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Genetic analyses of GII.17 norovirus strains in diarrheal disease outbreaks from December 2014 to March 2015 in Japan reveal a novel polymerase sequence and amino acid substitutions in the capsid region

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A novel GII.P17-GII.17 variant norovirus emerged as a major cause of norovirus outbreaks from December 2014 to March 2015 in Japan. Named Hu/GII/JP/2014/GII.P17-GII.17, this variant has a newly identified GII.P17 type RNA-dependent RNA polymerase, while the capsid sequence displays amino acid substitutions around histo-blood group antigen (HBGA) binding sites. Several variants caused by mutations in the capsid region have previously been observed in the GII.4 genotype. Monitoring the GII.17 variant's geographical spread and evolution is important.

The present study uses complete genome sequences and phylogenetic and in silico analyses to characterise GII.P17 norovirus strains contributing to gastroenteritis outbreaks in Japan from December 2014 to March 2015.

Norovirus outbreaks from October 2014 to March 2015 in Japan

In Japan, numbers of norovirus cases are reported to the Infectious Agents Surveillance Report (IASR) system, which is the national surveillance system overseen by the National Institute of Infectious Diseases (http://www.nih.go.jp/niid/ja/iasr-noro.html). In the six months from October 2014 to March 2015, a total of 2,133 norovirus cases in the country were reported to IASR, including 373 cases caused by genotype GII.4, 146 cases caused by GII.3 and 100 cases caused by GII.17. Other genotypes (GI.2, GI.3, GI.6, GI.7, GII.2, GII.7, GII.12, GII.13 and GII.14) were also detected in this season. Although for most of the six months of the 2014/15 winter season GII.4 norovirus predominated, the number of GII.17 cases presented a dramatic increase compared to the previous winter, whereby only three GII.17 cases had been detected from October 2013 to March 2014. In the previous five years, the average and standard deviation (SD) numbers of norovirus cases during the same months were moreover 2,727±340 for all norovirus cases, 589±256 for GII.4 cases, 130±216 for GII.3 cases and 1±1 for GII.17 cases. The first GII.17 cases in the 2014/15 winter season were observed in December 2014. In the subsequent months, more cases with this genotype continued to occur across the whole country. A sharp rise in GII.17 cases was moreover noted between January (n=11 cases) and February (n=55 cases) 2015, making GII.17 the most prevalent genotype in March (n=31 cases) 2015.

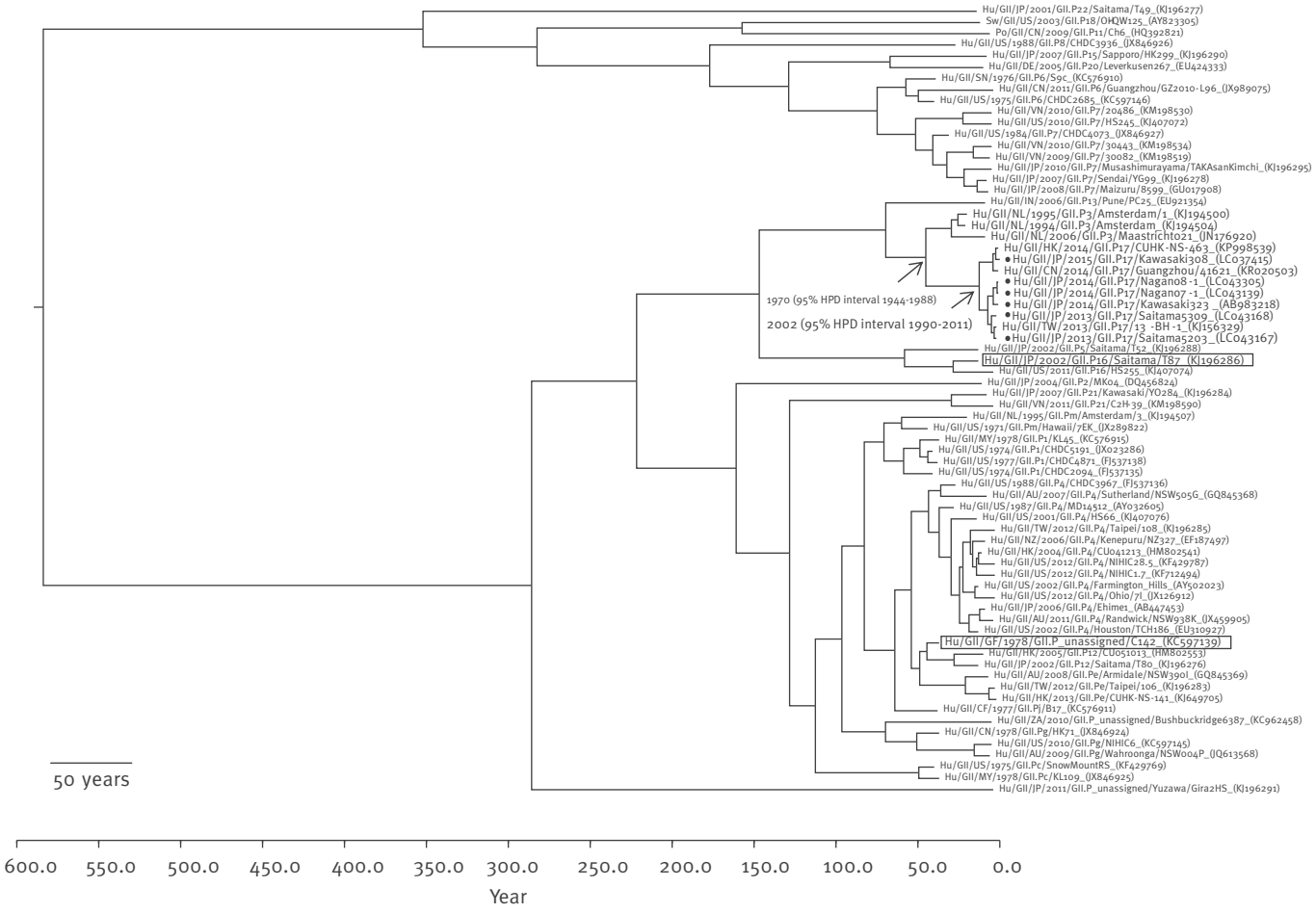
To investigate the GII.P17 norovirus strains responsible for outbreaks in Japan, and to also look for any changes in the viral genome of strains circulating between 2013 and 2015, six specimens from the Kanagawa, Nagano and Saitama prefectures that were available for further characterisation were used in this study.

