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Mumps outbreak in the Federation of Bosnia and Herzegovina with large cohorts of susceptibles and genetically diverse strains of genotype G, Bosnia and Herzegovina, December 2010 to September 2012

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A mumps outbreak reported from the Federation of Bosnia and Herzegovina involved 7,895 cases between December 2010 and September 2012. This was the largest outbreak in the country since the introduction of the measles, mumps and rubella vaccine in 1980. The highest disease incidence was found among 15 to 19 year-olds. About 39% (3,050/7,895) of cases reported to be unvaccinated; the vaccination status of 31% (2,426/7,895) was unknown. A seroprevalence study among 150 asymptomatic contacts to mumps cases showed that about one third (45/150) were susceptible to mumps. Among 105 clinically suspected mumps patients hospitalised at the Clinical Centre of the University of Sarajevo, orchitis (60% of all males: 51/85) and meningitis (9%: 9/105) were the most common complications. Among 57 outbreak sequences obtained for the small hydrophobic gene, eight different variants of genotype G viruses were identified. The outbreak affected mainly age groups comprising individuals who were not vaccinated during or after the Bosnian war, as well as cantons with single dose immunisation policies until 2001. In addition to issues related to vaccination of individuals, differential responses to vaccines and vaccine strains, waning of antibodies and potentially also the genetically diverse variants of genotype G may have compounded the size and duration of the outbreak. Our report emphasizes the need for supplementary immunisation programmes in particular for adolescents and young adults.

Introduction

Mumps is an infection with acute onset of unilateral or bilateral self-limited swelling of the parotid or other salivary glands, which lasts at least two days. The incubation period ranges from 12 to 25 days after exposure to the virus. Transmission from one person to another mostly occurs before and within five days of parotitis onset [1]. The disease is caused by mumps virus (MuV) and can be associated with complications such as aseptic meningitis, encephalitis, hearing loss, orchitis, oophoritis, mastitis or pancreatitis [1]. There is no specific treatment and vaccination is the only effective measure to prevent the disease. The presence of specific immunoglobulin M (IgM) in the serum confirms recent mumps infection. Reverse transcription-polymerase chain reaction (RT-PCR) is becoming increasingly popular for laboratory investigation of clinically suspected cases and provides also genotype information of circulating strains [1]. Currently, 12 different genotypes of mumps virus are recognised and genotype G seems to be the most prevalent genotype in Europe [2].

The combined measles, mumps and rubella (MMR) vaccine is in use in Bosnia and Herzegovina (B&H) since 1980 [3,4]. From 1980 to 1992 one dose of vaccine was given at 12 months of age and if this opportunity was missed, before the age of 14 years. The Republic of Srpska (RS) and some cantons of the second main entity of B&H, the Federation of Bosnia and Herzegovina (FB&H) (Bosnian Podrinje, Central Bosnia, Herzegovina-Neretva, Sarajevo, Tuzla, Una-Sana, Zenica-Doboj) continued to use the one-dose schedule until 2001. The remaining cantons (Canton 10, Posavina, West Herzegovina), two of which border Croatia, adopted in 1992 a two-dose schedule with the first dose given at 12 months and the second dose at seven years and no later than 14 years of age. In such

cantons, individuals born between 1981 and 1992 were given an opportunity for a second dose of vaccine. Since 2001, the two-dose MMR vaccination schedule is implemented throughout B&H [5], whereby cantons, which had a single dose schedule until 2001, offer two doses to individuals born from 2001 onwards.

The Public Health Institute of FB&H manages the national immunisation programme in FB&H and each canton is responsible for its implementation at the level of local health centres. Mumps is one of ten infectious diseases against which vaccination is compulsory and free of charge in FB&H. The immunisation status is checked by looking at the vaccination records when children enter kindergarten, when they start primary school and again during periodic revisions in preparation of the yearly immunisation plan and/or supplementary immunisation activities. Incompletely immunised children are vaccinated in catch-up campaigns.

The war between 1992 and 1995 disrupted MMR vaccination across the entire territory of B&H and deficiencies in the programme persisted up to several years after the war [3]. For instance, periodic shortages of vaccine supply and interruptions in the cold chain were reported. In addition, many refugees did not have any medical records. Before the introduction of vaccination against mumps, outbreaks occurred every three to four years and later every seven to eight years [4]. Between December 2010 and September 2012 a higher disease incidence with peaks in April 2011 and January 2012 was observed. This report describes this epidemic and investigates its causes.

Methods

Any patient with acute onset of unilateral or bilateral tender, self-limited swelling of the parotid or other salivary gland, lasting two or more days and without other apparent cause was considered a clinically suspected mumps case [6]. Suspected mumps cases are normally reported by medical practitioners to the Institute for Public Health of FB&H, which investigates outbreaks and reports to the Ministry of Health. Epidemiological data of 7,895 clinically suspected mumps cases reported between December 2010 and September 2012 were collected by the Institute for Public Health. The vaccination status of patients less than 18 years-old was checked in their medical records kept at the local health centre. For older patients the immunisation status was either checked in their medical records or self-reported.

Serological study

Serum was collected from 221 individuals between four and 64 years of age (mean: 21 years) who were clinically suspected mumps cases (n=71) or asymptomatic contacts to mumps cases (n=150) from the cantons of Sarajevo, Central Bosnia and Zenica-Doboj. All sera were tested with the Siemens Enzygnost Anti-Parotitis Virus kits for mumps-specific IgG and IgM antibodies.

Hospitalisation and complications

Between April 2011 and September 2012, 105 clinically suspected mumps cases from Central Bosnia and Sarajevo cantons were hospitalised at the Clinical Centre of the University of Sarajevo. Throat swabs were collected from all 105 patients and their medical records were checked.

Molecular and phylogenetic analysis of the mumps virus outbreak strains

RNA was extracted from the throat swabs of the 105 patients hospitalised at the Clinical Centre of the University of Sarajevo using the QIAamp Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions.

For reverse transcription, 5 µl of RNA and random hexamers were used in a total volume of 20 µl. PCRs were performed to amplify a genetic region comprising the small hydrophobic (SH) gene using previously described primers [7]. The genetic sequence was either obtained by one PCR (first PCR) or by two consecutive PCRs, whereby the first PCR was followed by a nested PCR. Starting material included 1 µl of cDNA for the first PCR, or 1 µl of the first PCR-product, for the nested PCR. The amplification steps consisted of an initial incubation for 5 minutes at 94°C, followed by 39 cycles of 94°C for 30 seconds, 56 or 52°C (first PCR) and 58 or 52°C (nested PCR) for 1 minute and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. PCR products were analysed in a 1.5% agarose gel and only samples negative after the first PCR were further amplified by nested PCR. PCR-positive samples were sequenced using nested primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

Sequences were analysed by SeqScape Software v2.5 (Applied Biosystems, USA), BioEdit version 7.0.9.0 [8] and molecular evolutionary genetics analysis (MEGA)4 software [9]. Neighbour-joining phylogenetic trees based on the Kimura 2-parameter model were constructed using 316 nucleotide (nt) sequences comprising the complete SH gene. The recommended set of reference sequences [2] and some identical or similar sequences obtained by basic local alignment search tool (BLAST) were included in the phylogenetic analysis. Nt sequences of the SH gene were translated into the corresponding amino acid sequences with BioEdit. Statistical tests for significance were done using SPSS 15.0 (IBM, USA).

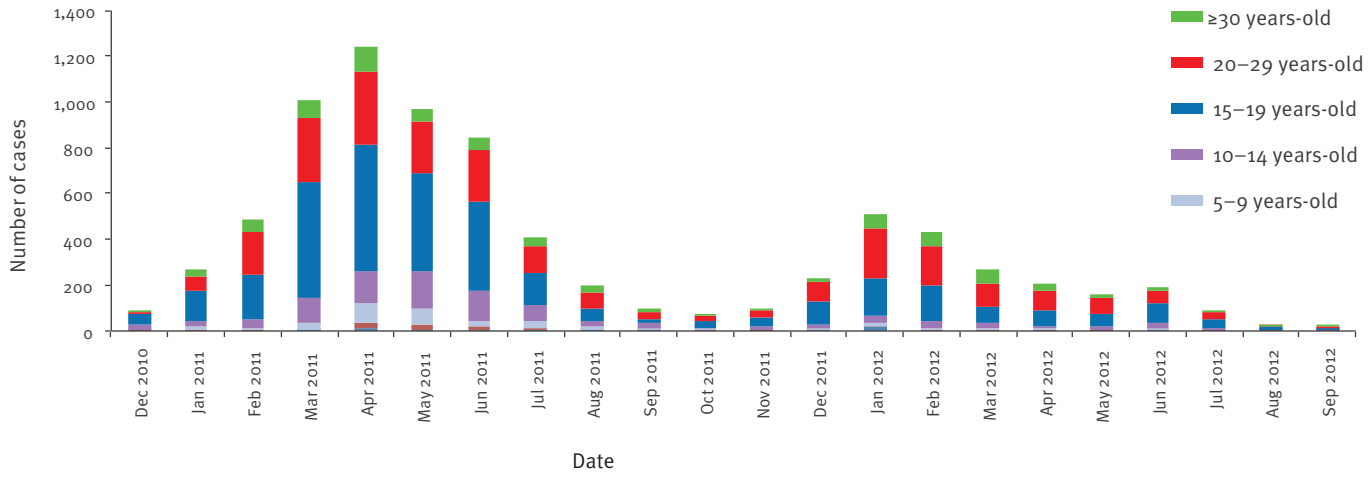
Results

Outbreak description

In the period from December 2010 to September 2012, a mumps outbreak including 7,895 cases was observed in FB&H. The outbreak consisted of two distinct epidemic waves, with one epidemic peak observed in April 2011 (n=1,240 cases) and another in January 2012 (n=509) (Figure 1).

Figure 1

Number of mumps cases, by age group, in an outbreak presenting two epidemic waves in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010–September 2012 (n=7,895)



Overall, the majority of cases reported (82%: 6,481/7,895) were located in three cantons: Central Bosnia (n=2,434 cases), Zenica-Doboj (n=2,215) and Sarajevo (n=1,832). These cantons had a one dose vaccination schedule until 2001. In contrast, Canton 10, and the cantons of Posavina and West Herzegovina, all, which had a two dose schedule since 1992, included only a total of 59 reported cases.

During the first wave of the outbreak, between December 2010 and the end of October 2011, 5,677 mumps cases were reported, mainly in the cantons of Central Bosnia, Zenica-Doboj, Sarajevo and Herzegovina-Neretva (n=5,534 cases in total for the four cantons) [3]. The second epidemic wave from November 2011 to September 2012, accounted for an additional 2,218 cases. During the second wave, cases continued to occur in the four previously most-affected cantons, but several hundred cases were also reported from Una-Sana (n=355) and Tuzla (n=450) cantons. Una-Sana and Tuzla cantons, which reported only sporadic cases during the first wave in 2011, observed the highest disease incidence at the beginning of 2012, during the second epidemic wave.

Considering the whole outbreak, the age of the cases ranged from less than one year to 64 years with a median age of 19 years. The age group comprising 15 to 19 year-olds was most affected during the first epidemic wave in 2011, while the highest case numbers were observed in 20 to 29 year-olds during the second wave (Figure 1). For the entire outbreak, nearly 82% (6,455/7,895) of the cases were older than 14 years (Figures 1 and 2). More males (63%: 5,005/7,895) than females were affected overall and in each age group, except for the one to four year-olds (Figure 2). Among the 20 to 29 year-old cases there were even more than twice as many males than females (Figure 2).

Mumps cases reported during the outbreak were mostly unvaccinated (39%: 3,050/7,895) or had an unknown vaccination status (31%: 2,426/7,895). The other cases had been vaccinated with either one dose (15%: 1,217/7,895) or with two doses of mumps-containing vaccine (15%: 1,202/7,895).

