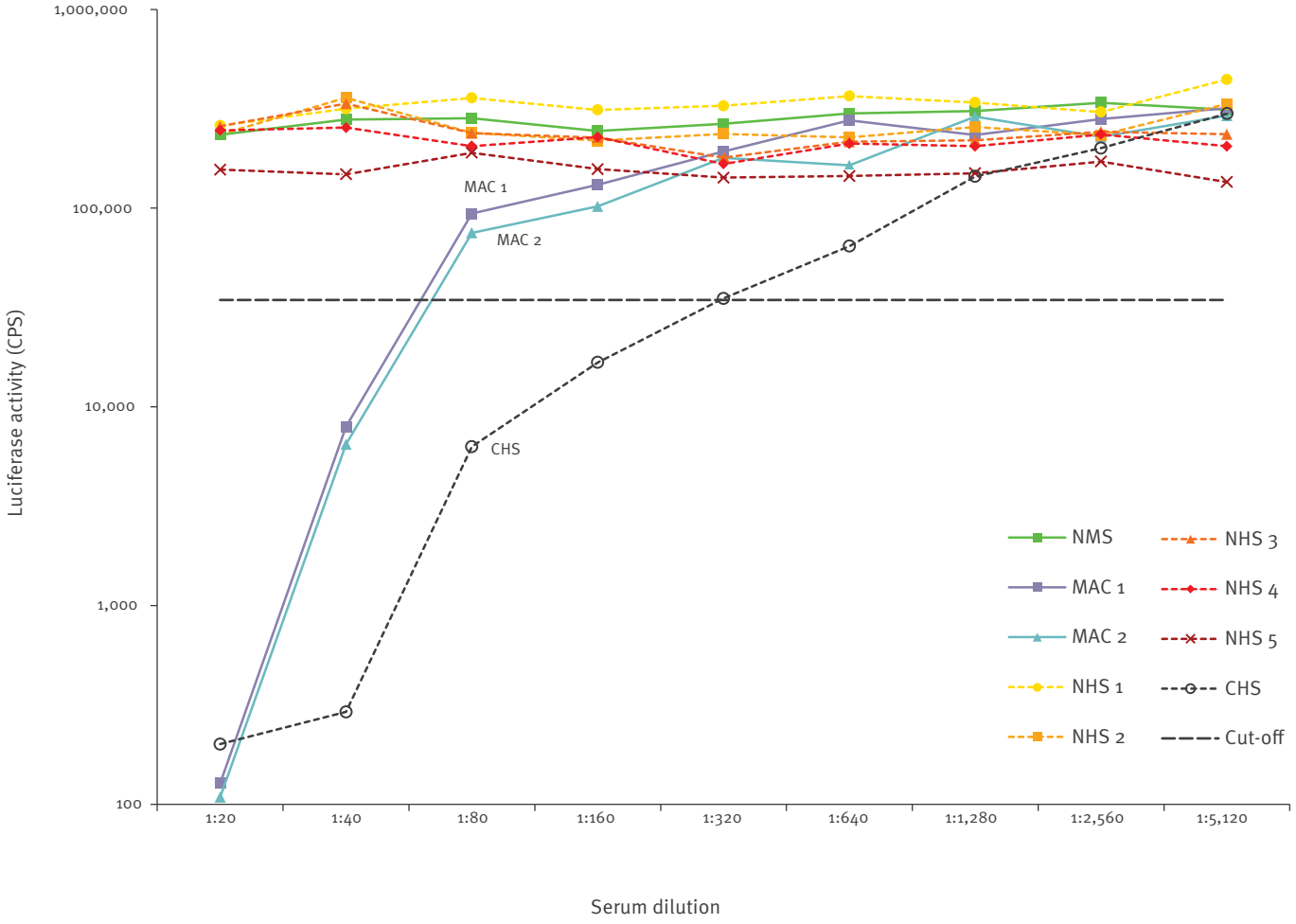
Discussion

Of 1,968 human and animal sera tested by MERS-CoV MN and 325 human and animal sera tested by MERS-CoV ppNT assays, only sera from dromedary camels had any neutralising antibody activity to the MERS-CoV. Of the 110 camel sera, 93.6% were seropositive by MERS-CoV MN test and 98.2% were seropositive by MERS-CoV ppNT test. The antibody titres were very high in MN as well as ppNT, suggesting that the virus infecting these camels was MERS-CoV virus itself or a very closely related virus.

It is known that dromedary camels host bovine coronaviruses (BCoV) which are lineage A beta-coronaviruses. However cross-neutralisation between MERS-CoV (lineage C beta-coronavirus) and BCoV was excluded by Reusken and colleagues in their study of sera from dromedary camels [10]. Furthermore, BCoV is antigenically closely related to the human coronavirus OC43. Human beta-coronavirus lineage A viruses OC43 and

Figure 1

MERS-CoV spike protein pseudoparticle neutralisation, human and animal samples from Egypt and Hong Kong, 2012–13 (n=9)



CPS: counts per second; MERS-CoV: Middle East respiratory syndrome coronavirus;

As positive controls, we used a convalescent human serum (CHS) from a patient with MERS, kindly provided by Dr C Drosten (Institute of Virology, University of Bonn Medical Centre, Bonn, Germany) and sera from two experimentally infected macaques (MAC1, MAC2), kindly provided by Bart Haagmans (Erasmus University Medical Center, Rotterdam, the Netherlands). As negative controls we used serum from a non-infected control macaque (NMS) and five human sera (NHS 1–5) from Hong Kong. The horizontal dotted line represents the 90% reduction in luciferase activity which represents the cut-off for positivity in the assay. Each batch of assays had the cut-off determined with reference to a serum-free negative control, and the data represented here are a compilation of two experiments. Thus the cut-off line is a representative indication based on an average of cut-offs used in seperate experiments.

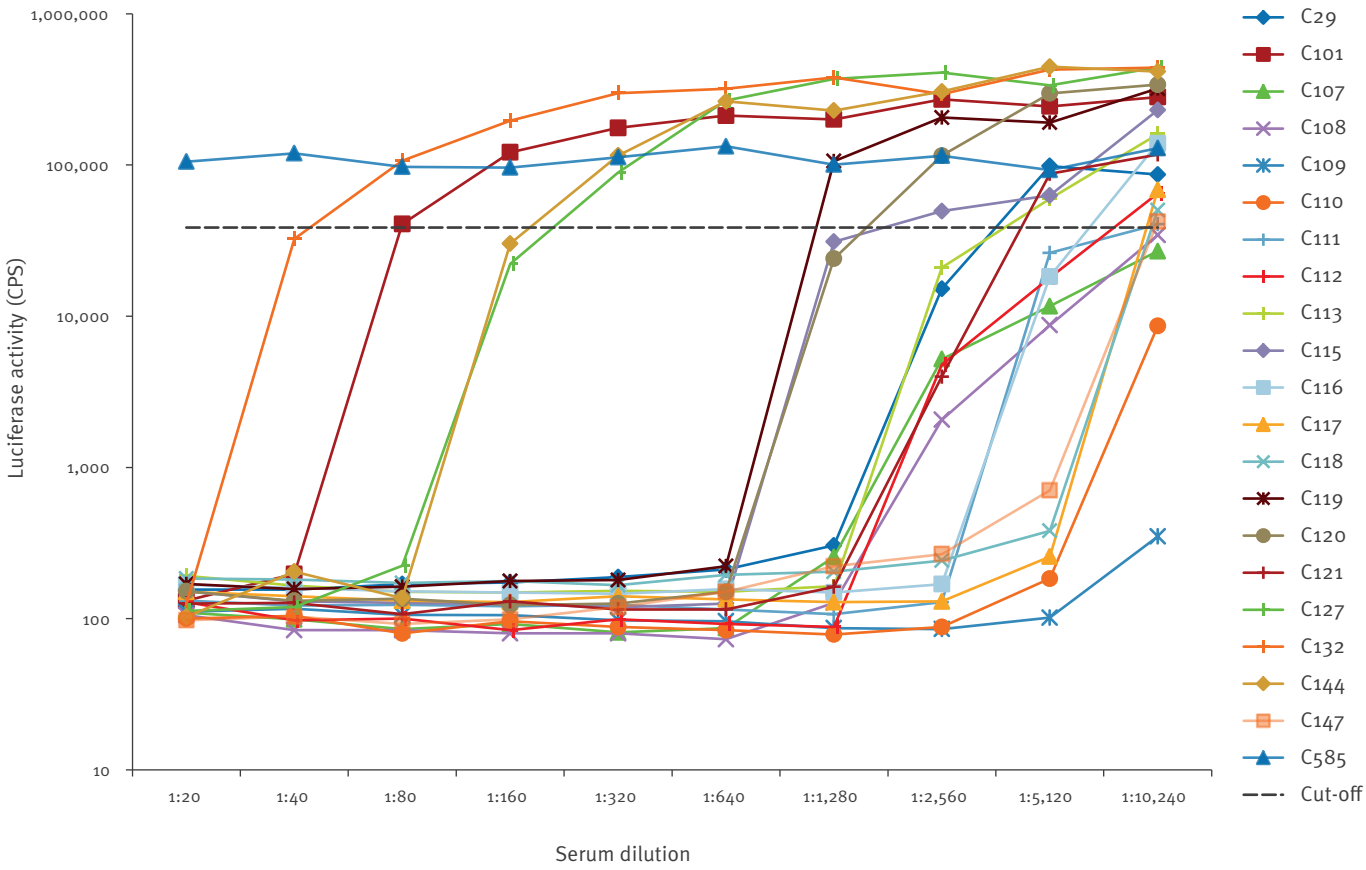
HKU1, and alpha-coronaviruses (229E and NL63) are ubiquitous respiratory viruses infecting humans and the panel of human sera of different ages tested can be expected to have varying levels of antibody to these viruses. The lack of any MERS-neutralising activity in the human sera we studied also indicates that the MN and ppNT assays are specific for MERS-CoV. The lack of cross-reactivity with convalescent serum from patients with SARS provides additional evidence of the lack of cross-reactivity in the MERS-CoV serology assays. Furthermore, it is notable that the camel sera with high antibody titres to MERS-CoV did not cross-react with SARS-CoV, a beta-coronavirus of lineage B. Taken together these data indicate that a MERS-CoV or a highly related virus is endemic in dromedary camels imported for slaughter in Egypt. These findings provide independent confirmation of the results recently reported by Reusken et al. who found very high antibody titres to MERS-CoV in dromedary camels [10].

The dromedary camels sampled in our study were those brought to abattoirs for slaughter in Cairo and in the Qalyubia governorate in the Nile Delta region. These animals were sourced from other East African countries such as Sudan and held in Egypt for some time prior to slaughter. Thus it is unclear where the animals originally acquired the infection. Considering the similar data from dromedary camels in Oman and the Canary Islands [10], it is likely that this coronavirus is widespread in North and East Africa and the Arabian peninsula.

There is substantial movement of people between Egypt and Saudi Arabia and other states on the Arabian peninsula, and thus it is possible that people may get infected, either as part of their travel to endemic areas or through zoonotic transmission within the country. There is also much movement of livestock across these Middle Eastern countries. The lack of antibody to MERS-CoV in sera of people resident in Egypt indicates

Figure 2

MERS-CoV spike protein pseudoparticle neutralisation on selected sera from dromedary camels, Egypt, June, 2013 (n=21)



CPS: counts per second; MERS-CoV: Middle East respiratory syndrome coronavirus; MN: microneutralisation; ppNT: pseudoparticle neutralisation.

Sixteen sera found to be positive and five sera found to be negative in the MERS-CoV MN screening assay were titrated in the MERS-CoV ppNT assay. The sera used are shown in Table 2. The horizontal dotted line represents the 90% reduction in luciferase activity which represents the cut-off for positivity in the assay.Each batch of assays had the cut-off determined with reference to a serum-free negative control and the data represented here are a compilation of two experiments. Thus the cut-off line is a representative indication based on an average of cut-offs used in seperate experiments.

that this infection is not common in Egypt, either as an infection acquired through travel or as an occasional zoonotic infection.

