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Imported case of MERS-CoV infection identified in China, May 2015: detection and lesson learned

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At the end of May 2015, an imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection was confirmed in China. The patient is in a stable condition and is still undergoing treatment. In this report, we summarise the preliminary findings for this imported case and the results of contact tracing. We identified 78 close contacts and after 14 days of monitoring and isolation, none of the contacts presented symptoms and all tested negative for MERS-CoV

Case report

On 27 May 2015, the Chinese Ministry of Health was notified by the World Health Organization (WHO) of a suspected case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection who had travelled from South Korea to Guangdong province, China, one day earlier. The case is a man in his 40s (Patient C) who was symptomatic during his travel but had not revealed his history of close contact with a confirmed MERS case during his stay in South Korea. The person was identified on 28 May. Medical staff wearing personal protective equipment (PPE) accompanied him to the closest hospital where he was treated in isolation in a negative pressure room. To reduce the risk of further transmission, strict infection control measures have been taken in hospital. Case investigation revealed that the man had been exposed to the first confirmed MERS case in South Korea (Patient A) who shared a ward with the father (Patient B) of Patient C. After confirmation of MERS-CoV as the cause of illness of Patient A on 20 May, contact tracing confirmed MERS-CoV in Patient B on 21 May. Patient C began feeling unwell on the same day because of back pain but he had no respiratory symptoms. On 25 May, his sister (Patient D) was reported to be the fourth confirmed MERS case in South Korea. On the same day, a temperature of 38.7 °C was recorded for Patient C. The next day, on 26 May, against medical advice, the man travelled by plane from South Korea to Hong Kong directly, and then took two consecutive buses from Hong Kong airport to his destination in Guangdong province, mainland China. He stayed at Hotel A overnight and on the following day he attended meetings all day and spent the night in Hotel B until he was identified and placed in isolation. According to his quarantine form, he had fever (39.7 °C) but did not report his history of exposure to a MERS case upon entry into Hong Kong. Once identified and admitted to hospital on 28 May, clinical examination revealed a temperature of 39.5 °C and non-productive cough. Over the following three days, his condition worsened. Chest x-ray showed that he had bilateral hilar infiltrates. He received ribavirin twice a day for two days, and once daily afterwards. As of 18 June, the patient remains under treatment in stable condition in hospital and would be discharged if there is no viral shedding observed for three consecutive days. The Figure illustrates the timeline of events for this case.

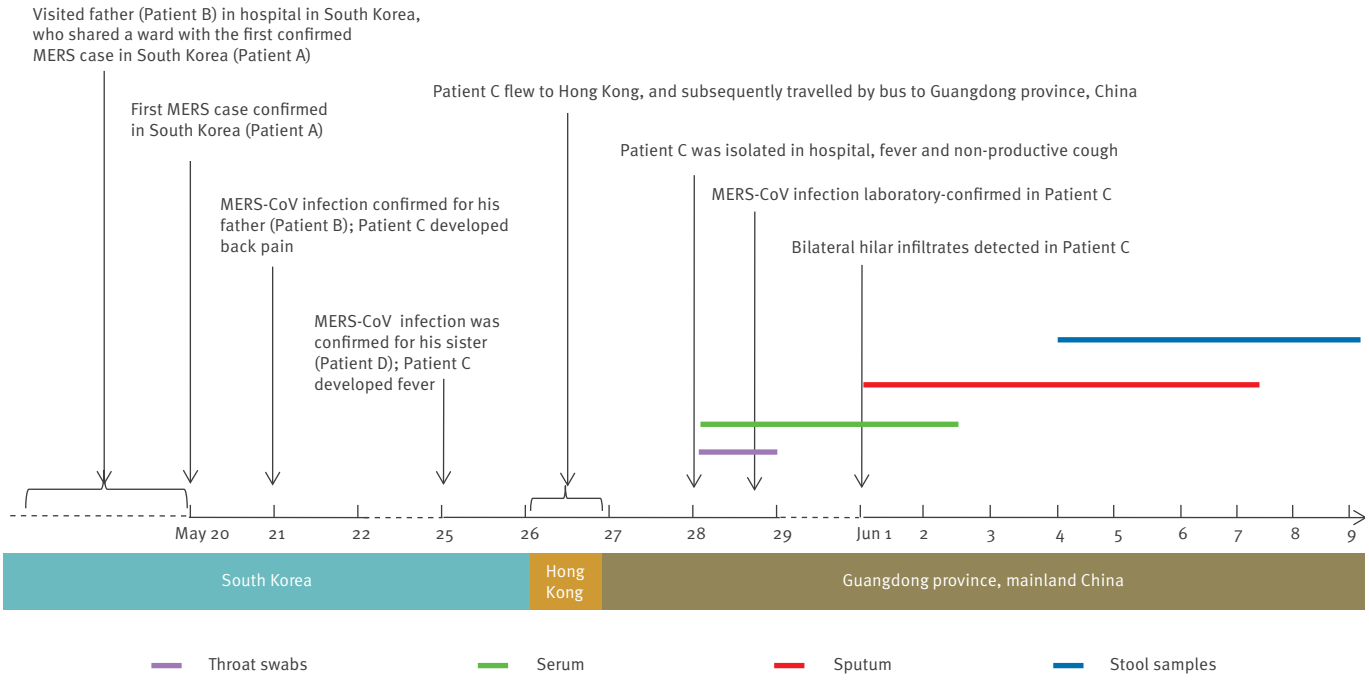
Laboratory findings

Diagnosis of MERS-CoV infection was performed based on the real-time RT-PCR method, using a target upstream of envelope gene (upE) as a screening test and the open reading frame (ORF) 1b gene as the confirmatory assay. MERS-CoV infection was firstly confirmed in Guangdong Centers for Disease Control (CDC) on 28 May and re-confirmed on 29 May by the national CDC in Beijing. Genome sequences were shared for assessment of possible virological changes through collaborations with the Collaborative Management Platform for detection and Analyses of (Re-) emerging and foodborne outbreaks in Europe (COMPARE) project (www.compare-europe-eu). Extensive follow-up sampling was carried out to monitor the evolution of the infection (Table).

MERS-CoV RNA was detected over eight days after fever onset, in serum samples, but only in the first four

Figure

Timeline of events for the first imported case of MERS-CoV infection (Patient C) identified in China, May–June 2015



MERS-CoV: Middle East respiratory syndrome coronavirus.
The horizontal lines indicate the days when MERS-CoV was detected in various samples.

days in throat swabs. Sputum was collected and the result of the test was positive for MERS-CoV from Day 7 when pneumonia was detected. We tested two faecal samples and obtained positive results on Day 10 and 15. Sample collection and testing are still ongoing.

Contact tracing

Contact tracing was conducted immediately after the confirmation of MERS-CoV infection. According to the guidelines of the National Health and Family Planning Commission of the People's Republic of China (PRC), stewards and passengers seated two rows in front and behind the case and persons who had prolonged (>15 min) face-to-face contact with the confirmed case, in any community settings (hotels, restaurants, conference rooms) were all defined as close contacts. They were included on the mandatory contact investigation list, because the case was symptomatic and potentially contagious. On 28 May, a press release was issued to inform the general public about the MERS case. Information including the travel routes of this case and preventive measures was made public via TV channels and Internet to facilitate efficient contact tracing. As of 2 June, a total of 78 close contacts including hotel staff (n=27), company employees (n=19), restaurant waiters (n=13), bus passengers (n=13), plane passengers (n=6) (passengers who stayed in Hong Kong were monitored by the Hong Kong Centre for Health Protection) were identified and monitored in isolation for 14 days after their last contact with the confirmed case. After 14 days of isolation, none of the contacts presented symptoms compatible with MERS-CoV infection. Throat swabs were collected on their first and last day of quarantine and all were negative for MERS-CoV. Strict infection control measures have been taken in hospital; healthcare workers used PPE during management of this patient and therefore, they were not considered as close contacts. However, for safety reasons, serum samples were collected from 53 healthcare workers on 10 June; all were MERS-CoV negative and the follow-up is still ongoing.

Discussion

MERS-CoV is a newly emergent subgroup C betacoronavirus, with a high mortality of ca 40% [1]. As of 31 May 2015, at least 1,150 laboratory-confirmed cases including 431 related deaths have been reported to WHO [2]. Although the majority of cases occurred in countries in the Arabian Peninsula, MERS cases involving international travel occurred in at least 15 countries [3-5].

The WHO risk assessment for MERS-CoV indicated that cases will continue to be exported to other countries as a result of international travelling [2]. Here, we reported the first imported case of MERS-CoV infection identified in mainland China, related to the ongoing MERS cluster in South Korea. The first identified MERS case in South Korea was a traveler returning from the Arabian Peninsula. Unlike the situation for previously reported travel-associated MERS cases, onward transmission

has been recently observed in South Korea, suggesting that human-to-human transmission could occur in countries outside the Arabian Peninsula and that these countries should also maintain a high level of vigilance. The WHO has published guidelines for case investigation including contact tracing since the first identification of MERS-CoV infection [6]. Control measures including quarantine of suspected cases, which have been proven effective in preventing the further spread of acute infectious diseases, may be hampered in countries with no supporting regulation in place. Therefore, in our view, without appropriate legislation, it may be more difficult to implement these recommendations. As illustrated by this incident that required a massive public health effort, infectious diseases are a global issue. While no contacts became infected in China, the spread in South Korea shows that secondary infection does constitute a risk. Therefore, until more is understood about the epidemiology and factors contributing to the spread of MERS-CoV, we believe that mandatory close monitoring and investigation of all close contacts are crucial.

This incident highlights vulnerabilities and gaps of our surveillance system, not all of which can be addressed. The early presentation of MERS-CoV or other emerging infections may not be specific [7]. Fever was observed when the case arrived in Hong Kong, but without active reporting of the previous high-risk exposure, it was reasonable not to initiate further investigation. There was no health check for this case at the entry point in mainland China, since MERS-CoV-related inquiry at entry point of mainland China mainly targets travellers returning from the Middle East. The increased number of countries outside the Arabian Peninsula affected by MERS-CoV highlights the need for enhanced awareness on the presence of the virus in travellers with fever from countries with ongoing epidemics. During our investigation, we observed sometimes people preferred not to disclose their history of exposure to a MERS case because of insufficient knowledge on the disease and its associated risks, or on the public health actions around it. Education of the public about MERS-CoV including symptoms, transmission modes, infection and prevention measures and risks, are critical to prevent the possible spread of MERS-CoV.

In this study, MERS-CoV RNA was detected in throat swabs only in the first two days of sampling after hospitalisation (four days after fever onset), while increased viral loads were observed in sputum seven days after fever onset when pneumonia was detected. This was consistent with previous studies that recommend that lower respiratory tract samples be given a high priority for MERS-CoV diagnosis especially in patients presenting late in their disease course with lower respiratory involvement [8,9]. We also obtained positive results when we tested stool and serum samples. Due to the possibility of viral shedding, comprehensive precautions for healthcare workers managing probable or confirmed MERS cases, are important. So far, data on MERS-CoV shedding were very rare and have shown different MERS-CoV detection profiles [10-12]. The complete viral load profiles from a large number of patients are essential for establishing infection control measures and their necessary duration. This can also be used to monitor possible early signs of virus change: the apparent deep respiratory tract tropism of MERS-CoV in this patient was an indication that the virus causing the large cluster in South Korea did not behave differently, as concluded from the initial sequence data (data not shown). Subtle changes in the virus-host interaction that would lead to increased replication in the upper respiratory tract could potentially lead to much more efficient transmission. Therefore, detailed virological monitoring, in addition to case and contact investigations, is crucial for monitoring evolution of emerging infectious diseases.

Table

Real-time RT-PCR results for an imported case of MERS-CoV infection, China, May–June 2015

Day after fever onset[a]	Threshold cycle (Ct) values of MERS-CoV upE and ORF1b gene							
	Throat swab		Serum		Sputum		Stool	
	ORF1b	upE	ORF1b	upE	ORF1b	upE	ORF1b	upE
D3	32	32	36	39	NA	NA	NA	NA
D4	34	35	35	36	NA	NA	NA	NA
D5	ND	ND	34	32	NA	NA	NA	NA
D6	ND	ND	36	36	NA	NA	NA	NA
D7	ND	ND	NA	NA	34	36	NA	NA
D8	ND	ND	36	35	30	32	NA	NA
D9	ND	ND	NA	NA	29	31	NA	NA
D10	ND	ND	ND	ND	25	24	35	31
D11	ND	ND	ND	ND	25	26	NA	NA
D12	ND	ND	ND	ND	27	28	NA	NA
D13	ND	ND	NA	NA	28	29	NA	NA
D14	NA	NA	ND	ND	NA	NA	NA	NA
D15	NA	NA	ND	ND	ND	ND	36	36

D: day; MERS-CoV: Middle East respiratory syndrome coronavirus; NA: not available; ND: not detected; ORF: open reading frame; upE: upstream of envelope gene.

[a] Day of fever onset (D0): 25 May 2015.

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Conflict of interest

None declared.

Authors' contributions

All authors contributed to gathering and analysis of the information. Lina Yi, Jie Wu and Changwen Ke drafted the manuscript.

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Emergence of a new GII.17 norovirus variant in patients with acute gastroenteritis in Jiangsu, China, September 2014 to March 2015

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From September 2014 to March 2015, 23 outbreaks of norovirus (NoV) acute gastroenteritis occurred in Jiangsu, China. Partial sequencing of the NoV capsid gene suggested that 16 of the 23 outbreaks were related to a new GII.17 variant. This variant was first detected in sporadic specimens in October 2014, and became predominant in February 2015. Analysis of the RNA-dependent RNA polymerase (RdRp), and complete capsid including the protruding domain P2 sequences confirmed this GII.17 variant as distinct from previously identified GII variants.

Norovirus (NoV) is a major cause of viral gastroenteritis and is the main aetiological agent ofoutbreaks of acute gastroenteritis [1]. It is estimated that each year NoV cause 64,000 episodes of diarrhoea requiring hospitalisation and 900,000 clinic visits among children in industrialised countries, and up to 200,000 deaths of children <5 years of age in developing countries [2].

NoVs are classified into six genogroups, GI–GVI, of which genogroup I, II, and IV are responsible for disease in humans [3,4]. Genogroups are subdivided further into genotypes. To date, based on RNA-dependent RNA polymerase (RdRp) and capid gene sequences, 31 and 22 genotypes of GII NoVs have been respectively determined [5]. Of these, GII.4 caused at least six epidemics of gastroenteritis worldwide over the past 20 years (1995–1996, 2002, 2004, 2006, 2009, and 2012) with the emergence and rapid global spread of viral variants [6]. In contrast, GII.17 NoV has rarely been reported as a major genotype causing diarrhoea.

In late 2014, the Emergent Public Health Event Information Management System (EPHEIM) in Jiangsu province observed an increase of NoV outbreaks compared with previous seasons. Data from these outbreaks indicated that this increase was associated with the emergence of a new variant of GII.17, which was rarely reported in China before 2014. Surveillance of NoV in both outbreak and sporadic cases was conducted from September 2014 to March 2015 to study the molecular epidemiology characteristics of GII.17-associated diarrhoea in Jiangsu province, China.

Methods

Surveillance of gastroenteritis

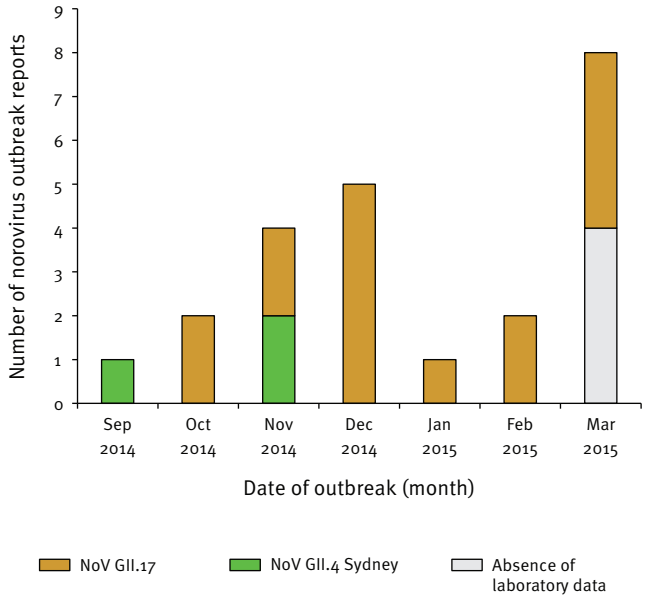
Outbreaks of gastroenteritis and the occurrence of sporadic cases were monitored through different surveillance systems.

Outbreak data were obtained from EPHEIM. An acute gastroenteritis outbreak was defined as ≥20 cases of vomiting and/or diarrhoea associated with a common exposure.

Data on sporadic cases of gastroenteritis in the September 2014 to March 2015 period originated from two surveillance systems in Jiangsu province. The first was the comprehensive surveillance of infectious diarrhoea, which was launched in March 2012 for epidemiological and aetiological surveillance of diarrhoea in children (≤15 years-old) and adult outpatients. This surveillance was conducted via 26 hospitals located in Nantong, Wuxi and Xuzhou, three cities chosen for their location in the southern, central and northern

Figure 1

Numbers of laboratory-confirmed norovirus (NoV) outbreaks per month in Jiangsu province, China, September 2014–March 2015 (n=23)



part of the province. Nantong, Wuxi and Xuzhou have a population of 7.298 million, 6.484 million and 8.591 million respectively, and totally account for 28% of the province's whole population (79.395 million). The second surveillance system for sporadic cases was that of diarrhoea of viral origin in Jiangsu province, which was launched in January 2006 for the epidemiological and aetiological surveillance of viral diarrhoea in children hospitalised in Suzhou Children's Hospital in Suzhou. This hospital is the second largest paediatric hospital in Jiangsu province.

Sporadic specimens that were laboratory-confirmed for any viral agent causing gastroenteritis were submitted to the laboratory of Jiangsu Provincial Center for Disease Control and Prevention (JSCDC) on a monthly basis for further analysis.

Testing samples for norovirus

For viral RNA extraction, a 10% (wt/vol) stool suspension in RNase-free water at a total volume of 1 ml was centrifuged for 5 min at 2,370xg. The supernatant was further processed with a MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems, CA, US) on an automated MagMAX Express24 Magnetic Particle Processor (Applied Biosystems, CA, US) according to the manufacturer's instructions.

The presence of NoV RNA was tested for GII NoVs by using the Qiagen Probe RT-PCR Kit (Qiagen, Hilden, Germany) on a 7500 real-time PCR platform (Applied Biosystems, Singapore) with primers (Cog2F/Cog2R) as described previously [7].

Molecular characterisation of the norovirus

RNA from NoV positive specimens was analysed by reverse transcription-polymerase chain reaction (RT-PCR) directed at the region C of the capsid gene (open reading frame (ORF)2; 344bp), using the previously described primers G2SKF/G2SKR [8].

Region A sequences of the RdRp gene in ORF1 were obtained by using a semi-nested GII-specific primer set (NV2F/G2SKR for a first-round PCR and p289IUB/G2SKR for a second-round PCR) [8-10], which amplified a region of 1,095 bp in the ORF1/ORF2 junction of the viral genome.

Extracted viral RNA was reverse transcribed to cDNA with a VN3T20 primer by using the Superscript III cDNA synthesis kit (Invitrogen, CA, US). ORF2 gene sequences encoding the major capsid protein viral protein 1 (VP1) were obtained by using a semi-nested PCR GII-specific primer set (COG-2F/VN3T20 in the first-round PCR and G2SKF/VN3T20 for the second-round PCR) [7,8,11].

The PCR products were purified and then sent to the Sangon Biotech (Shanghai) Company for sequencing. The nucleotide sequences data of GII.17 variants were deposited in GenBank under accession numbers KR270442–KR270449.

Preliminary genotypes were assigned by using the norovirus genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtool).

The phylogenetic analysis of aligned sequences was carried out using Molecular Evolutionary Genetics Analysis (MEGA) 5.1 [12]. The reliability of the phylogenetic tree was assessed by bootstrap sampling of 1,000 replicates, and genetic distances were calculated by Kimura's 2 parameter method [13].