Serology results

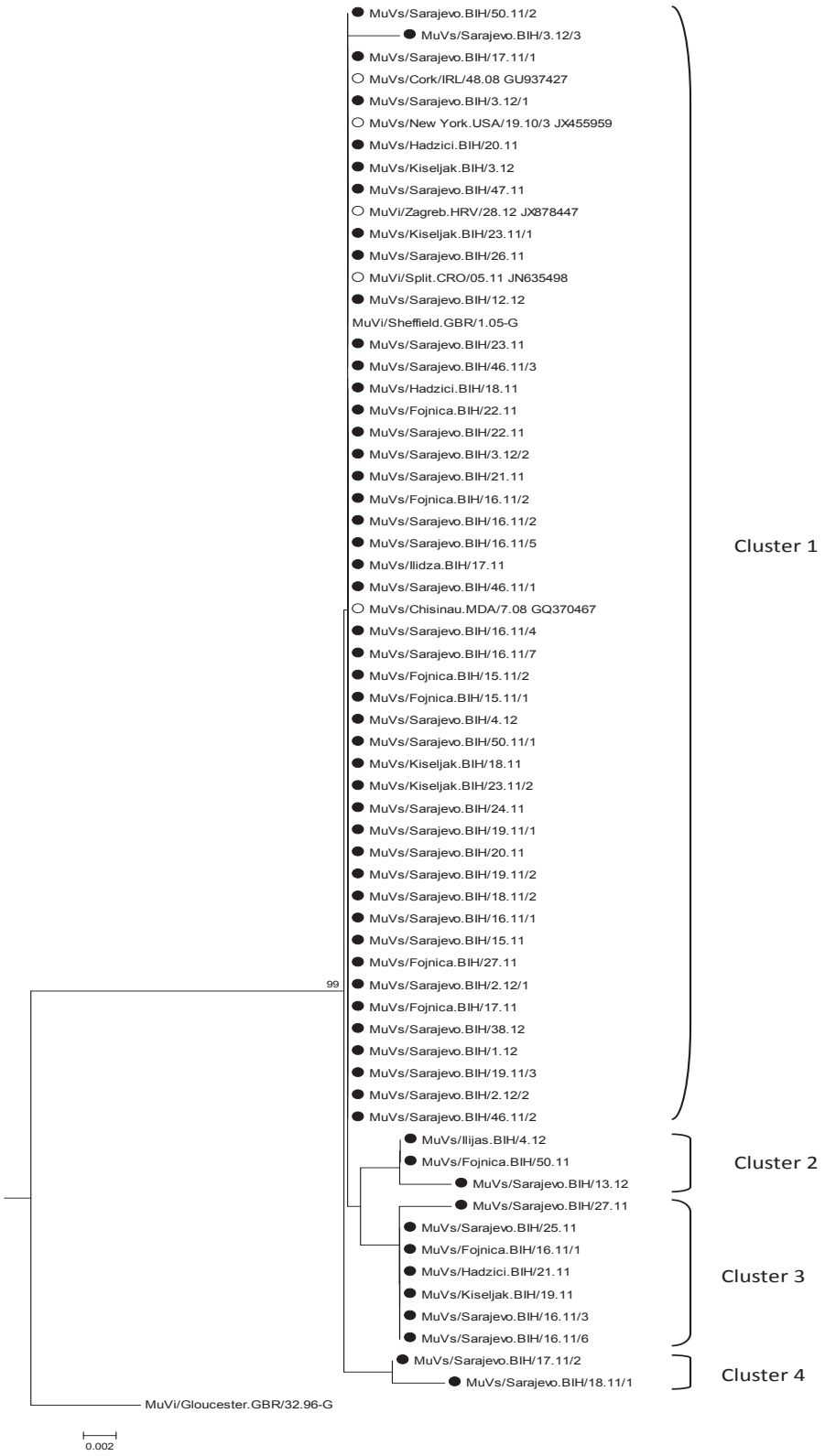
Among 71 patients with clinical symptoms consistent with mumps, 57 (80%) were IgM positive and IgG negative, two (3%) were positive for both IgM and IgG antibodies and 12 (17%) were only IgG positive. Among the 150 contacts without clinical signs or symptoms, five (3%) were IgM positive and IgG negative, 100 (67%) were only IgG positive and 45 (30%) were negative for both IgM and IgG antibodies. A positive serological test for mumps-specific IgM antibodies, with or without the presence of IgG, confirms recent infection. The presence of mumps-specific IgG antibodies indicates previous contact with mumps virus either by vaccination or natural infection.

Hospitalisation and complications

The median age of the 105 hospitalised patients was 20 years with a range of three to 64 years (Figure 3). The great majority were male (81%: 85/105). Similar to the overall cohort of reported cases, most of the hospitalised patients were unvaccinated (34%: 36/105) or had an unknown vaccination status (36%: 38/105) (Table). A total of 28% (29/105) were vaccinated with two doses of mumps containing vaccine and 2% (2/105) were vaccinated with one dose only (Table). The medical records documented serious complications such as orchitis (60% of all males: 51/85), meningitis (9%: 9/105) and orchitis and meningitis (2% of all males: 2/85) in hospitalised patients (Table and Figure 3). There was no statistically significant difference in the prevalence of complications between vaccinated and unvaccinated

Figure 4

Phylogenetic analysis of 57 mumps virus sequences of genotype G obtained during a mumps outbreak in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010–September 2012

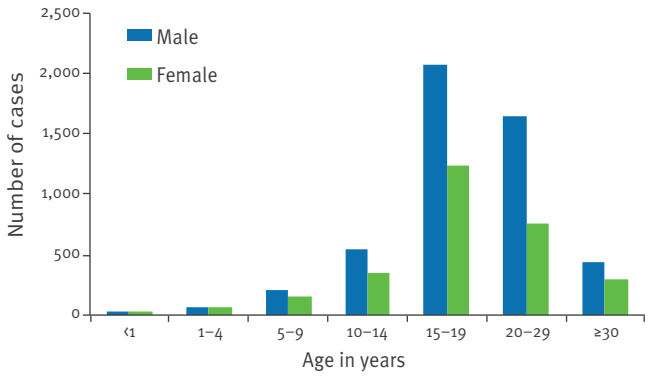


1

The Figure focuses on the genotype G cluster obtained within a larger phylogenetic tree, which was constructed with the neighbour-joining and Kimura 2 parameter methods, based on 316 nucleotide-long sequences comprising the SH gene, that were obtained in this study or in GenBank, and all World Health Organization reference sequences [2]. The sequences obtained during the outbreak are marked with full dots and the closest basic local alignment search tool (BLAST) fits among recently detected sequences are marked with open dots.

Figure 2

Number of mumps cases by age and sex in an outbreak in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010–September 2012 (n=7,895)



patients (p=0.723) or patients with unknown vaccination status (p=0.171) (Table).

Table. Characteristics of mumps patients hospitalised at the Clinical Centre of the University of Sarajevo during a mumps outbreak in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010–September 2012 (n=105)

Molecular characterisation of the mumps strains detected during the outbreak

A total of 58 of 105 throat swabs collected from suspected mumps patients were positive for MuV by PCR. Sequence information comprising the complete SH gene was obtained from 57 samples (GenBank accession numbers: HF912174 to HF912230). Eight different genotype G sequence variants forming four clusters were detected (Figure 4). Cluster 1 comprised the main outbreak variant (represented by 44 sequences), as well as an additional single sequence (MuVs/Sarajevo.BIH/3.12/3) which differed by one nt to the 44 others. A second cluster (cluster 2) of three sequences was found later during the outbreak (end of 2011 and in 2012); the sequence detected at the latest time point differed by a single nt from the other two. Cluster 3 comprised six identical sequences collected during week 16 to 25 (mid-April to second half of June) in 2011 and a sequence differing by one nt collected in week 27 (beginning of July). Cluster 4 comprised only two sequences differing by one nt from each other collected in weeks 17 and 18 (end of April to beginning of May) of 2011 (Figure 4). Overall, the outbreak sequences did not vary from each other by more than four nt. The maximum number of changes observed compared to the main variant was two nt. The main outbreak variant was identical to strains from Croatia (MuVi/Split.CRO/05.11, MuVi/Zagreb.HRV/28.12), the United States (US) (e.g. MuVs/New_York.USA/19.10/3), Ireland (MuVs/Cork/IRL/48.08) and Moldova (MuVs/Chisinau.MDA/7.08). No sequences identical to any of the other seven variants were found in GenBank.

Some of the genetically diverse variants had predicted amino acid substitutions in the SH protein: all sequences in cluster 3 had Leu20Met in the predicted SH protein; the two sequences in cluster 4 showed His40Tyr and sequence MuVs/Sarajevo.BIH/18.11/1 in addition Phe49Ser; sequence MuVs/Sarajevo.BIH/3.12/3 in cluster 1 had a predicted His50Tyr change.

The 13 patients infected with genetically diverse virus variants showed no symptoms different from the patients infected with the main strain. Variant strains were found in five different locations of FB&H (Sarajevo, Ilijas, Fojnica, Hadzici and Kiseljak).

Discussion

Between December 2010 and September 2012, 7,895 mumps cases were registered in FB&H and about 7,700 additional cases in RS, leading to approximately 15,600 cases across B&H. This was by far the largest mumps outbreak in the country since the introduction of the vaccine in 1980 [3] and it was also one of the largest outbreaks reported from Europe since the period between 2008 and 2009 [10].

The outbreak affected mainly regions of B&H with a single dose schedule until 2001, which is in line with the down to 60% long-term population-based effectiveness of a single dose of mumps vaccine [11]. During the 1992 to 1995 war and several years after, immunisation activities were irregular [3] with large cohorts of susceptibles, especially in regions where only a single vaccination opportunity was provided. This also explains why most patients involved in the recent mumps outbreak (72%: 5,710/7,895) belonged to the age groups 15 to 19 and 20 to 29 years (born 1981–1997) and why the majority of the patients (69%: 5,476/7,895) reported to be unvaccinated or had an unknown vaccination status. The breakdown of community immunity as a result of young adults moving on to new school or work environments may have compounded the incidence in this age group. A high mumps incidence among adolescents and young adults who were unvaccinated or received only a single dose has been reported from several other European countries [10,12-14].

Also waning of protective levels of antibodies [10,15] even after two doses may have played a role. Since mumps outbreaks seem to occur even in highly vaccinated communities, a third dose of mumps-containing vaccine as a booster later in life is currently under discussion [14-17]. Whether the robustness and persistence of the immune response to the various vaccine strains used in B&H during the past years (Institute for Immunology, Zagreb, Croatia (L-Zagreb strain, 1980–1999); GlaxoSmithKline Beecham, Belgium (RIT 4385 strain,1999–2012); Aventis Pasteur, France (Urabe strain, 2007 only) [4]) differed as was observed previously [11,18] deserves further attention.

Table

Characteristics of mumps patients hospitalised at the Clinical Centre of the University of Sarajevo during a mumps outbreak in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010–September 2012 (n=105)

Vaccination status	Number of patients	Complications			
		Orchitis n(%)	Meningitis n (%)	Orchitis and meningitis n(%)	No complications n(%)
Vaccinated (2 doses)	29	16 (55)	4 (14)	1 (3)	8 (28)
Vaccinated (1 dose)	2	1 (50)	0 (0)	0 (0)	1 (50)
Unvaccinated	36	20 (56)	1 (3)	1 (3)	14 (39)
Unknown	38	14 (37)	4 (11)	0 (0)	20 (53)
Total	105	51 (49)	9 (9)	2 (2)	43 (41)