The MERS-CoV ppNT assay described here is a safe and specific assay for large scale seroepidemiological studies in a range of animal species, and such studies are urgently needed in regions where MERS-CoV cases have been detected as well as other regions. The HIV backbone used for pseudoparticle production is not replication-competent and the MERS-CoV pseudoparticles can therefore be produced and used in Biosafety Level 2 containment; in contrast, MN assays involve handling of the live MERS-CoV and require Biosafety Level 3 containment which is not always available in affected regions. Unlike immunoassays, there is no requirement for finding and optimising an enzyme-labelled anti-Ig conjugate for each species to be investigated. Furthermore, the MERS-CoV ppNT assay appears around 10 times more sensitive than the conventional MN assay (Figure 3, Table 2). The MN assay is a neutralisation assay based on $TCID_{50}$ rather than a plaque reduction assay, which perhaps makes it less sensitive than a plaque neutralisation assay. In any event, experience with influenza virus serology using pseudoparticle assays has shown that they are more sensitive than conventional MN assays for detecting neutralising antibodies. Thus MERS-CoV ppNT can be used as a screening assay, and positive sera can be retested for confirmation in a MERS MN tests.

Serological data does not provide proof that the virus infecting dromedary camels is the MERS CoV, and infection by a closely related coronavirus or a chimeric virus with a MERS-CoV-like spike protein cannot be ruled out until the dromedary camel virus is detected and genetically sequenced. However, it provides a strong impetus to attempt to seek the virus in specimens from these animals and to identify the MERS-related virus that appears to be infecting them. These serological studies also need to be extended to other domestic animals species to define the circulation of MERS-CoV or related viruses in animals in close contact with humans. Such studies should also include humans exposed to dromedary camels. It is important to note that waning antibody levels may result in false-negative serology results, and this is particularly relevant in mild or asymptomatic episodes of infection where the peak antibody titre may be lower and drop more quickly.

Table 2

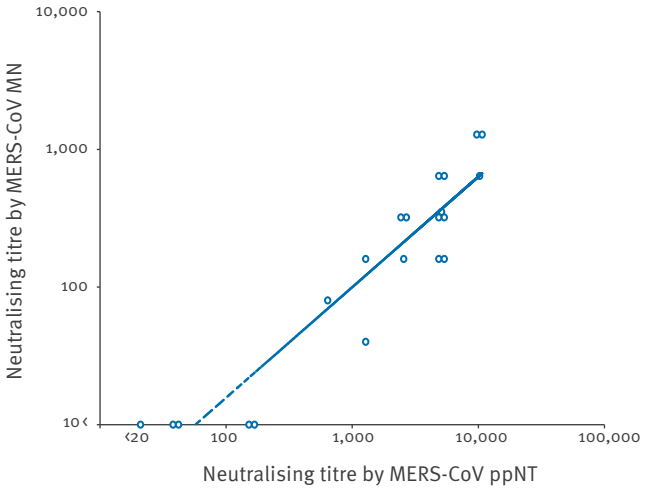
Antibody titres of selected sera from dromedary camels tested by microneutralisation for MERS-CoV and SARS-CoV and by MERS spike protein pseudoparticle neutralisation, Egypt, June, 2013 (n=21)

Camel sera	Antibody titres		
	MERS-CoV MN test	SARS-CoV MN test	MERS-CoV ppNT test
C101	<10 Negative	<10 Negative	40
C127	<10 Negative	<10 Negative	160
C132	<10 Negative	<10 Negative	40
C144	<10 Negative	<10 Negative	160
C585	<10 Negative	<10 Negative	<20 Negative
C29	320	<10 Negative	2,560
C107	160	<10 Negative	5,120
C108	160	<10 Negative	5,120
C109	640	<10 Negative	≥10,240
C110	≥1,280	<10 Negative	≥10,240
C111	320	<10 Negative	5,120
C112	320	<10 Negative	5,120
C113	320	<10 Negative	2,560
C115	160	<10 Negative	1,280
C116	320	<10 Negative	5,120
C117	640	<10 Negative	5,120
C118	640	<10 Negative	5,120
C119	80	<10 Negative	640
C120	40	<10 Negative	1,280
C121	160	<10 Negative	2,560
C147	≥1,280	<10 Negative	≥10,240

MERS-CoV: Middle East respiratory syndrome coronavirus; MN: microneutralisation; ppNT: pseudoparticle neutralisation; SARS-CoV: severe acute respiratory syndrome coronavirus.

Figure 3

Correlation of MERS-CoV antibody titres determined by MERS-CoV microneutralisation and MERS-CoV spike protein pseudoparticle neutralisation in selected sera from dromedary camels, Egypt, June, 2013 (n=21)



MERS-CoV: Middle East respiratory syndrome coronavirus; MN: microneutralisation; ppNT: pseudoparticle neutralisation.

The data used as those shown in Table 2. In the event of overlapping dots, their MN titre (X axis) was increased or decreased by 0.05% to slightly offset the overlap for ease of observation. The limit of detection in the MN and ppNT assays were titres of 10 and 20 respectively; and thus these values on the Y and X axis correspond to <10 and <20, respectively.

If the detection of MERS-CoV in insectivorous bats is confirmed [8] and if indeed the coronavirus we and others demonstrated to be common in dromedary camels is confirmed to be MERS-CoV, we will have a scenario of a virus reservoir in bats with a peridomestic animal such as the camel as intermediate host, which may in fact be the immediate source of human infection. It is notable that a number of index cases with MERS-CoV had a history of exposure to camels, although this is by no means universally the case. Given that the MERS-like coronavirus in camels appears to be ubiquitous, it remains to be explained why MERS in humans appears relatively rare. Coronaviruses are well known to mutate to markedly change virulence or host range. Examples are the emergence of the less pathogenic porcine respiratory coronavirus from virulent transmissible gastroenteritis virus of pigs, or virulent feline infectious peritonitis viruses emerging from low pathogenic feline coronaviruses [15]. Furthermore, the SARS-like virus detected in civets and other small mammals in live animal markets in southern China in 2002–03 initially appeared to infect humans, who appear to have seroconverted, but with minimal disease and onward transmission [16], while a few amino acid changes in the SARS-CoV spike protein allowed that virus to acquire efficient transmissibility and virulence in humans [17]. Thus, previous experience with animal and human coronaviruses highlights the public health urgency of investigations of MERS-CoV and MERS-CoV-like viruses in domestic and wild animals.

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Conflict of interest

None declared.

Authors' contributions

Pei-gang Wang developed the MERS-CoV pseudotype assay and carried out the tests. Ranawaka AMP Perera developed the MERS-CoV microneutralisation test and carried out the tests in BSL3 containment. Leo LLM Poon and Yi Guan provided advice on laboratory methods. Lewis YL Siu and Mingyuan Li carried out the MERS-CoV pseudoparticle assays. Mokhtar R. Gomaa, Rabeh El-Shesheny, Ahmed Kandeil, Ola Bagato, Mahmoud M. Shehata, Ahmed S. Kayed and Yassmin Moatasim collected human and animal sera in Egypt. Richard J. Webby and Mohamed A. Ali provided advice on field study design. Joseph SM Peiris and Ghazi Kayali designed and coordinated the study and wrote the manuscript. All authors reviewed and commented on the manuscript.

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Population susceptibility to North American and Eurasian swine influenza viruses in England, at three time points between 2004 and 2011

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Age-stratified sera collected in 2004, 2008 and 2010 in England were evaluated for antibody to swine influenza A(H3N2) and A(H1N1) viruses from the United States or Europe as a measure of population susceptibility to the emergence of novel viruses. Children under 11 years of age had little or no measurable antibody to recent swine H3N2 viruses despite their high levels of antibody to recent H3N2 seasonal human strains. Adolescents and young adults (born 1968–1999) had higher antibody levels to swine H3N2 viruses. Antibody levels to swine H3N2 influenza show little correlation with exposure to recent seasonal H3N2 (A/Perth/16/2009) strains, but with antibody to older H3N2 strains represented by A/Wuhan/359/1995. Children had the highest seropositivity to influenza A(H1N1)pdm09 virus, and young adults had the lowest antibody levels to A/Perth/16/2009. No age group showed substantial antibody levels to A/Aragon/RR3218/2008, a European swine H1N1 virus belonging to the Eurasian lineage. After vaccination with contemporary trivalent vaccine we observed evidence of boosted reactivity to swine H3N2 viruses in children and adults, while only a limited boosting effect on antibody levels to A/Aragon/RR3218/2008 was observed in both groups. Overall, our results suggest that different vaccination strategies may be necessary according to age if swine viruses emerge as a significant pandemic threat.

Introduction

Pigs are considered a mixing vessel for the reassortment of avian, swine and human influenza viruses. Recent events confirm their important role in the emergence of novel influenza viruses capable of causing a human pandemic [1]. Until the 1990s, classic swine influenza A(H1N1), the most commonly circulating swine influenza virus among pigs, remained genetically fairly constant [2]. However, by the late 1990s, different subtypes (H1N1, H3N2 and H1N2) had emerged and became predominant among North American pig herds [3]. These swine influenza A viruses acquired avian, human, and swine virus gene segments through reassortment [3,4] and various genetic lineages can be distinguished within each subtype [4]. In Europe, swine influenza is primarily caused by the aforementioned subtypes. However, their antigenic and genetic characteristics differ significantly from those found in North America and Asia [5,6]. Genetic diversity has been expanded through multiple introductions of influenza viruses from other animal hosts into pig herds, including from humans [7], most recently demonstrated with A(H1N1)pdm09 virus in Europe, Asia, and the Americas [6,8,9].

For this study of population susceptibility we chose two swine virus subtypes which have most recently caused outbreaks or sporadic cases in humans. These include representatives of swine influenza A(H3N2) viruses (swH3N2) recently isolated from human cases in the United States [10,11] and a swine influenza A(H1N1) viruses (swH1N1) isolated from a zoonotic infection in Europe [12].

The primary objective of this analysis was the improvement of the risk assessment of population susceptibility to currently circulating swine influenza viruses, with the proven ability to cause zoonotic infections.

Methods

We measured haemagglutination inhibition (HI) antibody prevalence to representative current and previous seasonal H3N2 and H1N1 strains, to which the population of the United Kingdom (UK) has been exposed, and compared it with HI antibody reactivity to influenza H3 and H1 strains of swine origin to which the UK population is very unlikely to have been exposed. We also determined vaccine-induced cross-reactive antibodies in pre- and post-immunisation sera.

Serum samples

We used a random selection of anonymised age-stratified residual serum aliquots collected in England

[14] from 1,982 individuals over three time periods as detailed in Table 1. Sera were collected from an age range of 0 to 89 years and stratified by birth cohorts. The 1,982 sera were grouped into panels according to time of serum sample collection (Table 1).

A small additional panel of anonymised children and adult sera before and after vaccination with 2010/11 trivalent inactivated influenza vaccine (TIV) was used to asses levels of vaccine-induced cross-reactive antibodies in children (3–14 years-old; 24 pairs) and adults (20–77 years-old; 24 pairs).

Viruses

Antigenic characterisation of virus isolates was performed using HI assays [13]. Virus strains used for H3N2 analysis were: A/Perth/16/2009 (human H3N2 virus, circulating from 2009 onwards); A/Wuhan/359/1995 (human H3N2 virus, circulating from the mid-1990s); A/Swine/Minnesota/593/1999 (A/sw/Minnesota/593/1999; genetic predecessor of swine H3N2 viruses, which have recently caused limited human infection in North America, kindly provided by Prof I. Brown at the Veterinary Laboratory Agency, UK); and A/Pennsylvania/14/2010 and A/Indiana/08/2011 (swine H3N2 viruses isolated from sporadic cases of human infection in the United States; both kindly provided by the World Health Organization Collaborating Centre (WHO CC) at the National Institute for Medical Research (NIMR), London, UK, who received the samples as part of the WHO Global Influenza Surveillance and Response System (GISRS) Pandemic Influenza Preparedness (PIP) Framework from the WHO CC at CDC, Atlanta), see also Table 2.