Results

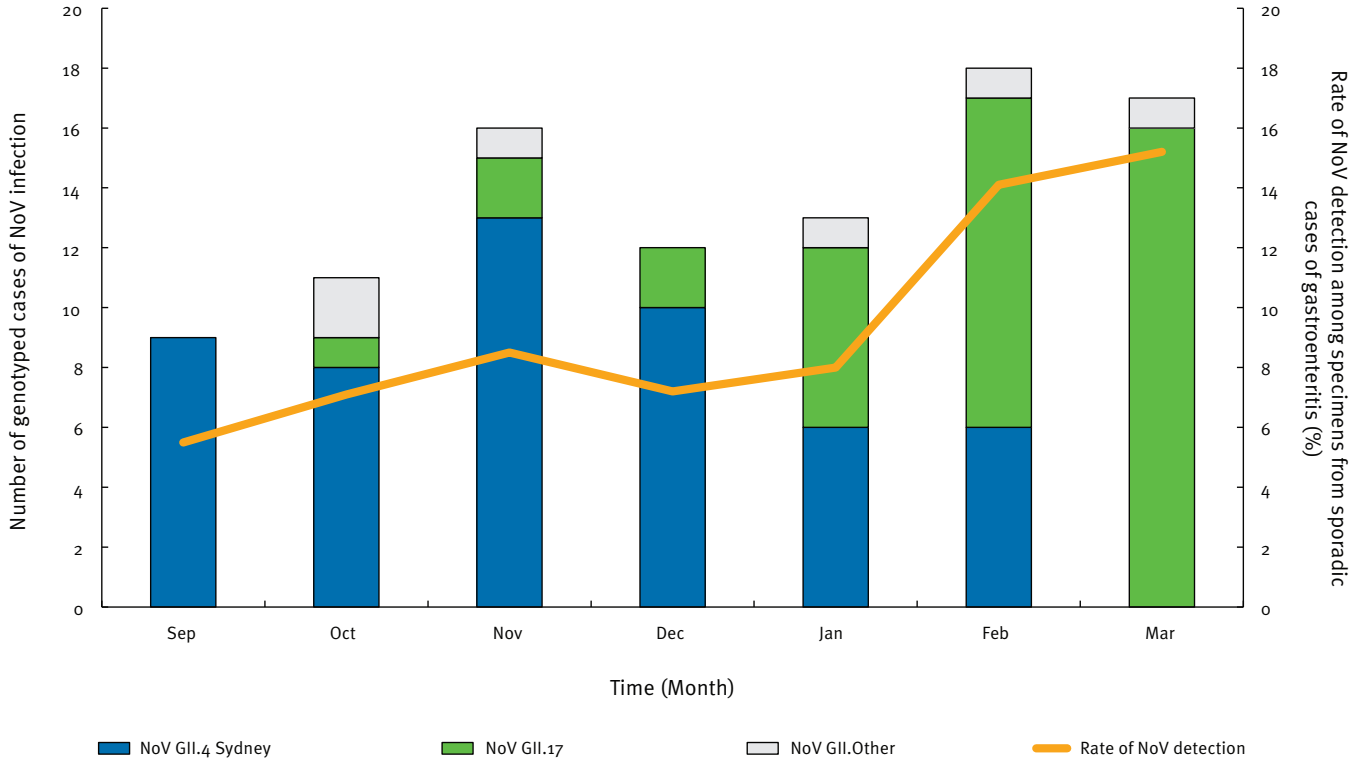
Epidemiological features and genotyping of noroviruses

From September 2014 to the end of March 2015, there were 23 norovirus laboratory-confirmed outbreaks reported to EPHEIM in Jiangsu province, substantially higher than during the same time period in the previous years 2013 to 2014. JSCDC received specimens from all outbreaks up to February 2015, however not from all the outbreaks in March 2015. This resulted in specimens being available from 19 of the 23 outbreaks. Sequence analysis of the Region C of the capsid gene from these 19 outbreaks (Figure 1) showed that three outbreaks (3/23) were caused by GII.4 Sydney, while the other 16 outbreaks (16/23) were all caused by a novel GII.17 variant. This novel variant was first identified in October 2014 and became the dominant strain from December 2014 to March 2015 (Figure 1).

During the study period, 132/1,077 sporadic specimens were positive for norovirus (79/783 outpatient,

Figure 2

Monthly distribution of norovirus (NoV) genotypes among sporadic NoV infections and monthly detection rate of NoV among specimens from sporadic cases of gastroenteritis, Jiangsu province, China, September 2014–March 2015



During the study period, 132/1,077 specimens obtained from sporadic cases of gastroenteritis were positive for NoV. The orange curve shows a plot of monthly detection rates of NoV among specimens.

Of the 132 specimens testing positive for norovirus, genotype was determined for a total of 95 samples. The chart vertical bars represent the total monthly number of specimens of each genotype.

and 53/294 hospitalised children), and partial capsid sequences (Region C) of 95 strains were sequenced, including 57/79 outpatient, and 38/53 hospitalised children. The sequencing of the 95 sporadic specimens resulted in the identification of five capsid genotypes (GII.2, GII.3, GII.13, GII.4 Sydney, and GII.17). A novel GII.17 variant was first identified in October 2014 with only one strain, compared with eight strains of GII.4 Sydney. GII.4 Sydney variant remained the dominant strain from September to December 2014 (9/9 in September, 8/11 in October, 13/16 in November, and 10/12 in December). In January 2015, the proportion of GII.4 Sydney decreased to 6/13, but that of the GII.17 variant increased to 6/13. In February 2015, the proportion of GII.17 variant further increased to 11/17 making it the predominant variant. This predominance continued in March 2015 when GII.4 Sydney was no longer detected. Along with the increased number of confirmed NoV GII.17 specimens, a higher detection rate of NoV among samples from sporadic cases of gastroenteritis was observed in February and March of 2015 (Figure 2).

Phylogenetic Analysis

The Region A and complete VP1 region of eight GII.17 variants (3 specimens from 3 respective outbreaks and 5 sporadic specimens) were further compared with other GII.17 strains by phylogenetic analysis.

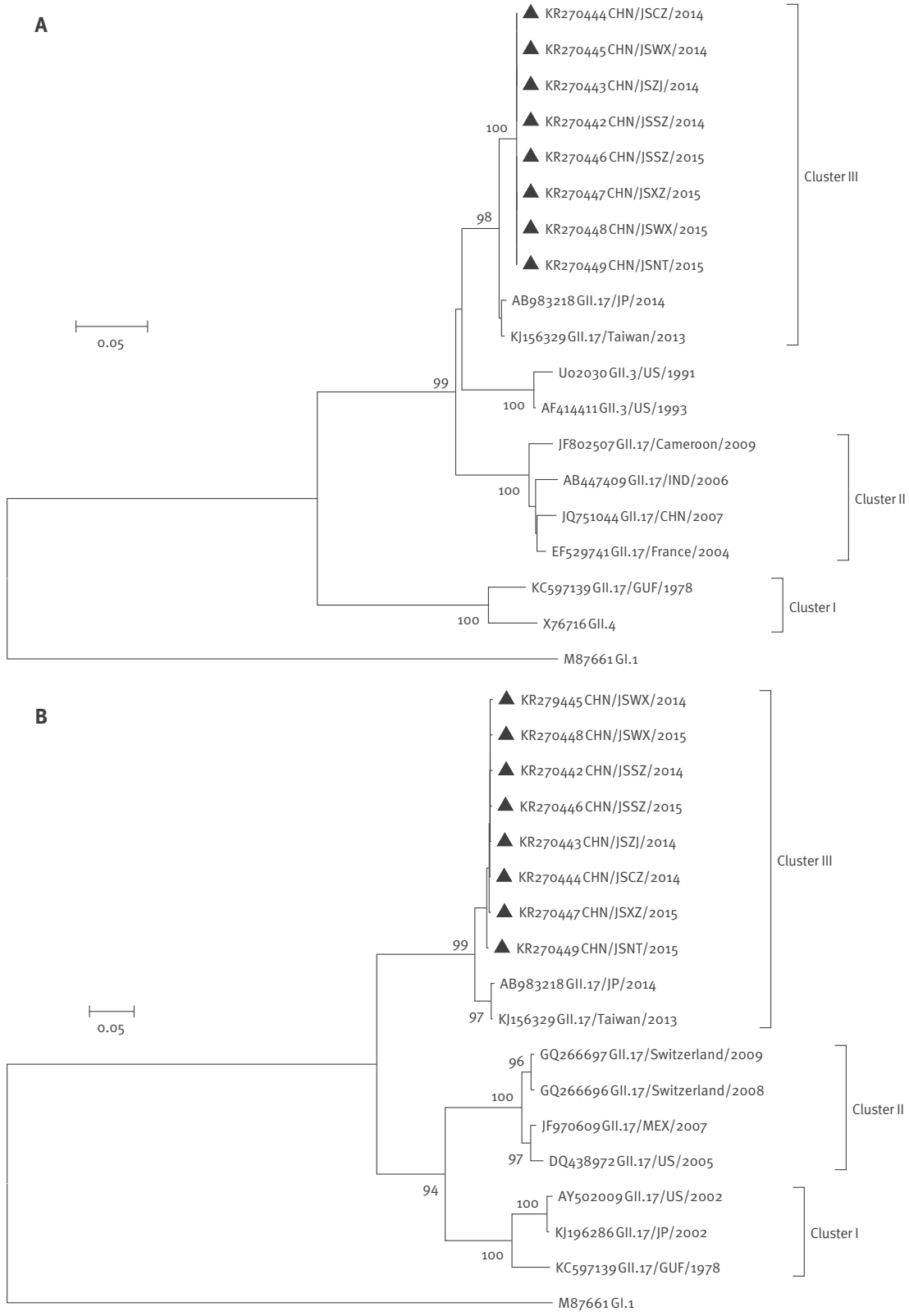
The GII.17 strains were segregated into three distinct genetic groups both in Region A (Figure 3a) and VP1 region (Figure 3b).

In the tree based on Region A, the GII.17 strain characterised in 1978 grouped with a GII.4 Rpdp, forming Cluster I. This cluster appeared to be ancestral to GII.17 sequences reported after 2003. The GII.17 variants identified in the period from 2004 to 2011 formed Cluster II and the variants originating from 2013 to 2015 formed Cluster III. Sequences from Cluster II and Cluster III shared a high nucleotide identity with GII.3 reference strains, especially those in Cluster III (Figure 3a).

In the tree based on the VP1 gene, the GII.17 variants from 1978 to 2002 formed Cluster I, those from 2005

Figure 3

Phylogenetic trees based on (a) Region A including the RNA-dependent RNA polymerase in open reading frame (ORF) 1 and (b) the complete major capsid protein viral protein 1 (VP1) in ORF 2



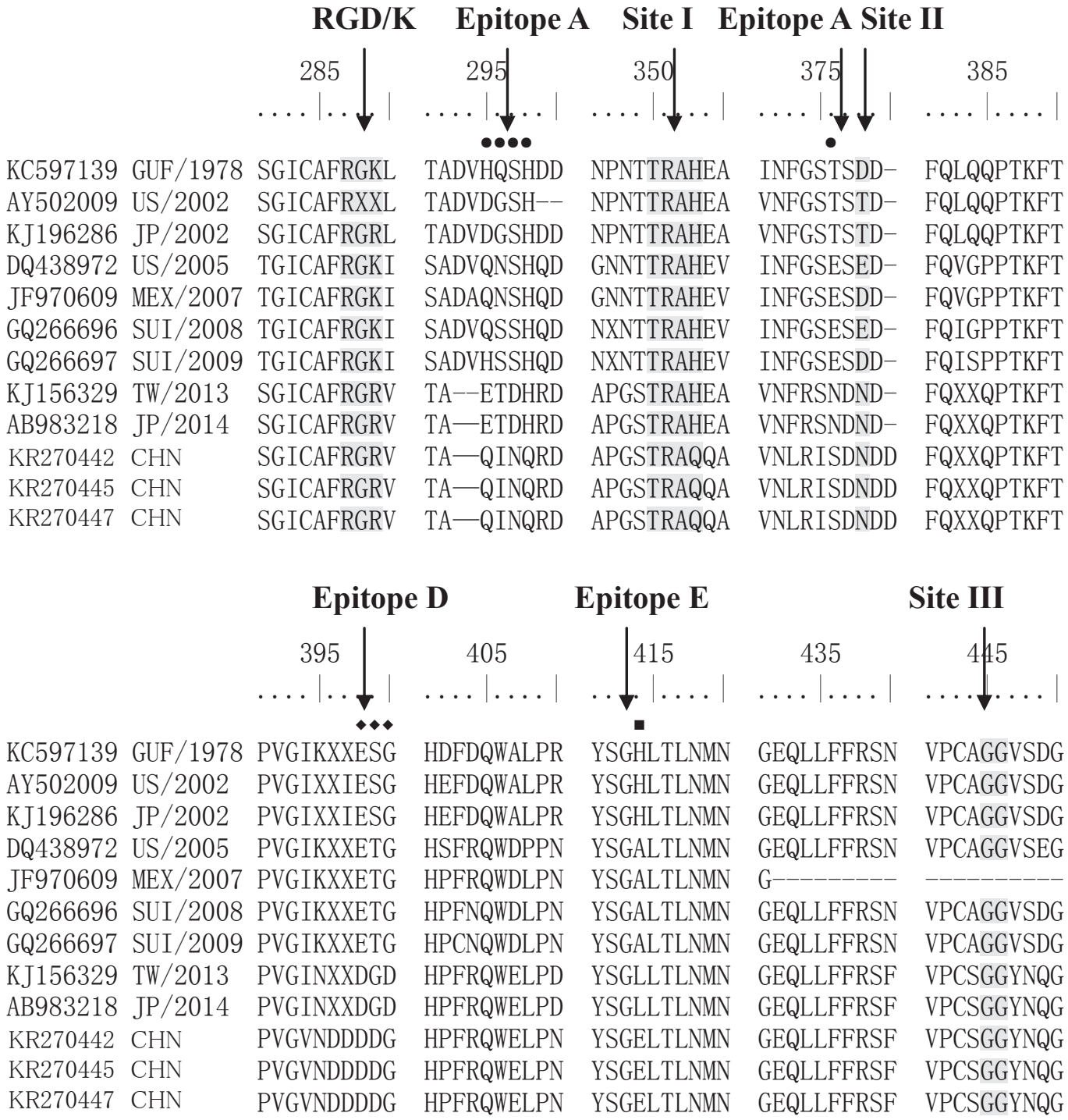
The scale bars indicate the number of nucleotide substitutions per site for the phylogenetic trees.

The numbers on the branches represent the percentage bootstrap support for the nodes after 1,000 iterations. Genbank accession numbers of all sequences used for the phylogenetic analysis figure in the respective trees.

In the two trees, the GII.17 respective VP1 and Region A sequences obtained from the eight strains characterised in the present study (Jiangsu province, China, October 2014–March 2015) are indicated by black triangles.

Figure 4

Alignment of sequences of viral protein 1 protruding P2 domain derived from norovirus (NoV) GII.17 strains



The VP1 amino acid numbering is based on the GII.17 prototype strain KC597139/GUF/1978. Dots indicate sequence identity among sequences presented in the alignment.

Amino acid positions corresponding to GII.4 predicted antibody binding regions epitope A, D, E [14] are marked by symbols: black circles, epitope A; black diamonds, epitope D; black square, epitope E.

Grey regions indicate the RGD motif [15] and site I, II, and III, which are putative histo-blood group antigen (HBGA)-binding sequences of GII NoV genotypes [16].

to 2009 formed Cluster II and those from 2013 to 2015 formed Cluster III.
All of the eight GII.17 variants reported here from Jiangsu province were in Cluster III and grouped with GII.17 strains from Taiwan (KJ156329, 2013) and Japan (AB983218, 2014) in both the polymerase- and VP1 region-based trees.

Twelve GII.17 capsid protein VP1 sequences from 1978 to 2014 were aligned, including nine GII.17 sequences released previously in the GenBank database and three sequences from representative strains obtained in our study. Sequence data showed that amino acid (aa) differences occurred mostly in the protruding P2 domain (Figure 4), particularly at aa 295–297, 376, 398–400 and 414 which form the predicted antibody binding regions in variant GII.4; epitope A (aa295–297, 376), epitope D (aa398–400) and epitope E (aa 414) [14]. In addition, some of the GII.17 strains, including the new GII.17 strains reported here, had a K289R mutation in the alert RGD/K-like motif, located at positions 287–289 [15]. In the three histo-blood group antigen (HBGA) binding sites, a single aa change (H353Q) at site I and a single aa (D380) insertion peripheral to site II occurred in the outbreak representative strains [16].

Discussion

Through the web-based surveillance system EPHEIM, increased levels of NoV activity were detected in late 2014 compared with previous season in Jiangsu province, China. Our findings suggest that this coincided with the emergence of a novel GII.17 variant, which caused most (16/23) of the NoV outbreaks reported between September 2014 and March 2015 in the province. Due to unavailability of laboratory data from four outbreaks in March, the number of GII.17-associated outbreaks could have been underestimated.

The novel variant was first noted in October of 2014, and spread rapidly throughout the province, causing an increasing number of outbreaks. During the course of the winter and early spring it became the predominant cause of NoV outbreaks (Figure 1), replacing the GII.4 Sydney variant starting from December 2014. Newly identified emerging variant that become predominant have been previously reported, in particular GII.4 variants, and these can also be associated with atypical increases in the incidence of acute gastroenteritis [17,18]. In Jiangsu province for example, an earlier dominant variant, GII.4-2006b, had been replaced by GII.4 Sydney in 2012/13 [19].

Based on the surveillance of NoV sporadic cases in this study, while the number of NoV GII.4 Sydney cases decreased in January 2015, sharp increases in the number of GII.17 cases were observed in February and March (Figure 2). In these two months the detection rate of NoV-positive specimens among specimens of sporadic cases of gastroenteritis also increased.

Phylogenetic analysis of GII variants, including GII.17 strains obtained in this and other studies, suggests that RNA recombination, a significant driving force in viral evolution [20,21] led to some characteristics of the novel GII.17 variant reported here. In the RdRp phylogenetic tree obtained in this study, the GII.17 strains formed three Clusters. Cluster I comprised the only GII.17 strain reported before the year 2000 as well as a GII.4 variant. Cluster II and Cluster III contained RdRp sequences of GII.17 strains, all found after 2003, and which additionally shared a high nucleotide identity with GII.3 reference strains characterised in the 1990s, especially those in Cluster III (Figure 3a). The fact that the only one GII.17 strain reported before 2000 had a GII.4 RdRp genotype, while most GII.17 NoVs detected in the 2000s possessed a GII.3-like RdRp genotype leads to hypothesise that the new GII.17 variant may be a recombinant strains with a GII.3-like RdRp gene and a GII.17 capsid gene. Interestingly, most GII.3 strains detected in the 2000s were recombinant strains, possessing a non-GII.3 RdRp genotype [22].

The VP1 protein P2 domain is the most exposed region of the viral particle and is well positioned to interact with potential neutralising antibodies and HBGA ligands. Mutations in this domain may have a significant effect on virus receptor binding and the host immune response to viral infection [23,24] and mutations in the P2 domain were observed in the new GII.17 variant. We speculate that, through the accumulation of mutations at several sites in the P2 domain, a new antigenic variant of the GII.17 lineage which gains the potential to escape herd host immunity could occur eventually. However, more studies, such as studies including virus-like particles (VLP)-HBGA binding assays, are needed to provide insights into the complex interaction between NoV GII.17 and their ligands.

The limitation of our study was that our results were not from nationwide surveillance but from Jiangsu province accounting for only 5.4% of the total China population. However, a similar situation to the one reported here was observed in 2012/13 with the emergence of the GII.4 Sydney variant, which was first detected in a NoV outbreak in late 2012, and soon afterwards led to large increases in NoV activity nationwide [19].

In conclusion, the new GII.17 variant which emerged in October 2014 appears to have subsequently increasingly caused NoV outbreaks in Jiangsu province, China. This study reveals that the variant presents a number of mutations in the P2 domain of VP1. Simultaneous dominance by GII.17 in outbreaks and sporadic infections indicates that this genotype might be established in Jiangsu Province. Nationwide surveillance for NoV outbreaks will be needed to understand epidemiological or outbreak trends related to the emergence of relatively rare GII.17 variants. Furthermore research into the mechanisms driving the evolution of NoV strains is also important for the development of effective prevention and control strategies.

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Conflict of interest

None declared.

Authors' contributions

Yefei Zhu, Miao Jin, Changjun Bao, and Fenyang Tang designed the study. Jianguang Fu, Jing Ai, Jun Zhang, Chao Shi, Qin Lin, and Zhaohu Yuan collected, analysed, and interpreted data. Jianguang Fu and Xian Qi characterised the specimens. Jianguang Fu, Jing Ai, and Yefei Zhu drafted the article. All authors reviewed and revised the first and final drafts of this manuscript.

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Detection of livestock-associated meticillin-resistant Staphylococcus aureus CC398 in retail pork, United Kingdom, February 2015

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Livestock-associated meticillin-resistant *Staphylococcus aureus* belonging to clonal complex 398 (LA-MRSA CC398) is an important cause of zoonotic infections in many countries. Here, we describe the isolation of LA-MRSA CC398 from retail meat samples of United Kingdom (UK) farm origin. Our findings indicate that this lineage is probably established in UK pig farms and demonstrate a potential pathway for the transmission of LA-MRSA CC398 from livestock to humans in the UK.

A survey was conducted in February 2015 to detect meticillin-resistant *Staphylococcus aureus* (MRSA) in retail meat products obtained from supermarkets in the United Kingdom (UK). A total of 103 (52 pork and 51 chicken) pre-packaged fresh meat products, labelled as being of UK farm origin, were purchased from supermarkets in five different locations (Locations A-E) in the UK. All meat products were frozen (-20 °C) and sent to the Department of Veterinary Medicine, University of Cambridge, for testing.