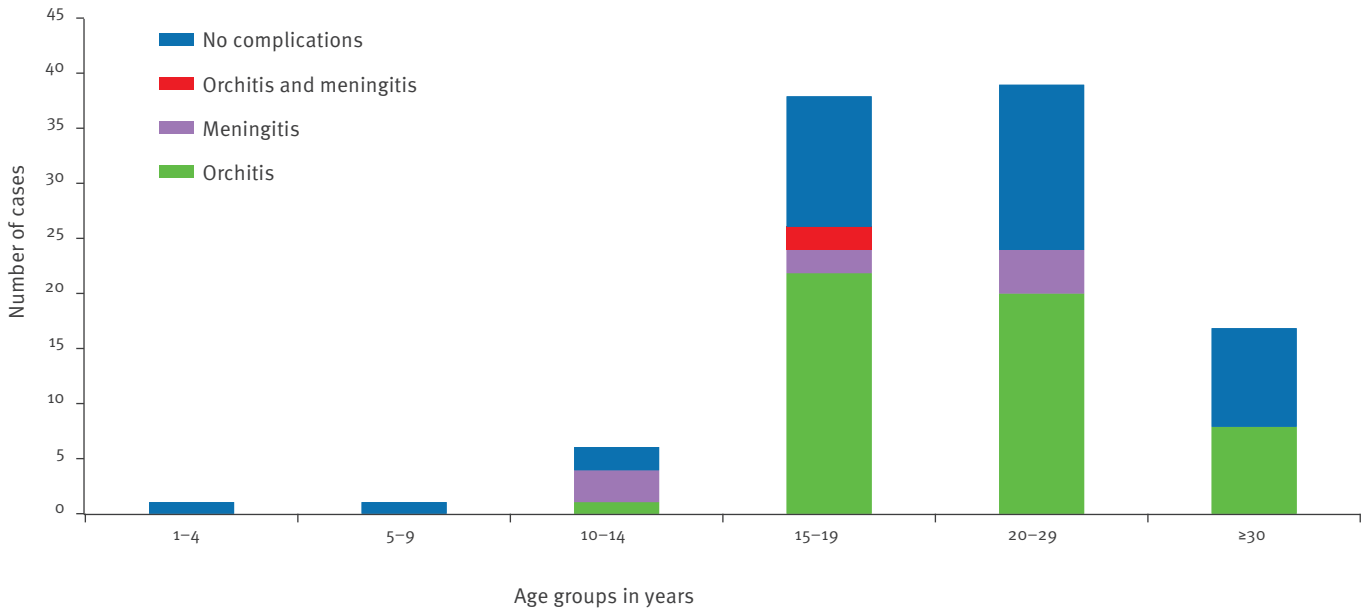
In response to the outbreak, all children less than 14 years of age who had received less than two doses of MMR vaccine were vaccinated, mumps cases were isolated, some schools with large numbers of cases were closed and the citizens were alerted via the media. However, the fact that each of the ten cantons of FB&H has its own health system, in addition to the anti-vaccination propaganda during the past few years [19,20] probably contributed to the overall rather weak and slow response to the outbreak.

Currently no mumps seroprevalence data are available for FB&H. The investigation of sera from a small number (n=150) of asymptomatic mumps patient contacts during the recent outbreak showed that about one third did not have specific IgG antibodies and were considered to be susceptible. A thorough seroprevalence study to identify main groups of susceptibles for targeted supplementary immunisation activities is warranted. Among the 71 clinically suspected mumps patients, 12 (17%) had IgG and no IgM antibodies, indicating that at least some of these patients may have been reinfected with mumps virus or infected after vaccination. In such cases virus detection should be attempted [21,22], but no samples besides serum were available from these patients.

The typical male complications may explain why more male patients in the 15 to 29 year age bracket were recorded. In fact, orchitis with or without meningitis was the most common complication in hospitalised patients. In contrast to a recently published study [21], we did not find any significant difference in disease severity between vaccinated and unvaccinated hospitalised patients. This may be due to low numbers

Figure 3

Complications observed among mumps patients hospitalised at the Clinical Centre of the University of Sarajevo according to age, mumps outbreak in the Federation of Bosnia and Herzegovina, Bosnia and Herzegovina, December 2010–September 2012 (n=105)



of patients in each category, but also to confounding effects of vaccine quality during and after the war.

SH gene sequences from 57 patients belonged to genotype G and eight different sequence variants were found. The main outbreak variant was identical to strains detected in the US in 2010 and in Croatia in 2011 and 2012. As nothing is known about the genetic variants of mumps virus present in FB&H before the outbreak, we can only speculate whether the virus was endemic in the country or whether it was introduced from the US or a European source, perhaps related to the earlier Ireland and Moldova 2008 cases.

Seven sequence variants were different from any of the sequences on GenBank. Since only 57 sequences from only two cantons were obtained while approximately 15,600 mumps cases were reported from throughout B&H, it is likely that the real sequence diversity is underestimated. Nevertheless, this diversity is already considerably higher than during a one-year outbreak in the US with 3,500 cases where 221 sequences differed by no more than a single nt [21]. Since at least cluster 1, 3 and 4 sequences were present already by the beginning of 2011 and genetically diverse strains were found in five different locations, the most probable scenario is that different variants were present in B&H already before the outbreak and/or correspond to multiple independent transmission chains. Some variation seems to have been generated during the epidemic since within clusters 2, 3 and 4 the strain collected at the latest time point was always the most diverse.

The differing strain from cluster 1 and all sequences from clusters 3 and 4 showed changes in the predicted amino acid sequence of the SH protein. Of note, the cluster 3 strains had a leucine to methionine substitution in position 20 of the predicted SH protein, which could potentially result in an N-terminal truncation. As the SH protein has been reported to inhibit tumour necrosis factor alpha-mediated apoptosis [23-25], it is conceivable that a truncated SH protein may have lost the ability to inhibit programmed cell death leading potentially to more severe clinical manifestations. In fact, six of the seven patients with the predicted leucine to methionine substitution in position 20 were hospitalised due to disease complications (4 had orchitis, 1 had orchitis and meningitis and 1 had meningitis). Further investigations are clearly warranted to elucidate the significance of the methionine substitution as well as the other predicted amino acid mutations.

It has been suggested that the gradual antigenic evolution of mumps viruses may undermine vaccine effectiveness [26]. A recent study showed indeed that the genotype A strain Jeryl-Lynn induced less neutralising antibodies against the common genotype G virus than against the vaccine strain [27]. The 13 patients who were infected with the genetically diverse variants in our study were between 18 and 43 years-old (mean: 25.2 years) and most of them reported that they had received two doses of vaccine (54%: 7/13) or that the vaccination history was unknown (31%: 4/13). Thus it is conceivable that waning of antibodies, vaccine quality issues, intensive exposure [21] or potentially an even lower neutralisation capacity against the present genotype G variants played a role in the outbreak.

In conclusion, we identified failures to vaccinate during and after the war and single dose immunisation policies in some cantons as important causes for the large and persistent mumps outbreak. Those may have been compounded by differential responses to vaccines and vaccine strains, waning of antibodies and potentially by the genetically diverse variants of genotype G. Molecular analysis of the mumps strains involved, revealed a level of diversity in the virus suggestive of several transmission chains, possibly as a result of long-term endemic circulation of mumps viruses in FB&H. Our study identified large cohorts of susceptibles and emphasises the need for supplementary immunisation activities in particular among adolescents and young adults who have received less than two vaccine doses.

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Conflict of interest

None declared.

Authors' contributions

MH was involved in the design of the study, drafted the article, contributed to the recruitment of study participants and management of their personal data, as well as participated in the analysis and interpretation of the results, the initiation of the study and the revision of the manuscript. AH was involved in interpretation of the results and the writing of the manuscript. JR was involved in the statistical analysis of epidemiological data. ZL was involved in the statistical analysis of epidemiological data. RB was involved in clinical data analysis. ADL contributed to the recruitment of study participants and the management of their personal data. AM performed clinical analysis and serological experimental work. ISB contributed to the recruitment of study participants and the management of their personal data. AS did all the molecular biology investigations. CPM was involved in the data interpretation and the revision of the manuscript. JMH contributed to the conception of the study, the interpretation of the results and the writing of the manuscript.

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Nosocomial outbreak of staphyloccocal scalded skin syndrome in neonates in England, December 2012 to March 2013

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Staphylococcal scalded skin syndrome (SSSS) is a blistering skin condition caused by exfoliative toxin-producing strains of *Staphylococcus aureus*. Outbreaks of SSSS in maternity settings are rarely reported. We describe an outbreak of SSSS that occurred among neonates born at a maternity unit in England during December 2012 to March 2013. Detailed epidemiological and microbiological investigations were undertaken. Eight neonates were found to be infected with the outbreak strain of *S. aureus*, of *spa* type t346, representing a single pulsotype. All eight isolates contained genes encoding exfoliative toxin A (*eta*) and six of them contained genes encoding toxin B (*etb*). Nasal swabs taken during targeted staff screening yielded a staphylococcal carriage rate of 21% (17/80), but none contained the outbreak strain. Mass screening involving multi-site swabbing and pooled, enrichment culture identified a healthcare worker (HCW) with the outbreak strain. This HCW was known to have a chronic skin condition and their initial nasal screen was negative. The outbreak ended when they were excluded from work. This outbreak highlights the need for implementing robust swabbing and culture methods when conventional techniques are unsuccessful in identifying staff carrier(s). This study adds to the growing body of evidence on the role of HCWs in nosocomial transmission of *S. aureus*.

Introduction

Staphylococcal scalded skin syndrome (SSSS) predominantly but not exclusively affects neonates and children under the age of five years. It results from infection with strains of *Staphylococcus aureus* containing genes encoding exfoliative toxin A (*eta*) and B (*etb*). Around 5% of all *S. aureus* produce exfoliative toxins. These toxins act in the zona granulosa of the epidermis, leading to a spectrum of illness ranging from mild localised blistering to extensive generalised lesions [1]. The prognosis in most cases is typically good if prompt antibiotic therapy is given [2].

The incidence rate of SSSS in England is unknown as no national clinical or laboratory surveillance system is in place. A review of the Staphylococcal Reference Unit database of Public Health England showed that around 50–100 cases of SSSS are confirmed in England each year, which equates to 0.94–1.88 cases/million/year (unpublished data). Incidence of SSSS probably varies among countries in Europe, with estimates ranging from 0.56 cases/million/year in France to 2.53 cases/million/year in the Czech Republic [3,4].

Outbreaks of SSSS have been infrequently reported in maternity and neonatal units [5-7]. Transmission in these outbreaks was interrupted by the identification and management of symptomatic infection or asymptomatic colonisation of healthcare staff shedding the outbreak strain and through reinforcing standard infection control measures [7,8].

Four neonates born within one week of each other at a maternity unit in England developed SSSS in 2013. The first affected neonate in the outbreak was born in December 2012. All four neonates were readmitted at the age of 8–9 days and were found to be infected with tetracycline-resistant *S. aureus* with genes encoding exfoliative toxin A and B. For the purpose of the investigation, the putative exposure period for acquisition of the outbreak strain in the neonate was the time from birth till discharge home. The outbreak period spanned

from the birth of the first affected neonate to the date of symptom onset in the last affected neonate (in March 2013). Day 0 of the outbreak period refers to the day before the birth of Case 1. An Outbreak Control Team (OCT), chaired by the Director of Infection Prevention and Control, was convened, with representation from hospital and public health staff. The hypothesis for the cause of the outbreak was an exposure or exposures occurring in the maternity unit from birth until discharge home. We describe the investigation and control measures that led to the resolution of the outbreak.

Methods

Setting

The outbreak occurred in an acute hospital with approximately 100 maternity beds. The maternity unit performs about 4,400 deliveries per year and has a dedicated labour ward with two operating theatres. Two dedicated wards for antepartum and postpartum care, with a total of about 40 beds, are located at a short distance from the labour ward but within the main hospital site. The maternity unit has about 300 doctors, nursing, midwifery and support staff.

Clinical investigations

During the outbreak period, all neonates presenting with clinical scalded skin syndrome were identified by senior paediatric clinicians and were promptly notified to key members of the OCT. Umbilical swabs, along with swabbing of infective lesions when present, were performed in all suspected cases. Digital photography of skin lesions was also undertaken.

Epidemiological investigations

As part of case finding, the hospital microbiology records were interrogated to check whether tetracycline-resistant *S. aureus* had been isolated in the laboratory in the previous six months. Using a standardised questionnaire tool, data were collected from the medical case records of the mother and neonate on key variables such as demographics, ward locations, chronological course, antenatal, perinatal and postnatal interventions for the mother and neonate, and key staff involved in providing clinical care. Social network analysis software (i2 Analysts Notebook 8 – i2 Ltd) was used to identify staff with a high level of contact with the affected neonates, with data extracted from in-depth case note review.

Environmental investigations

Throughout the outbreak period, an enhanced programme of environmental audit was undertaken to assess standards of cleanliness and disinfection. Limited environmental sampling was undertaken on day 68 from a variety of sites (n=28) such as baby cots, trolleys, incubators, resuscitaire equipment and internal and external air vents. A formal review of infection control practices and the maternity wards was conducted by a team of specialists in infection control.