Viruses used for H1N1 analysis were: NIBRG122 (reverse genetics virus of A/England/195/2009, the influenza A(H1N1)pdm09 UK prototype strain, provided by the National Institute for Biological Standards and Control (NIBSC)) and A/Aragon/RR3218/2008 (swine H1N1 virus isolated from a sporadic human case in Spain in 2008 [12], kindly provided by the National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain).

The NIBRG122, A/Perth/16/2009, A/Wuhan/359/1995, A/Aragon/RR3218/2008, A/sw/Minnesota/593/1999 and A/Pennsylvania/14/2010 viruses were grown in embryonated hens' eggs. A/Indiana/08/2011 was cultured in MDCK cells.

Serological methods and analysis

Antibody titres were measured by HI [14,15]. All assays were performed using turkey red blood cells (0.5%), with the exception of the analysis with A/Perth/16/2009 virus, for which we used guinea pig red blood cells (0.5%) according to WHO recommendation [16]. Undetectable titres (<8) were assigned a value of 4. Age-related geometric mean titres (GMTs) with 95% confidence intervals (CI) as well as proportion of participants with HI titre ≥32 (defined as seropositive) were calculated. Data were analysed by birth cohorts according to primary influenza exposure (before 1957, exposed to H1N1; 1957–68, exposed to H2N2; 1968–99 exposed to H3N2; from 2000 onwards, representing the very young). Pearson's correlation coefficient (*r*) was used to compare responses between log10 assay titres.

Table 1

Characteristics of serum panels for influenza serosusceptibility analysis, England, 2004–11 (n=1,982)

Panel name	Time of collection	Number of samples		Age Ranges			Analysed with	
		Total	By birth cohort	Birth cohorts	Age at collection (years)	Year of birth	H1N1 subtype	H3N2 subtype
2004 panel	June 2004	687	176	Pre-1957	1–80	1924–2003	A/England/195/2009[a], A/Aragon/RR3218/2008	Not analysed
			87	1957–1967				
			304	1968–1999				
			48	After 2000				
2008 panel	Jan 2008 to April 2009	1,179	588	Pre-1957	0–87	1921–2009	A/England/195/2009[a]	Not analysed
			67	1957–1967				
			314	1968–1999				
			209	After 2000				
2010 panel	Autumn 2010 and spring 2011	116	33	Pre-1957	0–89	1922–2011	A/England/195/2009[a], A/Aragon/RR3218/2008	A/Wuhan/359/95, A/sw/Minnesota/593/99, A/Pensylvania/14/10, A/Indiana/08/2011, A/Perth/16/09
			13	1957–1967				
			49	1968–1999				
			22	After 2000				

[a] The reverse genetics derivative, NIBRG122, was used.

Table 2

Source of viruses and sequence information used in this study (n=25)

Virus	Virus provided by	Sequence Source		Originating laboratory	Submitter
		GenBank	GISAID EpiFlu		
A/Wuhan/359/95	WHO CC National Institute for Medical Research, London	JX518888	NA	NA	NA
A/Moscow/10/99	WHO CC National Institute for Medical Research, London	DQ487341	NA	NA	NA
A/Johannesburg/33/94	WHO CC National Institute for Medical Research, London	CY121349	NA	NA	NA
A/Panama/2007/99	WHO CC National Institute for Medical Research, London	DQ487340	NA	NA	NA
A/Perth/16/2009	WHO CC National Institute for Medical Research, London	GQ293081	NA	NA	NA
A/England/215/2011	NA	JX518887	EPI393290	NA	Centre for Infections, HPA, London, United Kingdom
A/Swine/Minnesota/593/99	Veterinary Laboratories Agency, Weybridge, UK				
	AF251427	NA	NA	NA	
A/Kansas/13/2009	WHO CC for Reference and Research on Influenza, CDC, Atlanta	NA	EPI244297	Kansas Department of Health and Enviroment	WHO CC for Reference and Research on Influenza, CDC, Atlanta
A/Wisconsin/12/2010	WHO CC for Reference and Research on Influenza, CDC, Atlanta	NA	EPI291898	Evanston Hospital and NorthShore University	WHO CC for Reference and Research on Influenza, CDC, Atlanta
A/Pennsylvania/14/2010	WHO CC for Reference and Research on Influenza, CDC, Atlanta	NA	EPI291865	Pennsylvania Department of Health	WHO CC for Reference and Research on Influenza, CDC, Atlanta
A/Indiana/08/2011	WHO CC for Reference and Research on Influenza, CDC, Atlanta	NA	EPI344405	Indiana State Department of Health Laboratories	WHO CC for Reference and Research on Influenza, CDC, Atlanta
A/California/07/2009	National Institute Biological Standards and Control, HPA	ACP44189	NA	NA	NA
A/Swine/Iowa/00239/2004	NA	ABV25643	NA	NA	NA
A/New Jersey/11/1976	NA	ACU80014	NA	NA	NA
A/swine/Wisconsin/1/1961	NA	AAD25302	NA	NA	NA
A/swine/Iowa/15/1930	NA	ABV25634	NA	NA	NA
A/South Carolina/1/1918	NA	AAD17229	NA	NA	NA
A/Puerto Rico/8/1934	NA	ABO21709	NA	NA	NA
A/Roma/1949	NA	ABN59434	NA	NA	NA
A/New Caledonia/20/1999	NA	CAC86622	NA	NA	NA
A/Brisbane/59/2007	NA	ACA28846	NA	NA	NA
A/duck/Italy/69238/2007	NA	ACJ14445	NA	NA	NA
A/mallard/Alberta/35/1976	NA	AAD25304	NA	NA	NA
A/Aragon/RR3218/2008	Instituto de Salud Carlos III, Madrid-Majadahonda, Spain	NA	EPI393289	Instituto de Salud Carlos III, Madrid-Majadahonda, Spain	Centre for Infections, HPA, London, United Kingdom
A/swine/England/WVL7/1992	NA	ACO25133	NA	NA	NA

CDC: Centres for Disease Control and Prevention; HPA: Health Protection Agency; NA: not applicable; UK: United Kingdom; WHO CC: World Health Organization Collaborating Centre.

For analysis of vaccine sera, immunogenicity end points included group GMTs and geometric mean fold changes (GMTR) from pre- to post-vaccination with 95% CI, the proportion of participants with HI titre ≥32 ('seroprotection rate' when evaluating vaccine antigens), and the proportion of seroconverting individuals ('seroconversion rate'; SCR); showing four-fold increase in post- compared with pre-immunisation titres or from HI titre <8 before immunisation to at least 32 after immunisation.

Sequencing of full-length haemagglutinin and phylogenetic analysis

Virus RNA was extracted, underwent RT-PCR, and amplified products were sequenced [13,17]. Accession numbers for GenBank and the Global Initiative on Sharing All Influenza Data (GISAID) are listed in Table 2. Phylogenetic trees were constructed using deduced amino acid sequences with a neighbour-joining algorithm, available in the MEGA 4.0.1 software (http://www.megasoftware.net).

Results

Cross-reactivity of H3N2 viruses

The classical swine lineage virus A/sw/Minnesota/593/1999 showed some reactivity with ferret post-infection antiserum raised to human seasonal viruses from the mid-1990's, suggesting some antigenic similarity between swine and human viruses co-circulating during this period (Table 3).

Figure 1 shows the genetic relationships between haemagglutinin (HA) protein sequences of representative human H3N2 and swH3N2 lineages, including some from human infections with North American swine H3N2 viruses detected since 2009. A/sw/Minnesota/593/99 clusters with human viruses from the mid-1990s, since this virus is a representative from the swine triple reassortant lineage that arose in 1998 and includes an HA gene from human origin. The human lineage further separates into two branches of viruses isolated before or after 1998.

Of 59 residues located at antigenic sites, current human and swine North American H3N2 viruses differ at ca. 16 positions (73% identity at antigenic sites, 89% for the entire HA protein (data not shown). The highest pairwise identity between current North American swine viruses and human H3N2 viruses included in this analysis is shown with A/Wuhan/359/95 (78–83% identity at antigenic sites, 94% for the entire HA), which is consistent with this virus being an ancestor for the HA segment of recent and classic North American swH3N2 viruses.

Age stratified reactivity of human sera to seasonal H3N2 viruses shows a profile consistent with exposure

Table 3

Antigenic analysis of influenza A(H3N2) viruses (seasonal, swH3N2 and swH3N2 variant influenza strains) (n=11)

		A/Perth/16/2009	A/England/215/2011	A/Panama/2007/99	A/Moscow/10/99	A/Wuhan/359/95	A/Johannesburg/34/94	A/Pennsylvania/14/2010	A/Wisconsin/12/2010
		H3N2	H3N2	H3N2	H3N2	H3N2	H3N2	swH3N2	swH3N2
A/Perth/16/2009	H3N2	**2,560**	5,120	<	<	<	<	<	<
A/England/215/2011	H3N2	640	**2,560**	<	<	<	<	<	<
A/Panama/2007/99	H3N2	<	<	**2,560**	5,120	20	<	<	<
A/Moscow/10/99	H3N2	<	<	1,280	**10,240**	<	<	<	<
A/Wuhan/359/95	H3N2	<	<	<	<	**2,560**	160	<	<
A/Johannesburg/33/94	H3N2	<	<	<	<	<	**2,560**	<	<
A/Pennsylvania/14/2010	swH3N2	<	<	<	<	<	<	**5,120**	2,560
A/Wisconsin/12/2010	swH3N2	<	<	<	<	<	<	640	**2,560**
A/Kansas/13/2009	swH3N2	<	<	<	<	<	<	2,560	320
A/Indiana/8/2011	sw(H3N2)v	<	<	<	<	<	<	2,560	5,120
A/sw/Minnesota/593/99	swH3N2	<	<	<	<	160	160	<	<

sw(H3N2)v: variant of recent swH3N2 viruses, which acquired the M gene of the A(H1N1)pdm09 virus.

Haemagglutination inhibition titres for seasonal H3N2 viruses, novel swH3N2 viruses causing sporadic human infections, and swH3N2 viruses with post-infection ferret antiserum. < denotes a titre <40.

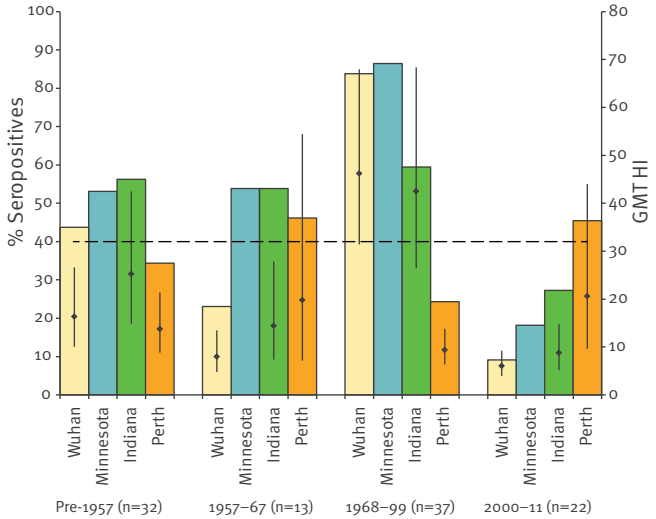
to different circulating strains according to birth cohort (Figure 2). Seropositivity with A/Perth/16/2009, the virus included in the TIV and the most recently circulating H3N2 virus in England, shows the least variation across different ages. The youngest age cohort (born after 2000) and those born between 1957 and 1967 had the highest number of seropositive individuals to this strain, while younger adults born between 1968 and 1999 showed the highest number of seropositives to the previously circulating H3N2 A/Wuhan/359/1995 virus.

Cross-reactive antibody levels to swH3N2 viruses were lowest in children (born after 2000) and older adults (born before 1968) for the two viruses used in the analysis (Figure 2), with the lowest GMTs for the recent swine virus isolate A/Indiana/08/2011 (GMT=9; 95% CI: 5–15) found in the youngest age cohort. However, the two groups with lowest overall GMT seem to differ in susceptibility. We found significantly (p=0.04, Fisher's exact test) fewer seropositives in those 12 years-old and younger (6/22=27% with A/Indiana/08/2011) compared to adults born before 1968 (25/45=56%). Highest levels of cross-reactive antibodies to swH3N2 strains were found in individuals born between 1968 and 1999. The susceptibility profile for the A/Wuhan/359/95 virus was very similar to that of an ancestor strain for swH3N2, A/sw/Minnesota/593/1999.