Preparation and testing of meat samples

The preparation of meat samples followed the European standard ISO 6887–2:2003 [1]. After thawing, the exterior packaging was disinfected before the meat was removed. A 10 g sample of meat was excised, mixed with 225 ml of 6% w/v NaCl Nutrient Broth (P and O laboratories, UK) and homogenised using a Stomacher (Stomcher80 Laboratory System, Seward Ltd, UK) for two minutes. Enrichment for *S. aureus* was performed as previously described [2]. Identification of potential MRSA colonies (blue colour) was confirmed by subculture on MRSA Brilliance 24 plates (Oxoid, Baskingstoke, UK) which were subsequently screened for *mecA*, *mecC* and *femB* by multiplex PCR as described previously [3]. Potential MRSA colonies subjected to PCR testing initially yielded two *mecA* positive cultures (samples C7 and D8). Three colonies from subcultures from each of these original samples were *spa* typed as described previously [4] which yielded a single *spa* type from one sample and two different *spa* types from the other.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all three isolates was analysed using the VITEK 2 system (bioMérieux, Basingstoke, UK) in accordance with the manufacturer's instructions using a Staph AST-P635 card with results interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [5]. Antimicrobial susceptibility results (Table 1) showed that all three isolates were phenotypically MRSA and were additionally resistant to tetracycline and trimethoprim.

Genomic analyses

Genomic DNA of all three *S. aureus* isolates was extracted from overnight cultures grown in TSB at 37 °C using the MasterPure Gram Positive DNA Purification Kit (Cambio, Cambridge, UK). Illumina library preparation was carried out as described by Quail et al. [6] and Mi-Seq sequencing was carried out following the manufacturer's standard protocols (Illumina, Inc., San Diego, CA, US). Genomes were assembled de novo from Fastq files with Velvet [7]. The draft sequences for C7–1, C7–2 and D8 had a total of 38, 22 and 31 contigs, respectively. Comparative genomics were carried out using WebACT and viewed with the Artemis comparison tool (ACT) [8]. The presence of antibiotic resistance genes was identified using the ResFinder-1.3 Server [9] and by BLAST [10] against the assemblies. Nucleotide sequences of isolates C7–1, C7–2 and D8 have been

Table 1

Antimicrobial susceptibility characteristics of meticillin-resistant *Staphylococcus aureus* CC398 from retail pork samples, United Kingdom, February 2015 (n = 3)

Isolate	Benzylpenicillin	Cefoxitin	Oxacillin	Ciprofloxacin	Clindamycin	Erythromycin	Tetracycline	Trimethoprim
C7–1	R	R	R	R	S	S	R	R
C7–2	R	R	R	S	R	S	R	R
D8	R	R	R	R	S	R	R	R

R: resistant; S: susceptible.

Results of testing using a VITEK 2 system (bioMérieux, Basingstoke, UK) using a Staph AST-P635 card (testing for susceptibility to cefoxitin, benzylpenicillin, oxacillin, gentamycin, ciprofloxacin, clindamycin, erythromycin, linezolid, daptomycin, teicoplanin, vancomycin, tetracycline, fusidic acid, mupirocin, chloramphenicol, rifampicin, and trimethoprim). All three isolates were susceptible to gentamycin, linezolid, daptomycin, teicoplanin, vancomycin, fusidic acid, mupirocin, chloramphenicol and rifampicin. Breakpoints were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

deposited in the European short read archive with accession numbers ERR902083, ERR902084 and ERR902085, respectively.

Multilocus sequence typing using the assembly sequences found that all three isolates belonged to sequence type ST398 and carried a composite staphylococcal cassette chromosome *mec* (SCC*mec*) V(5C2 and 5)c element including the cadmium and zinc resistance gene *czrC* [11]. All isolates lacked the *lukS-PV* and *lukF-PV* genes encoding Panton-Valentine leukocidin and the human-associated immune evasion cluster genes *sak*, *scn* and *chp* (often carried by the phage φSa3) [12]. All three isolates carried an extra copy of the von Willebrand factor-binding protein (vWbp) gene, *vwb* previously found on pathogenicity island SaPIbov5 in a ST398 isolate which confers the ability to clot ruminant plasma [13]. Genomic analysis demonstrated the presence of the tetracycline resistance genes *tet*(M) and *tet*(K) in addition to *mecA*, in all three isolates, together with other resistance determinants which varied between isolates and matched their antimicrobial susceptibilities (Tables 1 and 2). Three canonical single nt polymorphisms (canSNP) shown by Stegger et al. [14] to distinguish between human and livestock clades of ST398 had the livestock associated nt in all three positions for all three of the isolates.

Discussion

Here we describe the first isolation of LA-MRSA ST398 from retail meat originating from farms in the UK. Recent reports of CC398 isolates from horses [15], dairy cattle [2], poultry [16], and pigs [17,18] indicate that this lineage is widely distributed in the UK. In many countries LA-MRSA CC398 represents an occupational risk for those in close contact with livestock,

Table 2

Molecular characteristics of meticillin-resistant *Staphylococcus aureus* CC398 from retail pork samples, United Kingdom, February 2015 (n = 3)

Isolate	Location	Meat type	MLST	*spa* Type	SCC*mec* type	φSa3	canSNP 748	canSNP 1002	canSNP 3737	*tet*(M)	*tet*(K)	Other
C7–1	C	Pork sausage	ST398	t011	V(5c2 and 5)c	Neg	LA	LA	LA	Pos	Pos	*blaZ dfrK*
C7–2	C	Pork sausage	ST398	t034	V(5c2 and 5)c	Neg	LA	LA	LA	Pos	Pos	*blaZ dfrG spc linB aad9*
D8	D	Pork mince	ST398	t034	V(5c2 and 5)c	Neg	LA	LA	LA	Pos	Pos	*blaZ dfrG aadD lnu(B) erm(C) linB cadR merR*

LA: livestock-associated; MLST: Multilocus sequence typing; Neg: negative; Pos: positive.

The φSa3 phage is associated with human ST398 isolates which carries a cluster of human immune evasion genes [14]. The columns headed canSNP748, canSNP1002 and canSNP3737 refer to canonical SNPs described by Stegger et al. [14] associated with human- or livestock-associated lineages. The antimicrobial resistance genes were identified using the ResFinder-1.3 Server [9].

particularly pigs and veal calves. For example, significantly higher rates of CC398 MRSA nasal carriage by humans in contact with pigs (farm workers, abattoir workers, veterinarians) have been shown in epidemiological studies [19-22]. Other studies have revealed an association between clinical disease resulting from LA-MRSA CC398 infection and recent contact with pigs or pig farms [23-27]. As with other MRSA, LA-MRSA CC398 may be responsible for serious illness following wound or surgery site infections. They may also contribute to increased healthcare costs due to screening, isolation of carriers, and decolonisation. Adequate cooking (heating above 71°C) and hygienic precautions during food preparation should minimise the likelihood of human colonisation via contaminated pork. Still our finding of LA-MRSA CC398 in pork identifies a potential pathway from farms to the wider population. Cuny et al. [28] identified thawing liquid of broiler chicken carcasses as having greater numbers of bacteria which may represent an increased risk for frozen meats. Our study did not examine the thaw water separately and also failed to find ST398 in poultry samples which suggests that this lineage may be present in the UK at lower rates than in continental Europe; however, further studies are required to establish this.

While human contamination of carcasses or meat products in the abattoir or at the meat packing plant may occur, there is evidence that the ST398 isolates are of animal origin. The isolates carried tetracycline resistance genes, lacked the human virulence phage, φSa3, possessed the three canonical SNPs previously shown to identify animal lineages and copies of the von Willebrand factor-binding protein (vWbp) gene associated with livestock [13,14]. The ST398 isolates all came from processed pork (sausages and minced pork) likely to comprise meat from multiple carcases. Testing of these meat products used a highly sensitive method of detection of bacterial contamination and so the numbers of MRSA present may be low. It cannot be ruled out that the meat packing plants from which the MRSA from this study originated also handle imported meat. If this were the case, it is conceivable that cross-contamination might have occurred between non-UK to UK sourced meat. Further phylogenetic studies are required to provide evidence to examine that possibility.

Conclusions

This is the first description of LA-MRSA CC398 in retail meat products in the UK. The presence of a lineage capable of colonising a wide range of host species with a zoonotic potential make this finding of significance for both human and animal health. Furthermore, the presence of LA-MRSA CC398 in the human food chain demonstrates in addition to the established risk through direct contact with animals a possible further pathway for the transmission of antimicrobial resistance from livestock to the broader human population, and not just via those with direct contact with farm animals.

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Conflict of interest

None declared.

Authors' contributions

Nazreen F Hadjirin performed laboratory work and wrote the manuscript, Elizabeth M Lay collected samples and performed laboratory work, Gavin K Paterson performed some of the laboratory work and contributed to the manuscript, Ewan M Harrison performed some of the analysis and contributed to the manuscript, Sharon J Peacock edited the manuscript, Julian Parkhill edited the manuscript, Ruth N Zadoks edited the manuscript, Mark A Holmes designed the study, supervised the laboratory work, undertook some of the analysis and edited the manuscript.

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Systematic review of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccines in children

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In 2010, increased febrile convulsions (FC) occurred after administration of inactivated trivalent influenza vaccine (TIV) in Australia. We systematically reviewed the rates of fever, FC and serious adverse events (SAEs) after TIV, focussing on published and unpublished clinical trial data from 2005 to 2012, and performed meta-analysis of fever rates. From 4,372 records in electronic databases, 18 randomised controlled trials (RCTs), 14 non-randomised clinical trials, six observational studies and 12 registered trials (five RCTs and seven non-randomised) were identified. In published RCTs, fever ≥ 38 °C rates after first dose of non-adjuvanted TIV were 6.7% and 6.9% for children aged 6–35 months and ≥ 3 years, respectively. Analysis of RCTs by vaccine manufacturer showed pooled fever estimates up to 5.1% with Sanofi or GlaxoSmithKline vaccines; bioCSL vaccines were used in two non-randomised clinical trials and one unpublished RCT and were associated with fever in 22.5–37.1% for children aged 6–35 months. In RCTs, FCs occurred at a rate of 1.1 per 1,000 vaccinated children. While most TIVs induced acceptably low fever rates, bioCSL influenza vaccines were associated with much higher rates of fever in young children. Future standardised study methodology and access to individual level data would be illuminating.

Introduction

is a common respiratory viral infection with a substantial disease burden in children younger than five years, of whom between nine and 45 per 10,000 need hospital admission each year in developed countries [1-4]. Vaccination is the leading strategy to combat influenza. The recommendations for influenza vaccination have been progressively expanded and now include all healthy children aged six months and older in the United States (US) and several European countries [5,6]. The United Kingdom's (UK) Joint Committee on Vaccination and Immunisation (JCVI) recommended vaccination of all children two to 17 years of age with live attenuated influenza vaccine (LAIV) from the 2013/14 season onwards, although implementation was being staggered, commencing with two and three year-old children in the first year [7]. In Australia, TIV is funded nationally for any child older than six months with medical conditions predisposing to severe influenza, and in one state (Western Australia) also for healthy children aged six to 59 months [8].

In 2010, an unexpected and marked increase in fever and febrile convulsion (FC) rates in Australian children younger than five years was detected following receipt of the seasonal inactivated trivalent influenza vaccine (TIV). Influenza vaccination for children five years and younger was briefly suspended. The increase in FC (estimated to be between five and seven events per 1,000 vaccinated children) was related only to one brand of TIV, manufactured by bioCSL (Fluvax and Fluvax Junior) [9]. Despite its subsequent deregistration for children younger than five years, public concerns about vaccine safety have persisted, leading to markedly lower influenza vaccine uptake, especially in Western Australia [10]. Published data documenting the frequency and severity of fever after TIV in children are sparse. Furthermore, the age bands reported and fever cut-off values used vary widely, with limited application of standardised definitions such as those from the Brighton Collaboration [11]. We therefore systematically reviewed the evidence for influenza vaccine safety in children to examine the rates of fever, FCs and serious adverse events (SAEs as per standard definition [12]) associated with contemporary TIVs. We also aimed to assess the effect of age, vaccine type

(adjuvanted or not) and vaccine manufacturer on the frequency of these adverse events.

Methods

An electronic literature search, without language restriction, was performed using Medline, Embase, Cochrane Library databases, LILACS, SCOPUS, and Web of Science for studies published between January 2005 and March or April 2012. Our focus was on contemporary vaccines hence our restriction to this publication period. Both controlled vocabulary and text-word terms were used, including 'immunization', 'influenza vaccines', 'influenza, human', 'safety', 'fever', 'seizures, febrile', 'adverse event/effect', 'product surveillance, post-marketing', 'Guillain–Barré syndrome', together with 'child' or 'infant.' A listing of the specific databases, search strategy and coverage dates are available from the corresponding author upon request. In addition, a search was performed within Clinicaltrials.gov, a globally used registry, for phase 2, 3 or 4 clinical trials using TIV in a paediatric population.

We included randomised controlled trials (RCTs), non-randomised clinical trials (with or without a control group) and observational studies. Studies were included if they (i) involved the use of inactivated seasonal TIV, administered intramuscularly, in at least one study arm; (ii) involved healthy children up to 17 years of age; and (iii) presented safety data in an extractable format. Studies were excluded if they only involved children younger than six months or only populations with chronic illness and/or immunocompromise. We analysed data by age band, study design, vaccine type and vaccine manufacturer, where possible. Dose 1 and dose 2 data were analysed separately. Febrile convulsion rates and SAEs were noted, if documented.

The quality of RCT studies was assessed by examining bias using the Cochrane Collaboration's tool for assessing risk of bias [13]; non-randomised clinical trials were assessed by the Effective Public Health Practice Project (EPHPP) Quality Assessment tool, as this better encompassed variation [14,15].

Meta-analysis was conducted on fever data using the Brighton Collaboration case definition of ≥ 38 °C from any source (axillary, oral or rectal) [11]. Due to variability in study methods and a lack of placebo-controlled studies, we conducted a proportion meta-analysis of fever rates using similar single-arm data from trials (StatsDirect statistical software version 2.7.9) to calculate pooled fever proportions. This method has been used previously in systematic reviews across different disciplines [16-21]. A random effects model with the DerSimonian–Laird method was used to account for variability in study design and results. The I^2 statistic was used as a measure of heterogeneity of pooled estimates [13].

We conducted sensitivity analyses of meta-analyses to see if exclusion of high-risk RCTs, or those non-randomised clinical trials rated as weak, reduced heterogeneity. If heterogeneity was unchanged, then all available studies were used for analysis.

Results

Of the 4,372 studies initially identified (Figure), 18 RCTs [22-39], 14 non-randomised clinical trials [40-53], and six observational studies [54-59] were eligible for inclusion. The clinical trial registry search yielded 12 additional relevant studies (five RCTs and seven non-randomised trials). We found substantial variation in study methods, fever definitions, age of participants, year of study, length of follow-up for solicited adverse events, vaccine types and brands.

Characteristics of randomised controlled trials

In the 18 randomised control trials (Table 1), a total of 22,484 subjects were enrolled, of whom 16,474 received TIV and had safety data collected. Multiple study designs were encountered in terms of comparison groups; for non-adjuvanted TIV, comparison with placebo was only found in one study [33]. Five studies examined adjuvanted vaccines (MF59 or virosomal adjuvant) in at least one study arm [30,31,34,35,39].

Classification of fever varied across studies, but a majority of studies [22,25,27,29-31,34,35,37,38] provided data on fever ≥ 38 °C. We used these studies for meta-analysis of fever rate and one additional study [39], where we assumed a fever definition of ≥ 38 °C based on two similar studies by the same lead author [31,35].

Study quality varied using the Cochrane Collaboration's tool for assessing risk of bias. Five studies were assessed as being at low risk of bias [26,31,33-35]. Ten studies had medium risk of bias [22,24,25,27-30,32,38,39], and three studies had high risk [23,36,37]. Sensitivity analyses limited only to low-risk studies were not feasible; there were too few studies, and two did not use a fever definition of ≥ 38 °C.

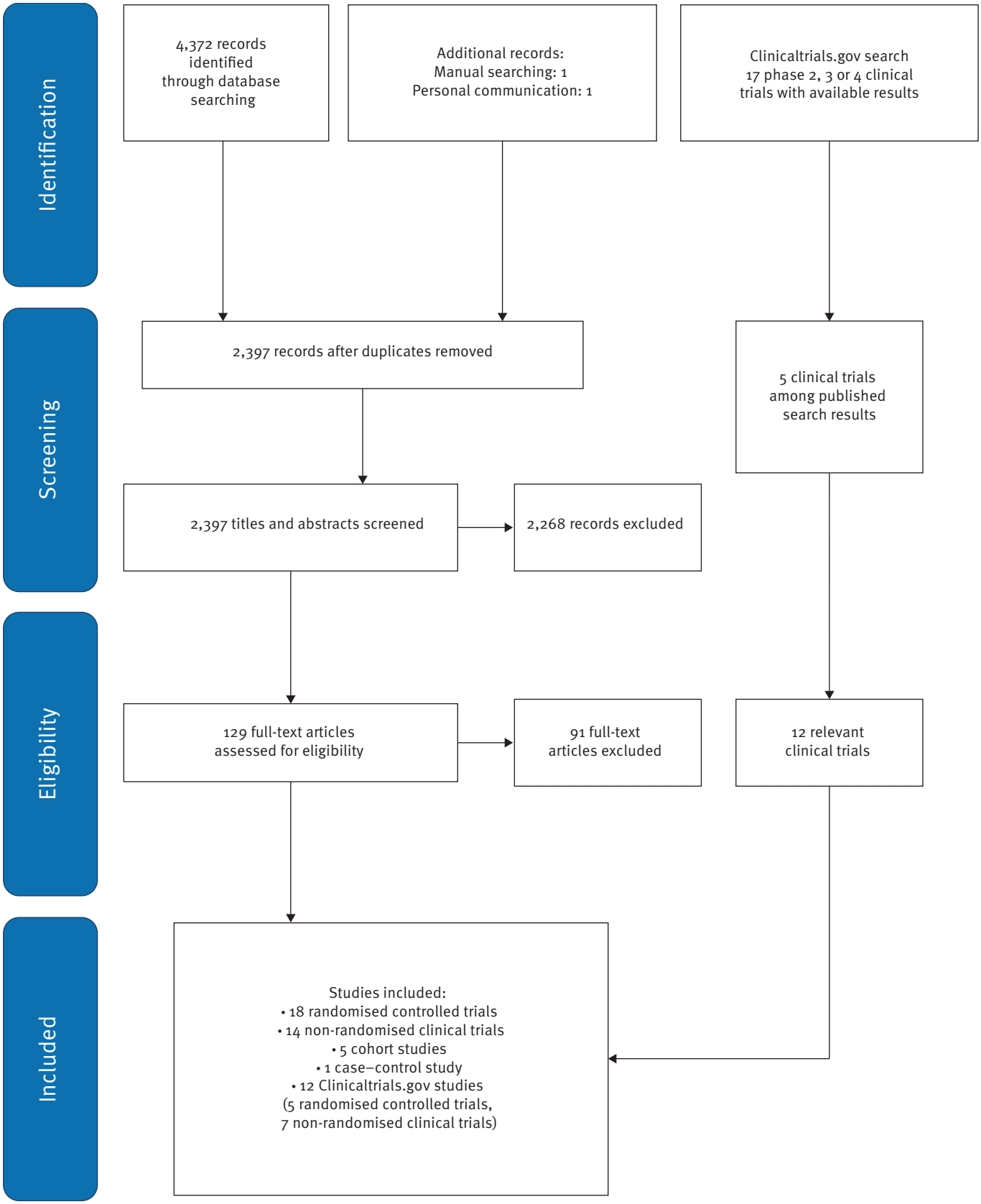
Characteristics of non-randomised clinical trials

Fourteen non-randomised trials were identified (Table 2). Of the 8,119 total participants, 7,901 received TIV and had safety data available. Two studies [48,52] were follow-on studies from previous RCTs. Most used within-study age cohorts for comparison and/or had no control group [40,42,44-47,49,50,53]. For fever meta-analysis, we used five studies with fever defined as ≥ 38 °C [40,41,48,49,52] and two [47,53] where fever was ≥ 37.5 °C axillary or ≥ 38 °C orally (still meeting the Brighton Collaboration criteria [11]).

Overall, a high risk of bias was observed due to lack of randomisation and open-label study designs, without blinding in most studies. In addition, many studies were lacking control groups. Five studies [41,43,48,49,51] were assessed as being of 'moderate' strength while nine studies were 'weak' [40,42,44-47,50,52,53].

Figure

Results of literature search for fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children, and studies analysed



Adapted from PRISMA 2009 Flow Diagram [73].