Neonatal screening investigations

To determine if asymptomatic colonisation with the outbreak strain was occurring, prospective umbilical swab screening of all neonates born at the maternity unit was undertaken at the time of first discharge to home between days 29 and 35 (phase 1). This exercise was repeated between days 62 and 71 (phase 2).

Staff screening investigations

Surveillance of the hands of all maternity staff was conducted and those with known or active dermatitis (identified by occupational health records, staff members volunteering information at the time of assessment and infection control staff identifying skin problems at assessment) were referred to occupational health for clinical assessment and swabbing as appropriate.

Staff known to have been involved in the care of the affected neonates were identified and screened for *S. aureus* nasal carriage in a targeted screening exercise undertaken between days 22 and 78. Subsequently, mass screening of all maternity staff was instigated on day 81, with a multiple site (nose, throat and hair-line) swabbing approach. All staff voluntarily complied with the request for being screened.

Microbiological investigations

Charcoal swabs were used to sample lesions from suspected cases and for screening of staff and neonates. Swabs from cases and staff in the targeted screening exercise were cultured by direct inoculation onto colistin-nalidixic acid (CNA) agar [9]. In the mass screening exercise, the pooled swabs were initially pre-enriched overnight in a brain–heart infusion before inoculation onto CNA agar. Identification and confirmation of *S. aureus* was by colony appearance, followed by latex agglutination and Gram staining [10]. Antibiotic sensitivities were conducted at the local hospital laboratory (where outbreak occurred) using the automated VITEK 2 system (bioMérieux, Inc. Hazelwood, United States).

All isolates of *S. aureus* were referred to the Staphylococcal Reference Service (Public Health England, Colindale) for detailed characterisation including the following: detection of 14 toxin genes (exfoliative toxins A, B and D; detection of enterotoxins A–E and G–J; detection of toxic shock syndrome toxin and Panton–Valentine leucocidin, *Staphylococcus* protein A gene (spa) typing and pulsed-field gel electrophoresis [11].

Infection control measures

During the course of the investigations, the following control measures were implemented: (i) daily hand hygiene audits in maternity and paediatric wards and tailored interventions to achieve 100% compliance with hospital standards; (ii) enhanced environmental cleaning of all clinical areas including theatres; (iii) named individual responsibility for cleaning and equipment disinfection in theatres and other clinical areas; (iv) decommissioning of the birthing pool on the labour

Table

Clinical and microbiological characteristics of neonates with staphylococcal scalded skin syndrome born at a maternity unit in England, December 2012–March 2013

Case	Duration of stay in hospital post-birth	Age when admitted with SSSS	Birth	Swab site	Tetracycline sensitivity	*spa* type	PFGE pattern	Exfoliative toxin genes
1	17 hours	9 days	Vaginal delivery	Umbilicus, face, groin	Resistant	t346	Pattern A	*eta,etb*
2	2 days, 4hrs	9 days	Elective C-section	Umbilicus, groin	Resistant	t346	Pattern A	*eta,etb*
3	16 hours	8 days	Vaginal delivery	Umbilicus, face, groin	Resistant	t346	Pattern A	*eta,etb*
4	1 day, 23 hours	9 days	Elective C-section	Groin	Resistant	t346	Pattern A	*eta,etb*
5	4 days, 12 hours	Not admitted	Emergency C-section	Umbilicus	Resistant	t346	Pattern A	*eta,etb*
6	4 days, 0 hours	10 days	Emergency C-section	Umbilicus, skin	Sensitive	t346	Pattern A	*eta*
7	2 days, 16 hours	9 days	Emergency C-section	Umbilicus, skin, nose	Sensitive	t346	Pattern A	*eta, etb*
8	10 hours	6 days	Vaginal delivery	Neck, groin	Sensitive	t346	Pattern A	*eta*

PFGE: pulsed-field gel electrophoresis; SSSS: staphylococcal scalded skin syndrome.

ward; (v) removal of any communal products, such as baby bathing liquid and cotton wool; (vi) joint environmental and infection control inspections by the infection control team, with instruction or intervention provided where areas of poor practice were identified; (vii) immediate isolation and prompt treatment of neonates admitted with suspected SSSS in paediatric wards; (viii) prompt decolonisation of staff with octenidine (0.3%) wash and mupirocin (2%) nasal ointment when *S. aureus* was isolated in screening, without awaiting strain characterisation; (ix) letters sent by email to community health professionals (via service managers), informing them about the outbreak and advising them on the need to refer suspected cases for urgent hospital assessment; and (x) informing all maternity and paediatric staff of the outbreak and reminding them of the importance of infection control measures.

Case definition

All clinically suspected cases were included in the outbreak from day 20, when the first meeting of the OCT was held following declaration of an outbreak. The case definition was reviewed and refined periodically by the OCT over the course of the outbreak to ensure that it remained a robust tool to delineate outbreak and non-outbreak cases based on clinical, epidemiological and microbiological information.

At the conclusion of the outbreak, the final case definition was agreed on: an SSSS case was a neonate born after day 0 of the outbreak at the maternity unit of the hospital, presenting with clinical scalded skin syndrome and infected with *S. aureus* genes *eta* and *etb* or *eta*, of *spa* type t346 belonging to multilocus sequence type clonal complex 15 (MLST CC-15) with a specific pulsotype (designated pattern A).

Results

Eight neonates met the outbreak case definition. Their key characteristics are presented in the Table. All cases were born at full-term and developed skin lesions predominantly in the perineum or periumbilical area or both within 10 days of birth. Seven were admitted to the hospital for administration of intravenous antibiotics. Case 5 was identified in phase 1 of the neonatal screening exercise and had developed a mild clinical illness by the time microbiological characterisation confirmed infection with the outbreak strain. This case was managed with oral antibiotics in the community. All neonates recovered uneventfully.

On the basis of the exposure period of the neonates in the maternity unit, the outbreak occurred in three distinct clusters – four cases in the first cluster between days 1 and 11, three cases in a second cluster between days 23 and 31 and a final 'cluster' of one case on day 88 (Figure).

Two mothers reported skin infections (breast lesion and caesarean wound infection) but their wound swabs were negative for *Staphylococcus*. No other illness among the mothers was reported.

A review of records of the microbiology laboratory did not reveal any tetracycline-resistant *S. aureus* isolates among neonates in the six months before the outbreak. Epidemiological investigations did not identify any common antenatal, intranatal or postnatal exposures. Possible exposures in the community (outside of the maternity unit) were considered and discarded due to lack of epidemiological evidence.

Neonatal screening investigations

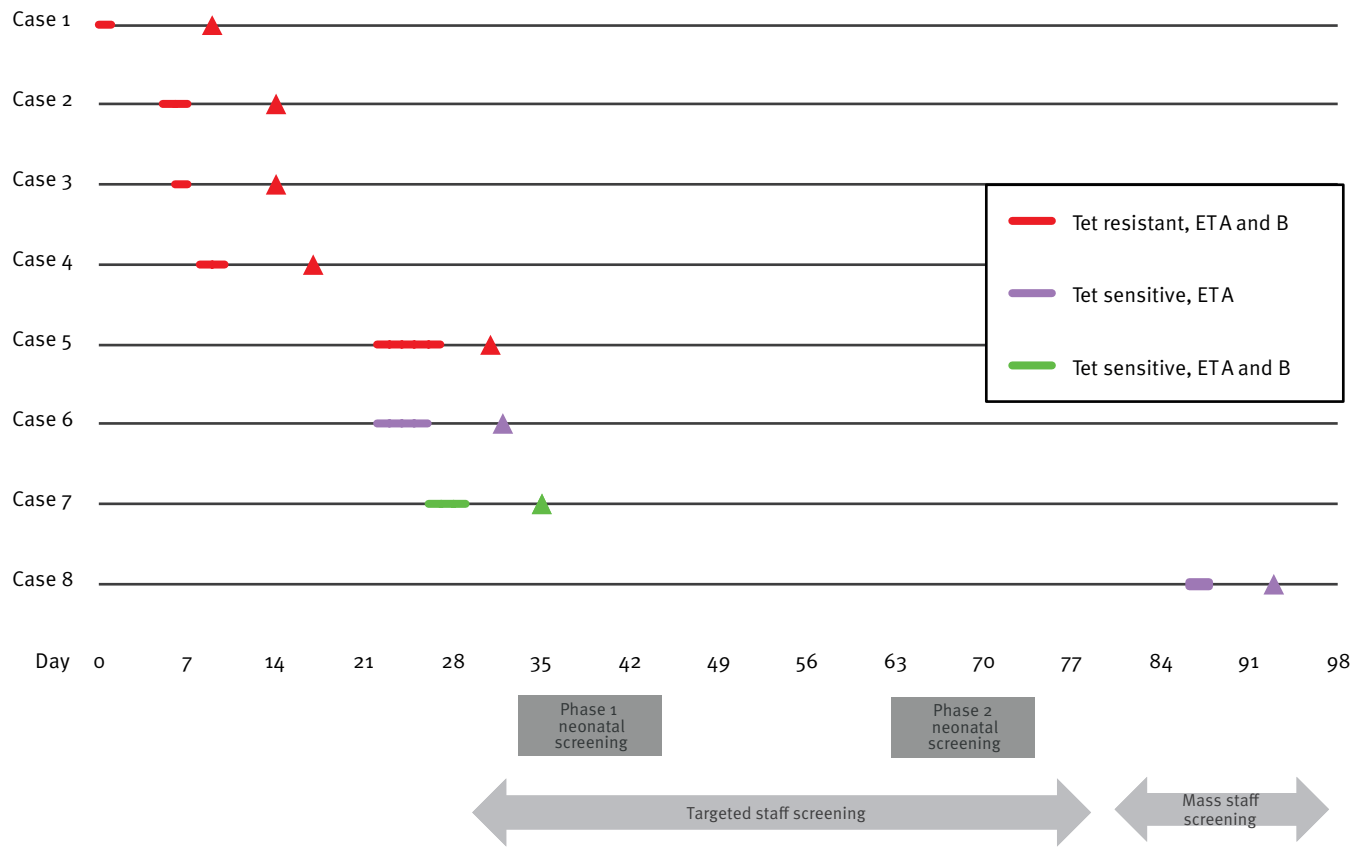
In phase 1 (neonatal screening at discharge), 19/47 screened neonates were found to be colonised with *S. aureus*, of whom one (Case 5) was identified as having the outbreak strain. In phase 2 (the repeat screening), 30/67 neonates were found to be colonised with *S. aureus*, but none had the outbreak strain.

Staff screening investigations

Surveillance of staff members' hands identified three healthcare workers (HCWs) with dermatitis, who were

Figure

Timeline of staphylococcal scalded skin syndrome outbreak, England, December 2012–March 2013



ET: exfoliative toxin; Tet: tetracycline.

The coloured bars indicate the time from birth to discharge for each case. The triangles represent the date of admission of the neonate to hospital with symptoms of staphylococcal scalded skin syndrome. Case 5 was not admitted.

referred to occupational health. Among these, one HCW had active skin lesions but a swab taken from the HCW's hand was negative for *Staphylococcus*. The other two HCWs were not swabbed as they did not have active dermatitis at the time of the assessment.

Targeted screening of 80 staff members using nasal swabs revealed tetracycline-sensitive staphylococcal carriage in 17 staff members. These 17 isolates were unfortunately discarded, as tetracycline resistance was initially considered as a marker for the outbreak strain. Once it became apparent that tetracycline resistance was not a reliable marker (as later cases were tetracycline sensitive), rescreening of the 17 staff members revealed the continued presence of *S. aureus* in 14 of them, but none had the outbreak strain.

A total of 20 staff members with the highest level of interaction with the cases were identified from a network analysis of the first seven cases. Since all staff members, except one, in this list had already been screened in the targeted screening exercise, the OCT decided to progress to mass screening of all maternity staff.

Mass screening of 217 maternity staff revealed the presence of *Staphylococcus* in 46 (21%). Among these, a HCW (HCW A) was found on day 89 to carry the outbreak strain. Network analysis confirmed that among all staff, HCW A had had the second-highest level of interaction with the affected neonates. Of interest, HCW A was one of three HCWs with known dermatitis reviewed by the occupational health department within a week of recognition of the outbreak but did not undergo microbiological screening as they had no active skin lesions. HCW A also underwent nasal swab screening in the initial targeted screening exercise on day 22, but *S. aureus* was not isolated.