We observed the strongest correlation between A/Wuhan/359/1995 and A/sw/Minnesota/593/1999 (r=0.80) and weaker correlation between A/Wuhan/359/1995 and A/Indiana/08/2011 (r=0.69) as well as between A/sw/Minnesota/593/1999 and A/Indiana/08/2011 viruses (r=0.5). By contrast, we found no evidence for the pairwise correlations of antibody titres between A/Perth/16/2009 and any of the other H3N2 strains used.

Figure 2

Reactivity in age-stratified sera to different influenza A(H3N2) viruses, England, 2010/11

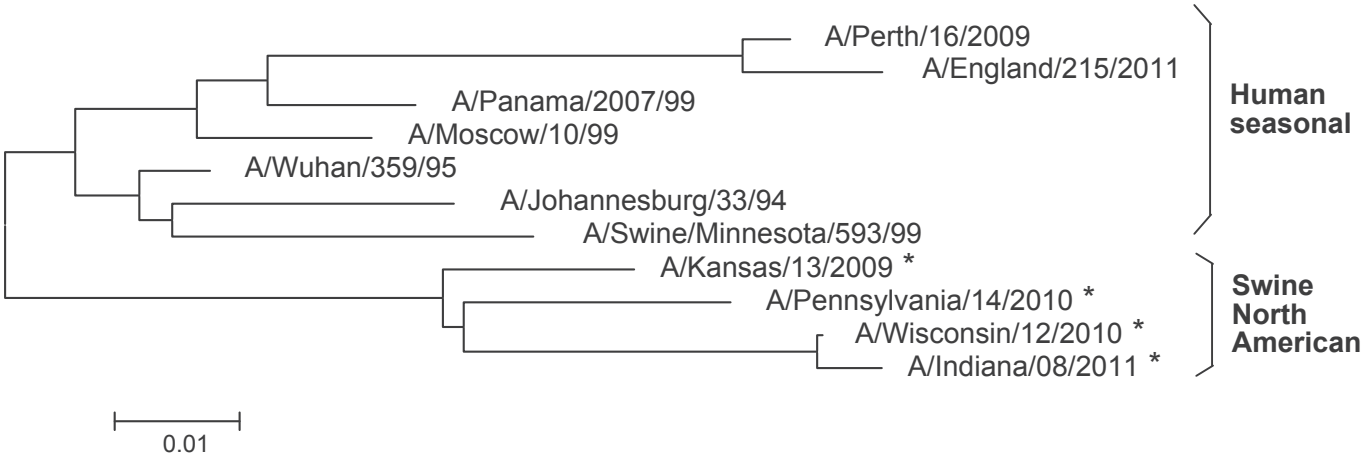


CI: confidence interval; GMT: geometric mean titre; HI: haemagglutination inhibition.

Proportion with HI titre ≥32 (% seropositives) by exposure-related age group for influenza A(H3N2) influenza viruses. The figure shows the results of the analysis of the 2010 panel (Table 1) with four influenza A(H3N2) viruses. The percentage of seropositives for the viruses are depicted in yellow for A/Wuhan/395/1995, blue for A/sw/Minnesota/593/1999, green for A/Indiana/08/2011 and orange for A/Perth/16/2009, while GMTs for analysis with A/Indiana/08/2011, A/Wuhan/395/1995 and A/Perth/16/2009 are illustrated as diamonds in each bar with their 95% CI shown as vertical lines. Cut-off for seropositivity is shown as dotted line. Numbers of samples in each age group are given below the bars. Due to low available serum volume, HI with A/sw/Minnesota/593/1999 virus was started at 1:16 dilution point for all samples and we could therefore not determine GMTs for this analysis.

Figure 1

Phylogenetic tree showing the relationship between human, swine and avian full length haemagglutinin sequences from influenza A(H3N2) viruses



Swine viruses isolated from humans are denoted with *. Mid-point rooted trees were constructed with a neighbour-joining algorithm, using MEGA 4 software.

Cross-reactivity of influenza A(H1N1) viruses

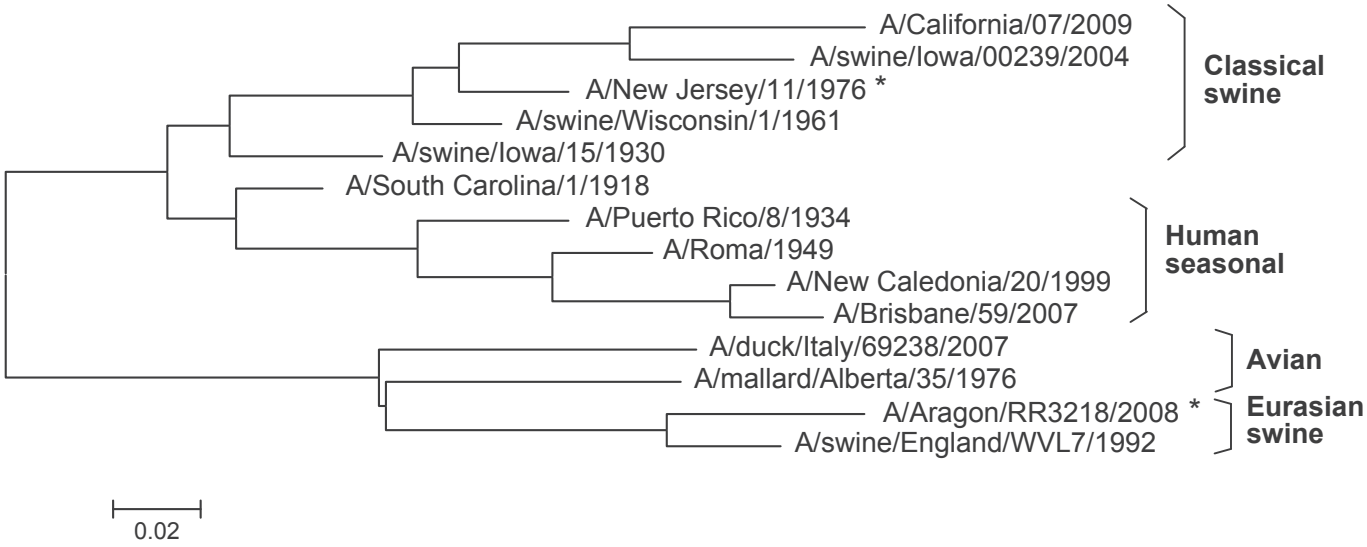
Ferret antiserum raised to human seasonal H1N1 virus strains showed no cross-reactivity with viruses from either the classical or Eurasian swine lineages. The prototype A(H1N1)pdm09 virus A/California/7/2009 from the classical swine lineage showed no reactivity with antiserum raised to either human seasonal H1N1 viruses or Eurasian swine viruses (data not shown and described elsewhere [18]). The recent Eurasian swine virus A/Aragon/RR3218/2008, that caused one sporadic human infection in 2008, had no reactivity with human seasonal virus antiserum.

Figure 3 shows the phylogenetic relationships between the HA sequences of representative human, swine and avian H1N1 viruses isolated since 1918. The pandemic virus A/California/07/2009 has its closest relationship with recent classical swine viruses, which have been circulating in North America and other regions since 1930 [19]. The branch most distal to A/California/7/2009 contains viruses isolated from pigs in Europe including A/Aragon/RR3218/2008, a swine virus isolated from a zoonotic infection in Spain. These viruses are closely grouped with H1N1 viruses of avian origin. These so called Eurasian swine viruses have been circulating in swine since 1979 [20], were entirely derived from avian viruses, and have not yet been detected in North America. These observations clearly show that the HA gene from A/Aragon/RR3218/2008, an Eurasian avian-like swine virus, is genetically distant and has a different ancestor from the A(H1N1)pdm09 virus than their swine counterpart (classical swine lineage) circulating in North America. The observed lack of antigenic relatedness between A/Aragon/RR3218/2008 and the A(H1N1)pdm09 virus is further supported by the fact that, out of 50 residues located at antigenic sites, the two viruses differ at 16 positions (74% identity for the entire HA gene). Only antigenic site Sa is conserved between them. These findings also reveal that, for H1N1 viruses, amino acid differences are present throughout the HA, unlike current swine and human H3N2 viruses, where divergence is located mostly at antigenic sites. Whole-genome analysis showed sequence identities around 80–85% between PB2, PB1, PA, NP and NS genes of A/California/7/2009 and A/Aragon/RR3218/2008.

We compared antibody levels in panels collected at different time points (Figure 4). Cross-reactive antibody levels to H1N1 viruses depended on the collection period. In 2004 (2004 panel), antibody levels to influenza A(H1N1)pdm09 virus were lowest in individuals born after 1999 and highest in individuals aged 37 to 47 at the time (born between 1957 and 1967). After the 2007/08 winter (2008 panel), dominated by influenza A(H1N1) virus circulation, all age groups showed increases in reactive antibody levels to A(H1N1)pdm09 virus. This was most evident in those born before 1957, whilst only moderate increases were observed in those born between 1957–99, and the smallest increase noticed in the youngest age group. After the emergence and wide circulation of the A(H1N1)pdm09 virus (2010 panel), significant increases in antibody levels to this virus were observed in all age groups. The youngest age groups had the highest titres overall (GMT=124, 95% CI: 65–236) against this virus and the highest percentage of seropositive individuals (91%), while the number of seropositives in the older age groups was at least 45% even in the group with the lowest percentage overall, those born before 1957.

Figure 3

Phylogenetic tree showing the relationship between human, swine and avian full length haemagglutinin sequences from influenza A(H1N1) viruses



Swine viruses isolated from humans are denoted with *. Mid-point rooted trees were constructed with a neighbour-joining algorithm, using MEGA 4 software.

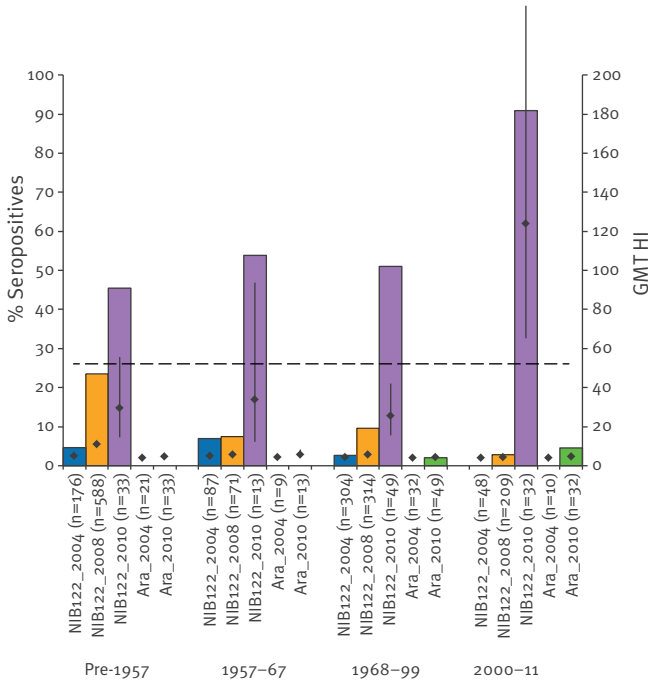
In contrast, we did not identify substantial time-dependent changes of cross-reactive antibody to the A/Aragon/RR3218/2008 strain in 2004 and 2010 panels, and age-related seropositivity suggests high level of susceptibility in all age groups.

Response to trivalent influenza vaccines

Analysis of a small additional panel of anonymised children (3–14 years-old; 24 pairs) and adult sera (20–77 years-old; 24 pairs) before and after vaccination with 2010/11 trivalent inactivated influenza vaccine, showed that children had higher levels of antibody to currently circulating influenza A strains prior to vaccination, which was consistent with our age-stratified cohort analysis, while no significant differences were identified between children and adults for the influenza B component of the TIV (data not shown).

Figure 4

Reactivity in age-stratified sera to influenza A(H1N1) viruses, England, 2004–11



CI: confidence interval; GMT: geometric mean titre; HI: haemagglutination inhibition.