Genotyping GII.P17 strains

From the norovirus GII.17 specimens collected from January 2013 to March 2015, six available specimens (i.e. Kawasaki308 (collected in February, 2015), Kawasaki323 (collected in March, 2014), Nagano7–1 (collected in August, 2014), Nagano8–1 (collected in August, 2014), Saitama5203 (collected in April, 2013) and Saitama5309 (collected in July, 2013)) were selected for full genome analyses by next-generation

Figure 1

Time-scaled phylogenetic tree obtained with the Bayesian Markov chain Monte Carlo method of RNA-dependent RNA polymerase (RdRp) sequences (1,283 nt) of norovirus GII strains



The phylogenetic analysis includes the nucleotide sequences of six Japanese GII.P17-GII.17 strains (indicated by black dots). The oldest GII.17 strain (C142) detected in 1978 and a previous Japanese GII.17 (Saitama T87) detected in 2002 are indicated by rectangular boxes. The arrows point to some nodes, for which the node ages are indicated with 95% highest posterior density (HPD) intervals. The scale bar represents time in years.

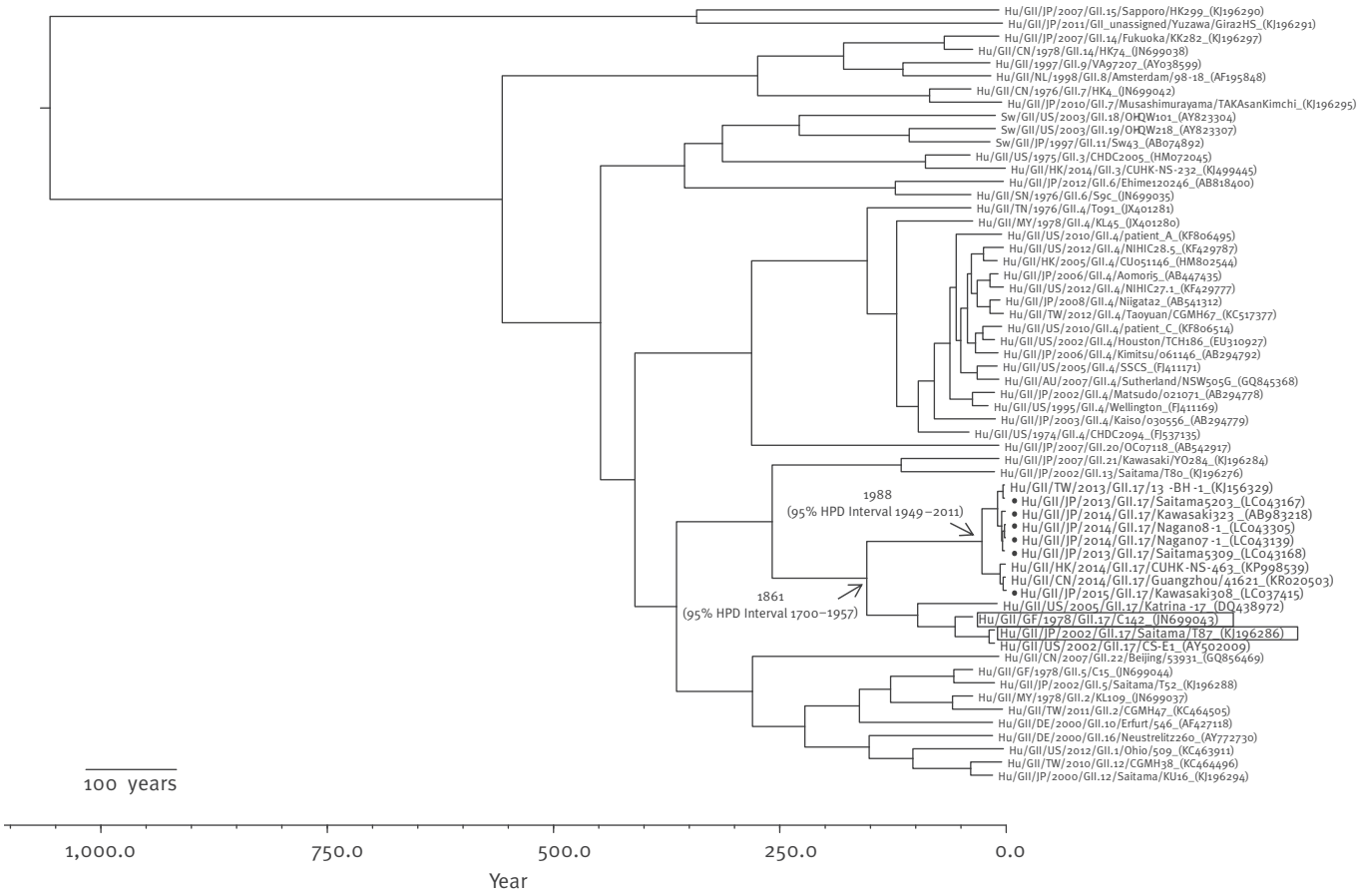
sequencing as described [1,2]. The data analysis was performed with CLC Genomics Workbench v8.0.1 (CLC Bio). Contigs were assembled from the obtained sequence reads by de novo assembly. The nucleotide sequences for the GII.P17 strains in this study were deposited in GenBank and assigned accession numbers AB983218, LC037415, LC043139, LC043167, LC043168, and LC043305. When the Norovirus Genotyping Tool was used (http://www.rivm.nl/mpf/norovirus/typingtool) [3], the capsid genotypes of all six strains were assigned to GII.17, but the RNA-dependent RNA polymerase (RdRp) genotypes of some strains could not be assigned to any known genotype in the database. This observation suggested the genetic novelty of the virus in this region. Upon first noticing this with the sequence of Kawasaki323 strain in June 2014, we sought the advice of NoroNet, who coordinate norovirus nomenclature through a global network of research scientists, clinicians and public health officials [4]. After discussions with them, this variant was assigned to the RdRp genotype, GII.P17, in August, 2014. Finally, we named the emergent variants of norovirus as Hu/GII/JP/2014/GII.P17-GII.17.

Phylogenetic and molecular dating analyses

The phylogenetic analyses, the time of most recent common ancestor (tMRCA), and the divergence times were estimated for emergent GII.P17-GII.17 variants, along with other representative norovirus GII strains, by the Bayesian Markov chain Monte Carlo (MCMC) method implemented in Bayesian Evolutionary Analysis Sampling Trees (BEAST) v1.8.1 [5]. As a result of the marginal likelihood calculation in the four clock (strict clock, uncorrelated lognormal relaxed clock, uncorrelated exponential relaxed clock and random local clock) and demographic models (constant size, exponential growth, logistic growth and expansion growth), the datasets were analysed using the Tamura and Nei 1993 (TN93) + Gamma + Proportion Invariant (for RdRp) and the generalised time-reversible (GTR) + Gamma + Proportion Invariant (for capsid) nucleotide substitution models, with an uncorrelated exponential relaxed clock model under a constant size

Figure 2

Time-scaled phylogenetic tree obtained with the Bayesian Markov chain Monte Carlo method of full length capsid gene (virus protein 1 gene) sequences of norovirus GII strains



The phylogenetic analysis includes the nucleotide sequences of six Japanese GII.P17-GII.17 strains (indicated by black dots). The oldest GII.17 strain (C142) detected in 1978 and previous Japanese GII.17 (Saitama T87) detected in 2002 are indicated by rectangular boxes. The arrows point to some nodes, for which the node ages are indicated with 95% highest posterior density (HPD) intervals. The scale bar represents time in years.

tree prior. Convergence was assessed by the effective sample size (ESS) after a 2% burn-in. Only parameters with an ESS above 150 were accepted. Phylogenetic analysis using the maximum likelihood (ML) method showed no differences of topology to the Bayesian MCMC trees (data not shown).