On finding that HCW A carried the outbreak strain, they were promptly excluded from the clinical environment. Screening of the remaining maternity staff was discontinued following identification of HCW A with the outbreak strain.

Decolonisation of HCW A was attempted: the first round failed. Following that, screening of their family contacts confirmed the presence of outbreak strain in household members. A second attempt at decolonisation of HCW A, along with their family members, also failed. Following this, HCW A was redeployed to a non-clinical role in the maternity unit around day 180.

Routine clinical and laboratory surveillance led to the recognition on day 347 of a neonate, born on day 338 at the maternity unit, with clinical SSSS and infected with the outbreak strain (tetracycline-sensitive *S. aureus*, encoding *eta* and *etb* gene, *spa* type t346 and pulsotype pattern A). Despite lack of evidence that HCW A had had any contact with the affected neonate, HCW A was further redeployed outside the maternity unit following this case. No further cases with the outbreak strain have been reported to date (August 2014).

Environmental investigations

All environmental swabs were negative for *S. aureus*. Improvements were made in cleaning, disinfection and infection control procedures as a result of the joint environmental and infection control inspections and the external review of infection control practices.

Non-outbreak case

One neonate was confirmed with SSSS associated with a different strain: eta positive, spa type t2649 (belonging to MLST-CC88) and a different pulsotype (designated pattern B). Of note, a staff member (HCW B) involved in the care of this neonate, with a previous negative nasal screen, was found to carry the same strain of *S. aureus* on rescreening. HCW B was promptly excluded and successfully decolonised. No further neonates or staff members were identified with this non-outbreak strain to date (August 2014).

Discussion

To the best of our knowledge, this is the first reported outbreak of SSSS in neonates in a maternity setting in England. Investigations were complicated by the phenotypic and genotypic variation observed in the outbreak strain, specifically the loss of tetracycline resistance and/or the *etb* gene in the isolates from the last three cases, most likely reflecting plasmid loss over time [12]. Furthermore, the wide variation in the antenatal, perinatal and postnatal exposures among affected neonates and mothers precluded targeting investigations and interventions at a specific ward, staff group or procedure.

Network analysis software was used in this outbreak to evaluate complex interactions between patients and HCWs. Network analysis is highly dependent on the quality of the data sources (i.e. recording of staff–patient interactions in case records) and the technical skills to analyse and interpret the data are sparse. While the software assists in determining the most likely path of interaction, interpretation becomes difficult when there are non-sequential interactions. Despite these limitations, this technology may have application for the investigation of outbreaks where the links between cases and staff exposures are difficult to ascertain with conventional epidemiological techniques.

Individuals with skin lesions are more prone to be colonised with and likely to shed *Staphylococcus* [13-15]. Asymptomatically colonised HCWs with chronic skin conditions have been previously identified as sources of outbreaks of staphylococcal infection in nurseries and hospitals [6,7,16-19].

HCW A reported a history of chronic dermatitis and was previously known to occupational health. At the time of the outbreak, HCW A was not known to have any difficulties in practising routine hand hygiene measures required for providing clinical care. In retrospect, a key lesson learnt from this outbreak was that HCWs with known or active skin conditions should be screened microbiologically as well as clinically, as part of nosocomial staphylococcal outbreak investigations among neonates. HCW A underwent nasal swabbing in the targeted screening exercise on day 22 but cultures for *S. aureus* were negative. Possible explanations for a negative culture include intermittent colonisation or shedding, low-level carriage or inadequate sampling methodology [20-23]. Multisite swabbing with pooled, enrichment culture was ultimately successful in isolating the outbreak strain from HCW A, thus concluding the outbreak investigations. On the basis of microbiological and epidemiological evidence, it is likely that HCW A had a key role in transmission of the pathogen in this outbreak. However, the source of the outbreak strain in HCW A remains obscure as HCW A had been employed in the same clinical role in the hospital for many years and had not been implicated in previous outbreaks.

Screening of all neonates was undertaken in two distinct short periods, to understand if asymptomatic infection with the outbreak strain was occurring. Among 114 neonates screened at the time of discharge, 49 (43%) were already colonised with *S. aureus* and one neonate was identified with the outbreak strain. These figures are consistent with previous studies demonstrating a staphylococcal carriage rate of 30–90% in neonates in hospitals [24-26]. Despite identifying only a single case in this instance, screening of neonates at discharge can provide valuable insight into assessing transmission rates and provide a degree of assurance on the effectiveness of infection control measures.

As all neonates had lesions centred in the umbilicus and perineum, umbilical cord care procedures were reviewed. It was noted that no specific interventions were undertaken in cord care, in line with standard national practice [27]. Antiseptics have been recommended in the past, but current consensus does not support the routine use of antiseptic agents, particularly in high-income countries [28].

The limitations of this study must be noted. First, the actual number of cases could have been higher. Despite our letters advising community health professionals to refer suspected cases to hospital, it is possible that some mild cases could have been managed in the community and not reported to the OCT. Second, umbilical swab screening of the neonates in the two separate phases was incomplete as all neonates discharged in the two screening phases could not be screened due to operational reasons. Nevertheless, the random nature of bias in this screening exercise is unlikely to substantially alter the rates of staphylococcal carriage found in this cohort. Third, the role of the inanimate environment in propagating this outbreak cannot entirely be excluded as environmental swabbing was limited to areas thought to be high-risk by the infection control team and was conducted late in the outbreak, following substantial improvements in cleaning and disinfection procedures.

Despite the multiple challenges encountered, continued investigations ultimately led to the identification and hence exclusion of the HCW carrying the outbreak strain thus successfully resolving the outbreak. This outbreak highlights the value of implementing robust swabbing and culture methods when conventional techniques are unsuccessful in identifying staff carrier(s). Furthermore, this outbreak adds to the growing body of evidence on the role of HCWs in the nosocomial transmission of *S. aureus*, prompting the need to reassess occupational health screening policies to monitor the health of healthcare staff, especially those with chronic dermatitis.

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Conflict of interest

None declared.

Authors' contributions

KP, AB, LM, SL, AT and FT were key members of the Outbreak Control Team and were involved in agreeing the strategic and operational approach to investigations and management of the outbreak. AK provided expert advice on outbreak strategy and oversaw detailed molecular characterisation of the isolates. ML provided significant input in to outbreak management strategy and led the review of infection control practices at the maternity unit. RP and SN led on the epidemiological investigations. KP wrote the first draft of the manuscript and all authors contributed to the final version.

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Investigations into the emergence of pertactin-deficient *Bordetella pertussis* isolates in six European countries, 1996 to 2012

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Pathogen adaptation has been proposed to contribute to the resurgence of pertussis. A striking recent example is the emergence of isolates deficient in the vaccine component pertactin (Prn). This study explores the emergence of such Prn-deficient isolates in six European countries. During 2007 to 2009, 0/83 isolates from the Netherlands, 0/18 from the United Kingdom, 0/17 Finland, 0/23 Denmark, 4/99 Sweden and 5/20 from Norway of the isolates collected were Prn-deficient. In the Netherlands and Sweden, respectively 4/146 and 1/8 were observed in a later period (2010–12). The Prn-deficient isolates were genetically diverse and different mutations were found to inactivate the *prn* gene. These are indications that Prn-deficiency is subject to positive selective pressure. We hypothesise that the switch from whole cell to acellular pertussis vaccines has affected the balance between 'costs and benefits' of Prn production by *Bordetella pertussis* to the extent that isolates that do not produce Prn are able to expand. The absence of Prn-deficient isolates in some countries may point to ways to prevent or delay the spread of Prn-deficient strains. In order to substantiate this hypothesis, trends in the European *B. pertussis* population should be monitored continuously.

Introduction

Introduction of whole cell pertussis vaccines (WCVs) 50 to 60 years ago has greatly reduced the pertussis burden worldwide [1]. However, despite a vaccine coverage between 80% and 96%, pertussis has resurged in many countries [2,3]. In particular, in the last few years, high incidences have been observed in the Netherlands, the United Kingdom (UK) and the United States (US) [4-6]. Several causes have been proposed for the resurgence of pertussis, including waning immunity and pathogen adaptation [7].

In the 1990s, less-reactive acellular pertussis vaccines (ACVs) replaced WCVs. ACVs contain between one and five pertussis proteins: (i) pertussis toxin (Ptx); (ii) pertactin (Prn); (iii) serotype 2 fimbriae (Fim2); (iv) serotype 3 fimbriae (Fim3); and (v) filamentous haemagglutinin (FHA). All ACVs contain Ptx. The effectiveness of the different available WCVs was highly variable [8], which complicates evaluation of the protection that ACVs induce compared with that of WCVs. However, recently, several studies have indicated that the immunity induced by ACVs is less long-lasting compared with effective WCVs, resulting in a higher pertussis burden, particularly in children aged 6 to 10 years [9-12].

The effect of waning immunity may have been aggravated by pathogen adaptation [7], which is reflected in large shifts in *Bordetella pertussis* populations [13-15]. Observed adaptations include antigenic divergence between circulating strains and vaccine strains, and the emergence of strains with a novel promoter for Ptx (*ptxP3*) [16]. In addition, most recently, strains have emerged that do not express Prn, hereafter named Prn-deficient strains [17-22]. Isolates with the *ptxP3* promoter increased in frequency in the 1980s and at present they predominate in many countries, reflecting a global selective sweep [6,13,16,23-26]. The *ptxP3* isolates produce higher amounts of Ptx in vitro (1.6-fold increase), when compared with the *ptxP1* isolates they replaced [16]. It has been suggested that this may result in a more effective suppression of host immunity [16]. In agreement with this, *ptxP3* isolates reach higher bacterial densities than *ptxP1* isolates in mice [27]. Prn-deficient isolates emerged around 2000 and have been found in Finland, France, Japan and the US [18-20,22]. In addition, less frequently, isolates were found that

Table 1

Year of introduction, vaccine composition and vaccination coverage of acellular pertussis vaccines in six European countries

Country	Year of ACV introduction[a,b]	Primary series Age <13 months		Childhood booster Age 13 months–10 years		Adolescent booster Age 11–20 years		Reference
		ACV[c]	Vaccination coverage	ACV[c]	Vaccination coverage	ACV[c]	Vaccination coverage	
Denmark	1997	1	>90%	–	–	–	–	[39]
	2003	–	–	1	>80%	–	–	
Finland	2003	–	–	3	–	–	–	[20]
	2005	2 or 3	>99%	2 or 3	–	3	–	
Netherlands	2001	–	–	3 or 5	>95%	–	–	[40]
	2005	3 or 5	>95%	–	–	–	–	
Norway	1998	3	>90%	–	–	–	–	[41]
	2006	–	–	2	90%	–	–	
Sweden	1996	1, 2 or 3	>98%	–	–	–	–	[40,42]
	2007	–	–	2 or 3	90%	–	–	
United Kingdom[b,d]	2000	3	>90%	–	–	–	–	[43]
	Oct 2001	–	–	3 or 5	–	–	–	
	2004	5	–	3 or 5	–	–	–	

ACV: acellular pertussis vaccine; FHA: filamentous haemagglutinin; *Fim2*: serotype 2 fimbriae; *Fim3*: serotype 3 fimbriae; Prn: pertactin; Ptx: pertussis toxin.

Dashes are used where data are not applicable.

[a] The year in which ACVs were introduced as the primary series and as booster.

[b] In the United Kingdom, an ACV3 was used temporarily for the primary series until 2001. Thereafter, the whole cell pertussis vaccines was used again for the primary series, with addition of an ACV3 or ACV5 childhood booster in October 2001. Subsequently, in 2004 the ACV5 was introduced permanently for the primary series.

[c] Vaccine compositions are shown. ACV1: Ptx monocomponent; ACV2: Ptx, FHA; ACV3: Ptx, FHA, Prn; ACV5: Ptx, FHA, Prn, *Fim2*, *Fim3*.

[d] For the purposes of this article, the United Kingdom is considered as one country.

do not produce FHA or Ptx. Hegerle et al. reported one isolate lacking both Prn and FHA, and one isolate lacking Ptx [17,19]. Bouchez et al. have suggested that the emergence of such isolates is related to the introduction of ACVs [17,28]. Prn-deficient isolates have been isolated from pertussis patients and seemed to be as virulent as isolates expressing Prn in infants less than six months of age as well as in a mouse model [17,29]. A better understanding of the epidemiology and virulence of Prn-deficient isolates is crucial for the development of next-generation pertussis vaccines.