Proportion with HI titre ≥32 (% seropositives) and GMT (95% CI) by exposure-related age group for H1N1 influenza viruses. The figure shows the analysis of three serum panels, collected at different time points (Table 1) with two influenza A(H1N1) viruses. The colouring of the bars indicates, which serum panel and virus were used in an analysis: the percentage of seropositives for the analysis with the NIBRG122 virus (reverse genetics derivative of A/England/195/2009) are indicated in blue (2004 panel), white (2008 panel) and green (2010 panel), while purple bars were used for the analysis with A/Aragon/R3128/2008 of 2010 panel (percentage of seropositives for analysis with A/Aragon/R3128/2008 in the 2004 panel is zero for all four age cohorts). The GMTs for the analysis with the NIBRG122 and A/Aragon/RR3128/2008 viruses are illustrated as diamonds in each bar with their 95% CI shown as vertical lines. Numbers under bars represent the number of samples in each age group. Cut-off for seropositivity is shown as dotted tine.

For influenza A(H3N2) viruses, we observed in children higher pre-vaccine GMTs with the currently circulating seasonal strain A/Perth/16/2009 (GMT=27; 95% CI: 13–55) and four to five times lower titres to recent swH3N2 viruses (A/Pensylvania/14/2010) and A/Wuhan/359/1995, while adults had higher titres to A/Pensylvania/14/2010 and A/Wuhan/359/1995 (GMT=40 and 34; 95% CI: 21–74 and 19–60, respectively), but significantly lower titres to A/Perth/16/2009. Children and adults showed comparable titre increases post vaccination, which were highest for the vaccine virus A/Perth/16/2009 (11.8 and 8.6-fold; 95% CI: 7.3–19.1 and 4.3–17.2, respectively). In both, GMTRs to A/Pensylvania/14/2010 and A/Wuhan/359/1995 viruses were three to four times lower than responses to A/Perth/16/2009. The seroconversion rates were generally higher in children than in adults; in both, rates with the vaccine virus A/Perth/16/2009 were almost twice as high as with the A/Pensylvania/14/2010 and A/Wuhan/359/1995 viruses.

For influenza A(H1N1) viruses, we observed clear differences in pre-vaccine titres for the currently circulating A/California/7/2009 virus, which were highest in children (GMT=76; 95% CI: 45–130) and significantly lower in adults (GMT=9; 95% CI: 5–16). Both age groups had only negligible titres against the A/Aragon/RR3218/2008. Comparing responses to A/California/7/2009 and cross-reactive antibody responses to A/Aragon/RR3218/2008 viruses, similar SCRs and GMTRs for both age groups were observed with the vaccine strain (SCR=96 and 63; GMTR=17.4 and 13.1, for children and adults respectively), while we observed two- to threefold lower SCR and six- to eightfold lower post-vaccine GMTRs with A/Aragon/RR3218/2008 virus.

Discussion

For our serological analysis, we chose three swine influenza isolates from the United States (US) representative of the recent limited human-to-human transmission of swH3N2 viruses in the US, together with historic and recent seasonal H3N2 strains. The swH3N2 viruses included an early isolate, A/sw/Minnesota/593/1999, closely resembling the ancestry of swH3N2 strains, which began circulating in North American pigs in 1998 [21], as well as two strains isolated from recent human cases A/Pennsylvania/14/2010 and A/Indiana/08/2011, the latter of which had acquired one of the eight gene segments (M gene) from the influenza A(H1N1)pdm09 virus [10,22]. We also selected a swine influenza A(H1N1) strain which had caused a sporadic human infection in 2008 in Spain [12] and compared serological responses with those to the A(H1N1)pdm09 virus. The diversity of these swine viruses was shown both in genetic analysis and antigenic characterisation.

Analysis of susceptibility to influenza A(H3N2) swine viruses

We found little evidence for reactive antibodies to North American swH3N2 viruses in children born in

England after 1999, despite moderate levels of antibody to the recent circulating human A/Perth/16/2009 H3N2 strain. This strongly suggests susceptibility of this age group to infection with North American swH3N2 virus. These data predict a high attack rate and greatest impact in young age groups, if these swH3N2 viruses were to emerge as a novel pandemic strain, analogous to the A(H1N1)pdm09 virus. The data are consistent with recently published results from the US [23], Canada [24] and Norway [25], and the observation that the cases identified so far have been mainly in children (ca. 90% in individuals younger than 18 years) [22,26]. They also suggest antibodies induced to the most recently circulating human H3N2 strains lack cross-reactivity with the investigated North American swH3N2 viruses.

Individuals born between 1968 and 1999 (aged 13–44 years in 2012) had the highest level of antibody to swH3N2 viruses, but the lowest level of antibody to the recent H3N2 seasonal A/Perth/16/2009 strain. This also supports the conclusion that antibody reactive with swH3N2 viruses occurs as a result of exposure to older H3N2 strains, either because of antigenic relatedness of older H3N2 strains to swH3N2 viruses or because of an increase in cross-reactive antibodies induced with increasing age. Cross-reactive antibodies in humans seem to correlate with exposure to H3N2 viruses circulating during the 1990s (e.g. A/Wuhan/359/1995 virus). We assume that cross-reactive antibodies in those born between 1968 and 1999 reflect extensive exposure to H3N2 variants circulating in that period and conform to previous observations that the highest attack rates following emergence of antigenic drift variants occur in the youngest age groups. Similar to surveillance data from the US [27] for the last two decades, variants of influenza A(H3N2) were the most commonly circulating strains in Western Europe with multiple drift variants recognised during this period [28-30]. Together, this suggests that the cumulative antibody responses to these H3N2 variants are a consequence of cross-reactivity to swH3N2 viruses, rather than arising from recent exposure to A/Perth/16/2009.

The data also suggest the importance of priming with an antigenically closely matched virus for later protection from a drifted strain – similar to observations in the 2009 pandemic, where individuals which had been exposed to historic H1N1 strains (dating from 1918 to 1956) early in their life seemed to be protected from infection with A(H1N1)pdm09 [14].

The assumption that cross-reactive antibody levels correlate with exposure to H3N2 viruses circulating during the 1990s is supported by the results from phylogenetic analysis (Figure 1) and antigenictiy work in ferrets (Table 3), which together point to similarity of seasonal human viruses of the 1990s and the swH3N2 viruses causing the recent zoonotic cases in the US. One of the influenza strains used in this study (A/Swine/Minnesota/593/1999) dates back to the emergence of influenza A(H3N2) in North American pigs and predates antigenic drift resulting from continuous circulation in pig herds. This isolate shares antigenic epitopes with human H3N2 viruses circulating at the same time, such as A/Wuhan/359/1995. We observed a close match of seroreactivity with A/Wuhan/359/1995 and A/Swine/Minnesota/593/1999 viruses.

In individuals born before 1968 (aged 44 years and older in 2012), antibody titres to A/Perth/16/2009 were of similar level, indicating a similar overall exposure to a recently circulating variant. However, compared to antibody levels in individuals in the 1957–67 birth cohort, we observed lower reactivity with swH3N2 viruses and A/Wuhan/359/1995 in these older adults despite greater likelihood of cumulative exposure to influenza A(H3N2) viruses. We assume that lower levels of cross-reactive antibody to swH3N2 in these individuals could be a result of priming with H3N2 viruses which emerged during the pandemic 1968, or childhood exposure to other, non-H3 influenza subtypes as suggested elsewhere [24]. Nevertheless, the overall GMTs suggest that significant numbers of individuals in England (ca. 50%) may currently be protected from swH3N2 infection.

We also determined the ability of pre- and post-immunisation sera from children and adults immunised with 2010/11 TIV to react with viruses of swine origin as a measure of whether vaccination with seasonal influenza vaccines produces cross-reactive antibodies capable of providing partial protection to emerging zoonotic swine influenza infections. Vaccination with contemporary TIV shows clear evidence of boosting reactivity to swH3N2 viruses after seasonal influenza vaccination. Although boosting was equally efficient in children and adults, vaccination is likely to be most beneficial to the younger age groups because of their generally lower cross-reactive baseline titres.

Analysis of susceptibility to influenza A(H1N1) European swine viruses

We found no evidence of significant pre-existing immunity to a recent Eurasian swH1N1 isolate (A/Aragon/RR3218/2008) in any age group (Figure 4). These findings are consistent with the substantial genetic (Figure 3) and antigenic divergence of this virus from the previous seasonal and current A(H1N1)pdm09 viruses. Baseline immunity analysis in 2009 [14] together with influenza surveillance data [31] point at the importance of priming with historic seasonal H1N1 strains for protection from infection with a newly emerging virus, i.e. A(H1N1)pdm09 [32]. In contrast, the genetic and antigenic divergence of previous and current seasonal H1N1 viruses as compared to the Eurasian swH1N1 points to a lack of priming in the English population.

However, whole genome sequencing data show that this virus has NA and M genes which are similar to those of the A(H1N1)pdm09 virus, with 90% and 94% of sequence identity, respectively, consistent with the

finding that these genes in the 2009 pandemic viruses had originated from the Eurasian lineage of swine viruses [33]. Vaccination with contemporary TIV shows only a limited boosting effect on antibody levels to A/Aragon/RR3218/2008 in both children and adults, and could indicate an inability of current commercial vaccines to protect against swH1N1viruses of the Eurasian lineage.

Our study has several limitations. We used a cut-off value of titres ≥32, while it is unclear whether this titre would indeed confer protection on an individual level, especially for zoonotic infections to which whole populations are immunologically naïve.

This analysis is based on HI data. It has been speculated that neutralisation assays are more likely to detect antibody arising from previous exposure or vaccinations with related strains, which are undetectable by HI [34]. This could have resulted in an underestimation of cross-reactive antibodies. We are also unable to predict the possible contribution of cell-mediated responses to protection. Furthermore, our analysis was opportunistic and intended to be indicative. We used samples available to us, but had only limited numbers of samples with enough remaining volume for this analysis, as the material from the Public Health England serum archive had been used extensively for the UK seasonal seroepidemiology programme. As a result, the described serum panels vary significantly in sample number and the study was underpowered to detect significant differences between adults and children for the analysis of cross-reactive responses post TIV for vaccine trials or by birth cohort in the three population-based serosusceptibility panels (Table 1), especially with the low seroprevalence of antibodies to A/Aragon/RR3218/2008.

The analysis described here has been performed over a period of three years. An identical standard operating procedure was followed throughout; together with use of appropriate and consistent control sera, this should have kept variability of the results to a minimum and allow their comparability.

Although A/Wuhan/359/1995 seems to be an ancestor strain of the investigated swH3N2 viruses, our antigenic characterisation (using ferret sera) indicates that it is not a precise antigenic match. However, seroprevalence data from our human cohort indicate that this virus might be closely related to a shared ancestor. Finally, for the swH1N1 of the Eurasian lineage we selected only one isolate; it is possible that use of other strains might lead to slightly different conclusions regarding cross-protection. However, the phylogenetic data show that viruses in this lineage are significantly distant from previous seasonal H1N1 viruses and the currently circulating A(H1N1)pdm09 viruses (Figure 3), suggesting that the observed lack of cross-reactivity is a universal feature for this group of viruses.

Conclusions

These data and the implied susceptibility to infection in different population subgroups highlight the importance of regular risk assessment of emerging swine origin viruses and virus-specific response planning. Vaccination and control strategies need to target individuals in society who appear to have least protection from infection. The observed differences in seroreactivity when analysing representative swine viruses from different geographical origin and two subtypes, both of which had recently caused infection in humans, emphasise the necessity of regular surveillance activities and interaction between animal and human health agencies.

The data presented here show that swH3N2 and swH1N1 subtypes have a different age-related pattern of potential susceptibility in the human population studied, which is again different from the variant H1N1 subtype that caused the 2009 pandemic. Recommendations for pandemic preparedness need to be adjusted accordingly to take into account virus subtype and source of origin. At a global level, epidemiology of influenza virus in pigs is very complex and diverse. Similarly, recommendations for vaccination with TIV to induce cross-reactive antibody will depend on the nature of the emerging strain and age-dependent priming history in the population.