Table 1a

Characteristics of randomised controlled trials included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Ages	Comparison groups	Enrolment period and location	TIV recipients evaluable for safety	Vaccines used	Vaccine manufacturer	Antigen dose per strain	Length monitoring solicited AE	Length SAE monitoring	Definition of fever	Method of measurement	Risk of bias assessment	Fever rate recordeda
Englund 2005 [22]	6 – 23 months	1. Standard schedule: 2 doses autumn 2. Previous year priming schedule: spring then autumn dose 3. Non-randomly allocated standard schedule	Apr – Jun 2003 United States	259	TIV	Aventis-Pasteur (Sanofi)	15 µg/0.5mL	5 days	6 months	≥ 38 °C	Axillary	Medium	6.7–8.0%
Hu 2005 [23]	6 months – 3 years, 6 – 12 years, 16 – 60 years, › 60 years	1. Fluviral 2. Vaxigrip	Mar – Sep 2004 China	785	Fluviral: TIV Vaxigrip: TIV	Fluviral: Shire Biologics. Vaxigrip: Aventis-Pasteur (Sanofi)	15 µg/0.5 mL	3 days	3 days	Not stated	Not stated	High	5.2–6.3% (6 months–3 years)
Ashkenazi 2006 [24]	6–71months	1. LAIV 2. Inactivated TIV	Oct 2002 9 European countries	1,086	LAIV TIV	LAIV: Wyeth Pharmaceuticals TIV: Aventis Pasteur	15 µg/0.5mL	11 days	To end of study	≥ 37.5 °C axillary or ≥ 38 °C Rectal	Axillary or rectal	Medium	21.4% (TIV) 23.5% (LAIV)
Walter 2006 [25]	6–23months	1. Spring–autumn schedule 2. Standard autumn 2-dose schedule	Apr – Jun 2004 United States	462	TIV	Aventis Pasteur (Sanofi)	15 µg/0.5mL	5 days 3 days for fever	6 months post last vaccine	≥ 38 °C	Axillary	Medium	3.8%
Belshe 2007 [26]	6–59 months	1. LAIV 2. Inactivated TIV	Oct 2004 16 countries	4,173	Fluzone: TIV Vaxigrip: TIV Flumist: LAIV	Fluzone and Vaxigrip: Aventis-Pasteur (Sanofi) LAIV: Medimmune	LAIV: 107 FFU/antigen TIV: not stated	42 days	Median 219 days (180 days after last vaccine)	› 37.8 °C	Oral, axillary or rectal	Low	2% (TIV measured Day 2 only) 5.4% (LAIV measured Day 2 only)
Chiu 2007 [27]	3–18 years	1. Intradermal TIV 2. Intramuscular TIV	Oct – Nov 2005 Hong Kong	56	Fluarix: TIV	GSK	15 µg/0.5mL	3 days	Not stated	› 38 °C	Not stated	Medium	7.1% (intramuscular route)
Zhu 2008 [28]	3–12 years; 18–59 years; › 60 years	1. Influvac 2. Agrippal	2005 China	300	2005–2006: Influvac TIV; 2005–2006: Agrippal TIV	Influvac:Solvay / Abbott Agrippal: Novartis	15 µg/0.5mL	3 days	4 weeks	Not stated	Not stated	Medium	4.0–4.5%
King 2009 [29]	6–59 months	1. Standard TIV 2. Recombinant TIV:	Oct – Nov 2006 United States	156	Fluzone: TIV FluBlok: recombinant TIV	TIV: Sanofi FluBlok: Protein Sciences Corporation	TIV: 15 µg/0.5mL	7 days	180 days	≥ 38 °C	Not stated	Medium	5.3% (standard TIV)
Marchisio 2009 [30]	1–5 years	1. Virosomal-ATIV 2. No treatment	Oct 2006 Italy	90	Inflexal V: virosomal ATIV	Berna Biotech	15 µg/0.5mL	7 days	Not stated	≥ 38 °C	Rectal	Medium	3.3%
Vesikari 2009 [31]	6–35 months	1. MF59 ATIV 2. TIV	Nov 2006 – Aug 2007 Finland	269	Fluad: MF59 ATIV Vaxigrip TIV	Fluad: Novartis Vaxigrip: Sanofi	15 µg/0.5mL	7 days	6 months	≥ 38 °C	Not stated	Low	4.3% TIV 6.9% ATIV
Baxter 2010 [32]	6 months–18 years for safety	1. Fluarix 2. Fluzone	Nov 2006 – Oct 2007 United States	3,325	Fluarix: TIV Fluzone: TIV	Fluarix: GSK Fluzone: Sanofi	15 µg/0.5mL	4 days (0–3)	6 months post first vaccine	≥ 37.5 °C	Axillary	Medium	7.4–7.5%
Cowling 2010 [33]	6–15 years	1. Vaccinated household 2. Placebo household	Nov – Dec 2008 Hong Kong	71	Vaxigrip: TIV	Sanofi Pasteur	15 µg/0.5mL	4 days	10 months	≥ 37.8 °C	Not stated	Low	1.4%
Esposito 2010 [34]	6–35 months	1. 2 doses of 0.50 mL 2. 2 doses of 0.25 mL	Oct 2008 – May 2009 Italy	65	Inflexal V: virosomal ATIV	Crucell	15 µg/0.5mL	14 days	Not stated	≥ 38 °C	Rectal	Low	7.0–9.1%
Vesikari 2010 [35]	6–35 months; 3–8 years; 9–17 years	1. H5N1-MF59 ATIV 2. MF59 ATIV	Sep – Nov 2007 Finland	137	Aflunov H5N1 Fluad: MF59 ATIV	Novartis	7.5 µg/0.5 mL H5N1 15 µg/0.5 mL TIV	7 days	Not stated	≥ 38 °C	Axillary	Low	12.5% (6–35 months, MF59 ATIV group)

Table 1b

Characteristics of randomised controlled trials included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Ages	Comparison groups	Enrolment period and location	TIV recipients evaluable for safety	Vaccines used	Vaccine manufacturer	Antigen dose per strain	Length monitoring solicited AE	Length SAE monitoring	Definition of fever	Method of measurement	Risk of bias assessment	Fever rate recordeda
Hoft 2011 [36]	6–35months	1. TIV/TIV 2. LAIV/LAIV 3. TIV/LAIV 4. LAIV/TIV	2005 – 2007 United States	14	Fluzone: TIV	Sanofi Pasteur	15 µg/0.5mL	14 days	7 months	›37.5 °C	Axillary	High	7.1%
Kang 2011 [37]	6 months–17 years	1. Green Cross TIV 2. Fluarix TIV	Sep – Nov 2008 Korea	282	Green Cross: TIV Fluarix: TIV	Green Cross Fluarix: GSK	15 µg/0.5mL	7 days	Not stated	≥38 °C	Axillary	High	0–3.1%
Skowronski 2011 [38]	6–23 months	1. Full dose 0.5 ml x 2 2. Half dose 0.25 mL x 2	Sep – Dec 2008 Canada	252	Vaxigrip: TIV	Sanofi Pasteur	15 µg/0.5mL	4 days (0–3)	45 days	≥38 °C	Axillary	Medium	2.3% (half dose group)
Vesikari 2011 [39]	6–71 months	1. MF59 ATIV 2. TIV 3. Active placebo -MenC or tickborn encephalitis vaccine	2007 – 2009 Germany and Finland	4,692	2007–08: Fluad MF59 ATIV and Agrippal S1 TIV. 2008–09: Fluad and Influsplit SSW TIV	Fluad: Novartis Agrippal S1: Novartis Influsplit SSW: GSK	15 µg/0.5mL	7 days	Year 1: 6 months Year 2: 12 months	Not stated	Not stated	Medium	13.3% (TIV) 15.3% (ATIV) 13.3% (control)

Adj: adjuvanted; AE: adverse event; ATIV: adjuvanted trivalent influenza vaccine; FFU: fluorescence focus assay units; GSK: GlaxoSmithKline; LAIV: live attenuated influenza vaccine; SAE: serious adverse event; TIV: non-adjuvanted trivalent influenza vaccine.

[a] Where multiple doses were administered, fever is listed for the first dose. Rates are for the youngest age group within the study unless otherwise stated.

Adverse events following immunisation

Fever

Pooled estimates of fever obtained using proportion meta-analysis of studies are shown in Table 3 and Table 4.

Non-adjuvanted vaccines in children six to 35 months of age

The pooled proportion estimate of fever was 6.7% (95% confidence interval (CI): 3.0–11.8) after first dose of TIV based on five eligible RCTs [22,29,31,38,39]. None of these RCTs had a high risk of bias. Analysis of five non-randomised clinical trials [40,41,47,49,53] provided higher first-dose fever estimates of 17.7% (95% CI: 11.3–25.2), largely due to the inclusion of two studies of bioCSL vaccines [47,53] that reported higher rates of post-vaccination fever. Rates after second doses are listed in Table 3 and Table 4.

Non-adjuvanted vaccines in children three to 17 years of age

There were only two eligible two-dose RCTs in this age group [29,39]. The pooled proportion estimate of fever for children three years and older was 6.9% (95% CI: 5.2–8.7) for dose 1. Meta-analysis of non-randomised clinical trials revealed more fever, 15.1% (95% CI: 13.3–17.0), again due to the inclusion of studies using bioCSL vaccines [47,53]. Second doses caused lower rates of fever.

Adjuvanted vaccines

Three RCTs used Fluad (Novartis), an MF59-adjuvanted vaccine which remains investigational and unlicensed in the paediatric age group, and included children aged from six months to 17 years [31,35,39]. Two of these studies [31,35] had low risk of bias and one was medium risk [39]. Point estimates of fever were higher than corresponding values for non-adjuvanted vaccines; however confidence intervals were wide due to the limited number of subjects. For children six to 35 months of age, first-dose pooled fever estimates were 11.9% (95% CI: 6.8–18.3). Data were more limited on children three years and older with pooled fever rates of 10.3% (95% CI: 1.1–27.0). Again, second doses elicited less fever. A small single non-randomised clinical trial reported fever rates of 16.0% for age 16–35 months, and 11.1% for age 36–48 months [48].

Direct within-study comparison between MF59-ATIV and non-adjuvanted TIV fever rates in two RCTs [31,39] showed significantly higher fever rates only in the subset of children aged 36–71 months in the ATIV group compared with the TIV group in one study (17.5% and 6.7%, respectively, for dose 1, $p < 0.001$) [39]. Two small studies of Inflexal V (Berna Biotech) virosomal-adjuvanted vaccine [30,34] showed pooled fever rates of 5.5% (95% CI: 1.3–12.3) (Table 3).

Post-vaccination fever, analysis by vaccine manufacturer

Fever estimates were calculated for Sanofi Pasteur, GlaxoSmithKline (GSK), Novartis, and bioCSL vaccines. Studies were grouped together, despite some variation in definition of fever, to maximise the number of studies evaluated. Data were analysed within age bands of six to 35 months and three to 17 years; data for dose 1 and 2 were analysed separately where possible. Data presented below covers non-adjuvanted vaccines. As MF59-adjuvanted (Novartis) and virosomal-adjuvanted (Berna Biotech) vaccines were produced by single manufacturers, corresponding data for adjuvanted vaccines are listed within the adjuvanted sections of Table 3 and Table 4.

Randomised studies

RCTs using Sanofi Pasteur products (Vaxigrip, Fluzone) [22-26,29,31-33,36,38], GSK's Fluarix [27,32,37], and Novartis's Agrippal [28] were examined (Table 3). Overall, fever rates were comparable between these brands of vaccine. For Sanofi products, in the age bands six to 35 months and three to 17 years, pooled first-dose fever rates were 5.1% and 4.4% respectively. Fever estimates were 4.7% (95% CI: 0.9–11.1) for GSK's vaccine and 4.0% (95% CI: 1.5–10.5) for Novartis's vaccine (analysis by age bands was not possible). Where applicable, high-risk studies were excluded, but this did not change heterogeneity.

Non-randomised studies

Fever rates were relatively high in Sanofi studies after the first dose in young children aged six to 35 months (16.9%; 95% CI: 12.6–21.6), but lower in three to eight year-old children (0.4%; 95% CI: 0–2.4). GSK studies did not allow analysis by these age bands; the average childhood fever rate was 5.6% (95% CI: 2.9–9.1).

In contrast, markedly higher fever rates were reported in the two studies of bioCSL vaccine [47,53]. Both were uncontrolled clinical trials and had different age cohorts. Pooled estimates of fever were elevated after the first dose in children aged six to 35 months and three to eight years (26.4%; 95% CI: 21.0–32.3 and 18.8%; 95% CI 15.9–21.9, respectively). Children nine years and older had a considerably lower fever rate (5.0%; 95% CI: 3.3–7.7). For second doses, fever rates were high for children aged six to 35 months (19.4%; 95% CI: 15.3–23.9) and were elevated, to a lesser extent, for three to eight year-old children (9.7%; 95% CI 7.7–11.9). Second-year booster doses of bioCSL vaccine with two vaccine strain changes, described in one study [47], showed even higher rates of fever, both in those aged six to 35 months (39.5%; 95% CI: 28.4–51.4) and in those aged three to eight years (27.0%; 95% CI 21.0–33.8) (Table 4).

Serious adverse events (SAEs)

'Serious adverse events' were not routinely defined in studies but was we assumed them to be the standard definition commonly used in clinical trials [12].

Randomised Studies

Among 15 RCTs of adjuvanted and non-adjuvanted vaccines [22,24-26,28-35,37-39] with 14,668 vaccinated individuals, 14 possibly or probably related SAEs were documented. Proportion meta-analysis yielded a pooled SAE rate of 1.2 per 1,000 vaccinated children. SAEs, where specifically described, included suspected allergic reactions to the vaccine, febrile and afebrile seizures after vaccination, new-onset diabetes, gait disorder, pneumonia, wheezing and viral gastroenteritis. A death was reported in one TIV recipient [26], deemed unrelated to the vaccination.

Non-randomised studies

Eight related SAEs were reported in non-randomised clinical trials among 7,655 vaccinated children (pooled estimate: 1.85 events per 1,000) [40,41,43-53]. SAEs described included post-vaccination fever requiring hospitalisation, bronchial hyperreactivity, bronchopneumonia, dysentery diarrhoea and distension of the abdomen, increased respiratory secretions, fever and vomiting or one FC and vomiting. One unrelated death was reported [51].

Febrile convulsions

Randomised studies

Using similar proportion meta-analysis of vaccinated study arms, we calculated an FC rate of 1.1 per 1,000 (95% CI: 0.51–1.9) using three large RCTs [26,32,39] (n = 7,439 children up to 59 or 71 months of age) that specifically reported FC as adverse events, and six RCTs (1,207 children aged up to 59 months) [22,25,29,31,34,38] that reported no related SAEs and by assumption, no FC. One of the three studies that reported on FC [32] included one vaccine-related seizure within a subset of 1,496 children aged 6–59 months (0.67 events per 1,000 children). Another study [26] reported two vaccine-related FCs among 4,173 children aged six to 59 months following TIV administration (0.48 events per 1,000). A third study [39], the only one incorporating a non-TIV control group, found similar FC rates in three study arms of non-adjuvanted TIV (2.82 per 1,000; n = 1,770), MF59 ATIV (2.59/1,000; n = 1,934) and active control vaccine (4.05/1,000; n = 988) in children six to 71 months of age. However, no comment was made if these FCs were causally related to vaccination.

Non-randomised studies

Two vaccine-related FCs were recorded in two non-randomised clinical trials (in total 2,269 evaluable children, 854 aged between six months and three years) [47,53]. Both studies used bioCSL TIV and had high rates of fever, particularly in younger vaccine recipients, compared with other non-randomised study results. Rates were not calculated due to the unavailability of denominator data within the susceptible age range.

Estimates of fever from unpublished clinical trial data (Clinicaltrials.gov)

Results from unpublished clinical trials are summarised in Table 5 and Table 6. Insufficient information on study methodology precluded detailed comparisons between studies. Temperature definitions were largely unavailable. There were five RCTs, of which three were double-blind RCTs (NCT00464672, NCT00764790, NCT00959049). One of these, an RCT (NCT00959049) which was unpublished at the time of our literature search [60], directly compared Afluria (bioCSL) with Fluzone (Sanofi) across several age bands. It was conducted in the US between September 2009 and May 2010 and defined fever as either ≥ 37.5 °C axillary or ≥ 38 °C oral. Afluria was associated with significantly higher rates of fever compared with Fluzone for first doses in children aged six to 35 months (37.1% vs 13.6%, respectively, $p < 0.0001$) and three to eight years (21.8% vs 9.4%, respectively, $p = 0.0001$). There were no significant differences in fever following second doses or after single doses in children aged nine to 17 years.

Fever rates in other RCTs ranged from 6.2 to 10.7% for children aged six to 35 months, 0–11.0% in children aged three to eight or nine years, and 0–3.8% in children aged nine or 10 to 17 years. Seven small non-randomised clinical trials were identified, all using Sanofi vaccine. Age ranges were variable, precluding detailed comparison. Fever rates varied widely (Table 6).

Observational studies: cohort studies and case–control studies

The six included observational studies [54-59] are summarised in Table 7. A study of inactivated virosomal-adjuvanted TIV (Inflexal V) in 966 vaccinated children reported fever in 0.52%, without comparison data from the unvaccinated cohort [54]. One retrospective case–control study assessed safety outcomes within 42 days after TIV in 13,383 children (3,697 vaccinated children aged six to 23 months, with three age- and sex-matched controls) from a US medical group patient database [55]. No significant associations were detected for any condition, including fever or seizures, except for pharyngitis and second TIV doses.

A large population-based retrospective cohort study investigated the safety of TIV in children six to 23 months of age [56]. It examined the risk of medically attended events (MAE) after TIV in 45,356 children (69,359 vaccinations) from 1991 to 2003. Using a case–crossover method, MAE in four risk windows post vaccination was compared with two control periods, one before and one after receiving TIV. No significant associations between TIV vaccination and any MAE, including FCs, were found. Another retrospective cohort study examined children aged 24 to 59 months in the US Vaccine Safety Datalink (VSD) over four influenza seasons (2002–06) [57]. Risk of fever and SAEs was examined in 66,283 children (91,692 doses). Similar case–crossover analysis showed no SAEs associated

Table 2a

Characteristics of non-randomised clinical trials included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Study design	Ages	Comparison groups	Enrolment period and location	TIV recipients evaluable for safety	Vaccines used	Vaccine manufacturer	Antigen dose per strain	Length monitoring solicited AE	Length SAE monitoring	Definition of fever	Method of measurement	EPHPP quality assessment tool rating	Fever rate recorded[a]
Mitchell 2005 [40]	Uncontrolled prospective study	6–35 months	1. 6–23 months 2. 24–36 months	2003/04 season United States	31	Fluzone: TIV	Sanofi-Pasteur	15 µg/0.5mL	3 days	Not stated	›38 °C	Rectal	Weak	10.5% (6–23 months)
Englund 2006 [41]	Open-label clinical trial	6–24 months	1. Vaccine primed 2. Vaccine naïve	Sep – Oct 2004 United States	100	Not stated	Aventis-Pasteur (Sanofi)	Not stated	5 days	6 months	≥38 °C	Axillary	Moderate	2.8 – 10.9%
Neuzil 2006 [42]	Uncontrolled prospective open label study	5–8 years	1. Healthy unvaccinated children	2004/05 season United States	232	Not stated	Sanofi-Pasteur	15 µg/0.5mL	5 days (0–4)	Not stated	≥37.8 °C	Not stated	Weak	0.4%
Avila Aguero 2007 [43]	Controlled open-label trial	6–35 months	1. Healthy children 2. High-risk children, unvaccinated 3. High-risk, previously vaccinated	2001/02 Costa Rica	218	Imovax Grippe (Vaxigrip): TIV	Sanofi-Pasteur	15 µg/0.5mL	30 days	Throughout study	≥37.1 °C	Axillary	Moderate	17.3% (healthy children)
Schmidt-Ott 2007 [44]	Uncontrolled open-label prospective phase IV study	6–13 years	1. Subjects 6–9 years: 2 vaccine doses 2. Subjects 10–13 years: 1 vaccine dose	Nov 2005 – Mar 2006 Germany	224	Influsplit SSW or Fluarix: TIV	GSK	15 µg/0.5mL	4 days	Not stated	≥37.5 °C	Axillary	Weak	2.7% (6–‹9 years)
Chai 2008 [45]	Uncontrolled clinical trial	›6 months	1. 6 months–3 years 2. 6–13 years 3. 18–60 years 4. ›60 years	2005/06 season China	764	TIV	Chinese manufacturer	15 µg/0.5mL	3 days	3 days	≥37.6 °C	Not stated	Weak	9.0% (6 months–3 years)
Kunzi 2009 [46]	Uncontrolled clinical trial	6 months–6 years	1. Children 6 months-6 years	2006/07 seasonGermany	405	Inflexal V: Virosomal ATIV	Crucell, Berna Biotech	15 µg/0.5mL	4 days	Not stated	Not stated	Not stated	Weak	5.3%
Nolan 2009 [47]	Uncontrolled prospective open-label clinical trial	6 months–8 years	1. 6months–‹3 years 2. ≥3 years–‹9 years	Mar 2005 – June 2006 Australia	293	Fluvax: TIV	bioCSL	15 µg/0.5mL	7 days (0–6)	6 months after last vaccine	≥37.5 °C axillary or≥38 °C oral	Oral or axillary	Weak	22.5% (6 months – ‹3 years)

Table 2b

Characteristics of non-randomised clinical trials included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Study design	Ages	Comparison groups	Enrolment period and location	TIV recipients evaluable for safety	Vaccines used	Vaccine manufacturer	Antigen dose per strain	Length monitoring solicited AE	Length SAE monitoring	Definition of fever	Method of measurement	EPHPP quality assessment tool rating	Fever rate recorded[a]
Vesikari 2009 [48]	Observer-blind follow-on study from previous RCT	16–47 months	1. Previous MF59 x 2. ATIV booster 2. Previous Split TIV x 2. TIV booster	2007/08 season Finland	89	Fluad: MF59 ATIV Vaxigrip: TIV	Fluad: Novartis Vaxigrip: Sanofi-Pasteur	15 µg/0.5mL	7 days	6 months	≥ 38 °C	Axillary	Moderate	8.7% (TIV, 16–35 months) 16.0% (ATIV, 16–35 months)
Walter 2009 [49]	Controlled clinical trial	6–12 weeks; 6 months	1. 6–12 week-old infants 2. 24–36 week-old infants	Apr – Aug 2005	393	Fluzone: TIV	Sanofi-Pasteur	15 µg/0.5mL	7 days	6 months	≥ 38 °C	Not stated	Moderate	18.2% (24–36 weeks)
Wang 2009 [50]	Uncontrolled clinical trial	› 6 months	1. 6–35 months 2. 3–11 years 3. 12–17 years 4. 18–60 years 5. › 60 years	2005/06 season China	2,794	Anflu: TIV	Chinese manufacturer	15 µg/0.5mL	7 days	7 days	≥ 37.6 °C	Not stated	Weak	5.3% (6–35 months)
D'Angio 2011 [51]	Controlled clinical trial	6–17 months	1. Full-term birth 2. Premature birth	2006/07, 2007/08 United States	83	Fluzone: TIV	Sanofi-Pasteur	15 µg/0.5mL	3 days (72 hours)	4–6 weeks after last vaccine	Not stated	Not stated	Moderate	14.7% (term group)
Walker 2012 [52]	Controlled open-label follow-on study	17 month-sAdj: adjuvanted; 13 years	1. Original study: non-adj H1N1 vaccine; given 1x TIV 2. Original study: adj H1N1 vaccine; given 1x TIV	Nov– Dec 2010 United Kingdom	295	Fluarix: TIV	GSK	15 µg/0.5mL	7 days	Not stated	≥ 38 °C	Axillary	Weak	13.6% (17 months – ‹ 5 years)
Lambert 2013 [53]	Uncontrolled prospective, multicentre, open-label clinical trial	6–17 years	1. 6–35 months 2. 3–8 years 3. 9–17 years	Mar– Jul 2009 Australia	1,976	Fluvax / Fluvax Junior: TIV	bioCSL	15 µg/0.5mL	7 days (0–6)	180 days after last vaccine	≥ 37.5 °C axillary or ≥ 38 °C oral	Oral or axillary	Weak	28.6% (6–35 months)

Adj: adjuvanted; AE: adverse event; ATIV: adjuvanted trivalent influenza vaccine; EPHPP: effective public health practice project; GSK: GlaxoSmithKline; LAIV: live attenuated influenza vaccine; RCT: randomised controlled trial; SAE: serious adverse event; TIV: non-adjuvanted trivalent influenza vaccine.