In this study, we investigated the emergence and spread of Prn-deficient isolates in Europe. To do so, we combine single nucleotide polymorphism (SNP) typing, antigen expression analysis and DNA sequencing with epidemiological data. The diversity of vaccination programmes in Europe (Table 1) offers a unique opportunity to study the effect, if any, of vaccination policy on the emergence of Prn-deficient strains. Ultimately, we aim to define conditions that could prevent or hamper the emergence of vaccine-adapted strains. This study provides a baseline for future European studies in which the occurrence and spread of Prn-deficient strains will be investigated.

Methods

Analysis of *Bordetella pertussis* isolates

Six countries provided a total of 665 strains isolated from nasopharyngeal swabs from confirmed pertussis patients for this study: Denmark (n=43), Finland (n=56), Netherlands (n=311), Norway (n=20), Sweden (n=197) and the UK (for the purposes of this article, the UK is considered as one country) (n=38) (Table 2). These countries were selected as they are members of European research programme for improved pertussis strain characterisation and surveillance (EUPERTSTRAIN) network and were able to contribute strains. The strains were isolated during 1996 to 2012. In the countries studied, primary immunisation with ACVs was introduced between 1996 and 2005 (Table 1). Furthermore, in these countries, different ACVs and different vaccine strategies were used and the vaccine coverage varied (Table 1). Strains were grouped into three periods, 1996–2006, 2007–09 and 2010–12 (periods I, II and III, respectively). Bacteria were cultured on Bordet Gengou agar plates supplemented with 15% sheep blood and incubated at 35 °C for 3 to 4 days.

PCR and DNA sequencing

DNA isolation was performed using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Zwijndrecht,

Table 2

Bordetella pertussis isolates typed by single nucleotide polymorphisms and analysed for pertactin and pertussis toxin production in six European countries, 1996–2012

Country	Period I 1996–2006			Period II 2007–2009			Period III 2010–2012			Periods I–III 1996–2012			
	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	Total number of strains provided
Denmark	20	0	–	23	23	0 (0)	0	0	–	43	23	0 (0)	**43**
Finland	38	0	–	16	17	0 (0)	0	0	–	54	17	0 (0)	**56**
Netherlands	34	82	0 (0)	16	83	0 (0)	4	146	4 (3)	54	311	4 (1)	**311**
Norway	0	0	–	20	20	5 (25)	0	0	–	20	20	5 (25)	**20**
Sweden	27	80	0 (0)	25	99	4 (4)	0	8	1 (13)	52	187	5 (3)	**197**
United Kingdom[a]	20	0	–	18	18	0 (0)	0	0	–	38	18	0 (0)	**38**
Total	**129**	**162**	**0 (0)**	**118**	**260**	**9 (3)**	**4**	**154**	**5 (3)**	**261**	**576**	**14 (2.4)**	**665**

Prn: pertactin; Ptx: pertussis toxin; SNP: single nucleotide polymorphism.
Dashes are used where a percentage is not applicable.

[a] For the purposes of this article, the United Kingdom is considered as one country.

the Netherlands), following the manufacturer's instructions for Gram-negative bacteria. For multi-antigen sequence typing (MAST), the pertussis toxin promoter (*ptxP*), regions 1 and 2 of the pertactin gene (*prn*), the genes for serotype 2 fimbriae (*fim2*) and serotype 3 fimbriae (*fim3*) were sequenced in order to determine polymorphisms [16,30].

To determine the location and size of the mutation causing non-expression of Prn, four sets of primers were selected for PCR amplification of the *prn* gene (Table 3). Prn PCR products were visualised and sized by capillary electrophoresis on a QIAxcel instrument (Qiagen Benelux), using the QIAxcel high-resolution kit, QX DNA size marker 250–8,000 base pairs (bp) and QX alignment markers 15/8,000 bp. Each type of mutation was verified in at least one isolate by Sanger sequencing using an ABI 3700 DNA sequencer (Perkin-Elmer Applied Biosystems).

Identification of vaccine antigen-deficient isolates

A microsphere-based Multiplex Immunoassay (Luminex, Austin, TX, US) [31] was developed to measure the expression of Ptx and Prn. Isogenic *ptx* (Bo085) and *prn* (B1686) knockout strains were used as negative controls [32,33]. Analyses of these negative controls resulted in values <5 ng/ml. Therefore, isolates with antigen concentrations <5 ng/ml were considered deficient isolates. All deficient isolates were tested at least twice, starting from independent cultures. In addition, Prn deficiency was verified with an immunoblot using the monoclonal antibody Prn Ab 62, E4D7 [34] (Figure 1).

Single nucleotide polymorphism typing and clustering analysis

SNP typing was used to establish genetic relationships between *B. pertussis* isolates [35,36]. A total of 113 SNPs that are useful for discriminating between isolates were selected after comparing whole genome data of a selection of 74 worldwide isolates (data not shown). In order to study the origin and spread of European Prn-deficient isolates, SNP typing was performed on 261 isolates from all six countries using the Sequenom technology (Sequenom, San Diego, CA, US) [35]. At least 40 randomly selected strains (using the function RAND in Microsoft Excel) per country were typed, if available. A tree was constructed in Bionumerics 6•6 (Applied Maths, Sint-Martens-Latem, Belgium), treating the SNPs as character data and using the neighbor-joining algorithm. The vaccine strains Bo006 (from the Netherlands, 1950, *ptxP2*), Bo499 (ACV strain 10-536, 1951, *ptxP2*) and Bo005 (from the Netherlands, 1950, *ptxP1*) were included as outgroups.

Table 3

Primers used for the characterisation of mutations leading to non-expression of pertactin in *Bordetella pertussis* isolates in six European countries, 1996–2012

PCR	Primer name	Primer sequence (5' to 3')	Positiona	Reference
1	prn-SPF1	TCC CTG TTC CAT CGC GGT G	1098036–1098054	[44]
	prn-SPR1	CCT GAG CCT GGA GAC TGG	1100878–1100895	
2	prn-BF	AGC TGG GCG GTT CAA GGT	1099489–1099506	[45]
	prn-BR	CGG AT T CAG GCG CAA CTC	1100006–1100024	
3	prn-D1F	CAT GAA ATC CGC CAT CCG CT	1095775–1095794	[this work]
	prn-D1R	ACT GGC CCC CAA CAC AGA CA	1098750–1098769	
4	prn-D2F	TAC TTT TGC TGC GCC CAT T	1098015–1098033	[this work]
	prn-D2R	CTT GAT GGT GGT TCC GCT G	1098283–1098301	

F: forward primer; R: reverse primer.

[a] Position of the primer, relative to the origin of replication of reference strain Tohama (National Center for Biotechnology Information (NCBI) reference sequence NC_002929).

Results

Frequency of pertactin-deficient isolates in six European countries

Expression of Prn was assessed by a multiplex Immunoassay assay and immunoblotting (Figure 1) for 576 of the 665 strains from the six countries, of which 14 (2.4%) were shown to be Prn deficient (Tables 2 and 4). All 576 strains tested positive for Ptx production. For period I (1996–2006), only strains from the Netherlands (n=82) and Sweden (n=80) were available for this analysis. One Prn-deficient isolate from Finland was obtained in period I: this isolate was not included in the Prn-deficiency frequency analysis because no other Finnish isolates were analysed for Prn expression in that period. For period II (2007–09), between 17 and 99 strains from each of the six countries were analysed for Prn expression. Prn-deficient strains were observed in Norway (5/20 strains) and Sweden (4/99), but not in Denmark (0/23), Finland (0/17), the Netherlands (0/83) or the UK (0/18). As for period I, for period III (2010–12) only strains from the Netherlands (n=146) and Sweden (n=8) were available for this analysis. In that period, 4/146 Dutch strains and 1/8 Swedish strains were found to be Prn deficient. Thus, Prn-deficient isolates in the frequency analysis were detected in periods II and III. All isolates produced Ptx. The vaccination status of 14 of the 15 patients from whom the Prn-deficient isolates were recovered was unknown (Table 4). Therefore, we cannot relate the infection with a Prn-deficient strain to the degree of immunisation.

Mutations causing non-expression of pertactin

Previous studies observed five types of mutations that inactivate the *prn* gene: inversion, deletion of (part of) the gene, insertion of IS*481* into *prn*, insertion of a single nucleotide resulting in a frameshift mutation, or a SNP resulting in the introduction of a stop codon [17-22] (Figure 2, Table 5). We used PCR and sequencing of the *prn* gene to determine the cause of non-expression of the Prn-deficient isolates we analysed (Table 4). PCR fragment analysis showed that three Prn-deficient isolates (B3545, B3748, B3865) had an insertion of approximately 1 kilobase pair (kb) proximal to region 2 (Figure 3). We assumed this is the result of an insertion of IS*481* into this region and no further sequencing analysis was performed on these isolates. Sequence data of the remaining 12 Prn-deficient isolates showed that in two isolates, the promoter region was inverted as described by Pawloski et al. [22]. Another three isolates (B3582, B3640, B3891) had an IS element inserted proximal to one of the two repeat regions of the *prn* gene, designated region 2 (Figure 2). This mutation has also been found previously in French Prn-deficient isolates [17]. Sequence data of a sixth isolate (B3771), which originated from Finland in period I, contained a deletion of 49 bases in the signal sequence. This isolate was not included in the Prn-deficiency frequency analysis because no other Finnish isolates were analysed for Prn expression in period I. The seventh isolate, B3876 from The Netherlands, contained a SNP at position 223 that introduces a premature stop codon in the *prn2* gene. An eighth isolate (B3658), originating from Norway, also contained a SNP that introduces a premature stop codon in *prn2*, but at position 1,273. Sequencing of the *prn* gene around position 1,273 showed that the other four Norwegian Prn-deficient isolates also harboured this SNP.

Phylogenetic relationships of European pertactin-deficient *Bordetella pertussis* isolates

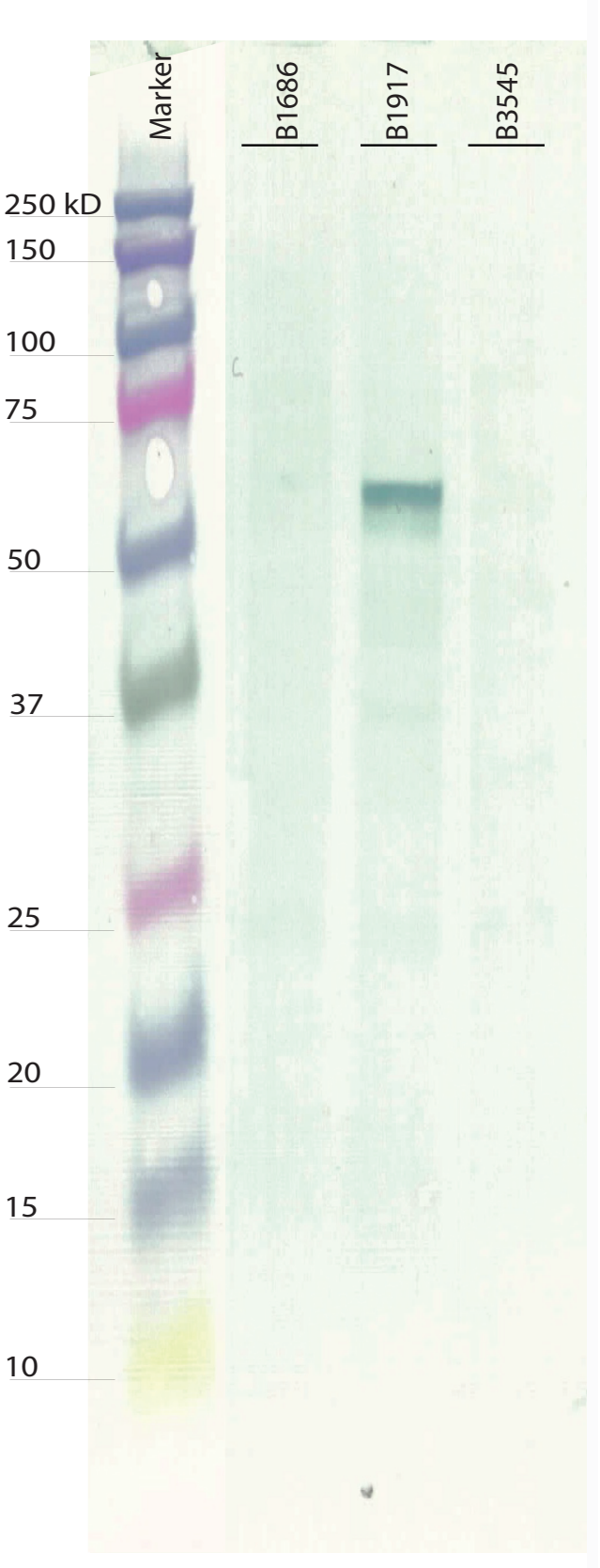
Typing of 261 isolates from the six different European countries using 113 SNPs allowed us to distinguish 20 STs.