In terms of the RdRp gene, the emergent Japanese GII.P17 belonged to a single cluster including other strains detected in Asia in 2013 (Taiwan: KJ156329) and in 2014 (Guangzhou: KR020503; Hong Kong: KP998539). The tMRCA for the emergent GII.P17 cluster was estimated to be 2002 (95% highest posterior density (HPD) interval: 1990–2011

This suggests that the emergent GII.P17 has been circulating around Asia for ca 13 years (Figure 1). In terms of the RdRp gene, the emergent GII.P17 was most closely related to GII.P3, and evolved from a common ancestor in 1970 (95% HPD interval: 1944–1988). Nearly 32 years elapsed between the tMRCA of the emergent GII.P17 cluster, and the divergence of emergent GII.P17 strains from the GII.P3 cluster (Figure 1). Interestingly, the oldest GII.17 (C142 detected in French Guiana in 1978) and the oldest Japanese GII.17 (Saitama T87 detected in 2002) had genotype combinations that were different from the emergent GII.17 forms in this study, the GII.P_unassigned-GII.17 for C142 and GII.P16-GII.17 for Saitama T87 (Figure 1). These results suggested that capsid GII.17 genotype evolved by exchanging the RdRp gene through at least two recombination events.

In terms of the capsid gene, the emergent GII.P17-GII.17 strains also belonged to a single cluster that was different from the cluster formed by the older GII.17 strains (Figure 2). Notably, the emerging GII.17 cluster diverged from the old GII.17 cluster around the year 1861 (95% HPD interval: 1700–1957), yet the tMRCA was in the year 1988 (95% HPD interval: 1949–2011) (Figure 2). The two independent clusters formed by the old and emerging GII.17 strains partially arose from changes in the amino acids of the major epitopes (Figure 2 and Table). Thus, we thought that the emerging GII.17

Table

Amino acid substitutions in the major epitopes of virus protein 1 between novel GII.17 variants and previously identified GII.17 strains of norovirus

Name of strains[a]	Amino acid number (major epitopes)[b]																																									
	217–225 (I)									291–298 (II)										359–363 (III)					371–379 (IV)										390–396 (V)							
C142	P	P	S	V	E	S	K	T	K	T	A	D	V	H	Q	S	H	D	D	S	S	Q	F	V	F	G	S	T	S	D	–	D	F	Q	I	K	V	E	S	G	–	H
Saitama T87	.	.	.	.	.	.	.	.	.	.	.	.	.	D	G	.	.	D	D	.	.	.	.	.	.	.	.	.	.	T	–	.	.	.	.	.	I	.	.	.	–	H
CS-E1	.	.	.	.	.	.	.	.	.	.	.	.	.	D	G	.	.	D	D	.	.	.	.	.	.	.	.	.	.	T	–	.	.	.	.	.	I	.	.	.	–	H
Katrina-17	.	.	.	.	.	.	.	.	.	S	.	.	.	Q	N	.	.	Q	D	.	T	.	.	.	.	.	.	E	.	E	–	.	.	.	.	.	I	.	T	.	–	H
Saitama5203[c]	.	.	.	.	.	.	.	.	.	.	.	E	T	–	–	D	.	R	D	.	P	.	.	.	.	R	.	N	D	N	–	.	.	.	.	N	D	D	G	D	–	H
Saitama5309[c]	.	.	.	.	.	.	.	.	.	.	.	E	T	–	–	D	.	R	D	.	P	.	.	.	.	R	.	N	D	N	–	.	.	.	.	N	D	D	G	D	–	H
13-BH-1	.	.	.	.	.	.	.	.	.	.	.	E	T	–	–	D	.	R	D	.	P	.	.	.	.	R	.	N	D	N	–	.	.	.	.	N	D	D	G	D	–	H
Kawasaki323[c]	.	.	.	.	.	.	.	.	.	.	.	E	T	–	–	D	.	R	D	.	P	.	.	.	.	R	.	N	D	N	–	.	.	.	.	N	D	D	G	D	–	H
Nagano7-1[c]	.	.	.	.	.	.	.	.	.	.	.	E	T	–	–	D	.	R	D	.	P	.	.	.	.	R	.	N	D	N	–	.	.	.	.	N	D	D	G	D	–	H
Nagano8-1[c]	.	.	.	.	.	.	.	.	.	.	.	E	T	–	–	D	.	R	D	.	P	.	.	.	.	R	.	N	D	N	–	.	.	.	.	N	D	D	G	D	–	H
CUHK-NS-463	.	.	.	.	.	.	.	.	.	.	.	Q	I	–	–	N	Q	R	D	.	P	.	.	.	L	R	I	S	D	N	D	.	.	.	V	N	D	D	D	D	G	H
Guangzhou41621	.	.	.	.	.	.	.	.	.	.	.	Q	I	–	–	N	Q	R	D	.	P	.	.	.	L	R	I	S	D	N	D	.	.	.	V	N	D	D	D	D	G	H
Kawasaki308[c]	.	.	.	.	.	.	.	.	.	.	.	Q	I	–	–	N	Q	R	D	.	P	.	.	.	L	R	I	S	D	N	D	.	.	.	V	N	D	D	D	D	G	H

New GII.P17-GII.17 variants are highlighted in red. Previous GII.17 strains are highlighted in black. Dots indicate sequence identity among sequences presented. A dash represents the relative deletion of an amino acid at a certain position, compared to other sequences in the alignment.