[a] Where multiple doses were administered, fever is listed for the first dose. The youngest age group is shown unless otherwise stated.

Table 3

Pooled estimates of fever proportions from randomised controlled trials of inactivated trivalent influenza vaccine in children

Fever in randomised controlled trials	Age	Dose	Number of children	Single study fever proportion (%)	Overall fever estimate[a] (%)	95% CI	I^2
Non-adjuvanted vaccines							
	6–35 months	Dose 1 [22,29,31,38,39]	1,543	NA	6.7	3.0–11.8	87.7
		Dose 2 [22,29,31,38,39]	1,501	NA	7.6	3.5–13.0	87.6
	3–17 years	Dose 1 [29,39]	795	NA	6.9	5.2–8.7	NA[b]
		Dose 2 [29,39]	775	NA	5.4	1.2–12.1	NA[b]
Adjuvanted vaccines							
MF59 adjuvanted	6–35 months	Fluad Dose 1 [31,35,39]	1,286	NA	11.9	6.8–18.3	74.7
		Fluad Dose 2 [31,35,39]	1,261	NA	10.4	4.2–18.9	86.4
	3–17 years	Fluad Dose 1 [35,39]	913	NA	10.3	1.1–27.0	NA[b]
		Fluad Dose 2 [35,39]	894	NA	9.0	0.3–27.2	NA[b]
Virosomal adjuvanted	6 months–5 years	Inflexal V Dose 1 [30,34]	112	NA	5.5	1.3–12.3	NA[b]
		Inflexal V Dose 2 [30,34]	112	NA	5.5	1.3–12.3	NA[b]
Vaccine manufacturers							
Sanofi (Vaxigrip, Fluzone)	6–35 months	Dose 1 [22,29,31,36,38]	558	NA	5.1	2.8–8.1	42.2
		Dose 2 [22,29,31,36,38]	548	NA	4.3	2.8–6.2	0
	3–17 years	Dose 1 [23,29,33]	162	NA	4.4	1.2–9.2	32.8
		Dose 2 [29]	18	0	NA	0–18.5[c]	NA[d]
GSK (Fluarix)	6 months–17 years	Combined doses [27,32,37][e]	2,151	NA	4.7	0.9–11.1	79.7
Novartis (Agrippal)	3–12 years	Dose 1 [28][d]	100	4.0	NA	1.1–9.2[c]	NA[d]

CI: confidence interval; NA: not applicable.

[a] Overall fever estimate calculated from studies using 38 °C fever definition for non-adjuvanted and adjuvanted vaccine analyses. Analysis by vaccine manufacturer used any fever definition. Random-effects proportion meta-analysis performed.

[b] I^2 not calculated due to low numbers of studies.

[c] Calculated confidence interval of a single proportion.

[d] Single study data. No meta-analysis performed.

[e] Only combined dose data available.

Table 4

Pooled estimates of fever proportions from non-randomised clinical trials of inactivated trivalent influenza vaccine in children*

Fever in non-randomised clinical trials	Age	Dose	Number of children	Single study fever proportion (%)	Overall fever estimate[a] (%)	95% CI	I^2
Non-adjuvanted vaccines							
	6–35 months	Dose 1 [40,41,47,49,53]	1,253	NA	17.7	11.3–25.2	85
		Dose 2 [40,41,47,49,53]	1,046	NA	11.7	5.4–19.9	89.9
	3–17 years	Dose 1 [47,53]	1,420	NA	15.1	13.3–17.0	NA[b]
		Dose 2 [47,53]	781	NA	9.7	7.7–11.9	NA[b]
Adjuvanted vaccines							
MF59 adjuvanted	16–35 months	Fluad [48][c]	25	16.0	NA	4.5–36.1[d]	NA[c]
	36–48 months	Fluad [48][c]	18	11.1	NA	1.4–34.7[d]	NA[c]
Vaccine manufacturer							
Sanofi (Fluzone, Vaxigrip, Imovax Grippe)	6–35 months	Dose 1 [40,41,49]	287	NA	16.9	12.6–21.6	4.3
		Dose 2 [40,41,49]	280	NA	6.2	0.0–21.0	90.9
	3–8 years	Dose 1 [42][c]	232	0.4	NA	0–2.4[d]	NA[c]
		Dose 2 [42][c]	232	1.3	NA	0.3–3.7[d]	NA[c]
GSK (Influsplit SSW / Fluarix)	17 months–13 years	Combined doses [44,52][e]	627	NA	5.6	2.9–9.1	65.3
bioCSL (Fluvax / Fluvax Junior)	6–35 months	Dose 1 [47,53]	854	NA	26.4	21.0–32.3	NA[b]
		Dose 2 [47,53]	766	NA	19.4	15.3–23.9	NA[b]
		Booster dose [47][c]	76	39.5		28.4–51.4[d]	NA[c]
	3–8 years	Dose 1 [47,53]	1,022	NA	18.8	15.9–21.9	NA[b]
		Dose 2 [47,53]	781	NA	9.7	7.7–11.9	NA[b]
		Booster dose [47][c]	196	27.0	NA	21.0–33.8[d]	NA[c]
	9–17 years	Dose 1 [53][c]	398	5.0	NA	3.3–7.7[d]	NA[c]

CI: confidence interval; NA: not applicable.

[a] Overall fever estimate calculated from studies using 38 °C fever definition for non-adjuvanted and adjuvanted vaccine analyses. Analysis by vaccine manufacturer used any fever definition. Random-effects proportion meta-analysis performed.

[b] I^2 not calculated due to low numbers of studies.

[c] Single study data. No meta-analysis performed.

[d] Calculated confidence interval of a single proportion.

[e] Dose 1 and 2 treated as separate groups within analysis.

Table 5a

Unpublished clinical trials from Clinicaltrials.gov included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Phase	Study design	Ages	Comparison groups	Study period and location	TIV recipients evaluable for safety	Vaccine type (whole, split, subunit)	Vaccine manufacturer	Length monitoring solicited AE	Length monitoring unsolicited AE	Length SAE monitoring	Definition of fever	Method of measurement
Randomised studies													
NCT00391391	2	RCT – open-label	6–35 months; 3–8 years	1. Fluzone intradermal 2. Fluzone IM	Oct 2006 – Oct 2007 United States	517	Split vaccine	Sanofi Pasteur	7 days	6 months after last vaccination	6 months after last vaccination	≥ 37.5 °C oral or ≥ 38 °C rectal (exclusion criteria)	NR
NCT00464672	3	RCT, double-blind	3–8 years; 9–17 years	1. Novartis vaccine 2. Comparator	Apr 2007 – Dec 2007 Argentina	1,200	Subunit	Novartis	7 days	Day 21–216 post vaccination	Until Day 216	NR	NR
NCT00764790[a] [74]	3	RCT, double-blind	6–35 months	1. Fluarix 2. Fluarix, half dose 3. Fluzone	Oct 2008 – Mar 2009 5 countries	3,256	Fluarix split; Fluzone split	GSK : Fluarix Sanofi-Pasteur: Fluzone	4 days	28 days post vaccination	6 months	NR	NR
NCT00943202[a] [75]	2	RCT, open-label	Primed 6–35 months; primed 3–9 years; 10–18 years	1. Day 0: H1N1; Day 21: H1N1; Day 42: TIV 2. Day 0: H1N1 + TIV; Day 21: H1N1 3. Day 0: H1N1; Day 21: H1N1 + TIV 4. Day 0: TIV; Day 21: H1N1; Day 42: H1N1	Aug 2009 – May 2010 United States	262	Licensed seasonal trivalent influenza vaccine	Sanofi Pasteur: H1N1	8 days	21 days post last vaccination	8 months post first vaccination	› 37.8 °C axillary or 38.3 °C oral	Axillary or oral
NCT00959049 [60]	3	RCT, double-blind	6 months–18 years	1. bioCSL: Afluria in 3 age cohorts 2. Sanofi: Fluzone in 3 age cohorts	Sep 2009 – May 2010 Unites States	1,468	Afluria split Fluzone split	BioCSL: Afluria Sanofi: Fluzone	7 days post vaccination	30 days	6 months after last vaccination	≥ 37.5 °C axillary or ≥ 38 °C oral	Axillary or oral

Table 5b

Unpublished clinical trials from Clinicaltrials.gov included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Phase	Study design	Ages	Comparison groups	Study period and location	TIV recipients evaluable for safety	Vaccine type (whole, split, subunit)	Vaccine manufacturer	Length monitoring solicited AE	Length monitoring unsolicited AE	Length SAE monitoring	Definition of fever	Method of measurement
Non-randomised studies													
NCT00831675	4	Non-randomised, open-label, parallel assignment	6–<36 months	1. 6–<12 months, healthy 2. 12 months–<36 months, healthy	Sep 20 04 – Apr 2006 United States	30	Split	Sanofi: Fluzone	4 days (day 0–3)	42 days post vaccination	42 days post vaccination	NR	NR
NCT00258817	4	Non-randomised, open-label, parallel assignment	6 months–<36 months	1. Vaccine naïve, 2 doses 2. Vaccine primed, 1 dose	Oct 2005 – Aug 2007 United States	30	Split	Sanofi: Fluzone	4 days (day 0–3)	2 weeks after last vaccine	2 weeks after last vaccine	≥ 38 °C	NR
NCT00389857	4	Non-randomised, open-label, parallel assignment	6 months–<36 months	1. Vaccine naïve, 2 doses 2. Vaccine primed, 1 dose	Oct 2006 – July 2008 United States	31	Split	Sanofi: Fluzone	4 days (day 0–3)	2 weeks after last vaccine	2 weeks after last vaccine	NR	NR
NCT00561002	4	Non-randomised, open-label, parallel assignment	6 months–<36 months	1. Vaccine-naïve/inadequately primed ≤ 1 previous dose: given 1 dose now 2. Vaccine-primed 2 previous doses: given 1 dose now	Oct 2007 – Jun 2008 Unites States	32	Split	Sanofi: Fluzone	4 days (day 0–3)	2 weeks after last vaccine	2 weeks after last vaccine	NR	NR
NCT00755274	4	Non-randomised, open-label, parallel assignment.	6–<59 months	1. Vaccine primed ≥ 2 previous doses: given 1 dose now 2. Vaccine-naïve/inadequately primed ≤ 1 previous dose: given 2 doses now	Sep 2008 – Jan 2009 United States	32	Split	Sanofi: Fluzone	4 days (day 0–3)	2 weeks after last vaccine	2 weeks after last vaccine	NR	NR
NCT00885105	3	Non-randomised, open-label, parallel assignment	6–<11 months	1. Previous study 2x Fluzone at 2 months: given 2 doses Fluzone 2. Fluzone naïve: given 2 doses Fluzone	Oct 2005 – Sep 2007 United States	242	Split	Sanofi: Fluzone	8 days (day 0–7)	6 months post vaccination	6 months post vaccination	NR	NR
NCT00390884	4	Non-randomised, open-label, parallel assignment	11–14 months	1. Fluzone primed: previous study Fluzone 2 doses; given 2 doses Fluzone 2. Fluzone naïve: previous study placebo 2 doses; given 2 doses Fluzone	Oct 2006 – Sep 2008 United States	173	Split	Sanofi: Fluzone	8 days (day 0–7)	2 months post vaccination	2 months post vaccination	NR	NR

AE: adverse event; GSK: GlaxoSmithKline; NR: not recorded; RCT: randomised controlled trial; SAE: serious adverse event; TIV: non-adjuvanted trivalent influenza vaccine.

[a] Studies published after our literature search and review.

with TIV in healthy children, however, fever was significantly associated with TIV within the window between Day 1 and 14 (incidence rate ratio (IRR) = 1.71; 95% CI: 1.64–1.80).

One retrospective observational cohort study in children in Western Australia (WA) from 2010 reported on the rate of fever seen with bioCSL TIV [58]. Data linkage of TIV-associated FC cases and vaccine exposure recorded in the Australian Childhood Immunisation Register, was added to data obtained from vaccine providers or primary caregivers. A high rate of FC, 3.3 per 1,000 vaccine doses, was documented during the 49-day vaccination programme, with 62 of 63 FC associated with bioCSL TIV, all occurring after a first dose, with a median time of 7 hours from vaccination to symptom onset. In children younger than five years, FCs were significantly more associated with bioCSL TIV than with Solvay's Influvac ($p < 0.0001$).

Subsequent to the reporting of excess FC rates post TIV in Australia, another VSD study was conducted in the US during the 2010/11 influenza season, examining Day 0 to 1 after TIV administration and examined 206,174 children aged six to 59 months who received at least one dose of vaccine [59]. None received bioCSL vaccine as its recommendation had been removed. While the main finding was of increased FC with concurrent TIV and 13-valent pneumococcal conjugate vaccine (PCV13), adjustment for PCV 13 still yielded a statistically significant increase in seizures following TIV by itself (IRR = 2.4; 95% CI: 1.2–4.7). The risk difference estimate was maximal at 16 months of age with 12.5 vaccine-attributable seizures per 100,000 doses.

Discussion

Our study summarises fever and FC data from multiple clinical trials, reporting group (not individual) safety outcomes following TIV receipt. Using published RCT data, we have found a reassuringly low pooled rate of fever ≥ 38 °C after non-adjuvanted TIV, which was similar to most non-bioCSL vaccines in observational studies conducted during 2010 when safety concerns arose due to bioCSL TIV [61-63].

Limited pooled data on investigational MF59-ATIV showed higher fever rates compared with non-adjuvanted vaccines. However in the two RCTs [31,39] with direct comparison of MF59-ATIV and TIV, fever rate differences were non-significant between adjuvanted and non-adjuvanted vaccine groups, apart from a subset of children aged 36 to 71 months in one study where the MF59-ATIV recipients had higher fever [39]. The same RCT [39] found no differences in fever rate between MF59-ATIV and TIV in younger children aged six to 35 months. However, it also recorded the highest fever rates in the non-adjuvanted arm for this age group (13.3% and 13.4% for doses 1 and 2, respectively) relative to all other non-adjuvanted vaccine study arms in our meta-analysis; this may have contributed to the absence of observable difference in fever between MF59-ATIV and TIV. In addition, the European Medicines Agency (EMA) raised concerns, after site inspections, that this study was not conducted in accordance with guidelines on good clinical practice (GCP), and therefore did not grant marketing approval for the Novartis MF59-ATIV used [64,65].

Non-randomised clinical trials were of lower quality, often being uncontrolled. Pooled fever estimates for non-adjuvanted vaccines were higher than those from RCTs, probably due in part to the inclusion of reactogenic bioCSL vaccines [47,53], although other manufacturers' vaccines also recorded higher fever rates than in RCT studies.

A recent systematic review of fever by Kaczmarek et al. following dose 1 of inactivated TIV, reported a similar rate (8.0%) for any fever in children aged six to < 36 months after non-adjuvanted TIV, using weighted average weekly risk [66]. However, our study, by using a proportion meta-analysis method, allowed inclusion of a broader range of studies. We used the Brighton Collaboration's fever definitions (≥ 38 °C) and analysed fever in a number of additional settings: adjuvanted vaccine studies, older children (36 months and older), fever after second doses of vaccine and by vaccine manufacturer.

Most non-bioCSL brand TIVs had low rates of fever in RCT analyses. However, bioCSL TIVs had significantly higher fever after first doses in children aged six months to eight years, across three studies conducted from March 2005 through to May 2010, particularly in an RCT (NCT00959049) comparing bioCSL's Afluria and a comparator TIV [60], subsequently published after our literature search and review (Table 6). Observational studies from 2010 in Australia and New Zealand documented similar findings comparing bioCSL TIV to other manufacturers [58,62].

Our findings on SAE and FC rates are considerably limited by the absence of studies using within-study placebo controls, which precludes calculation of true vaccination-related rates. However, analysing TIV-vaccinated arms, we found that vaccination-related SAEs were uncommon. Our calculated FC rate from published RCT data (no bioCSL studies available) was 1.1 per 1,000 children six to < 72 months-old and vaccinated with non-adjuvanted TIV. However, it was unclear in one study if all FC reported were causally related to TIV [39]; the actual rate may be lower. The same study showed no difference in FC rates between TIV and the non-TIV, active control arm [39]. We could not calculate FC rates in the clinical trials with bioCSL vaccine, but two observational studies conducted since 2010 reported FC rates of 3.5–4.4/1,000 doses for bioCSL Fluvax/Fluvax Junior compared with no FCs after 4,720 doses of Solvay vaccine (Influvac) or 3,213 doses of non-bioCSL TIV [58,62]. Furthermore, a 2010 investigation by the Therapeutics Goods Administration (TGA)

Table 6

Fever estimates from unpublished trials identified at Clinicaltrials.gov following administration of inactivated trivalent influenza vaccine in children

Study code	Fever definition	Age	Dose	Fever rate study vaccine % (denominator)	Fever rate comparator vaccine % (denominator)
Randomised controlled trials					
NCT00391391[a]	>37.5 °C			Fluzone intramuscular	Fluzone intradermal
		6–35 months	Dose 1	10.3% (97)	10.3% (97)
		6–35 months	Dose 2	9.3% (97)	6.2% (97)
		3–8 years	Dose 1	11.0% (163)	6.3% (160)
		3–8 years	Dose 2	8.6% (163)	10.0% (160)
NCT00464672	ND			Novartis vaccine	Comparator vaccine
		3–8 years	Dose 1	3.0% (402)	1.5% (199)
		3–8 years	Dose 2	2.5% (396)	2.5% (197)
		9–17 years	Dose 1	0.3% (400)	2.0% (199)
NCT00764790[b]	ND			Fluarix – GSK	Fluzone – Sanofi Pasteur
		6–35 months	Any dose	6.2% (1,080)	6.6% (1090)
NCT00943202[c]	≥ 37.8 °C			TIV as first vaccine	TIV as third vaccine
		6–35 months	Fever after TIV	10.7% (28)	9.4% (32)
		3–9 years	Fever after TIV	2.0% (51)	0.0% (49)
		10–17 years	Fever after TIV	3.8% (53)	0.0% (49)
NCT00959049 [60]	≥ 37.5 °C axillary or ≥ 38 °C oral			Afluria – BioCSL	Fluzone – Sanofi
		6–35 months	Dose 1	37.1% (229)	13.6% (228)
		6–35 months	Dose 2	14.6% (96)	13.6% (110)
		3–8 years	Dose 1	21.8% (252)	9.4% (255)
		3–8 years	Dose 2	5.9% (68)	6.4% (78)
		9–17 years	Dose 1	6.3% (254)	4.0% (250)
Non randomised studies					
NCT00831675	ND	6–11 months	Dose 1	0.0% (12)	
		6–11 months	Dose 2	8.3% (12)	
		12–35 months	Dose 1	16.7% (18)	
		12–35 months	Dose 2	16.7% (18)	
NCT00258817	≥ 38 °C			Vaccine naïve	Vaccine primed
		6–35 months	Dose 1	6.7% (15)	13.3% (15)
		6–35 months	Dose 2	33.3% (15)	
NCT00389857	ND			Vaccine naïve	Vaccine primed
		6–35 months	Dose 1	0.0% (14)	5.9% (17)
		6–35 months	Dose 2	7.1% (14)	
NCT00561002	ND			Vaccine naïve	Vaccine primed
		6–35 months	Dose 1	17.4% (23)	22.2% (9)
		6–35 months	Dose 2	13.0% (23)	
NCT00755274	ND			Vaccine naïve	Vaccine primed
		6–59 months	Dose 1	25.0% (8)	8.3% (24)
		6–59 months	Dose 2	25.0% (8)	
NCT00885105	ND			Fluzone (Sanofi) naïve	Fluzone (Sanofi) primed
		6–10 months	Dose 1	25.0% (130)	25.0% (112)
		6–10 months	Dose 2	14.0% (130)	14.0% (112)
NCT00390884	ND			Fluzone (Sanofi) naïve	Fluzone (Sanofi) primed
		11–14 months	Dose 1	10.5% (57)	15.5% (116)
		11–14 months	Dose 2	15.8% (57)	17.2% (116)

ND: not defined; TIV: trivalent influenza vaccine.