Phylogenetic analysis revealed a closely related European *B. pertussis* population (Figure 4). The tree topology showed two distinct clades containing *ptxP1* and *ptxP3* isolates. In addition, a *fim3*.1 and a *fim3.2* cluster could be observed within the *ptxP3* clade (Figure 4).

The 12 European Prn-deficient isolates included in the phylogenetic analyses were found in four different STs: ST13, ST14, ST17 and ST19 (Figure 4). These STs were among the predominant types and contained 70% (184/264) of the isolates analysed.

Figure 1

Immunoblot to confirm absence of pertactin (69 kD) expression in *Bordetella pertussis* isolates in six European countries, 1996–2012



The result of isolate B3545 (fourth lane) is shown here as an example. A Kaleidoscope marker (161-0375, Bio-Rad, Hercules, US) was loaded in the first lane. B1686 is an isogenic pertactin knock-out strain, which was used as a negative control. A commonly used reference strain, B1917, was used as a positive control.

All Prn-deficient isolates analysed in this study were found in the *ptxP3* clade but were associated with both *fim3*.1 and *fim3*.2 alleles. ST14 contains two types of *prn* mutations (Table 4). In contrast, the five Prn-deficient isolates that belong to ST13 all harboured the same mutation in *prn*: a SNP resulting in premature translational termination of *prn*. All five isolates were from Norway. The *prn* mutation, in which IS*481* was inserted proximal to region 2, was found in three STs that were dispersed across the *ptxP3* clade. This mutation was associated with both the *fim3*.1 and *fim3*.2 alleles. Thus, identical mutations were observed in different STs, while within a single ST, different mutations were found.

Discussion

The diversity in immunisation programmes in Europe offers an opportunity to explore the effect of vaccination strategies on adaptation of *B. pertussis* in populations with a vaccine coverage between 80% and 96%. In this study, the overall mean of Prn-deficient strains from six European countries isolated between 2007 and 2012 was 3.4% (14/414 isolates). Bouchez et al. suggested that the emergence of French Prn-deficient isolates was driven by the switch from WCVs to ACVs [17]. Indeed, Prn-deficient isolates were rarely found before the introduction of ACVs, when WCVs were used [37]. Compared with the mean of Prn-deficient strains found in the six countries studied here (3.4%), higher percentages of Prn-deficient isolates were observed in France (13% in 2011), Japan (32% in 2005–09) and the US (53% in 2012) [18,19,22]. In Japan, ACVs were introduced in 1981 [18], and in the US, the first ACV was marketed in 1991. Thus, these countries have a longer history of ACV immunisation compared with the six countries included in this study, where ACVs vaccines for the primary series were introduced between 1996 and 2005 (Table 1). Interestingly, the early Prn-deficient strains found in this study were mainly from Norway and Sweden, where ACVs for the primary series were introduced in 1996 and 1998, respectively, which is earlier than in the UK (in 2000), Finland and the Netherlands (both in 2005) (Table 1). Notably, in France an ACV booster was introduced in 1998, while the WCV for the primary series was replaced with an ACV in 2004 [29]. The timing of these changes is similar to that in Norway which, as in France, has a relatively high percentage of Prn-deficient strains. In Denmark, an ACV was introduced relatively early in 1997, while Prn-deficient strains were not detected. Unlike the vaccine in the other five countries in this study, which use ACVs containing Prn (Table 1), the Danish ACV contains Ptx only. As the Danish vaccine does not induce Prn antibodies, it is conceivable that Prn-deficient strains will not emerge in Denmark, if Prn-deficiency does not contribute to strain fitness, although overflow of Prn-deficient strains from neighbouring countries is possible.

In 2007–09, five of the 20 Norwegian strains were Prn-deficient. Although the number of isolates tested was

Table 4

Pertactin-deficient *Bordetella pertussis* isolates analysed from four European countries, 2004–12 (n=15)

Isolate name	Patient's age	Patient's country of residence	Patient's vaccination status	Year isolate recovered	Sequence type[a]	Mutation[b]
B3771[c]	2 years	Finland	Vaccinated	2004	ST19	¤6: *prn2* (32_80Δ[49])
B3645	7 months	Norway	Unknown	2007	ST13	¤12: *prn2* (1273STOP:C>T)
B3652	14 years	Norway	Unknown	2008	ST13	¤12: *prn2* (1273STOP:C>T)
B3654	14 years	Norway	Unknown	2008	ST13	¤12: *prn2* (1273STOP:C>T)
B3545	Unknown	Sweden	Unknown	2008	ST17	*prn2* (PCRregion2::1kb)
B3657	2 months	Norway	Unknown	2009	ST13	¤12: *prn2* (1273STOP:C>T))
B3658	3 months	Norway	Unknown	2009	ST13	¤12: *prn2* (1273STOP:C>T)
B3594	1 month	Sweden	Unknown	2009	ST14	¤1: *prn2*(-20892_-75‹›[22kb])
B3582	0 months	Sweden	Unknown	2009	ST17	¤4: *prn2* (1613^1614::IS481[1049])
B3748	11 months	Sweden	Unknown	2009	ST17	*prn2* (PCRregion2::1kb)
B3640	13 years	Netherlands	Unknown	2010	ST14	¤4: *prn2* (1613^1614::IS481[1049])
B3865	Unknown	Netherlands	Unknown	2011	ST14	*prn2* (PCRregion2::1kb)
B3876	2 years	Netherlands	Unknown	2011	NI	¤10: *prn2*(223STOP:C>T)
B3891	Unknown	Sweden	Unknown	2011	NI	¤4: *prn2* (1613^1614::IS481[1049])
B3977	69 years	Netherlands	Unknown	2012	NI	¤1: *prn2*(-20892_-75‹›[22kb])

NI: not included in the SNP analysis; SNP: single nucleotide polymorphism; ¤: mutation number.

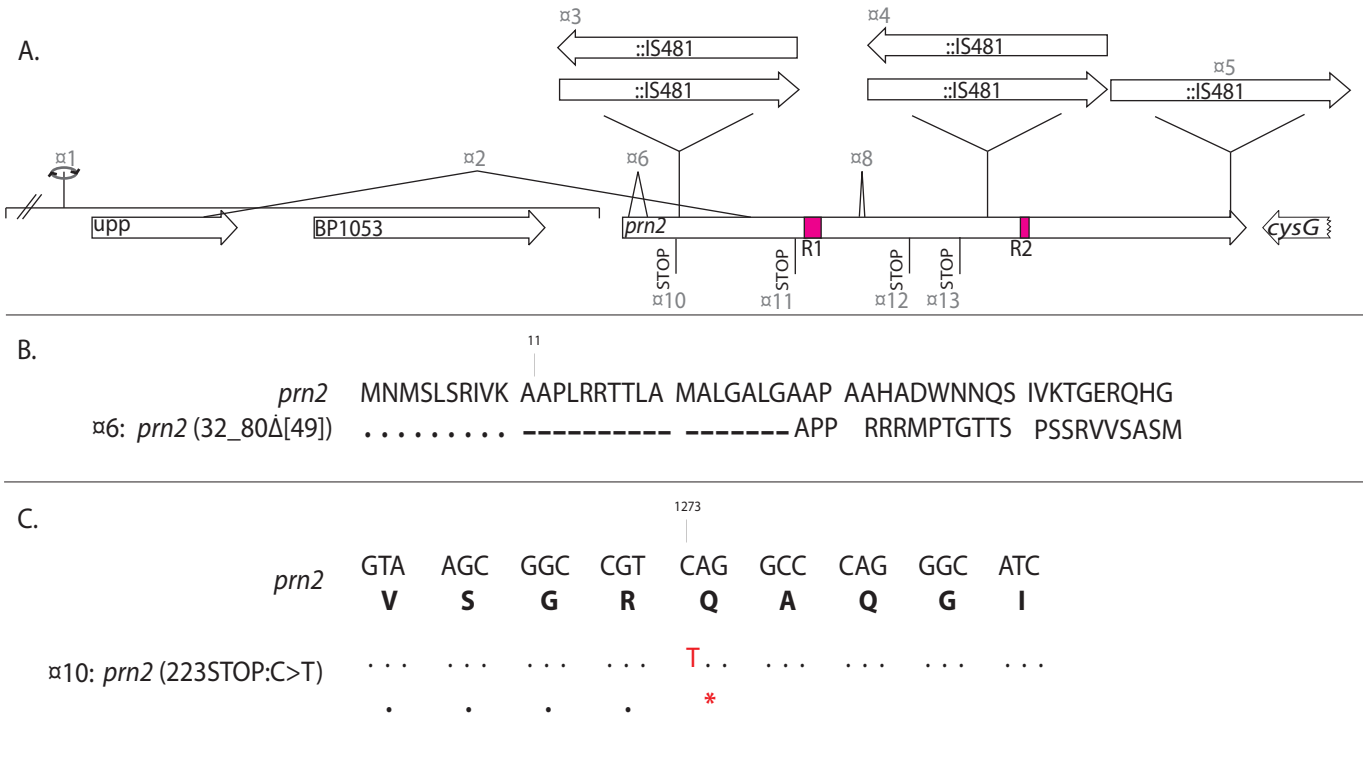
[a] Sequence type as presented in Figure 2A.

[b] Mutation causing the non-expression of pertactin. Numbers correspond to those in Table 5 and Figure 2A.

[c] This isolate was not included in the frequency analysis, because it was a single analysed Finnish strain that was isolated before 2007, but was included in SNP analysis and *prn* mutation analysis.

Figure 2

Mutations in *prn2* causing non-expression of *pertactin* in *Bordetella pertussis* isolates in six European countries, 1996–2012



A. *prn2* is shown with the surrounding genes. The arrowheads indicate the transcription direction. The variable regions 1 (R1) and 2 (R2) of *prn* are indicated in red. Numbers 1 to 13 refer to mutations (¤) in *prn2* and correspond to the numbers in Table 5.

B. Detailed representation of mutation ¤6, a deletion of 49 bases in the signal sequence: *prn2* (32_80-[49]): • identical bases/amino acids, - deleted bases/amino acids.

C. Detailed representation of mutation ¤10: a SNP leading to a stop codon: *prn2* (223STOP:C>T). Base and amino acid numbering is relative to the *prn2* initiation codon.

Table 5

Mutations in the *pertactin* gene causing loss of pertactin expression

Number of the mutation[a]	Name of the mutation[b]	Note	Reference
¤1	*prn2* (-20892_-75‹›[22Kb])	Inversion of about 22 kb in the promoter	[21, this work]
¤2	*prn2* (-1846_553Δ[2399])	Deletion of the first part of *prn2* and upstream *BP1053*	[16]
¤3	*prn2* (245^246::IS*481*[1049])	Insertion of IS*481* in *prn2*, between position 245 and 246	[20]
¤4	*prn2* (1613^1614::IS*481*[1049])	Insertion of IS*481* in *prn2*, between position 1,613 and 1,614	[16, this work]
¤5	*prn2* (2735^2736::IS*481*[1049])	Insertion of IS*481* in *prn2*, between position 2,735 and 2,736	[21]
¤6	*prn2* (32_80Δ[49])	Deletion in *prn2* signal sequence	[this work]
¤7	*prn2* (Δprn)	Deletion of the entire prn gene	[18]
¤8	*prn2* (1043_1067Δ[25])	25 bp deletion in first repeat region	[18]
¤9	*prn2* (Δ[89])	89 bp deletion at 5' of prn	[18]
¤10	*prn2* (223STOP:C›T)	SNP C›T, leading to a stop codon	[this work]
¤11	*prn2* (760STOP:C›T)	SNP C›T, leading to a stop codon	[21]
¤12	*prn2* (1273STOP:C›T)	SNP C›T, leading to a stop codon	[20, this work]
¤13	*prn2* (1479STOP)	SNP leading to a stop codon	[18]
¤14	*prn1* (1598^1599::IS*481*[1049])	Insertion of IS*481* in *prn1*, also found in reverse direction	[17]
¤15	*prn1* ΔSS	Deletion in *prn1* signal sequence	[17,19,21]

bp: base pairs; IS: insertion element; kb: kilobase pairs; SNP: single nucleotide polymorphism; ¤: mutation number.