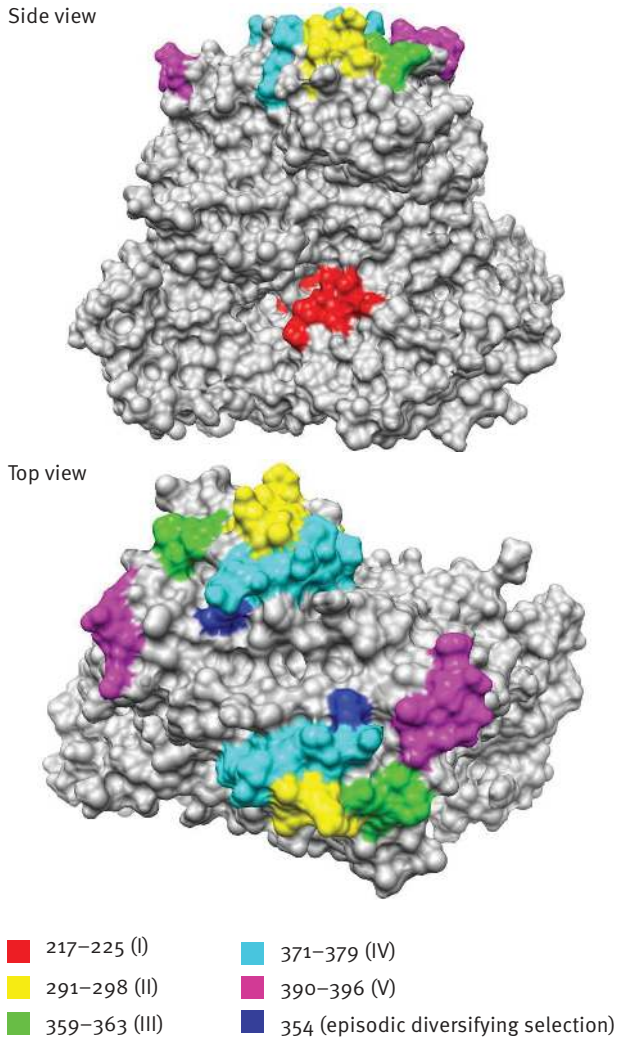
[a] The GenBank accession numbers of the sequences are as follow: C142 (JN699043); CS-E1 (AY502009); Katrina-17 (DQ438972); Saitama T87 (KJ196286); Kawasaki308 (LC037415); Kawasaki323 (AB983218); Nagano7–1 (LC043139); Nagano8–1 (LC043305); Saitama5203 (LC043167); Saitama5309 (LC043168).

[b] Amino acid numbering is based on the sequence of the Kawasaki323 strain.

[c] Japanese GII.P17-GII.17 strains in this study.

Figure 3

Structural model of the dimer formed by virus protein 1 (VP1) of Kawasaki323 GII.17 strain



The GII.17 VP1 model was constructed by homology modelling with the crystal structure of norovirus capsid (1IHM). Five predicted epitopes (I–V) are shown by each colour with the amino acid positions based on the sequence of Kawasaki323 strain. Amino acids with episodic diversifying selection are highlighted in dark blue.

strains may have variants of the capsid gene, as has been observed with some GII.4 strains [6].

Moreover within the emergent GII.P17-GII.17 cluster, diversification of strains further led to two sub-clusters in the capsid and RdRp genes, with changes to the amino acids of the major epitopes of the virus protein 1 (VP1) protein (Figures 1 and 2 and Table). One sub-cluster comprised the Kawasaki308 strain, while the other sub-cluster included the Kawasaki323 strain as well as all the other four GII.P17-GII.17 strains from this study. These results clearly suggest that the new GII.P17-GII.17 variants have different evolutionary histories than previously identified GII.17 strains, and that rapid evolution may occur within the emergent GII.P17-GII.17 variants. The GII.17 genotype may produce other variants whereby mutations lead to changes in the antigenicity of the P2 domain while still being constrained by host immunity, in the same way as has been observed for GII.4 [6].

Estimation of positive selection sites and B-cell epitopes in virus protein 1 sequence of the GII.P17-GII.17 variant

We analysed the evolutionary constraints on the GII.P17-GII.17 variants from host-immune pressure, based on the single likelihood ancestor counting (SLAC), fixed effect likelihood (FEL), internal FEL (IFEL), fast unconstrained bayesian approximation for inferring selection (FUBAR), random effects likelihood (REL) and mixed effects model of evolution (MEME) with only the GII.17 sequences in the dataset used in phylogenetic analyses for VP1 gene, but using another alignment [7]. One positive selection (V354W) (V, sub-cluster including Kawasaki323 strain; W, sub-cluster including Kawasaki308) was identified with the MEME analysis. This non-synonymous mutation was only observed in the sub-cluster of Kawasaki308 strain, suggesting that the selection was episodic but not pervasive. Moreover, the BepiPred and DiscoTope servers [8,9] predicted B-cell epitopes associated with humoral immunity at the amino acid positions 217–225 (I), 291–298 (II), 359–363 (III), 371–379 (IV), and 390–396 (V) in the VP1 protein of the Kawasaki323 strain (Table). Epitope I was conserved in all sequences within the GII.17 cluster, whereas the others (II-V) were variable with amino acid substitutions, deletions, and insertions not only between the novel GII.P17-GII.17 variant and old GII.17 strains, but also within the GII.P17-GII.17 variant cluster, between sub-clusters represented by the Kawasaki323 and Kawasaki308 strains (Table).

To identify these amino acid positions on the capsid structure, we calculated and constructed a capsid 3D model of the Kawasaki323 strain with the MODELLER 9.13 programme [10]. The epitopes were located on the exterior surface of the shell (epitope I) and the protruding 2 (P2) domains (epitope II-V), including the binding pocket for the histo-blood group antigens (Figure 3). Additionally, the mutation (V354W) (V, sub-cluster including Kawasaki323 strain; W, sub-cluster including Kawasaki308) associated with episodic positive selection was close to the 372R within the epitope IV (Figure 3). These results indicate that our GII.P17-GII.17 variants might have the potential to escape from host neutralising antibody by amino acid alterations of four putative B-cell epitopes in the P2 domain top, and to improve the binding capacity of the histo-blood group antigens. During the winter 2014/15, noroviruses similar to the Kawasaki308 strain became extremely prevalent in Japan and China [11], and this observation may imply a high infectivity of the strain because a number of mutations, including positive selection, were observed between strains detected in the 2013/14 and 2014/15 seasons.

Conclusions

During the season from October 2014 to March 2015, a novel norovirus variant GII.P17-GII.17 was prevalent in Japan from December 2014 onwards. While, in general, the total number of norovirus cases during this season was lower than previous years beyond a range of average±SD, the number of cases affected by the GII.17 genotype appeared to be higher and increased dramatically in February 2015, making this the predominant genotype in the country in March 2015. Further characterisation GII.17 available strains from January 2013 to March 2015 assigned these to the novel GII.P17-GII.17 variant. Because this variant was detected from a few cases in Japan and Taiwan, prior to becoming prevalent and causing outbreaks during the 2014/15 winter season in Japan and China [11], early surveillance of sporadic cases caused by this or any other potential variants may assist in anticipating outbreaks. Molecular and phylogenetic analyses conducted here show that the novel GII.17 variant has a different evolutionary history to previously identified GII.17 strains. As it might have the potential to spread globally in the near future, presumably by escaping host immunity as GII.4 variants do [6], monitoring trends in the geographical spread and evolution of the variant is important.