[a] Only data on intramuscularly administered vaccine group was used.

[b] Only groups with full dose were examined. Data from groups with half dose are not presented.

[c] Only groups with TIV administered alone are listed.

Table 7

Characteristics of observational studies included for analysis of fever, febrile convulsions and serious adverse events following administration of inactivated trivalent influenza vaccine in children

Reference	Study design	Study period	Location	Number of participants	Intervention	Main findings
Salleras 2009 [54]	Prospective cohort study	2004/05 season	Barcelona, Spain	1951 children 3–14 years-old; 966 received TIV	Inflexal V Virosomal adjuvanted vaccine	Only vaccinated cohort findings presented. Fever≥ 38 °C recorded in 0.52% of vaccinated cohort. Local redness in 4%. Systemic malaise in 0.72%. SAE not documented.
Goodman 2006 [55]	Retrospective case–control study	2002/03 and 2003/04 seasons	United States	13,383 including 3,697 TIV recipients aged 6–23 months at vaccination	TIV	Safety outcomes assessed within 42 days of TIV. Pharyngitis associated with dose 2 of TIV. No other associations detected including for fever or seizures.
Hambidge 2006 [56]	Retrospective cohort using self-control analysis	1991–2003	United States	45,356 children aged 6–23 months with 69,359 vaccinations	TIV	13 diagnoses less likely to occur within two weeks after TIV compared with control periods before/after this period. Positive association with non-infectious gastroenteritis in Emergency Department setting. No association with convulsions detected.
Glanz 2011 [57]	Self-controlled screening study	Oct 2002–Mar 2006	United States	66,283 children aged 24–59 months with 91,692 vaccinations from the Vaccine Safety Datalink	TIV	No association between any serious medically attended events to TIV post-vaccination period. Non serious associations detected for limb soreness, fever, and gastrointestinal tract symptoms
Armstrong 2011 [58]	Three-part study: 1. Descriptive/case–control study 2. Incidence study 3. Retrospective cohort study of AE after three brands TIV	1. Mar–Apr 2010 2. 2008–2010 3. 2010	Western Australia	1. 63 TIV-associated FC 2. Coded public hospital presentations for FC temporally related to TIV 3. Three groups of 120 children each who had received a different brand of TIV	TIV	1. 3.3 FC/1,000 doses of TIV. All occurred after first dose, with median onset 7 h post vaccine. CSL TIV 14.8 × higher risk of febrile reaction compared with alternative brand. 2. Pattern of elevated post-TIV FC not seen in years before 2010. 38 TIV temporally associated FC coded in 2010, one in 2009, nil in 2008 3. CSL-branded TIV (OR 8.9; 95%CI 3.1 to 25.7, p<0.0005) and younger age (p=0.024) associated with higher risk of "significant febrile adverse events" in logistic regression model.
Tse 2012 [59]	Near real-time surveillance study for FC using self-controlled risk interval and current vs historical vaccinee study designs	2010/11 influenza season	United States	206,174 children aged 6–59 months from the Vaccine Safety Datalink	TIV (not CSL brand)	Among children 6–59 months of age, the incidence rate ratio for TIV adjusted for concomitant PCV13 was 2.4 (95% CI: 1.2–4.7). Risk difference estimates were highest at 16 months (12.5/100,000 doses for TIV without concomitant PCV13) due to varying age-related baseline risk for seizures in young children.

AE: adverse event; CI: confidence interval; FC: febrile convulsion; OR: odds ratio; PCV13: 13-valent pneumococcal vaccine; TIV: trivalent influenza vaccine.

into bioCSL vaccine found FC rates of 5–7 per 1,000 doses [9].

Based on one study, MF59-ATIV was associated with 2.59 FCs per 1,000 vaccinated children aged six to 71 months, but this was not significantly different to control groups (non-adjuvanted TIV or active control vaccine) [39]. Further study of adjuvanted vaccines is warranted to investigate their safety profile, in terms of fever and FC.

Despite an observational study reporting a link between the 2010/11 US non-bioCSL TIV and FC on Day 0 to 1 [59] (mostly with concurrent PCV13), the absolute risk of TIV-related FC appeared low overall (a maximum of 12.5/100,000 doses), less than the risk seen after measles-mumps-rubella (MMR) vaccine (33/100,000) and similar to the risk after 13-valent PCV (13.7/100,000) [59,67]. A subsequent study of the 2011/12 US influenza season confirmed elevated fever after concurrent TIV and PCV13 on Day 0 to 1 and listed fever rates after TIV alone similar to our findings at 7.5% in children aged six to 23 months [68].

Proposed explanations for higher fever rates with bioCSL vaccines have included 2010 TIV strain changes and manufacturing methods. Investigations by bioCSL concluded that their method of manufacture retained more virus components due to less splitting of virus, compared with other manufacturers, and that characteristics of the three viruses included in the 2010 vaccine elicited an excessive immune response in young children [69,70]. However, all manufacturers used the same new strains in formulating the 2010 southern hemisphere vaccine without eliciting increased fever or FCs.

These results highlight the differences in the propensity to febrile events that may exist between different companies' TIVs. The single RCT (NCT00959049) comparing bioCSL TIV with a comparator vaccine in children most clearly demonstrates these important differences. This study was conducted in 2009/10 but only recently published in 2014 [60]. It was not yet completed when the bioCSL TIV problem emerged in April 2010. Access to individual level data of this study would offer valuable insights into fever following receipt of TIV.

The lack of clearly presented, publicly available, comparable data regarding the safety of influenza vaccines, particularly in young children, has been emphasised in a previous systematic review of influenza vaccination [71]. Few of the studies we examined were eligible for that systematic review due to the lack of placebo controls. Without such placebo-controlled studies, the true rate of adverse events due solely to TIV is difficult to ascertain accurately. Such studies are difficult to justify ethically as more and more countries recommend universal influenza vaccination of healthy children. Our study addressed as much data as possible, with sensitivity analyses, to provide the most comprehensive information by which to compare vaccines.

Limitations of this study are acknowledged, including the difficulty of comparing studies that have different methodology. By examining studies involving healthy children, we have maximised the comparability of studies, but the findings may not apply to children with chronic illness for whom TIV is specifically recommended. The majority of fever analyses showed substantial heterogeneity; I2 values ranged from 0% to 95.6% with most being larger than 50%. Bias assessment revealed that the majority of randomised studies had low to moderate risk of bias. A random-effects model for pooled fever estimates was used to provide an accurate estimate across variable studies. Our sensitivity analysis was not able to identify specific sources of heterogeneity based on assessments of study quality, but underlying study variability is the most likely cause.

Our analysis did not specifically take into account differing follow-up periods. Solicited AE follow-up periods longer than 48 hours result in the possibility of unrelated fever being captured. This highlights the need for consistent reporting in studies of post-vaccination fever rates occurring within specific timeframes, particularly the first 24 hours. Lastly, most pooled fever estimates involved overlapping confidence intervals, meaning that the point estimates of fever must be compared cautiously. However, where possible, we have compared similar types of vaccines, within set age ranges, and included studies that used Brighton Collaboration definitions of fever.

Conclusions and recommendations

This review provides a generally reassuring assessment on the safety of most TIVs which have low rates of fever or serious adverse events. There is, however, evidence that the bioCSL brand vaccines have been associated with higher rates of fever than comparable vaccines. This cannot be ascribed to the change in vaccine strains alone as the 2010 TIV made by other manufacturers was not highly reactogenic.

Although Tse et al. [59] found an association between early post-vaccination FCs and US 2010/11 non-bioCSL TIVs, containing strains identical to the 2010 southern hemisphere TIV, the risk was low and comparable to other routine immunisations.

We advocate prompt reporting and publication of clinical trial safety data for influenza vaccines. This is even more pertinent with the impending adoption of quadrivalent influenza vaccines (QIV) containing an additional influenza B strain, to ensure that reactogenicity is not increased. Closer scrutiny of the safety of each new season's vaccine formulations in children, for example through a period of active surveillance after TIV release each season, may facilitate the early detection and rapid response to any future safety signals

to minimise future impacts on the health of vaccinees and maintain confidence in immunisation programmes. The EMA is heading in this direction with requirements from 2014 to 2015 for vaccine manufacturers to implement systems for yearly enhanced safety surveillance to rapidly detect clinically significant changes in the frequency or severity of expected reactogenicity of influenza vaccines [72,73].

Furthermore, we believe public availability of individual-level data (of precise levels of fever over time) from both past and future vaccine trials as well as the use of standardised study methods, through stricter adherence to Brighton Collaboration case definitions and reporting recommendations for adverse events, is essential to enable effective comparison both between vaccines and over time.

Erratum *

The statement of conflict of interest was omitted in the original publication and added on 25 June 2015. In Table 4, a line was added between the data for GSK and BioCSL.

Conflict of interest *

J. K. Yin received an educational grant from Sanofi Pasteur for influenza economic research in 2012. R. Booy has received funding from bioCSL, Roche, Sanofi, GlaxoSmithKline (GSK), Novartis, and Pfizer to conduct sponsored research or attend and present at scientific meetings; any funding received is directed to a research account at the Children's Hospital at Westmead. C. Jones has received funding from GlaxoSmithKline (GSK) to attend and present at the New Zealand Infection and Immunisation Special Interest group in 2013.

Authors' contributions

Jean Li-Kim-Moy conceived and designed the study, was involved in screening of relevant studies, data collection, data analysis, data interpretation and writing of the manuscript. Jiehui Kevin Yin conceived and designed the study, was involved in screening of relevant studies, data collection, assisted in writing all sections of the paper, and revision of the manuscript. Harunor Rashid conceived and designed the study, was involved in screening of relevant studies, data collection, data analysis, and revision of the manuscript. Gulam Khandaker assisted with design of the study, was involved in screening of relevant studies, and revised the manuscript. Catherine King conducted the electronic literature search, assisted in writing the methods section, and revised the manuscript. Nicholas Wood, Kristine Macartney, and Cheryl Jones revised the manuscript and assisted in writing all sections of the manuscript. Robert Booy conceived, designed, and supervised the study; he was involved in data interpretation, writing of all sections of the paper, and revision of the manuscript.

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Waterborne outbreaks in the Nordic countries, 1998 to 2012

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A total of 175 waterborne outbreaks affecting 85,995 individuals were notified to the national outbreak surveillance systems in Denmark, Finland and Norway from 1998 to 2012, and in Sweden from 1998 to 2011. Between 4 and 18 outbreaks were reported each year during this period. Outbreaks occurred throughout the countries in all seasons, but were most common (n = 75/169, 44%) between June and August. Viruses belonging to the *Caliciviridae* family and *Campylobacter* were the pathogens most frequently involved, comprising n = 51 (41%) and n = 36 (29%) of all 123 outbreaks with known aetiology respectively. Although only a few outbreaks were caused by parasites (*Giardia* and/or *Cryptosporidium*), they accounted for the largest outbreaks reported during the study period, affecting up to 53,000 persons. Most outbreaks, 124 (76%) of those with a known water source (n = 163) were linked to groundwater. A large proportion of the outbreaks (n = 130/170, 76%) affected a small number of people (less than 100 per outbreak) and were linked to single-household water supplies. However, in 11 (6%) of the outbreaks, more than 1,000 people became ill. Although outbreaks of this size are rare, they highlight the need for increased awareness, particularly of parasites, correct water treatment regimens, and vigilant management and maintenance of the water supply and distribution systems.

Background

outbreaks remain an important public health concern, despite advances in water management and sanitation, even in industrialised countries, as large numbers of people can be infected within a short time period and some of the infections can be life threatening. While people depend on water to live, the supplies can remain vulnerable to contamination from animal and human faeces and provide an excellent environment for the survival and transmission of a range of infectious agents. The traditional paradigms of treatment have been challenged by emerging microorganisms, such as *Cryptosporidium*, which are resistant to chlorination at the concentrations used in drinking water treatment and require either advanced filtration or ultraviolet (UV) disinfection [1]. In addition, globalisation is changing the distribution of microorganisms [2]. High population density can generate stress on available water sources and sanitation systems.

Drinking water in the Nordic countries is mostly supplied by waterworks (either municipal or managed by private companies). In addition, there are also a considerable number of people who are supplied with water from single-household wells, mainly those living in remote rural areas or in summer houses or cabins in the countryside (Table 1). The water source for drinking water differs among the countries. In Denmark, all drinking water is obtained from groundwater, while in Norway surface water is the main source. In Sweden and Finland, surface water predominates as the source for large waterworks, while groundwater is the main source for medium- and small-sized waterworks (Table 1). Chlorination and UV radiation are the most frequently used disinfection methods for treating surface water (Table 1). Groundwater is usually not disinfected in the Nordic region. Drinking water regulations in all

Table 1

Drinking water: overview of water sources, waterworks, water treatment, Denmark, Finland, Norway and Sweden[a]

Variable	Denmark	Finland	Norway	Sweden
Water sources	Almost exclusively groundwater (›99%).	Large waterworks: – surface water 44% – groundwater 41% – artificial groundwater 15%. Medium-sized waterworks: – groundwater 92–95% – surface water 5%.	Surface water supplies 61% of the waterworks and 90% of the served population. Groundwater supplies 39% of the waterworks and 10% of the served population.	Surface water supplies 10% of the waterworks and 53% of the population. Groundwater supplies 85% of the waterworks and 23% of the population. Artificial groundwater supplies 24% of the population and 5% of the waterworks.
Waterworks	2,600 waterworks serving › 98% of the population; about 2% are served by small private facilities (such as private wells). 2/3 of population served by ‹100 major waterworks.	156 large waterworks supply 4.32 million people. ›700 medium-sized waterworks provide water to›500,000 people.	1,594 waterworks serving 4.34 million people, 88 % of the population: – 63% are municipal – 2% are intermunicipal – 35% are private. These waterworks serve 71%, 24% and 5% of the population supplied by waterworks, respectively.	1,750 waterworks supply 84% of the population. About 1,000,000 people are supplied by private wells in permanent households and about 1,000,000 by private wells in summer houses
Water treatment	Generally no disinfection for aeration and filtering.	Surface water: mainly chlorination and UV radiation. Groundwater: often no disinfection.	Mainly UV radiation (72% of the served population) and to a lesser extent chlorination (66% of the served population). 45% of the supplied population is served by waterworks using coagulation in addition to disinfection. About 7,000 people are served by waterworks with surface water without disinfection.	Mainly UV radiation and chlorination. 90% of the population connected to surface water supplies has coagulation in addition to disinfection. Sometimes in combination with ozonation and membrane filtration. About 400,000 people are served by groundwater waterworks without disinfection.

UV: ultraviolet.

[a] The table shows data from 2010 in Norway, 2012 in Denmark and Finland, and 2014 in Sweden.

four countries [3-7] follow the European Union Drinking Water Directive [8].

Municipal health, environmental and food safety authorities are responsible for outbreak detection, investigation and control. Medical practitioners who suspect an outbreak are obliged by law to report it to the municipal authorities. National public health institutes have a consulting role, providing assistance if needed, or a coordination role, if the outbreak affects more than one administrative region [9-12]. All four countries have national surveillance reporting systems in place that municipal authorities should use to notify waterborne outbreaks. All the systems are currently web-based.

In this study, we present information available on waterborne outbreaks notified between 1998 and 2012 in these countries to gain a better understanding of their scope and characteristics in the Nordic region.

Methods

We analysed data on all waterborne outbreaks notified between 1998 and 2012 (in Sweden, up to 2011) to the national outbreak surveillance systems in each of the four countries. Where data about the outbreaks were incomplete, local and regional authorities responsible for each outbreak investigation provided additional data to make the datasets as complete as possible.

In order to collect and systematise the data, a link to a web-based questionnaire designed using the Questback application [13] was sent to all four countries. The questionnaire included questions on number of cases, date of onset of symptoms of the first case, municipality of occurrence, microorganism(s) involved, water source (surface water, groundwater, other), type of water supply, (including municipal or private waterworks, single household, other), number of people supplied with a given water supply, water disinfection status, factors contributing to the outbreak (pollution of water source, failure of water treatment, failure of

Table 2

Overview of waterborne outbreaks, Denmark, Finland, Norway and Sweden, 1998–2012[a] (n = 175)

Country	Number of outbreaks	Outbreaks per year	Number of people involved	Total population in 2012
Denmark	4	0.27	660	5,426 million [27]
Finland	59	3.9	22,594	5,421 million [28]
Norway	53	3.5	10,483	5,033 million [29]
Sweden	59	4.2	52,258	9,555 million [30]

[a] For Sweden, 1998 to 2011.

water distribution system, other) and level of evidence of drinking water being the cause of the outbreak (strongly associated, probably associated and possibly associated, using the categories developed by Tillett et al. [14]).

Once the data were gathered through the Questback application, we carried out a descriptive analysis of the information.

Results

Outbreaks

A total of 175 waterborne outbreaks affecting 85,995 individuals were notified in the four Nordic countries during the study period (Table 2). Outbreaks occurred throughout the four seasons, but were mainly during June to August (75/169 outbreaks, 44%) and March to May (38/169 outbreaks, 22%) (Figure 1).

For six outbreaks, the season was not reported. The number of notified outbreaks varied from 4 to 18 outbreaks per year, affecting between 300 and 28,000 persons per year. Most of the outbreaks with known number of cases (130/170 outbreaks, 76%) had fewer than 100 persons involved. However, all countries except Denmark reported outbreaks with more than 1,000 persons per outbreak (11/170 outbreaks, 6%), including two outbreaks in Sweden in 2010 and 2011 with more than 20,000 persons involved each time (three-year period trends are shown in Figure 2).

Implicated microorganisms

The aetiology was known for 123 outbreaks (70% of all outbreaks). The microorganisms most frequently implicated were viruses belonging to the *Caliciviridae* family, involved in 51 outbreaks (41% of outbreaks with known aetiology). Of these, norovirus was the cause in 44 outbreaks while in seven outbreaks the specific type of calicivirus was not specified. The second most common microorganism involved was *Campylobacter*, which caused 36 outbreaks (29%). The 36 outbreaks involving other laboratory-confirmed microorganisms were caused by pathogenic *Escherichia coli* (8 outbreaks), *Francisella tularensis* (6 outbreaks), *Salmonella* (2 outbreaks) and *Shigella* and rotavirus (1 outbreak each), and parasites such as *Giardia* (5 outbreaks) and *Cryptosporidium* (4 outbreaks). There were nine outbreaks in which more than one microorganism was identified in samples from patients and/or water (Table 3).

In terms of number of outbreak cases reported, the following four groups of pathogens dominated as aetiological agent and contributed to more than 90% of all cases: *Cryptosporidium* (58%), viruses belonging to the *Caliciviridae* family (17%), *Campylobacter* (9%) and *Giardia* (7%) (Table 3).

Certain types of microorganisms were country-specific, such us *F. tularensis*, which was only notified in Norway, in six outbreaks.

Type of water supply, water source, disinfection status and contributing factors

Most of the outbreaks with known water supply were associated with waterworks (101/168 outbreaks, 60%). Of these, 62 were municipal waterworks and 39 were owned by private companies. Around 35% of outbreaks (58/168) occurred in single households. In addition, nine involved an outdoor open water source. Groundwater was the water source involved in most of the outbreaks with known water source (124/163 outbreaks, 76% of those with known water source) followed by surface water in 39 outbreaks (24%). The distribution of type of water supply and water source involved in outbreaks remained relatively stable during the study period (Figure 2). Outbreaks involving municipal waterworks with surface water as water source (17/175 outbreaks) accounted for the largest number of cases (67% of all cases (57,315/85,995)), followed by outbreaks involving municipal waterworks with groundwater as water source (42/175 outbreaks) with 23,816 cases (28% of all cases).