Globally, very few programmes exist that are based on interconnected animal and human health agencies. It is a clear recommendation from WHO that animal surveillance efforts should be enhanced beyond disease notification, with sharing of viruses between the human and animal sector to improve pandemic risk assessments [35].

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Reduction of the nosocomial meticillin-resistant *Staphylococcus aureus* incidence density by a region-wide search and follow-strategy in forty German hospitals of the EUREGIO, 2009 to 2011

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Meticillin-resistant *Staphylococcus aureus* (MRSA) disseminates between hospitals serving one patient catchment area. Successful prevention and control requires concerted efforts and regional surveillance. Forty hospitals located in the German EUREGIO have established a network for combating MRSA. In 2007 they agreed upon a synchronised strategy for screening of risk patients and a standard for transmission-based precautions (search and follow). The same year, the hospitals started synchronised MRSA prevention and annually reporting MRSA-data to the public health authorities. The median rate of screening cultures per 100 patients admitted increased from 4.38 in 2007 to 34.4 in 2011 (p<0.0001). Between 2007 and 2011, the overall incidence density of MRSA (0.87 MRSA cases/1,000 patient days vs 1.54; p<0.0001) increased significantly. In contrast, both the incidence density of nosocomial MRSA cases (0.13 nosocomial MRSA cases/1,000 patient days in 2009 vs 0.08 in 2011; p=0.0084) and the MRSA-days-associated nosocomial MRSA rate (5.51 nosocomial MRSA cases/1,000 MRSA days in 2009 vs 3.80 in 2011; p=0.0437) decreased significantly after the second year of the project. We documented adherence to the regional screening strategy resulting in improved detection of MRSA carriers at admission. Subsequently, after two years the nosocomial MRSA-incidence density was reduced. Regional surveillance data, annually provided as benchmarking to the regional hospitals and public health authorities, indicated successful prevention.

Introduction

Meticillin-resistant *Staphylococcus aureus* (MRSA) globally belongs to the most frequent causes of healthcare-associated infections [1]. In addition, the severity of MRSA infections is documented by studies estimating that patients with MRSA bloodstream infection (BSI) have a higher 30-day mortality compared to those with BSI due to meticillin-susceptible *S. aureus* (MSSA) [2].

In 2007, the annual burden of MRSA infections in European Union (EU) Member States, Iceland and Norway was estimated to comprise 171,200 cases including 12% BSI [3]. However, MRSA rates in Europe show remarkable differences: In 2010, Sweden reported the lowest proportion of invasive isolates resistant to meticillin (0.5% of all *S. aureus*) and Portugal the highest (52.2%) [4]. Such discrepancies were even reported when comparing directly neighbouring countries. In the German federal state of North Rhine-Westphalia (NRW), 57.6 MRSA bacteraemia episodes per 1,000,000 inhabitants were reported to public health authorities in 2010, whereas only a few kilometres across the Dutch–German border the rate of bacteraemia episodes was estimated to be 1.8 per 1,000,000 inhabitants in the Netherlands [5,6]. Hence, the prevalence of MRSA differs nationally and regionally. Moreover, it was found that there are substantial regional differences regarding the molecular subtypes of MRSA circulating in Europe [7] and that compared with other continents, which observed a shift of the major MRSA burden from healthcare institutions into the community, healthcare-associated (HA-) MRSA are still predominant in Europe [8].

The molecular epidemiology of MRSA in Europe indicates that the dissemination of MRSA is mainly governed either by direct transfer of patients between hospitals, nursing homes and other healthcare facilities or by indirect exchange of patients admitted consecutively to several facilities in one same catchment area [7]. Indeed, of 354 MRSA carriers identified at admission to German hospitals, 32 were directly transferred from other hospitals, 173 had been hospitalised for › 24 h in the previous six months and 58 were

residents of long-term care facilities [9]. Other studies have shown that patients once identified as MRSA carriers had a 44% probability of being re-admitted to the same or other regional hospitals whilst still carrying MRSA [10] and that of 1,032 known patients with previous MRSA carriage attending the Hannover Medical School hospital, 39% were re-admitted more than once during a 46-month period, 59% of which remained MRSA positive during all admissions [11]. Simulating the importance of inter-facility patient movements, it has been shown that especially small long-term care facilities with low patient turnover rates are most susceptible to sustaining high MRSA prevalence, especially when cooperating with large, high-prevalence hospitals [12]. A model based on patient-flow characteristics of 29 acute care hospitals cooperating in Orange County, California, revealed that in case of an outbreak (increase of MRSA prevalence to 15%) in a single hospital within the county, most other regional hospitals also experienced an increase of the MRSA prevalence (median increase of MRSA prevalence 1.8%). In this model, even outbreaks in single intensive care units affected the overall prevalence of MRSA in regional acute care hospitals [13].

In consequence, the referral of patients between regional hospitals might contribute to MRSA spread. Thus preventive standards for MRSA control implemented only locally in single hospitals may be not effective to reduce MRSA. The implementation of harmonised preventive standards in the regional healthcare cluster, where the nosocomial pathogen spreads predominantly is more likely to be successful. The hospitals of the Dutch–German border region EUREGIO have been confirmed to represent such a regional healthcare cluster using mathematical modelling [14]. Therefore, the governments of the 16 German *Bundesländer* (i.e. federal states) have recommended to address the problem of antimicrobial resistance in regional networks of hospitals and other institutions involved in patient care [15], and to apply regional approaches and concerted regional action to solve the MRSA problem [15,16]. In addition, the implementation of preventive interventions including screening of defined risk patients, single or cohort room isolation and the use of transmission-based precautions (gloves, gowns) when caring for MRSA colonised or infected patients [17,18] has been recommended for all German acute care hospitals. However, despite the availability of guidance [19], it was shown that for German hospitals it is challenging to implement preventive bundles sufficiently and in a standardised manner on a local and regional level [20]. To reinforce regional cooperation and overcome the problem of limited local implementation, all 40 hospitals in the Münsterland region in NRW are cooperating in the EU-funded Dutch–German EUREGIO MRSA-net (www.mrsa-net.eu) and EurSafety HealthNet (www.eursafety.eu) projects since 2005. Since these 40 hospitals form a connected healthcare cluster [14], and contain all hospitals in the region, they can be considered to form a single patient catchment area.

In 2007, hospitals agreed upon and started the implementation of a concerted strategy for identification of MRSA carriers by (pre)admission MRSA screening of risk patients, isolation of carriers, continued care and decolonisation even after transfer to another hospital or after discharge and collection of basic MRSA surveillance data for centralised analysis and benchmarking [9,15]. In this report, we analyse surveillance data collected between 2007 and 2011 resulting from the network with respect to changes in the implementation of MRSA screening and the number and incidences of imported and nosocomial MRSA cases.

Methods

The German part of the project region geographically comprises six German districts (codes DEA33, DEA34, DEA35, DEA37, DEA38 and DE94B, level3, according to the Nomenclature of Territorial Units for Statistics) [21] and is inhabited by 1.7 million people. In 2007, 40 hospitals were located in the region, treating about 360,000 patient cases during 2,500,000 inpatient days per year. Of 40 hospitals, 36 were acute care hospitals, while one was a rehabilitation clinic and three hospitals were specialised in psychiatry.

During a prevalence screening of all admissions and risk factor assessment in November 2006, 35.6% of patients admitted to the hospital had at least one MRSA risk factor [9]. In 2007, all hospitals started to systematically screen defined patients associated with any one of the known risk factors described previously [9], prior to or at admission to hospital. In 2008, the risk factors were slightly adapted according to a new German national recommendation [18]. In the project there was no harmonised microbiological protocol for performing the MRSA screening. Mostly a culture-based approach using chromogenic MRSA media was used. Positive screening was followed in all hospitals by the implementation of single or cohort room isolation, transmission-based precautions and decolonisation therapies as recommended in Germany [17]. Adherence to the recommendations was checked exemplarily by the local public health authorities during annual inspections.

Since 2007 standardised MRSA-related data were collected, based on a surveillance protocol adapted from that of the national German Nosocomial Infections Surveillance System to ensure comparability [22]. We analysed and reported all surveillance results and reported once a year to the participating hospitals in an anonymised feedback data set via the responsible public health offices.

Data collection

The collected surveillance data included the numbers of MRSA cases (colonisation and infection) classified as imported or nosocomial cases, the overall number of patient cases treated and the overall number of patient days as well as patient days of MRSA cases and the number of nasal swabs performed at admission. The

findings of at least each MRSA-detection based on the first positive isolates of all MRSA-inpatient cases were inserted in a database (Epi-MRSA®, Ridom GmbH, Münster, Germany or Excel®, Microsoft Inc., Redmond, USA). For each case, information on every first MRSA isolate was included in an annual report to the public health authorities with one exception: if a patient developed a MRSA bacteraemia during the hospital stay, this detection of MRSA-bacteraemia based on the positive MRSA blood culture isolate was included in the report. Each MRSA-detection (colonisation or infection) of isolates sampled more than three days after admission was classified as nosocomial unless the patient was a known MRSA carrier. A day, which an MRSA patient spent in hospital, was classified as MRSA-in-hospital day. In most hospitals, the MRSA-in-hospital days could be specified by counting the isolation days of MRSA cases.

Data analysis

We analysed the surveillance data of five years (2007–2011) and calculated the following parameters: (i) number of MRSA cases, classified as imported or nosocomial cases, (ii) screening rate (number of screening cultures/100 admissions), (iii) MRSA incidence at admission (imported MRSA cases/100 admitted cases), (iv) MRSA incidence density (MRSA cases/1,000 patient days), (v) nosocomial MRSA incidence density (nosocomial MRSA-cases/1,000 patient days), (vi) the mean daily MRSA-burden (MRSA-in-hospital days/100 patient days), (vii) MRSA-days-associated nosocomial MRSA rate (nosocomial MRSA-cases/1,000 MRSA days).

Time trends of MRSA parameters were analysed by Friedmann test ($p<0.05$ was considered significant). The percentage of nosocomial MRSA cases on all MRSA cases was assessed by Cochran Armitage test of linear trend ($p<0.05$). The correlation of MRSA parameters was done calculating the Spearman rank correlation coefficient ($p<0.05$). All statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, USA). Results of significance tests were discarded if the programme displayed an alert due to more than 10% of missing values in the respective dataset.

Results

In this study, we collected MRSA-related data of regional hospitals in the EUREGIO during a five-year period. While in 2007 and 2008, 38/40 hospitals (95%) participated in surveillance, the participation rate was 100% in 2009 (40/40 hospitals), 2010 (38/38 hospitals) and 2011 (37/37 hospitals). The number of participating hospitals changed from 40 to 37 because of acute care hospital fusions.

Overall, the implementation of the screening strategy as monitored by analysis of the median screening rate increased significantly ($p<0.001$) over time from 4.38/100 patients (interquartile range (IQR): 2.15–11.8) admitted in 2007 to 34.4/100 patients (IQR: 27.4–51.6) in 2011 (Table 1, Figure 1).

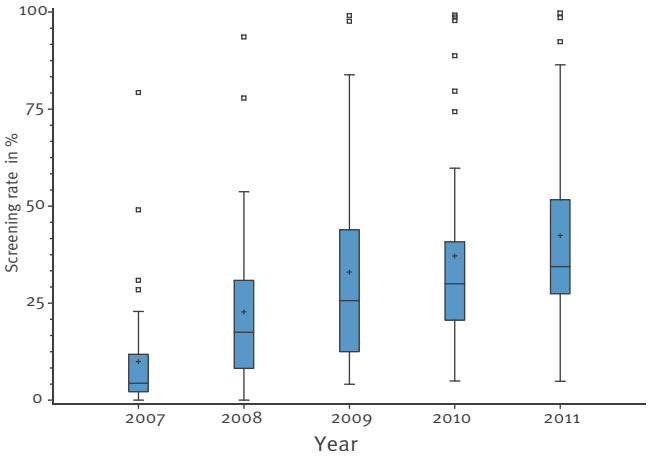
Table 2 shows the numbers of documented regional MRSA cases. In 2007, the total number of cases was 2,351. For 1,864 of these cases stratification into imported (1,481) and nosocomial (383) cases was possible (Table 2). Between 2007 and 2011, the overall number of cases increased significantly ($p<0.0001$). According to Cochran Armitage trend test, the percentages of imported cases on all MRSA cases increased significantly ($p<0.0001$) between 2007 and 2011, while the percentage of nosocomial cases on all MRSA cases decreased significantly ($p<0.0001$) (Table 2).