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Conflict of interest

None declared.

Authors' contributions

Yuki Matsushima and Hideaki Shimizu designed the study. Yuki Matsushima and Kazuhiko Katayama drafted the manuscript. Yuki Matsushima, Mariko Ishikawa and Koo Nagasawa performed bioinformatic analyses. Yuki Matsushima, Mariko Ishikawa, Tomomi Shimizu, Ayako Komane and Hideaki Shimizu conducted experiments using samples derived from Kawasaki City, Japan. Shizuko Kasuo performed experiments with samples collected in Nagano Prefecture, Japan. Michiyo Shinohara conducted experiments using samples derived from Saitama Prefecture, Japan. Kei Haga and Yen Hai Doan performed sequencing analyses of these strains using a next generation sequence system. Yuki Matsushima, Mariko Ishikawa, Hirokazu Kimura, Akihide Ryo, Nobuhiko Okabe, Yen Hai Doan, Kazuhiko Katayama and Hideaki Shimizu reviewed and revised the manuscript.

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Emergence of a novel GII.17 norovirus – End of the GII.4 era?

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In the winter of 2014/15 a novel GII.P17-GII.17 norovirus strain (GII.17 Kawasaki 2014) emerged, as a major cause of gastroenteritis outbreaks in China and Japan. Since their emergence these novel GII.P17-GII.17 viruses have replaced the previously dominant GII.4 genotype Sydney 2012 variant in some areas in Asia but were only detected in a limited number of cases on other continents. This perspective provides an overview of the available information on GII.17 viruses in order to gain insight in the viral and host characteristics of this norovirus genotype. We further discuss the emergence of this novel GII.P17-GII.17 norovirus in context of current knowledge on the epidemiology of noroviruses. It remains to be seen if the currently dominant norovirus strain GII.4 Sydney 2012 will be replaced in other parts of the world. Nevertheless, the public health community and surveillance systems need to be prepared in case of a potential increase of norovirus activity in the next seasons caused by this novel GII.P17-GII.17 norovirus.

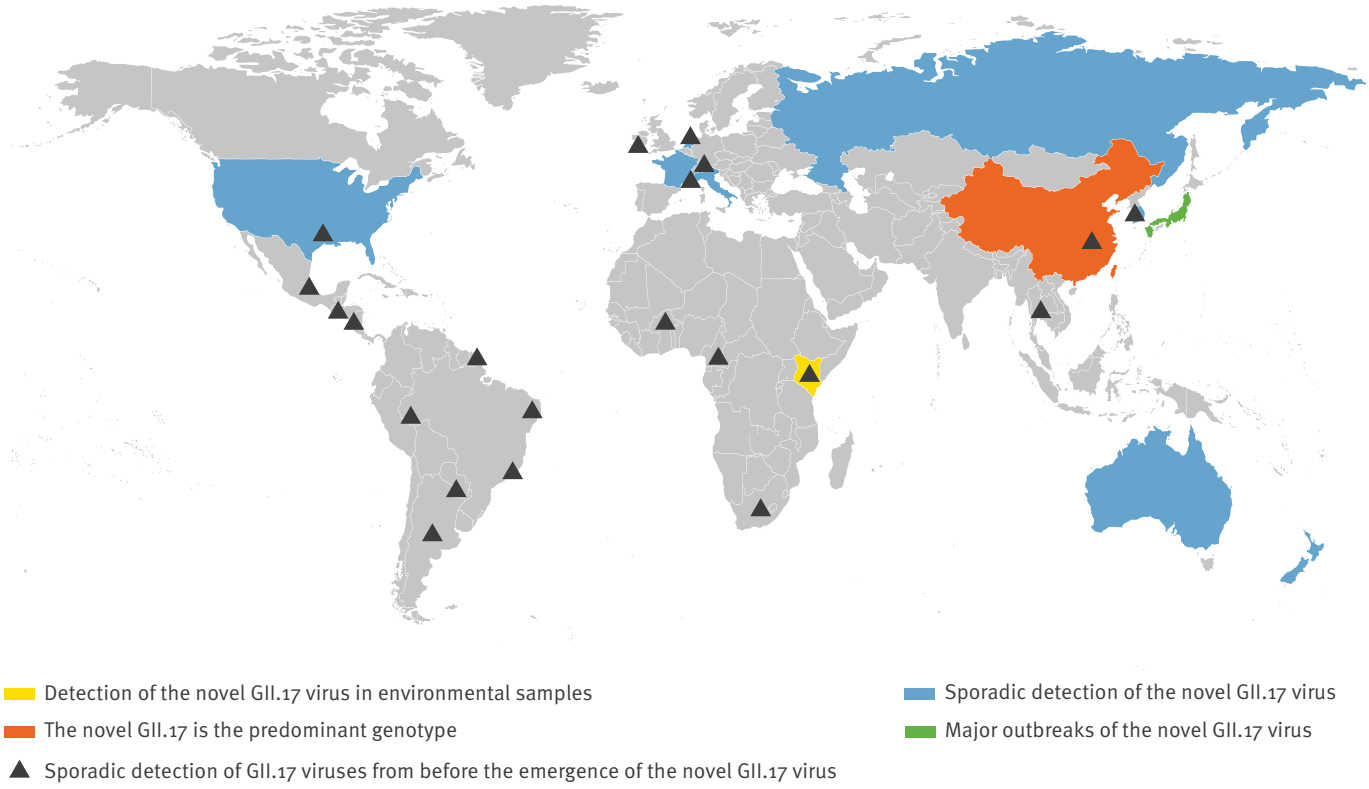
In this issue of *Eurosurveillance*, observations from Japan are reported on an unusual prevalence of a previously rare norovirus genotype, GII.17, in diarrheal disease outbreaks at the end of the 2014/15 winter season [1], similar to what was observed for China [2,3]. Norovirus is a leading cause of gastroenteritis [4]. Although the infection is self-limiting in healthy individuals, clinical symptoms are much more severe and can last longer in immunocompromised individuals, the elderly and young children [5,6].

The *Norovirus* genus comprises seven genogroups (G), which can be subdivided in more than 30 genotypes [7]. Viruses belonging to the GI, GII and GIV genogroups can infect humans, but since the mid-1990s GII.4 viruses have caused the majority (ca 70–80%) of all norovirus-associated gastroenteritis outbreaks worldwide [8-10].

GII.4 viruses can continue to cause widespread disease in the human population because they evolve through accumulations of mutations into so-called drift variants that escape immunity from previous exposures [11]. Contemporary GII.4 noroviruses also demonstrate intra-genotype recombination near the junction of open reading frame (ORF) 1 and ORF2, which is likely to foster the emergence of novel GII.4 variants [12]. In addition, the binding properties of GII.4 viruses have altered over time, resulting in a larger susceptible host population [13].