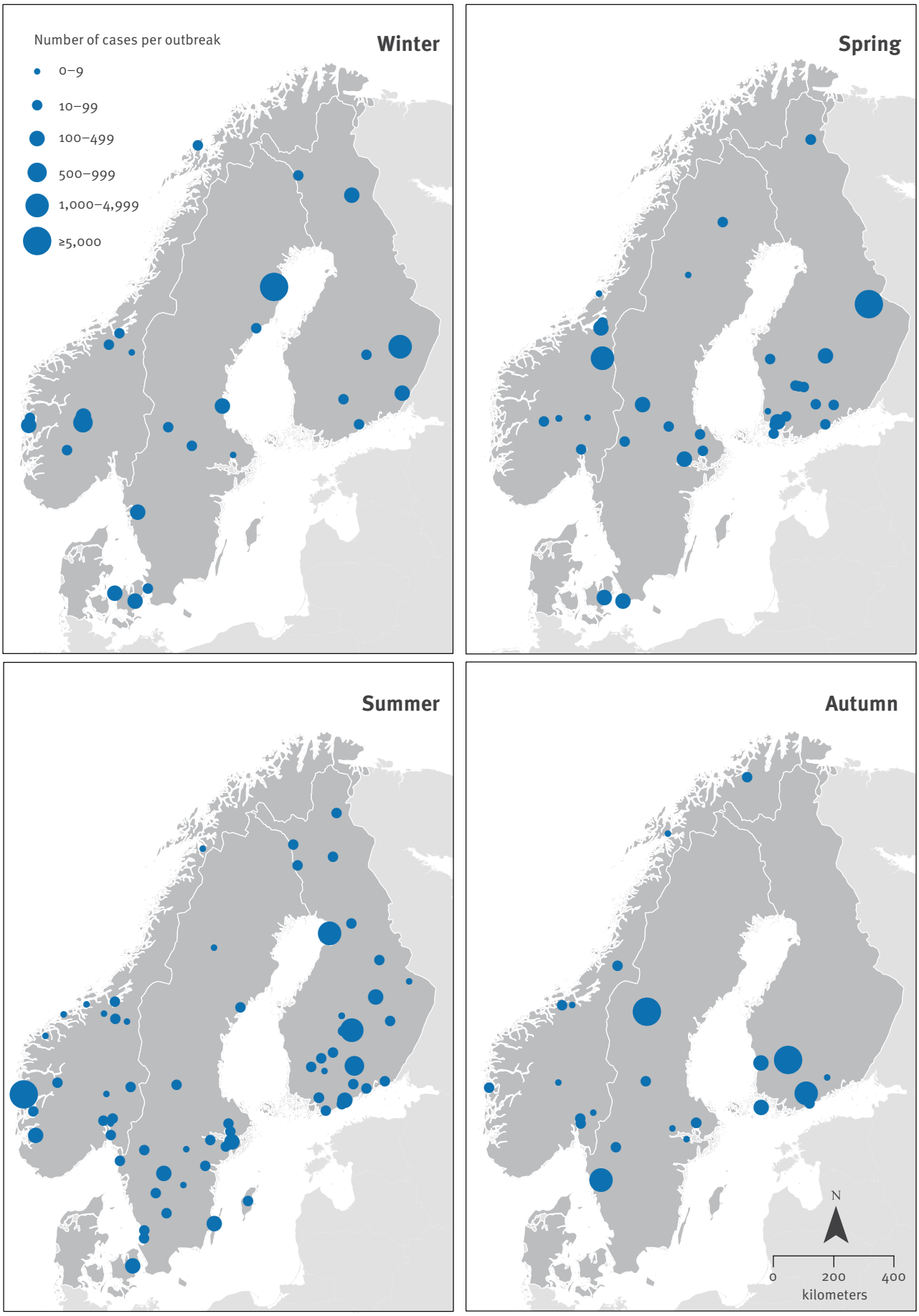
In 122 outbreaks, water had not been disinfected before the outbreak. All outbreaks that occurred in single households in which disinfection status was known (50 outbreaks) were caused by non-disinfected water. The most common contributing factor was contamination at the source (95 outbreaks). Failures in the distribution system accounted for 26 outbreaks (Table 4).

Level of association of outbreak with water

According to the classification developed by Tillett et al. [14], 32 outbreaks were classified as being 'strongly'

Figure 1

Seasonal distribution of waterborne outbreaks by size of outbreak, Denmark, Finland, Norway and Sweden, 1998–2012[a] (n = 169)

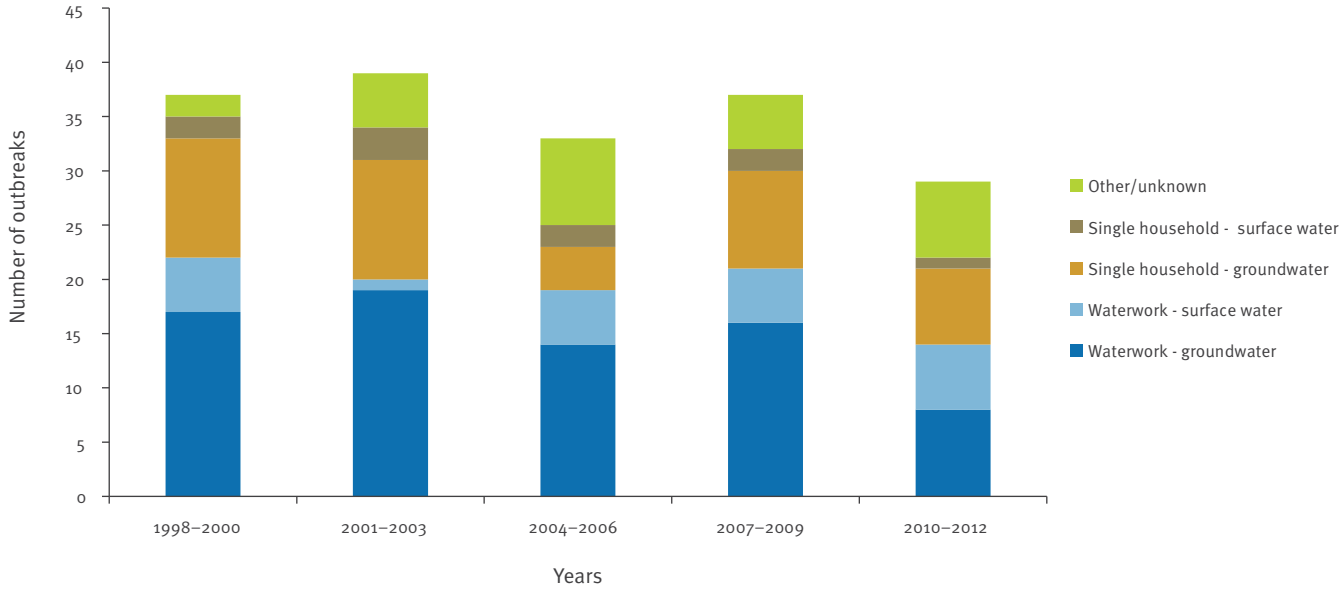


[a] For Sweden, 1998 to 2011.

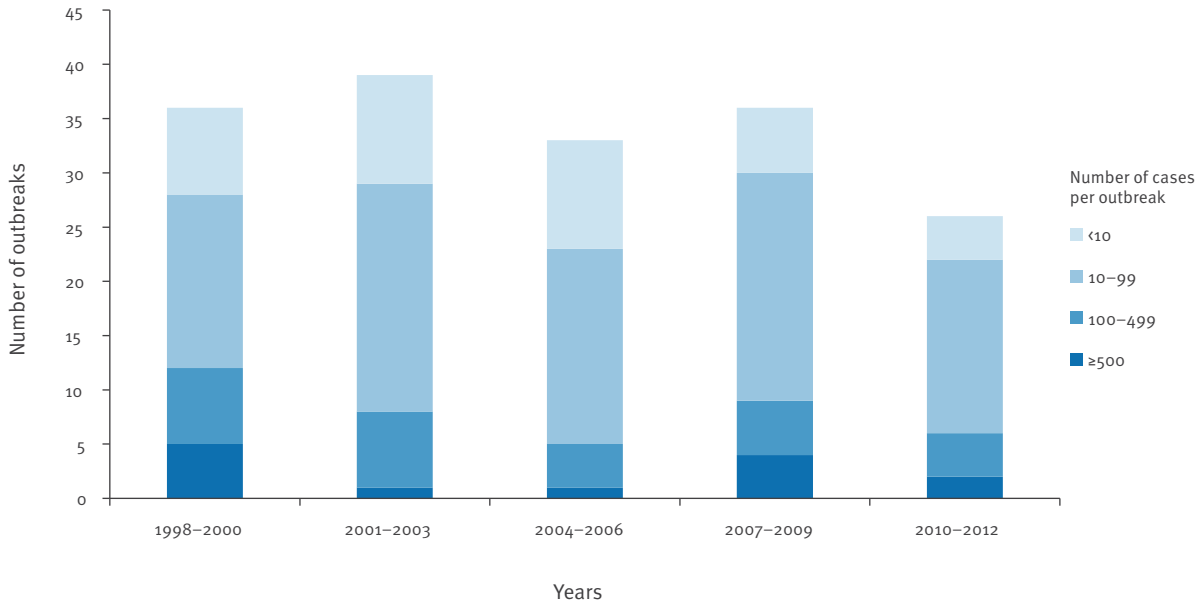
Figure 2

Waterborne outbreaks by three-year periods and (A) type of water supply and water source (n = 175) and (B) size of outbreak, Denmark, Finland, Norway and Sweden, 1998–2012[a] (n = 170)

A Type of water supply and water source



B Size of outbreak



[a] For Sweden, 1998 to 2011.

associated with water, 51 were classified as 'probably' associated and 56 as 'possibly' associated with water (Figure 3). The proportion of outbreaks with a known level of association was higher as the number of cases involved increased. A total of 36 outbreaks could not be classified due to missing information.

Discussion

In the 15-year period included in this study, a total of 175 waterborne outbreaks affecting thousands of people were notified in the Nordic countries. However, we consider the numbers presented to be an underestimation of the true occurrence. For example, outbreaks linked to municipal or inter-municipal waterworks are more likely to be recognised and reported than those

Table 3

Waterborne outbreaks by microorganism involved and year, Denmark, Finland, Norway and Sweden, 1998–2012[a] (n = 175)

Year	Number of outbreaks (number of patients involved) by microorganism											
	Caliciviridae	Campylobacter	Cryptosporidium	*Escherichia coli* (pathogenic)	Giardia	Rotavirus	Salmonella	Shigella	Francisella tularensis	Multiple microorganisms	Unknown	Total
1998	2 (2,500)	2 (2,216)	–	1 (unknown)[b]	1 (3)	–	–	–	–	–	1 (13)	7 (4,732)
1999	4 (238)	2 (14)	–	–	–	–	1 (55)	–	–	–	7 (664)	14 (971)
2000	5 (5,944)	4 (1,063)	–	–	1 (37)	–	–	–	–	1 (300)	5 (167)	16 (7,511)
2001	3 (698)	4 (1,069)	–	–	–	–	1 (3)	–	–	–	2 (37)	10 (1,807)
2002	5 (746)	4 (114)	–	–	–	–	–	–	1 (11)	1 (50)	5 (520)	16 (1,441)
2003	7 (291)	1 (3)	–	1 (8)	–	1 (140)	–	–	–	–	3 (101)	13 (543)
2004	3 (259)	3 (13)	–	–	1 (6,000)	–	–	–	–	–	4 (32)	11 (6,304)
2005	1 (45)	2 (300)	–	1 (16)	–	–	–	–	1 (2)	–	5 (144)	10 (525)
2006	1 (150)	2 (45)	–	1 (10)	–	–	–	1 (18)	1 (5)	2 (35)	4 (38)	12 (283)
2007	3 (90)	3 (1,613)	1 (28)	–	1 (13)	–	–	–	3 (27)	2 (6,513)	5 (2,431)	18 (10,715)
2008	1(2,000)	2 (20)[b]	–	1 (20)	1 (2)	–	–	–	–	–	4 (110)	9 (2,152)
2009	4 (436)	2 (210)	–	1 (4)	–	–	–	–	–	–	3 (67)	10 (717)
2010	5 (401)[b]	2 (275)	2 (27,000)[b]	–	–	–	–	–	–	1 (40)	2 (30)	12 (27,746)
2011	5 (57)[b]	3 (56)	1 (20,000)	1 (8)	–	–	–	–	–	1 (27)	2 (15)	13 (20,163)
2012	2 (170)	–	–	1 (15)	–	–	–	–	–	1 (200)	–	4 (385)
Total	**51 (14,025)**	**36 (7,011)**	**4 (47,028)**	**8 (81)**	**5 (6,055)**	**1 (140)**	**2 (58)**	**1 (18)**	**6 (45)**	**9 (7,165)**	**52 (4,369)**	**175 (85,995)**

Dashes indicate that there were no such outbreaks.

[a] For Sweden, 1998 to 2011.

[b] There was an outbreak with an unknown number of people involved. There were five such outbreaks in total

Table 4

Factors contributing to waterborne outbreaks by type of water supply, Denmark, Finland, Norway and Sweden, 1998–2012[a] (n = 175)

Contributing factors	Number of outbreaks (number of patients involved) by type of water supply						Total
	Single households	Municipal waterworks		Private waterworks		Other/ unknown	
		Groundwater	Surface water	Groundwater	Surface water		
Contamination at source	29 (579)	15 (11,410)[b,c]	6 (55,005)[b]	19 (934)[b]	1 (15)	12 (455)	82 (68,398)
Failures in the distribution system	–	11 (7,594)	3 (238)	–	–	2 (24)	16 (7,856)
Failures in water treatment	–	–	1 (4)	1 (unknown)[b]	–	–	2 (4)
Contamination of the water source plus failures in water treatment	2 (55)	–	1 (1,700)	–	–	–	3 (1,755)
Contamination of the water source plus failures in the distribution system	1 (16)	3 (2,549)	–	3 (117)	1 (100)	1 (360)	9 (3,142)
Contamination of the water source plus failures in the distribution system plus failures in water treatment	–	1 (35)	–	–	–	–	1 (35)
Unknown	26 (471)	12 (2,228)	6 (368)	9 (1,149)	3 (71)	6 (518)	62 (4,805)
Total	**58 (1,121)**	**42 (23,816)**	**17 (57,315)[d]**	**32 (2,200)**	**5 (186)**	**21 (1,357)**	**175 (85,995)**

Dashes indicate that there were no such outbreaks.

[a] For Sweden, 1998 to 2011.

[b] There was an outbreak with an unknown number of people involved. There were five such outbreaks in total.

[c] There were two outbreaks in this category with unknown numbers of people.

[d] Two outbreaks accounted for 54.7% (47,000) of all cases.

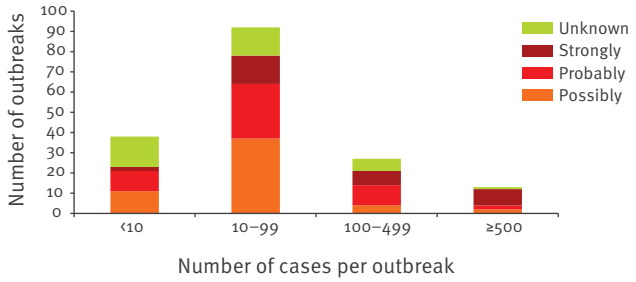
that involve a single-household water supply. Similarly, outbreaks caused by treatment failure or contamination of source water affecting all the persons supplied in the area are more likely to be recognised than outbreaks caused by failures in the water distribution system that affect only a small part of the population. Outbreaks of diseases with severe symptoms are also more likely to be identified as people are more likely to seek medical attention. Additionally, it is difficult to state whether the geographical differences in reported outbreaks reflect a real difference in risk between the regions or just differences in outbreak detection and reporting routines by the local authorities.

Viruses belonging to the *Caliciviridae* family, mainly noroviruses, and *Campylobacter* were the groups of microorganisms most frequently associated with waterborne outbreaks. The largest outbreak notified in Denmark of campylobacteriosis, affecting more than 200 people in the city of Køge in 2010 [15]. It was caused by a point source contamination, most probably in the central water supply system. One of the largest waterborne outbreaks reported in Norway, in the city of Røros in 2007 with 1,500 sick, was also caused by *Campylobacter* [16]. Several events that might have caused a fall in water pressure and influx of contaminated water into the water distribution system were identified as the main contributing factor to the outbreak in the environmental investigation. In addition, it was considered that faecal contamination from birds, containing *Campylobacter*, could have passed directly to a production well of groundwater from an uncovered extra service well (Arnulf Moseng, Røros municipality, personal communication, November 2010).

Outbreaks caused by parasites (*Giardia* and/or *Cryptosporidium*) were few but large in size. The largest outbreaks reported in Sweden and Norway were caused by these types of microorganisms. In Norway, a giardiasis outbreak occurred in 2004 in the city of Bergen, resulting in an estimated 6,000 cases. In this outbreak, leaking sewage pipes combined with insufficient water treatment for inactivation of parasites (only chlorination was used) in the water supply serving

Figure 3

Waterborne outbreaks by level of association with water[a] by size of outbreak, Denmark, Finland, Norway and Sweden, 1998–2012[b] (n = 170)[c]



[a] Known for 139 outbreaks.

[b] For Sweden, 1998 to 2011.

[c] Five outbreaks with an unknown number of cases.

the city centre was the likely cause [17]. In Sweden, a cryptosporidiosis outbreak at the end of 2010 in the city of Östersund that involved around 27,000 persons is the largest waterborne outbreak ever reported in Europe [18,19]. One suspected source was sewage water from a few households being discharged directly into a stream, which ran into a lake from which the drinking water was obtained. The second largest outbreak in Sweden was also caused by *Cryptosporidium* and occurred half a year later in Skellefteå, further north, affecting around 20,000 persons. The cause of the outbreak was unknown but it was considered to be partly related to the Östersund outbreak. The surface waterworks in both cities lacked sufficient barriers for parasites. The outbreaks resulted in increased awareness regarding barriers and risks for waterborne disease, and actions have been taken by national authorities and at municipal waterworks. The ability to detect *Cryptosporidium* and *Giardia* in primary diagnostic laboratories has also been identified as critical for being able to detect and respond to outbreaks. The occurrence of large outbreaks should stimulate health professionals to encourage routine detection of these pathogens in samples from patients with diarrhoea. The detection of only one *Cryptosporidium* outbreak before 2010 suggests it is likely that other outbreaks may have been missed.

Nine outbreaks involved multiple microorganisms. These types of outbreaks were mainly caused by contamination with sewage. In Finland, the largest outbreak reported occurred in 2007 in the city of Nokia, where *C. jejuni*, norovirus, *Giardia* and *Salmonella* were detected in drinking water [20]. Cross-connection between the waste water system and drinking water pipeline contaminated the drinking water distribution network.

In 52 outbreaks, 30% of the total, the microorganism involved was not identified. This could be related to problems associated with microbiological testing in outbreak settings. Microbiological analysis of water during an outbreak is challenging as the contamination is often of short duration, and by the time the outbreak is detected, the contamination episode is over. Technically, it is easier to find the relevant pathogen in patient stool samples than in water samples. However, few people with uncomplicated diarrhoea consult a clinician, and stool samples are not always requested. Epidemiological analysis of outbreaks requires sufficient case numbers to give statistically significant results. This reinforces the importance of encouraging patients to go to a doctor in order to get a stool sample taken during outbreak investigations.

A large proportion of outbreaks, although of small size, occurred in single households. This highlights the importance of correct protection of wells. If this cannot be achieved, disinfection of wells should be considered. The largest outbreaks were those in which drinking water was obtained from municipal waterworks supplied by surface water, followed by those involving municipal waterworks supplied by groundwater. It is important that the function of barriers in waterworks with surface water as their water source is evaluated and if necessary improved or supplemented by additional treatment steps. Water utilities also need to be encouraged to better protect groundwater sources to minimise the risk of contamination.

In a previous report on waterborne outbreaks in the Nordic countries, based on 17 years' data (1975 to 1992), a total of 143 outbreaks were recorded [21], lower than the total number reported in our study. This could be explained by the fact that surveillance systems in the Nordic countries have been further improved and developed during the last decades, including new and improved web-based outbreak notification systems [22]. In the previous report, the proportion of outbreaks in which groundwater and surface water were involved was similar, while in our study, groundwater was the source most commonly involved. In the previous report, Denmark was also the country with fewest outbreaks reported. *Campylobacter* and *Caliciviridae* viruses were the most frequent microorganisms reported in the previous study. The proportion of outbreaks with unknown microorganisms in our study was much lower (30% compared with around 60% in the previous report), likely due to improvements in methods and routines for microbiological analysis.

The aetiologies of waterborne outbreaks reported by other European countries differ from those of the outbreaks presented here. During a 10-year period (1992 to 2003), 69% of all waterborne outbreaks reported in Wales and England were caused by *Cryptosporidium* [23]. In the United States (1971–2006) and Canada (1974–2001), the most frequently reported microorganisms in outbreaks associated with drinking water were parasites, of which *Giardia* was the most common [24,25]. While noroviruses were the most frequently reported viruses in the United States,

Campylobacter was only the third most frequent bacteria associated with waterborne outbreaks, after *Shigella* and *Salmonella*, which are not very common waterborne pathogens in the Nordic countries. In Canada, *Campylobacter* was the most common bacteria reported. The reasons for the differences in the aetiologies of the outbreaks in these countries are not completely understood. It might be due to varying levels of endemicity of the diseases or different routines in sampling, laboratory procedures or reporting.

In only a few of the outbreaks included in our study was drinking water strongly associated with the outbreak. Denmark and Finland were the countries with the highest proportion of outbreaks with a strong association. In most of the notified outbreaks, water quality failure, water treatment problem or descriptive epidemiology suggested that water was involved. In only a few of the outbreaks was a pathogen identified in the water or an analytical epidemiological study confirmed an association with water: both are always needed for an outbreak to be classified as strongly associated with drinking water according the Tillett et al. criteria [14]. The lack of demonstrated association in an outbreak partly reflects the difficulties and limitations that investigators face when performing epidemiological, microbiological and environmental investigations in these settings. Most of the outbreaks reported were small and had few laboratory-diagnosed cases. It should be emphasised that in outbreak situations every effort needs to be made to confirm cases by laboratory identification and typing of isolates so that appropriate analytical epidemiological investigations can be undertaken.