From 2007 to 2011, the MRSA admission incidence (0.51 vs 1.09 MRSA cases/100 patients admitted), the MRSA-incidence density (0.87 vs 1.54 MRSA cases/1,000 patient days) as well as the mean daily MRSA-burden (1.30 vs 1.82 MRSA-in-hospital days/100 patient days) increased significantly ($p<0.0001$) (Table 1). These overall increases were due to increases in the years from 2007 to 2009. From 2009 to 2011, the MRSA admission incidence ($p=0.5796$), the MRSA incidence density ($p=0.6729$) and the mean daily MRSA-burden ($p=0.7327$) remained stable.

The incidence density of nosocomial cases decreased from 0.14 nosocomial MRSA cases per 1,000 patient days in 2007 to 0.08 per 1,000 patient days in 2011

Figure 1

Distribution of increasing screening rate at admission to identify meticillin-resistant *Staphylococcus aureus* (MRSA) carriers in German regional hospitals of the EUREGIO, 2007–2011 (n=40)



IQR: interquartile range.

Distribution of nasopharyngeal admission screening cultures per 100 patients admitted to the 40 German hospitals of the EUREGIO taking part in a concerted strategy for identification of MRSA carriers. For each year the number of screenings per 100 patients is shown in the boxplot with whiskers as minimum and maximum and outliers as squares below 1.5*IQR*25th percentile or above 1.5*IQR*75th percentile and far outliers above 3*IQR*75th percentile. 50% of hospitals reported a screening rate indicated in the rectangle with the mean (plus) and the median (band).

(Table 1). The MRSA-days-associated nosocomial MRSA rate decreased from 9.52 nosocomial MRSA cases per 1,000 MRSA days in 2007 to 8.14 in 2008 and to 5.51 in 2009. It subsequently increased to 7.77 in 2010 followed by a decrease to 3.80 in 2011 (Table 1).

The data quality allowed for specifying the significance of trend for these parameters from 2009 to 2011 only; other time periods were statistically excluded for lack of more than 10% of the data. From 2009 to 2011, the MRSA-admission incidence remained stable (p=0.5796) whereas the nosocomial MRSA-incidence density (p=0.0084) as well as the MRSA-days-associated nosocomial MRSA rate (p=0.0437) decreased significantly.

There was a moderate positive correlation between the mean daily MRSA-burden and the screening rate (Spearman rank correlation coefficient r=0.32710; p<0.0001) (Figure 2). Furthermore, there was a low negative correlation between the MRSA-days-associated nosocomial MRSA rate and the screening rate (Spearman rank correlation coefficient r=-0.23829; p=0.0017) (Figure 3). In Figures 2 and 3, the median and the IQR are plotted. In this way, hospitals beyond the 50% range may be graphically detected, stimulating inspection and, if necessary, intervention measures.

Discussion

According to the antimicrobial resistance surveillance in Europe in 2010, MRSA is the most important cause of antibiotic resistant healthcare-associated infections worldwide. In 11 of 28 European countries, the percentage MRSA-isolates per *S. aureus*-isolates in blood cultures is higher than in Germany. The *S. aureus* resistance to meticillin in Germany in 2010 is still reported as increasing [4].

Due to an active search and destroy policy in Dutch hospitals, the incidence of hospital-associated cases and the rate of nosocomial transmission have been kept at a low level since decades [23]. Within the framework of two Dutch–German preventive network projects (EUREGIO MRSA-net and EurSafety Health-net), we aimed to establish an adapted 'search and follow' strategy in hospitals in the German part of the Dutch–German border area [24]. This includes active search by region-wide screening for MRSA carriage at admission following the standardised implementation

Table 1

Admission screening rates and meticillin-resistant *Staphylococcus aureus* related rates in 40 hospitals in the EUREGIO, 2007–2011

Parameter	2007	2008	2009	2010	2011
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Screening rate (MRSA/100 patients admitted)	4.38 (2.15–11.8)	17.5 (8.19–30.9)	25.6 (12.5–43.9)	30.0 (20.6–40.9)	34.4 (27.4–51.6)
MRSA admission incidence (MRSA cases/100 patients admitted)	0.51 (0.39–0.79)	0.94 (0.60–1.24)	0.86 (0.60–1.34)	1.12 (0.75–1.39)	1.09 (0.70-1.35)
MRSA incidence density (MRSA cases/1,000 patient days)	0.87 (0.56–1.21)	1.37 (0.93–1.89)	1.62 (1.01–2.20)	1.63 (1.19–2.35)	1.54 (0.92–2.27)
Nosocomial MRSA incidence density (nosocomial MRSA cases/1,000 patient days)	0.14 (0.06–0.24)	0.15 (0.10–0.21)	0.13 (0.04–0.25)	0.13 (0.06–0.23)	0.08 (0.03–0.15)
Mean daily MRSA burden (MRSA-in-hospital days/100 patient days)	1.30 (0.86–1.95)	1.98 (1.53–2.67)	2.01 (1.39–2.62)	1.80 (1.58–3.24)	1.82 (1.27–2.96)
MRSA-days-associated nosocomial MRSA rate (nosocomial MRSA-cases/1,000 MRSA days)	9.52 (2.97–17.4)	8.14 (4.53–11.7)	5.51 (3.31–12.5)	7.77 (3.62–10.7)	3.80 (2.04–7.97)

IQR: interquartile range; MRSA: meticillin-resistant *Staphylococcus aureus*.

Table 2

Numbers of meticillin-resistant *Staphylococcus aureus* cases documented in 40 German hospitals in the EUREGIO, 2007–2011

Numbers of MRSA cases	2007	2008	2009	2010	2011
	n (%)	n (%)	n (%)	n (%)	n (%)
MRSA (total)	2,351 (100)	3,522 (100)	4,206 (100)	4,276 (100)	4,512 (100)
MRSA (stratified)[a]	1,864 (100)	3,263 (100)	3,759 (100)	4,150 (100)	4,347 (100)
Imported MRSA cases	1,481 (79)	2,807 (86)	3,262 (87)	3,641 (88)	3,992 (92)
Nosocomial MRSA cases	383 (21)	456 (14)	497 (13)	509 (12)	355 (8)

MRSA: meticillin-resistant *Staphylococcus aureus*.

[a] Number of MRSA cases with available information allowing stratification as imported or nosocomial.

of transmission-based precautions (hand hygiene, isolation and contact precaution) and decolonisation of MRSA carrier.

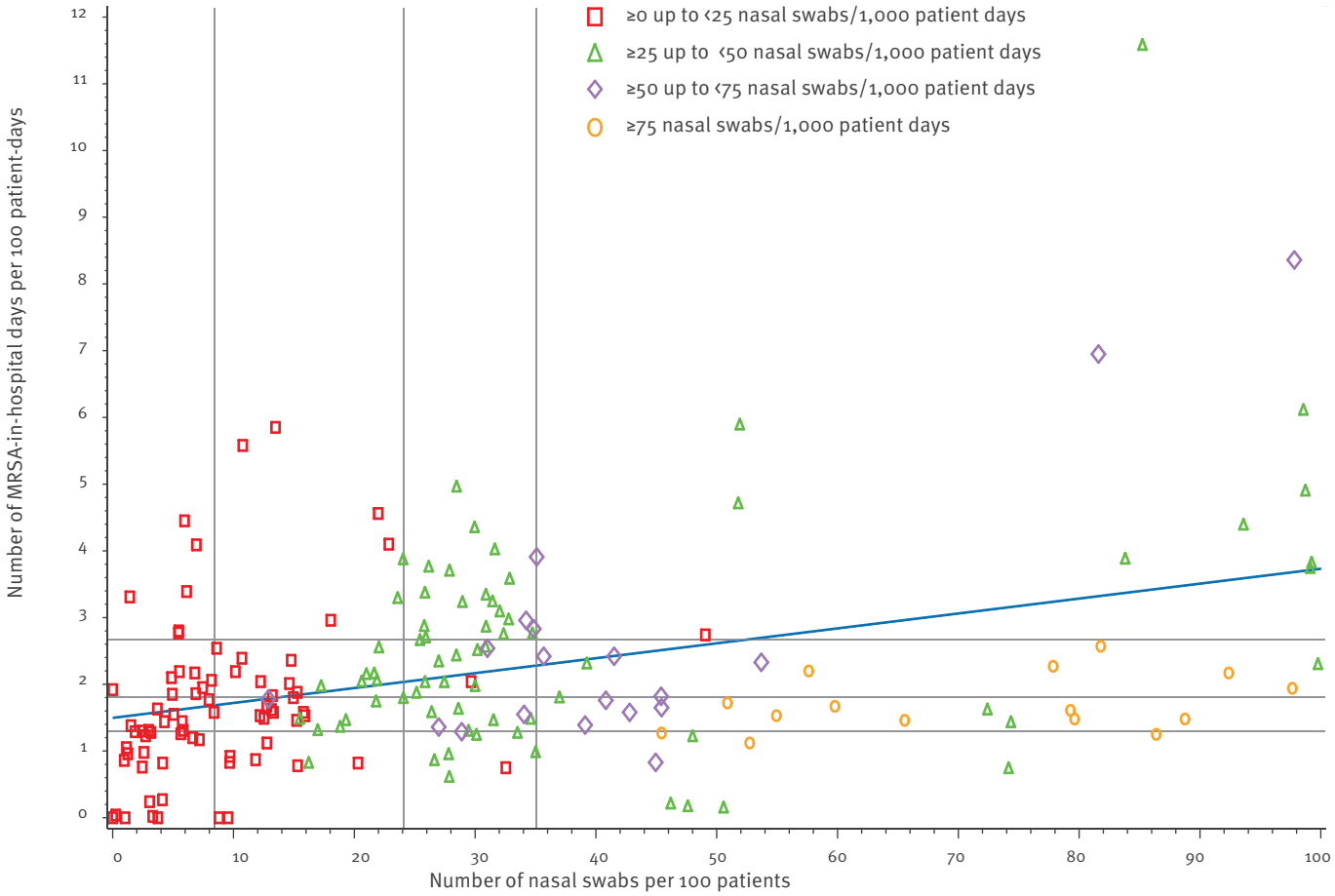
Here we present surveillance data obtained from this regional network aiming to prevent inter-institutional MRSA spread. We demonstrate that the network structures enabled the implementation of a risk-based admission screening approach. This was documented by a significant increase of nasopharyngeal MRSA screenings performed in the network hospitals after agreement upon a minimum standard for a screening regime. The aim of the network was to establish a screening of patients at a higher risk of MRSA carriage as defined by national German recommendations [17,18]. In 2011, the regional hospitals achieved a median screening rate of about 30%, which argues for successful implementation of the screening-policy. The latter is supported by the study in which the same hospitals have assessed risk factors for MRSA carriage among all patients admitted during a one-month period in 2006. A total of 35.6% of the patients exhibited at least one risk factor at admission. The observed admission MRSA prevalence was 1.6/100 patients [9].

The effect of the improved admission screening was demonstrated by an overall 92% increase of MRSA cases detected in the participating hospitals. Moreover, the MRSA admission-incidence nearly doubled (0.51 MRSA/100 patients in 2007 vs 1.09 MRSA/100 patients in 2011). This increase was not surprising as it is known that 69 to 85% of all MRSA cases are not detected if microbiological cultures are only performed for clinical reasons and screening is not implemented [25].

We assume that the risk-based screening strategy implemented in the participating hospitals still does not detect all MRSA cases, but, according to our data at least 68%, due to the observed admission-incidence after full implementation (1.09/100 patients) which is

Figure 2

Moderate positive correlation between the mean daily meticillin-resistant *Staphylococcus aureus* burden and the screening rate, stratified by screening extent, of 40 German regional hospitals, EUREGIO, 2007–2011



MRSA: meticillin-resistant *Staphylococcus aureus*.