Figure 1

World map showing areas where GII.17 norovirus strains have been detected, 1978–2015



Emergence and geographical spread of GII.17 genotype noroviruses

Viruses of the GII.17 genotype have been circulating in the human population for at least 37 years; the first GII.17 strain in the National Center for Biotechnology Information (NCBI) databank is from 1978 [14]. Since then viruses with a GII.17 capsid genotype have sporadically been detected in Africa, Asia, Europe, North America and South America (Table, Figure 1). The virus appears to be clinically relevant, as it has been associated with acute gastroenteritis (AGE) in children and adults, and with chronic infection in an immunocompromised renal transplant patient [15] and a leukaemia patient (unpublished data). In the United States (US), only four GII.17 outbreaks were reported between 2009 to 2013 through CaliciNet, with a median of 11.5 people affected by each outbreak [16]. In Noronet, an informal international network of scientists working in public health institutes or universities sharing virological, epidemiological and molecular data on norovirus, GII.17 cases were also sporadically reported in Denmark and South Africa during this period [17].

More widespread circulation of GII.17 was first reported for environmental samples in Korea from 2004 to 2006. This information was published in a report in 2010 by the Korean Food and Drug Administration (KFDA) and was cited by Lee et al. [18], but the original document describing this finding is not publicly available and there are no matching clinical reports. From 2012 to 2013 a novel GII.17 virus accounted for 76% of all detected norovirus strains in rivers in rural and urban areas in Kenya [19]. In the winter of 2014/15, genetically closely related GII.17 viruses were first detected in AGE outbreaks in the Guangdong province in China in schools, colleges, factories and kindergartens [3]. Sequence analyses demonstrated that 24 of the 29 reported outbreaks during that winter were caused by GII.17. A large increase in the incidence of AGE outbreaks was also reported; 29 outbreaks associated with 2,340 cases compared with nine outbreaks and 949 cases in the previous winter when GII.4 Sydney 2012 still was the dominant genotype [3].

During the same winter there was also an increase in outbreak activity in Jiangsu province, which could be attributed to the emergence of this novel GII.17 [2]. This triggered us to investigate the prevalence of GII.17 in other parts of the world by means of a literature study and by inviting researchers collaborating within Noronet to share their data on GII.17. Currently, in Asia, in addition to Guangdong and Jiangsu [2,3], the novel GII.17 is also the predominant genotype in Hong Kong (unpublished data) and Taiwan [20], while in Japan, a sharp increase in the number of cases caused by this novel virus has been observed during the 2014/15 winter season [1]. Related viruses have been detected sporadically in the US [21] (http://www.cdc.gov/norovirus/reporting/calicinet/index.html), Australia, France, Italy, Netherlands, New-Zealand and Russia (unpublished data, www.noronet.nl) (Figure 1). In France the novel GII.17 virus appeared at the beginning of 2013,

Table A

Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[a]	Year[b]	ORF1	ORF2	Study population	Proportions of typed strains or outbreaks[c]	Suspected source of infection	Description of the sequence (size)	Accession number	References
French Guiana	Single location	1978	GII.P4	GII.17	Children with AGE	1 strain	–	Partial genome (7,441 bp)	KC597139, JN699043	[14]
Brazil	Rio de Janeiro	1997 (1994–2008)	–	GII.17	Children with AGE	3/52 strains	–	5'-end ORF2 (300 bp)	JN600531	[31]
Kenya	Nairobi	1999–2000	–	GII.17	HIV positive children with or without AGE	1/11 strains	–	5'-end ORF2 (309 bp)	KF279387	[32]
France	Briançon	2004	GII.P13	GII.17	Child with AGE	1 strain	–	Partial ORF1/2 (1,361 bp)	EF529741	Data not shown
Paraguay	Asuncion	2004–2005	–	GII.17	AGE in children (<5 years)	5/29 strains	–	3'-end ORF2 (255 bp)	KC736582, KC736580, KC736578, KC736569	[33]
Brazil	States of Acre (Brazil)	2005 (2005–2009)	–	GII.17	AGE	2/62 strains	–	3'-end ORF2 (215 bp)	JN587118 JN587117	[34]
United States	Houston	2005	–	GII.17	AGE evacuees hurricane Katrina	Predominant genotype in an outbreak	Sewage	ORF2 and 3 (2,459 bp)	DQ438972	[35]
Argentina	Single location (Argentina)	2005–2006	–	GII.17	River samples	1/33 strains	–	–	–	[36]
Brazil	State of Rio de Janeiro	2005–2006 (2004–2011)	–	GII.17	Outbreaks of AGE	3/112 outbreaks	–	3'-end ORF2 (214 bp)	KJ179752, KJ179753, KJ179754	[37]
Nicaragua	Léon	2005–2006	–	GII.17	AGE	1 strain	–	5'-end ORF2 (244 bp)	EU780764	[26]
France	Sommières	2006	GII.P13	GII.17	AGE	1 strain	Foodborne	Partial ORF1/2 (1,056 bp)	EF529742	Data not shown
Thailand	Lopburi	2006–2007	–	GII.17	AGE	2 strains	–	5'-end ORF2 (209 bp)	GQ325666, GQ325670,	[38]
China	Wuhan	2007 (2007–2010)	GII.P13	GII.17	AGE	1/488 strains	–	Partial ORF1/2 (1,096 bp)	JQ751044	[39]
Mexico	Mexico City	2007	–	GII.17	–	–	Waterborne	5'-end ORF2 (1,337 bp)	JF970609	NCBI[d]
Switzerland	Zürich	2008	–	GII.17	Renal transplant patient	1/9 strains	–	ORF2 (1,599 bp)	GQ266696	[15]
Nicaragua	Léon	2008	–	GII.17	AGE in children (<5 years)	2/38 strains	–	5'-end ORF2 (244 bp)	EU780764	[40]
South Korea	Seoul	2010 (2008–2011)	–	GII.17	AGE	1/710 strains	–	5'-end ORF2 (209)	JQ944348	[41]
Brazil	Quilombola	2009 (2008–2010)	–	GII.17	Children (<10 years)	2/16 strains	–	3'-end ORF2 (215 bp)	JX047021, JX047022	[42]