[a] As presented in Figure 2, except for ¤14 and ¤ 15.

[b] The name is composed as follows: pertactin gene (prn) allele (position from open reading frame of the prn gene/type of mutation/[size of the mutation]).

x_y	from position x to y
x^y	between position x and y
‹›	inversion
::IS481	insertion of IS481
Δ	deletion
STOP:C›T	SNP (C to T) leading to a premature stop codon

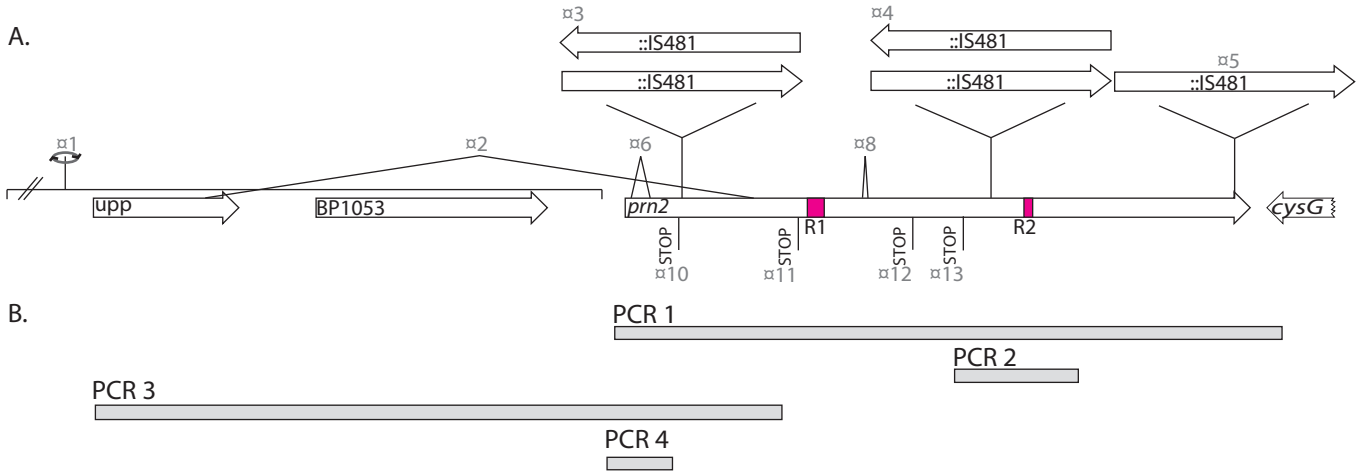
low, this proportion is relatively high compared with the overall mean of the six countries in this period (3.4%, 14/414). This implies that in Europe, besides France, Norway may also have elevated levels of Prn-deficient isolates. Comparisons of frequencies of Prn-deficient isolates should, however, be made with caution, as differences in the period investigated, vaccination policies and isolate collection are among the factors that can influence the outcome. It seems valid, though, to conclude that the fraction of Prn-deficient strains in several countries of Europe is relatively low, with the exception of France [19] and Norway, as stated above. In order to confirm these findings, further studies are required that include more isolates for each country. The analyses of larger amounts of Prn-deficient strains may also reveal if these strains are found in higher frequencies in particular age groups. Future studies should include more southern European countries.

Mutations that were previously found to inactivate the *prn* gene [17,19,21] were also observed in the Prn-deficient isolates analysed in this study. We found that the mutations in *prn* occurred several times independently in different lineages, which is an indication that it has been fixed in the population by positive selection. The European Prn-deficient isolates seem to have originated from distinct lineages, but within the diverse nature of the mutations causing non-expression, we also observed possible signs of clonal expansion of a Prn-deficient strain in Norway. Notably, the specific point mutation that occurred in five Norwegian isolates was also found in seven of 12 Prn-deficient isolates collected in Philadelphia in the US [21]. It was previously suggested that the spread of Prn-deficient isolates in Japan was the result of clonal expansion [18].

The production of bacterial virulence factors, such as pertactin, involves 'costs and benefits' for the bacteria. Costs include the energy used to express the virulence factors and activation of the host immune response against these antigens. Benefits comprise attachment to host tissues and manipulation of host defences. We hypothesise that the switch from WCV to ACV has affected the balance between these costs and benefits of Prn production to the extent that strains that do not produce Prn are able to expand. In support of this, our phylogenetic analysis indicates Prn-deficiency is subject to positive selection. We postulate that, because non-expression of one of the

Figure 3

Characterisation of the *prn* gene from pertactin-deficient *Bordetella pertussis* isolates by polymerase chain reaction, from six European countries, 1996–2012



C.

Marker	PCR1			PCR2		PCR3		PCR4	
	¤ 10-13	¤3, ¤4 or ¤5	¤6	¤ 10-13	¤4 not ¤3 or ¤5	¤ 10-13	¤6	¤ 10-13	¤6
	WT	:: [~1kb]	Δ[~100bp]	WT	:: [~1kb]	WT	Δ[~100bp]	WT	Δ[~100bp]
4000									
3000									
2000									
1000									
750									
500									
250									

bp: base pairs; kb: kilobase pair; PCR: polymerase chain reaction; WT: wild type.

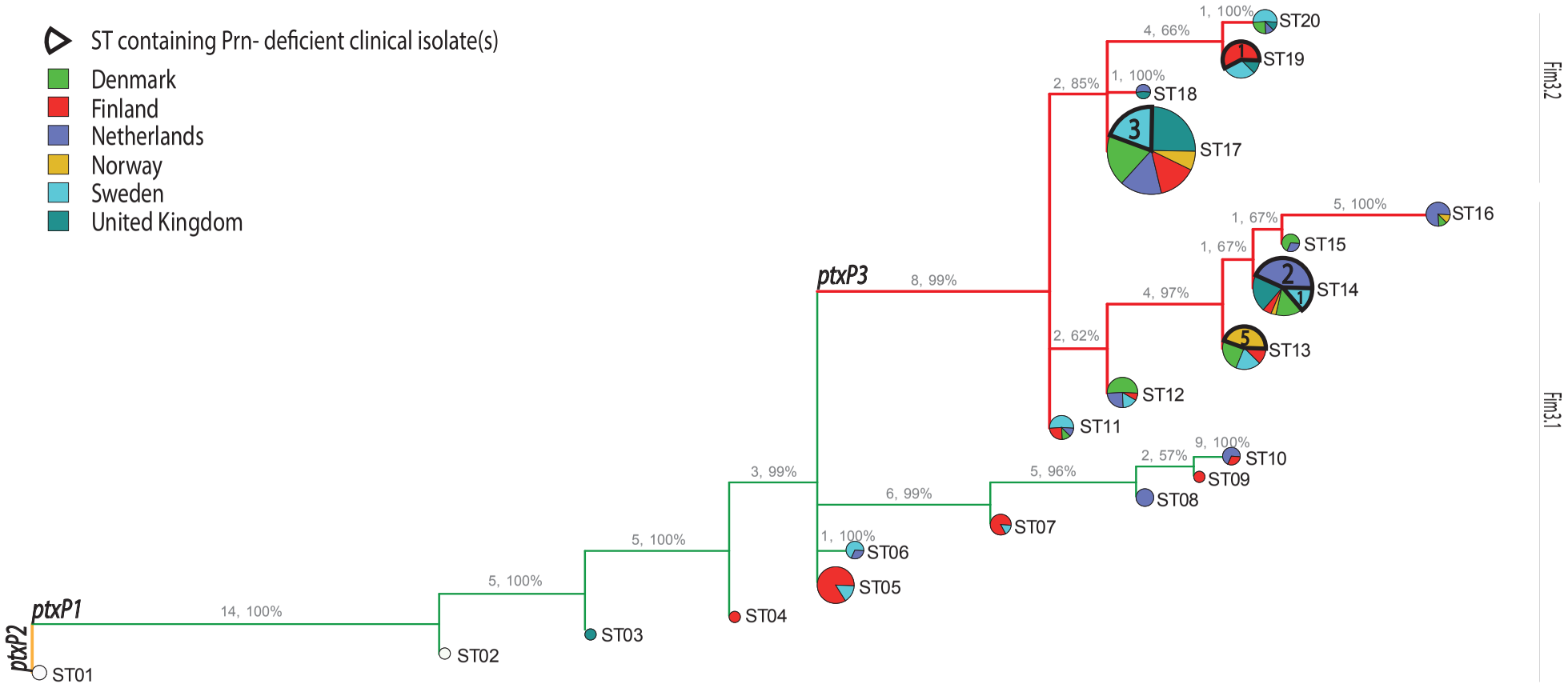
A. The *prn* gene and surrounding genes.

B. The grey bars represent the regions targeted in the PCR that were used to identify insertion and deletion mutations in *prn*. For the primers, see Table 3.

C. The PCR products of the WT *prn* allele were compared with PCR products derived from *Prn*-deficient strains. PCR fragments were sized by capillary electrophoresis. On the basis of the primers used and the size of the PCR fragments, the type of mutation was derived, as shown above the lanes. Designation of PCR fragments as in (B) and mutations as in Table 5. The first lane contains the size marker DNA (in bp). PCR2 distinguishes ¤4 from ¤3 or ¤5, which does not result from PCR1. Mutations 10–13 yielded WT products for the PCRs.

Figure 4

Genetic relationship between European *pertactin*-deficient *Bordetella pertussis* isolates



SNP: single nucleotide polymorphism; ST: sequence type.

A total of 113 predefined SNPs were used to construct a neighbor-joining tree of 261 European strains collected between 1998 and 2011. The STs are listed next to the branch tips and circle sizes are scaled to the number of isolates in each ST. Colours indicate the country from which the isolates originate. The bold sectors are STs containing Prn-deficient strains and the number in the centre of the segment represents the number of Prn-deficient isolates. The *ptxP* alleles are indicated next to the tree arms (*ptxP2*: orange line; *ptxP1*: green line; *ptxP3*: red line) and the *fim3* types are presented at the tips of the branches. Branch labels give the distance between the STs followed by the bootstrap values as percentages (200 replicates). ST01 and ST02 respectively consist of two and one vaccine strain(s), which are used as an outgroup.

vaccine antigens contributes relatively little to immune evasion of a broad immune response, vaccination with WCVs limits the advantage of Prn-deficient isolates. Conversely, if the immune response is directed against a limited number of antigens (one to five in the case of ACVs), non-expression of one of the vaccine antigens may confer a considerable selective advantage. This is especially the case when high antibody titres are induced, as is observed for ACVs [38]. However, further studies are required to substantiate this hypothesis.

The emergence of Prn-deficient strains raises questions about its role in pertussis. An analysis of clinical symptoms caused by Prn-positive and Prn-negative isolates in infants did not reveal major differences between the two groups, which suggests that lack of Prn in *B. pertussis* does not affect virulence in infants substantially [29]. Other important questions that should be addressed in the future are the effect, if any, of Prn-deficiency on the efficacy of pertussis vaccines and whether Prn-deficient strains prevail more in groups with a particular level of immunity. This study establishes a baseline for investigations into the emergence and spread of Prn-deficient strains in Europe.

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Conflict of interest

None declared.

Authors' contributions

FRM obtained funding and supervised the study. QH and JM organised the EUPERTSTRAIN 2 and 3 collections from the network members. Isolates, epidemiological data and isolate characteristics were provided by all authors. AZ, MvG, KH, HvdH, MB and FRM were responsible for the study concept and practical work. AZ and FRM analysed and interpreted data and wrote the manuscript. All authors were responsible for the critical revision of the manuscript.

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