Moderate positive correlation between mean daily MRSA burden (MRSA-in-hospital days/100 patient days) and screening rate (admission screenings/100 patients). Each sign represents a network hospital once a year from 2007 to 2011, with the respective colour and shape reflecting the degree of screening implementation in nasal swabs per 1,000 patient days. The grey lines represent the respective medians and interquartile ranges (IQR) of the MRSA-in-hospital days per 100 patient days and the nasal swabs per 100 patients.

lower than the admission burden found when screening all regional patients at admission (1.6 MRSA cases/100 patients) [9].

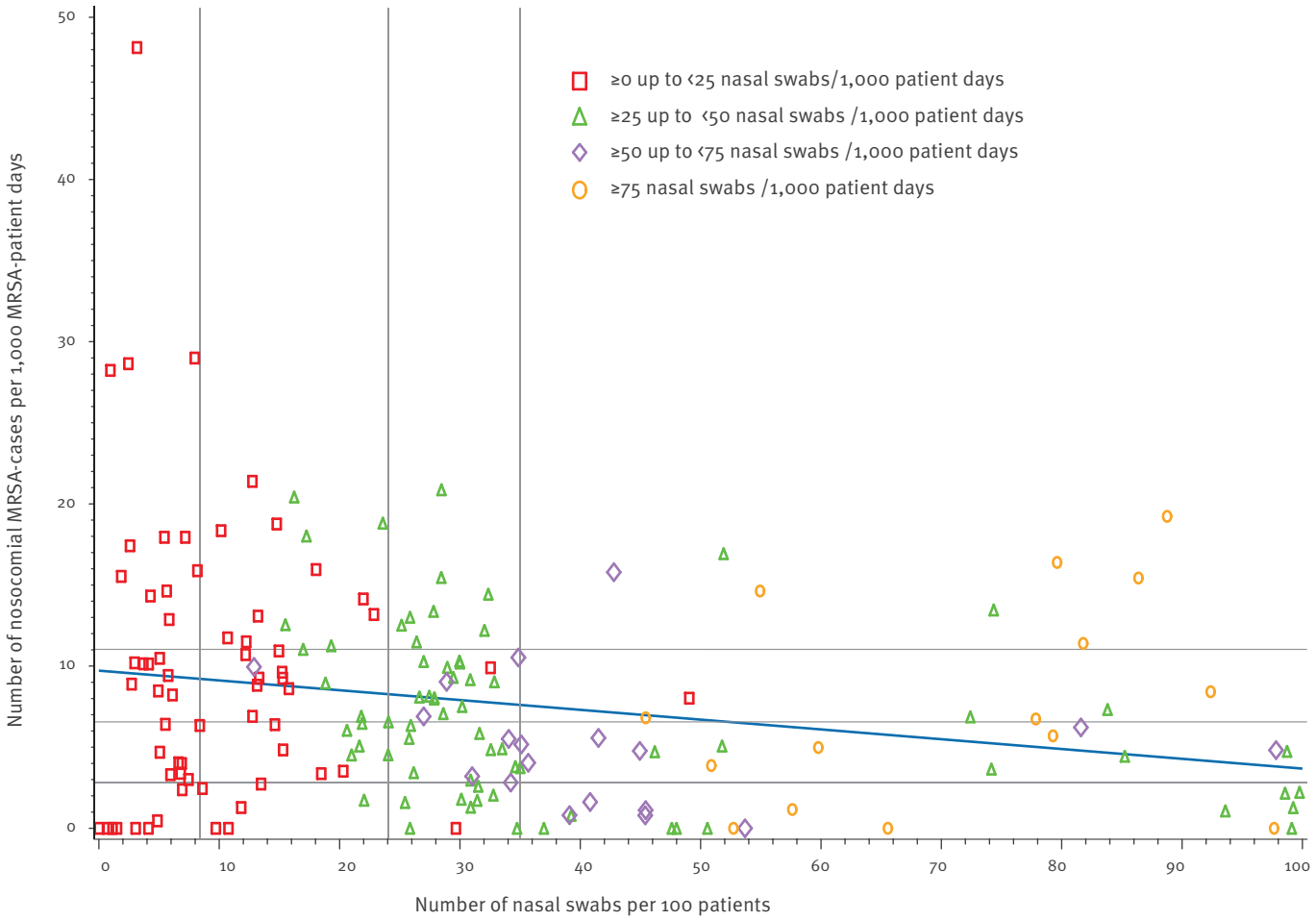
Furthermore in 2011, the MRSA admission incidence was higher compared to the median incidence of 0.83 MRSA/100 patients described in 302 of 2,041 German hospitals participating in a German nationwide surveillance programme [22,26]. Since data from admission prevalence screening studies do not indicate that MRSA was more endemic in the EUREGIO compared to other parts of Germany [27], this difference is most likely due to the fact that the median screening rate was 34.4/100 patients in the EUREGIO-hospitals, but much lower (9.39/100 patients) in hospitals taking part in the national surveillance programme. However, from 2009 to 2011, we observed a stabilisation of the MRSA-incidence and the mean daily MRSA burden, which indicates a saturation of excess MRSA detection due to enhanced screening.

The improved detection of MRSA at admission also led to a decrease in the proportion of nosocomial MRSA-cases within all MRSA cases. This is similar to results reported by other authors for 26 hospitals involved in the German nationwide MRSA surveillance from 2004 to 2006 [28], but has so far not been shown in a region-wide surveillance. From 2004 to 2009, the 111 hospitals, taking continuously part in the German national MRSA surveillance, reported a stable nosocomial MRSA incidence density of about 0.25 nosocomial MRSA cases per 1,000 patient days. A decrease was only observed in intensive care units [29]. The EUREGIO hospitals reported from 2007 to 2009 about 0.14 nosocomial MRSA cases per 1,000 patient days and even a significant reduction from 2009 to 2011.

The time-delayed reduction of nosocomial MRSA incidence density may be because the improved screening leads stepwise to better detection of MRSA cases in general, increasingly allowing better containment

Figure 3

Low negative correlation between the meticillin-resistant *Staphylococcus aureus* (MRSA)-days-associated nosocomial MRSA-rate and the screening rate, stratified by screening extent, of 40 German regional hospitals, EUREGIO, 2008–2011



Low negative correlation between the MRSA-days-associated nosocomial MRSA-rate (number of nosocomial MRSA-cases/1,000 MRSA-patient-days) and screening rate (admission screenings/100 patients). Each sign represents a network hospital once a year from 2007 to 2011, with colour and shape reflecting the degree of screening implementation in nasal swabs per 1,000 patient days. The grey lines represent the respective median and interquartile ranges (IQR) of the nosocomial MRSA-cases per 1,000 MRSA days and nasal swabs per 100 patients.

of the bacteria and more limiting spread in the hospital by MRSA-management measures like for example hand hygiene, contact precautions, and decolonisation efforts. The introduction of screening at admission and the increased awareness for MRSA in the hospitals might have led to more detection of nosocomial MRSA-cases as well as to fewer MRSA cases not classifiable as imported or nosocomial (21% in 2007 vs 4% in 2011). On the other hand, there might be less 'false' nosocomial MRSA-cases (imported MRSA-cases detected via an isolate sampled from an inpatient after three days of hospital stay). Additionally, it may be that the hospitals improved the quality of the submitted data at least from 2009 to 2011, after two years of participation in the surveillance system. Furthermore the nosocomial MRSA incidence density was low (about 0.14 MRSA cases/1,000 patient days) in the 40 EUREGIO-hospitals compared to 111 hospitals, which took part in the German national surveillance system (0.25 MRSA cases/1,000 patient days, stable from 2004 to 2009). The low number MRSA cases per 1,000 patient days in our hospitals reduced the probability for a (significant) decrease, which we could nevertheless demonstrate from 2009 to 2011.

For infection control staff, the advantage of improved MRSA detection at admission is that detection of nosocomial cases more reliably reflects cases of MRSA infection or colonisation which are caused by intra-institutional transmission of the pathogen. This is important because a high number of nosocomial MRSA cases per 1,000 patient days (i.e. a nosocomial MRSA-incidence in the range of the upper quartile of all hospitals taking part in a surveillance system) might be an indicator for deficits in infection control and/or for selection pressure due to use of antibiotics, which should be checked for possible improvements [30]. Locally, the knowledge of nosocomial MRSA cases enables more targeted reactions with respect to elucidating the transmission pathways and implementing measures appropriate to forestall further transmission.

Admission screening for MRSA carriage has been shown to be (cost-) effective in reducing both nosocomial infections and MRSA transmission on the wards, because it enables timely implementation of transmission-based precautions, isolation and decolonisation therapies [31,32]. Although, we did not assess to what extent isolation measures or decolonisation therapies were performed for the MRSA patients detected, applying a standardised panel of MRSA infection control measures in all participating hospitals was part of the network's quality goals and the implementation of these measures was controlled exemplarily by the regional public health authorities. Since nosocomial transmission is more probable in a hospital with a high mean daily MRSA burden than in a hospital with a low mean daily MRSA burden, the MRSA-days-associated nosocomial MRSA rate is used to compare hospitals as this rate takes into account the institutional mean daily MRSA-burden and thereby reflects the degree of transmission on the ward more reliably [33]. In this study, we observed a negative correlation between the slightly decreasing MRSA-days-associated nosocomial MRSA rate and the increasing screening rate. This also suggests that in those hospitals where screening was performed consistently it was followed by adequate hand hygiene, contact precautions, isolation of MRSA-carriers and subsequently led to a reduction of nosocomial transmissions.

Another important question related to the implementation of preventive strategies is whether they are effective in reducing the number of nosocomial MRSA cases. Many authors have investigated this issue with divergent results [31,34-36]. In this context, we found that from 2009 to 2011 the nosocomial incidence density decreased significantly from 0.13/1,000 patient days to 0.08/1,000 patient days. This indicates that after an implementation period, when MRSA carriers were detected at an early stage, the rate of nosocomial cross-transmission was reduced.

Besides screening, hand hygiene, isolation of MRSA-carriers and contact precautions the 'search and follow'-strategy in the EUREGIO implies a post-discharge MRSA-case management outside the hospital [15]. In this way after a reduction of the nosocomial burden, the overall burden of MRSA-carriage in the region may be reduced.

This study also has some limitations. Analysis of MRSA-surveillance data of all hospitals in a regional network cannot adjust for all existing differences between the hospitals including different specialities, difference in the presence of infection control staff, different patient populations and case-mixes. Especially in 2007 and 2008, there were missing data concerning MRSA days or classification of MRSA cases as imported or nosocomial. We addressed the latter by not calculating statistical differences in the absolute numbers of nosocomial cases, in the MRSA-admission incidence, in the nosocomial MRSA incidence density and in the MRSA-days-associated nosocomial MRSA rate assessed between 2007 and 2008. However, during the course of the study, the quality of data collection was enhanced and the statistical tests indicated robust performance. Furthermore, we cannot adjust for differences in further hygiene measures implemented in the participating hospitals (e.g. different participation in the German version of the World Health Organization (WHO) campaign 'Clean Care is Safer Care' [37]). In the future, the network will have to focus on a structured assessment of nosocomial MRSA infections rather than cases of colonisation and infection to monitor the clinical impact of MRSA.

In conclusion, we documented the successful implementation of a screening programme in hospitals participating in a regional prevention network. This approach led to a significant increase of MRSA cases, but, eventually to a significant reduction of nosocomial

MRSA-cases per 1,000 patient days. The data reflects a paradoxical situation ('MRSA-screening-paradox'): Initially, more MRSA carriers are found when more patients are screened. This may make some hospitals reluctant in establishing such a screening policy due to increasing and costly efforts to isolate patients in single rooms. However, only after few years, the nosocomial MRSA burden starts to reduce, which finally may encourage the hospitals to accept this burden of prevention. Since efforts of single hospitals may not change the MRSA-situation in the long run, this argues for establishing regional networks of healthcare providers sharing a common patient catchment population and synchronising prevention methods in the networks. Within such a network, surveillance data can be used for internal benchmarking as well as for validation and improvement of local standards. Thereby the network can support the work of local infection control personnel.

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