Table B

Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[a]	Year[b]	ORF1	ORF2	Study population	Proportions of typed strains or outbreaks[c]	Suspected source of infection	Description of the sequence (size)	Accession number	References
Cameroon	Southwestern region of Cameroon	2009	GII.P13	GII.17	Healthy children and HIV positive adults	4/15 strains	–	Partials ORF1/2 (1,024 bp)	JF802504–JF802507	[43]
Guatemala	Tecpan	2009	–	GII.17	Children after waterborne outbreak	1/18 strains	Waterborne	–	–	[44]
Burkina Faso	Ouagadougou	2009–2010	–	GII.17	AGE in children (<5 years)	1/36 strains	–	5'-end ORF2 (287 bp)	JX416405	[27]
Netherlands	Single location	2002–2007	–	GII.17	Nosocomial	3/264 strains	Nosocomial	–	–	[45]
South Korea	South Korea	2010	–	GII.17	Groundwater samples	2/7 strains	–	5'-end ORF2 (311 bp)	KC915021–KC915022	[18]
Ireland	Ireland	2010	–	GII.17	Influent waste water	4/24 strains	–	5'-end (302 bp)	JQ362530	[46]
South Africa	South Africa	2010–2011	–	GII.17	Waste water	9/69 strains	–	5'-end ORF2 (305 bp)	KC495680, KC495686, KC495672–KC495674, KC495664, KC495657, KC495655, KC495640	[47]
South Korea	Jinhae Bay	2010–2011	–	GII.17	Oysters	1 strain	–	–	–	[48]
Morocco	Oujda (Morocco)	2011	–	GII.17	AGE in children (<5 years)	1/42 strains	–	5'-end (205 bp)	KJ162374	[49]
South Africa	Johannesburg (South Africa)	2011	GII.P16	GII.17	AGE	–		Partial ORF1/2 (1,010 bp)	KC962460	[50]
Cameroon	Limbe	2011–2012	GII.P3	GII.17	Healthy adults and Children	4/100 strains	–	Partial ORF1/2 (653 bp)	KJ946403	[51]
Kenya	Kenya	2012–2013	–	GII.17	Surface water	16/21 strains	–	5'-end ORF2 (306 bp)	KF916584–KF916585, KF808227–KF808254	[19]
South Korea	Gyeonggi	2012	–	GII.17	AGE outbreak	1 strain	Waterborne	5'-end (205 bp)	KC413386 KC413399–KC413403	[22]
China	Guangdong province	2014–2015	–	GII.17	AGE outbreaks	24/29 outbreaks	–	5'-end (249 bp)	KP718638–KP718738	[3]
United States	Gaithersburg	2014	GII.P17	GII.17	AGE in child of 3 years	1 strain	–	Partial genome (7,527 bp)	KR083017	[21]
China	Jiangsu province	2014–2015	GII.P17	GII.17	Outbreaks of AGE	16/23 outbreaks	–	–	KR270442–KR270449	[2]

Table C

Overview of detected GII.17 norovirus strains worldwide, 1978–2015

Country	Geographical spread GII.17[a]	Year[b]	ORF1	ORF2	Study population	Proportions of typed strains or outbreaks[c]	Suspected source of infection	Description of the sequence (size)	Accession number	References
Japan	Japan	2014–2015	GII.P17	GII.17	Outbreaks of AGE	100/2,133 strains	–	Partial genome (7,534–7,555 bp)	AB983218, LC037415, LC043139, LC043167, LC043168, LC043305	[1]

AGE: acute gastroenteritis; HIV: human immunodeficiency virus; NCBI: National Center for Biotechnology Information; ORF: open reading frame.

[a] GII.17 detection location with study location between brackets (when different from GII.17 detection location).

[b] GII.17 detection year(s) with study years between brackets.

[c] Either the proportion of strains that was typed as GII.17 or the proportion of outbreaks that was caused by GII.17 is given.

[d] Information derived from the GenBank entry related to the accession number of the sequence.

but since then, it has not resulted in an increase in AGE outbreaks as observed in China, nor replaced the predominant GII.4 in the last seasons (data not shown).

Based on sequence analyses of the ORF1-ORF2 junction region, most diagnostic real-time transcription polymerase chain reactions (PCRs) will be able to detect this novel GII.17 virus, but it is not known whether the same holds true for immunoassays. However, only a small portion of norovirus outbreaks are typed beyond the GI and GII classification, therefore it is possible that GII.17 is more prevalent than we currently suspect.

Phylogenetic analyses and molecular characterisation of the novel GII.17 viruses

Phylogenetic analysis of the viral protein 1 (VP1) of GII.17 strains in the NCBI database demonstrated at least two clusters, with the novel Asian GII.17 strains grouping together with the GII.17 strains detected in the surface water in Kenya (Figure 2,[21]) and in an outbreak in 2012 in Korea [22]. Although the novel GII.17 clusters away from previously identified GII.17 strains, the amino acids changes in VP1 are not sufficient to separate it into a different genotype. For only a limited number of GII.17 strains the full VP1 has been sequenced, which demonstrated three deletions and at least one insertion compared with previous GII.17 strains (comprehensive alignments are given in Fu et al. and Parra et al. [2,21]). The majority of these changes could be mapped in or near major epitopes of the VP1 protein and potentially result in antigenic drift or altered receptor-binding properties [21]. Most publicly available GII.17 sequences only comprise the VP1, and most frequently the 5'-end of VP1 (C region), while most of the observed diversity within the GII.17 genotype is observed in the 3'-end of VP1 (D region) [23].

Previously, viruses with a GII.17 VP1 genotype contained a GII.P13 ORF1 genotype, although recombinants with an ORF1 GII.P16, GII.P3 and GII.P4 genotype have also been identified (Table). Sequence comparison showed that the ORF1 region of the novel GII.17 viruses was not detected before and cluster between GII.P3 and GII.P13 viruses [21]. Since this is the first orphan ORF1 sequence associated with GII.17, it has been designated GII.P17 according to the criteria of the proposal for a unified norovirus nomenclature and genotyping [24]. The novel GII.17 virus was termed Kawasaki 2014 after the first near complete genome sequence (AB983218) submitted to GenBank. Noronet provides a publicly available and widely used tool for the typing of norovirus sequences (http://www.rivm.nl/mpf/norovirus/typingtool). This typing tool was updated to ensure correct classification of both ORF1 and ORF2 sequences of the newly emerged GII.P17-GII.17 viruses.

The acquisition of a novel ORF1 could potentially result in an increase in replication efficiency and may – in part – explain the increase of the AGE outbreak activity. Histo-blood group antigens (HBGAs) function as (co-) receptors for noroviruses. Alpha(1,2)fucosyltransferase

Figure 2

Unrooted maximum likelihood phylogenetic tree based on the 5'-end of virus protein 1 (VP1) sequences (C region) of GII.17 noroviruses, available from the National Center for Biotechnology Information (NCBI)

The tree was estimated under the general time reversible model using PhyML. Bootstrap values above 70% are given. Sequences from Kenya are depicted in red and those from the recent outbreaks (2013–1015) reported in Asia in blue. The scale bar represents nucleotide substitutions per site.