Outbreaks of disease caused by contaminated drinking water still occur every year in the Nordic region, pointing to several emerging and persisting public health challenges associated with drinking water systems. Thus it is important to adopt the World Health Organization approach to water supply described in *Water Safety Plans* [26]. Although large outbreaks due to contaminated water are rare, they highlight the need for increased awareness in the public health sector, particularly of *Cryptosporidium*, correct treatment regimens (using coagulation, filtration and disinfection) and vigilant management and maintenance of water supply and distribution systems.

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Conflict of interest

None declared.

Authors' contributions

BGH was the main investigator in the study and drafted the manuscript. KN provided supervision and scientific coordination throughout the study. All authors provided scientific input. All authors participated in manuscript writing and revision. All authors read and approved the final manuscript.

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Outbreak of psittacosis in a group of women exposed to Chlamydia psittaci-infected chickens

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Eight cases of psittacosis due to *Chlamydia psittaci* were identified in May 2013 among 15 individuals involved in chicken gutting activities on a mixed poultry farm in France. All cases were women between 42 and 67 years-old. Cases were diagnosed by serology and PCR of respiratory samples. Appropriate treatment was immediately administered to the eight hospitalised individuals after exposure to birds had been discovered. In the chicken flocks, mainly *C. gallinacea* was detected, a new member of the family *Chlamydiaceae*, whereas the ducks were found to harbour predominantly *C. psittaci*, the classical agent of psittacosis. In addition, *C. psittaci* was found in the same flock as the chickens that the patients had slaughtered. Both human and *C. psittaci*-positive avian samples carried the same *omp*A genotype E/B of *C. psittaci*, which is widespread among French duck flocks. Repeated grassland rotations between duck and chicken flocks on the farm may explain the presence of *C. psittaci* in the chickens. Inspection by the veterinary service led to temporary closure of the farm. All birds had to be euthanised on site as no slaughterhouses accepted processing them. Farm buildings and grasslands were cleaned and/or disinfected before the introduction of new poultry birds.

Introduction

The members of the family *Chlamydiaceae* are Gram-negative obligate intracellular bacteria with a unique biphasic developmental cycle. Avian chlamydiosis, also called psittacosis, is a zoonosis caused by *Chlamydia psittaci*. More than 467 avian species can be affected by chlamydial infections [1]. In birds, clinical signs vary greatly in severity and depend on the species and age of the bird as well as the infecting strain involved. Zoonotic transmission mainly occurs via inhalation of infected excretions and discharges [2,3]. The spectrum of clinical manifestations in humans is wide and varies considerably, from inapparent to a mild influenza-like illness or serious atypical pneumonia, with occasionally fatal outcome [4]. Intermittent shedding by animal carriers represents an important path of infection for birds and humans. Avian strains of *C. psittaci* are currently divided into 13 genotypes of the outer membrane protein A (OmpA), designated A to F, E/B, 1V, 6N, MatI16, R54, YP84, CPX0308 [5]. A degree of host specificity can be noted, with genotype A being detected mostly in psittacines, B and E in pigeons, or C and E/B in ducks [6].

In domesticated birds, *C. psittaci* infections occur most commonly in turkeys and ducks. Recent studies reported frequent *C. psittaci* infections in European and Asian chickens [7-9]. While *C. psittaci* was until recently considered to be the sole causative agent of avian chlamydiosis, two new avian species, *C. avium* and *C. gallinacea*, have recently been described [10]. Based on currently available data, using both broad-range and specific diagnostic tools, it seems likely that *C. gallinacea* is widely disseminated among poultry and *C. avium* is frequently found in pigeons. PCR-based tools have been developed for their specific detection [11,12]. The aetiological importance of these new agents in humans or birds is at present not well understood.

Table 1

Diagnostic data and background information of psittacosis cases, France, May 2013 (n = 8)

Case	Day of first clinical signs	Day of hospitalisation	Clinical diagnosis / symptoms	Day of discharge	Serology		PCR *C. psittaci* (*incA*)			Case status	Participation in meal preparation	Underlying diseases
					Date	Result	Date	Material	Result	Geno typing		
1	24 May	26 May	Pneumonia	4 Jun	30 May	Negative	29 May	Sputum	Positive	NA	Confirmed	Both sessions	Type 2 diabetes
2	24 May	26 May	Fever	4 Jun	29 May	IgG 1/128	29 May	Sputum	Positive	NA	Confirmed	Both sessions	Rheumatoid arthritis
					11 Jun	Negative	3 Jun	Throat swab[a]	Negative	ND			
3	25 May	27 May	Pneumonia	6 Jun	30 May	Negative	3 Jun	Throat swab[a]	Negative	ND	Possible	Both sessions	None
4	25 May	27 May	Pneumonia	5 Jun	30 May	Negative	29 May	Sputum	Negative	ND	Possible	Both sessions	Type 2 diabetes
					ND	ND	3 Jun	Throat swab[a]	Negative	ND			
5	25 May	29 May	Fever, cough	6 Jun	10 Jun	Negative	29 May	Sputum	Positive	E/B - 859	Confirmed	Both sessions	None
6	26 May	29 May	Fever	6 Jun	30 May	Negative	29 May	Sputum	Positive	E/B - 859	Confirmed	Both sessions	Asthma
7	27 May	30 May	Fever, cough	5 Jun	30 May	IgG 1/64	3 Jun	Throat swab[a]	Negative	ND	Probable	First session	Cirrhosis
8	28 May	29 May	Pneumonia	6 Jun	29 May	Negative	ND	ND	ND	ND	Possible	Both sessions	None

NA: no amplification; ND: not done.

[a] Taken after the onset of the treatment.

In France, *C. psittaci* genotypes C and E/B are highly prevalent in duck flocks [13], and human cases linked to this species are not rare. The French reference centre for psittacosis, which provides passive surveillance, diagnosed 32 cases in 2012–13. For 17 of them, exposure to ducks could be clearly established. In the present paper, we report an outbreak of psittacosis due to *C. psittaci* in women who gutted chickens bred on a farm where also ducks were raised.

Methods

Epidemiological investigations

Case definition

In the present study, a patient with fever and/or respiratory symptoms who participated in the evisceration of chickens on 14 and/or 24 May was regarded as a possible case. A probable case was a possible case combined with an IgG titre›32. A confirmed case was a possible case with either positive detection of *C. psittaci* by PCR in a respiratory sample, or seroconversion, or a fourfold increase in IgG titre.

Questionnaire

After notification of a cluster of psittacosis cases to the public health authorities of the Department of Aquitaine, a telephone investigation was conducted. A questionnaire covering age, sex, date of onset of clinical signs, symptoms and travel activities within 15 days before illness onset was completed for each hospitalised person after they were discharged.

Microbiological investigations

Human samples

Aliquots of early serum from each patient were sent to the National Reference Centre for Chlamydiae (NRC, Bordeaux, France). Sputum samples from five patients were collected during their hospitalisation, as well as throat swabs from four patients after medication.

Direct detection of Chlamydia psittaci from human samples

Clinical samples were extracted by using the automated MagNA Pure DNA extraction (Roche Diagnostics, Meylan, France) [14] then analysed using a *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene [15] and a specific *inc*A real-time PCR protocol [16].

Serology

A commercialised micro-immunofluorescence test was used (Chlamydia MIF, Focus, Eurobio, France). This assay can distinguish between IgM and IgG subclasses. Each well contained four spots: one yolk sac control and three individual antigen spots consisting of elementary bodies of *C. psittaci*, *C. trachomatis* and *C. pneumoniae* suspended in a yolk sac matrix. Each run included a positive (murine hyperimmune serum) and negative control (human serum). For IgG, the serum

was serially diluted from 1/16. The reciprocal of the highest serum dilution yielding apple-green fluorescence was termed the serum endpoint titre. For IgM, only one serum dilution was tested (1/16) and the result was assessed qualitatively, i.e. positive or negative.

Traceback investigations

Animal samples

To identify the source of infection of the patients, a survey was conducted in all poultry flocks of the farm. On 10 June, samples were collected from all duck flocks (n = 4, denominated MD for mule duck) and chicken flocks (n = 3, denominated BC for broiler chicken) on the farm. In each sampled flock, 15 randomly selected animals were submitted to a double cloacal swabbing. Samples were transported on ice to the National Reference Laboratory for Avian chlamydiosis (NRL, Maisons-Alfort, France). One panel of swabs was stored in conservation buffer SPG [17] at – 80 °C until inoculated onto chicken eggs. The second panel was stored dry at – 80 °C until subjected to DNA extraction.

Direct detection of Chlamydiaceae in birds

The dry panel of cloacal swabs was subjected to DNA extraction using the QIAamp DNA Mini Kit, following the buccal swab protocol (Qiagen, Courtaboeuf, France). A *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene was used in this study [15]. All samples with a cycle threshold (Cq) › 38 were considered negative.

Inoculation onto chicken eggs

For cell culture, suspensions of cloacal swabs stored in conservation buffer at – 80 °C were inoculated onto seven day-old embryonated eggs as previously described [18].

Real-time PCR for detection of *Chlamydia psittaci* and *Chlamydia gallinacea*

All *Chlamydiaceae* PCR-positive samples from humans and animals were re-analysed using previously described real-time PCR assays for *C. psittaci* [16] and *C. gallinacea* [11]. In addition, a new *eno*A-based real-time PCR protocol for *C. gallinacea* was developed in this study. It uses primers *eno*A_F (5'-CAATGGCCTACAATTCCAAGAGT-3'), *eno*A_R (5'-CATGCGTACAGCTTCCGTAAAC-3') and probe *eno*A_P (5'- FAM-ATTCGCCCTACGGGAGCCCCTT-TAMRA-3') under standard cycling conditions.

DNA microarray and sequencing

A previously described DNA microarray capable of identifying all *Chlamydia* spp. [19] was recently extended to include the new species of *C. avium* and *C. gallinacea* [20]. This array version Chlamydia04 (Alere Technologies, Jena, Germany) was used throughout the study.

The *omp*A gene was partially amplified from human samples and animal isolates as described previously using primers CTU/CTL [21] or 191CHOMP/CHOMP371 [22].

Results

Recognition of the outbreak

In May 2013, eight cases of respiratory disease were reported to the public health authorities of Aquitaine, south-western France. As individuals had gutted about a hundred chickens for the preparation of two meals on a poultry farm on the days preceding the onset of clinical signs, they were suspected of psittacosis. The entire group that had participated in these activities on 14 and/or 24 May comprised 15 persons.

A summary of patient information and diagnostic testing is given in Table 1. In four cases, the presence of *C. psittaci* in sputum was demonstrated by real-time PCR. The eight hospitalised cases were treated with antibiotics (macrolides in association with cephalosporins for six days, then only macrolides for seven additional days), and all individuals quickly recovered. Throat swabs collected from four patients after the beginning of their treatment were all negative by PCR.

Finally, four confirmed cases, one probable case and three possible cases were identified. The relatively high DNA content in the samples from Patients 5 and 6 (Cq 28 and 30, respectively) allowed *omp*A sequencing, which revealed identical sequences with 100% homology to *C. psittaci* strain 06–859. This strain was assigned to *omp*A genotype E/B, subtype 859 [5].

Patient characteristics

All patients were hospitalised between 26 and 30 May, with onset of clinical signs recorded between 24 and 28 May (Figure). All were women aged between 42 and 67 years. All except one participated in the preparation of both meals. No previous travel was reported by any of them. All presented fever and two had cough. Headache, vomiting, asthenia, myalgia, dizziness and urinary tract infection was also reported. Type 2 diabetes, rheumatoid arthritis, asthma or cirrhosis were underlying diseases reported for five patients. Unfortunately, due to difficulties in communicating with women from this group, who spoke very little

Figure

Psittacosis case distribution by date of disease onset, France, May 2013 (n = 8)

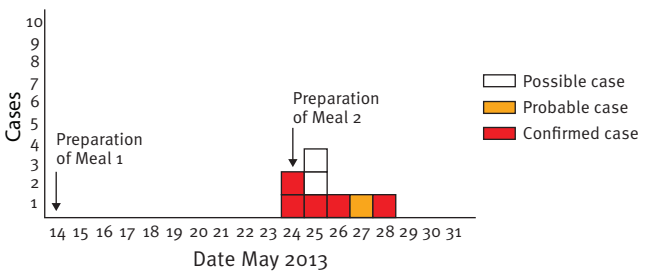

Table 2

Diagnostic data and background information on poultry flocks, psittacosis outbreak, France, May 2013 (n = 8)

Id Anses_Flock	Species	Age (days)	Number of animals	Arrival on the farm	PCR *Chlamydiaceae* CH23S)		PCR *C. psittaci* (*incA*)		PCR *C. gallinacea* (*enoA*)		Isolates	Species previously held on this field		
					Positive/ total	Cq values mean (range)	Positive/ total	Cq values mean (range)	Positive/ total	Cq values mean (range)		Species	Effective	Implementation date
13–1648_ BC1	Chicken	130	4,700	31/01/13	5/15	28.1 (19.2–37.7)	3/5	29.3 (17.7–37.1)	3/5	24.2 (22.4–25.3)	1 *C. psittaci*	Duck	2,550	08/11/12
13–1648_ BC2	Chicken	67	2,200	05/04/13	15/15	28.6 (24.9–37.7)	3/15	35.7 (34.0–36.7)	14/15	29.3 (25.4–37.5)	6 *C. gallinacea*	NA	NA	NA
13–1648_ BC3	Chicken	42	2,800	29/04/13	2/15	31.9 (27.1–36.8)	2/2	36.8 (36.5–37.1)	1/2	27.9	No isolate	NA	NA	NA
13–1648_ MD1	Duck	50	2,652	22/04/13	5/15	36.4 (32.9–37.8)	1/5	37.2	3/5	36.0 (33.2–38.4)	ND	Chicken	3,200	28/11/12
13–1648_ MD2	Duck	78	2,652	25/03/13	13/15	34.3 (30.7–37.6)	13/13	33.9 (29.5–37.8)	4/13	37.5 (36.5–37.9)	ND	Duck	2,552	21/12/12
13–1648_ MD3	Duck	34	2,040	07/05/13	12/15	34.8 (28.2–37.8)	4/12	30.2 (26.7–37.9)	7/12	36.8 (36.2–37.8)	ND	Chicken	2,700	02/11/12
13–1648_ MD4	Duck	70	2,941	03/04/13	6/15	35.4 (33.6–36.7)	4/6	33.9 (33.1–34.7)	3/6	37.7 (37.7–37.9)	ND	Duck	2,652	29/01/13

NA: not available; ND: not done.

French, only the eight patients attending a physician were questioned. Therefore, further epidemiological investigations within the group were not possible.

Examination of samples from poultry

In preliminary testing, swabs from five chickens were examined by real-time PCR, which revealed positivity for *Chlamydiaceae*. These findings prompted a more comprehensive investigation to include all flocks on the site, i.e. three broiler chicken (BC) flocks and four mule duck (MD) flocks. A summary of diagnostic data and information on flocks is given in Table 2. Interestingly, *C. psittaci* was detected in all flocks, as well as the recently introduced species of *C. gallinacea*. In terms of infected animal number and bacterial load, ducks were predominantly infected by *C. psittaci*, whereas chickens were predominantly infected by *C. gallinacea*, except for flock BC1, which also included one high shedder of *C. psittaci* among 15 animals tested. All *Chlamydiaceae*-positive samples were re-analysed with the extended chlamydial microarray that included *C. gallinacea*-specific probes. A very good correlation between real-time PCR and microarray was observed for samples having Cq < 35. No clinical signs were reported in any of these flocks.

Isolates were successfully cultured from BC1 (n = 1) and BC2 (n = 6) chicken flocks (Table 2). Using real-time PCR, the BC1 isolate was identified as *C. psittaci*, whereas the six BC2 isolates were *C. gallinacea*.

Comparison of human and animal samples

Partial sequencing of the *omp*A gene from the BC1 *C. psittaci* isolate revealed an identical sequence to those obtained from the two PCR-positive patients from whom sequencing was possible. This sequence was also obtained from two duck samples with sufficient DNA content (both from flock MD2). Analysis of the *omp*A sequences from the six *C. gallinacea* isolates of BC2 revealed two distinct groups, which suggests mixed *C. gallinacea* infection in this flock.

Farm management

Frequent rotations between duck and chicken flocks, with flocks sharing the same fields (Table 2), were characteristic for the management of the farm. Interestingly, ducks had previously been raised on the same field on which flock BC1 was established in January.

Discussion

Eight cases of psittacosis (four confirmed and four probable or possible cases) were identified among a group of 15 women who gutted chickens in a confined space on the days that preceded the onset of clinical signs. Initially, infection with Middle East respiratory syndrome coronavirus had been considered because one case had been identified in France in the same month [23]. However, this assumption was discarded in favour of psittacosis as these women suffered from pneumonia or influenza-like symptoms. Clinical signs of psittacosis are similar to those associated with other pathogens that cause pneumonia, so that

clinicians need to include *C. psittaci* in their differential diagnosis, especially when close contact with birds is reported. Knowledge of previous exposure to birds was crucial for the decision on medication of these patients, which included an early and adapted prescription of antibiotics.

In France, *C. psittaci* is widespread in poultry, particularly on duck farms [24], and the most severe human cases reported each year by the NRC are mainly related to ducks, less frequently to pigeons or psittacines. *C. gallinacea* is a newly described chlamydial species [10]. Recent surveys on the prevalence of *C. gallinacea* in poultry flocks in four European countries and China revealed a prevalence that could even exceed that of *C. psittaci* [11], at least for chickens and turkeys. These data were recently confirmed by a survey conducted on *Chlamydiaceae* prevalence in French slaughtered poultry birds, which revealed that *C. gallinacea* is mainly encountered in chickens and turkeys, while *C. psittaci* is most often detected in ducks [25]. On the farm investigated in this study, the same general observation was made, except that *C. psittaci* was also detected in chicken flocks BC1 and BC2, with one animal in BC1 identified as a high shedder (Cq = 17). Sequences of the *omp*A gene from DNA of patient samples and from the *C. psittaci* isolate obtained from BC1 were identical and homologous to the E/B genotype subtype 859. The same *omp*A sequence was obtained from swab samples collected from ducks, suggesting one single *C. psittaci* isolate may have been circulating on this farm and probably represented the origin of the human outbreak. This genotype is commonly identified in *C. psittaci* isolates from French ducks [18]. Interestingly, while chickens and ducks were reared separately on this farm, retrospective analysis of flock rotations showed that ducks had preceded BC1 chickens on the same field. The alternation of poultry species on grasslands probably explains the presence of *C. psittaci* in these chickens alongside *C. gallinacea*. Monitoring faecal shedding could be a way to track the persistence of *Chlamydiaceae* on animals as well as contaminations between flocks.

While *C. gallinacea* has also been detected in the chicken flock harbouring the birds gutted by the patients, DNA extracted from human samples were only positive for *C. psittaci*. The pathogenicity of *C. gallinaceae*, a recently discovered species, has yet to be defined [10]. The infectious dose seems to be a critical parameter for an active human infection. In flock BC1, *C. psittaci* was the more prevalent chlamydial agent in terms of bacterial load in infected birds, as very low Cq values were detected. *C. psittaci* antibodies were detected using micro-immunofluorescence testing in only two cases. This is in line with observations from experimental infection of animals, where the humoral immune response to *C. psittaci* infection was generally weak and did not emerge regularly [24]. New serological techniques based on specific oligopeptides are currently under development in order to differentiate chlamydial antibodies at species level [26]. Such a tool, if extended to include the recently described new species of *Chlamydia*, would be of great value, e.g. to assess the aetiological importance and zoonotic potential of *C. gallinacea*.

Following reports of this psittacosis cluster, the veterinary services made an on-site inspection on the farm and commissioned samples. Slaughtering activities were suspended and farm activities were temporary blocked. Several slaughterhouses were contacted and did not accept to process these poultry birds due to the known risk of psittacosis, so that the animals had to be euthanised on site. This series of events was an opportunity to test the national procedures in place for the emergency management of outbreaks of avian influenza. On the farm, buildings and grasslands were cleaned and/or disinfected and recommendations were given to the farmer on farming practices in order to limit the risk of a new outbreak.

In conclusion, this survey showed that, even if rare in French flocks, chickens can also harbour *C. psittaci*. Farming practices that include grassland rotations of different species should be avoided to prevent the transmission of pathogens from one avian species to another. All individuals involved in activities associated with live poultry birds, especially if done in a confined area, must wear appropriate protective clothing (masks and gloves). It is also important to keep in mind that *C. psittaci* as a zoonotic agent is generally highly prevalent in poultry birds, notably in ducks, despite the absence of clinical signs in carrier animals.

Conflict of interest

None declared.

Authors' contributions

RA, KL, KS wrote the manuscript. IC took part in the clinical management of the patient. RA, BdB, KS collaborated in diagnosis methods. LM, HR, PR, VS, VV collaborated on the public health investigation. FM, JLM collaborated on the veterinary investigations.
All authors approved the manuscript.

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