2 (FUT2) adds an alpha-1,2 linked fucose on HBGAs, and individuals lacking the FUT2 gene are referred to as 'non-secretors', while those with a functional FUT2 gene are called 'secretors'. Non-secretors have been shown to be less susceptible to infection with several norovirus genotypes [25]. In studies investigating the genetic susceptibility to norovirus genotypes, a secretor patient with blood type O Lewis phenotype Le^{a-b+} and a secretor patient with blood type B Lewis phenotype Le^{a-b-} were positive for previously identified GII.17 viruses and no non-secretors were found positive [26,27], suggesting that there could be genetic restrictions for GII.17 viruses in infection of humans. How the observed genetic changes have affected the antigenic and binding properties of the novel GII.17 strains, and hereby the susceptible host population, remains to be discovered.

Public health implications

Based on the emergence and spread of new GII.4 variants, we know that noroviruses are able to rapidly spread around the globe [28,29]. The novel GII.17 virus has been detected in sporadic cases throughout the world, but until now it has not resulted in an increase

in outbreak activity or replacement of GII.4 Sydney 2012 viruses outside of Asia. Following the patterns observed in the past years for GII.4 noroviruses and based on the data from China and Japan, an increase in norovirus outbreak activity can be expected if the currently dominant GII.4 is replaced by GII.17. Another possibility – however – would be some restriction to global expansion, as has been observed previously for the norovirus variant GII.4 Asia 2003 [29]. Such restrictions could be due to differences in pre-existing immunity, but could also be the result of differences between populations in the expression of norovirus receptors [29]. Based on current literature on the novel GII.17 virus there is no indication that it will be more virulent compared with GII.4. Nevertheless, the public health community and surveillance systems need to be prepared in case of a potential increase of norovirus activity by this novel GII.17 virus.

Conclusions

Understanding the epidemiology of norovirus genotypes is important given the development of vaccines that are entering clinical trials. Current candidate vaccines have targeted the most common norovirus genotypes, and it remains to be seen if vaccine immunity is cross-reactive with GII.17 viruses [30]. Contemporary norovirus diagnostic assays may not have been developed to detect genotype GII.17 viruses since this genotype was previously only rarely found during routine surveillance. These assays need to be evaluated and updated if necessary to correctly diagnose norovirus outbreaks caused by the emerging GII.17 virus. Norovirus strain typing ideally should include ORF1 sequences and the variable VP1 'D' region as well as metadata on the host, like clinical symptoms, immune status and blood group. This will allow us to better study and monitor the genetic disposition, pathogenesis, evolution and epidemiology of this newly emerged virus.

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Conflict of interest

None declared.

Authors' contributions

MG, JB, HV: compiling the data, drafting the manuscript; AP, FB, KT, MC, JM, JN, GR, ML, LDR, NI JH, VM, KAB, JV, PW: collecting field data, critical review of the manuscript; MK: initiation of study, providing data, critical review of the manuscript.

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Coincidental detection of the first outbreak of carbapenemase-producing *Klebsiella pneumoniae* colonisation in a primary care hospital, Finland, 2013

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In Finland, occurrence of *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-KP) has previously been sporadic and related to travel. We describe the first outbreak of colonisation with KPC-KP strain ST512; it affected nine patients in a 137-bed primary care hospital. The index case was detected by chance when a non-prescribed urine culture was taken from an asymptomatic patient with suprapubic urinary catheter in June 2013. Thereafter, all patients on the 38-bed ward were screened until two screening rounds were negative and extensive control measures were performed. Eight additional KPC-KP-carriers were found, and the highest prevalence of carriers on the ward was nine of 38. All other patients hospitalised on the outbreak ward between 1 May and 10 June and 101 former roommates of KPC-KP carriers since January had negative screening results. Two screening rounds on the hospital's other wards were negative. No link to travel abroad was detected. Compared with non-carriers, but without statistical significance, KPC-KP carriers were older (83 vs 76 years) and had more often received antimicrobial treatment within the three months before screening (9/9 vs 90/133). No clinical infections occurred during the six-month follow-up. Early detection, prompt control measures and repetitive screening were crucial in controlling the outbreak.

Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) pose a severe health threat causing potentially incurable infections because antimicrobial treatment options are limited due to the resistance. In the United States (US), *Klebsiella pneumoniae* carbapenemase (KPC) is the most common mechanism of carbapenemase resistance among *Enterobacteriaceae*, while metallo-beta-lactamases such as VIM, IMP and NDM are common especially in Asia [1]. Plasmid-encoded KPC was first identified in North Carolina in 1996 and has been increasingly reported worldwide, including in Israel since 2005 [1-3]. In the US, KPC strains first spread in short-stay acute care hospitals. Subsequently, KPC-producing *Enterobacteriaceae* were recognised in long-term care facilities and in a study in 2010/11, up to 30% colonisation prevalence has been detected e.g. in long-term acute care hospitals [4].

In 2013, most European countries detected CPE: nine countries reported sporadic cases or single or sporadic hospital outbreaks, 11 reported regional or national spread and in Greece, Italy and Malta, CPE were regularly isolated in most hospitals and have become endemic [5]. Most outbreak reports before 2013 were from acute care hospitals [6-8]. The source of CPE in the index case is described only in some of the reports and was typically related to hospital transfers from abroad [6,8,9]. Also in the Nordic countries, sporadic cases of CPE have been documented among repatriated hospital patients, causing some local spread or sporadic hospital outbreaks [10-12]. In Finland, transmission of CPE between two patients was suspected only once, in 2011 [11].

Here, we present the first outbreak in a Finnish primary care hospital in summer 2013 with nine patients colonised with KPC-producing *K. pneumoniae* (KPC-KP) strain ST512. We describe the outbreak control measures and risk factors for KPC-KP carriage.

Methods

Setting

The outbreak occurred in a 137-bed primary care hospital (Hospital A) in the Helsinki metropolitan area, Finland, responsible for post-acute care of a catchment population of 250,000. In addition, the area is served by a second hospital with tertiary care wards and three primary care wards (Hospital B). These wards provide acute care and transfer patients to Hospital A which has four wards: Ward A with 38 beds for geriatric patients, Ward B with 38 beds for orthogeriatric patients and rehabilitation, Ward C with 27 beds for rehabilitation of patients with cerebral insults and Ward D with 34 beds for terminal care and geriatric patients. Patients with carriage of multidrug-resistant organisms (MDRO; mainly meticillin-resistant *Staphylococcus aureus* (MRSA)) in this hospital are treated at Ward A in contact isolation. Usually, none or only few such patients are present and the ward therefore accepts also non-carriers in dedicated rooms to avoid empty beds.

Index case

The index case was detected on Ward A on 10 June 2013 after a urine specimen was taken for culture from an asymptomatic patient who had been staying on that ward since February 2013. A nurse took the sample without a prescription because the patient's catheter urine looked turbid. The culture grew *K. pneumoniae* resistant to meropenem. The suprapubic catheter had been inserted nine days earlier during an outpatient visit to the tertiary care hospital due to urinary obstruction. An earlier urine culture from this patient in March had been negative (information on the clinical indication for that culture is not available).

Screening and contact tracing

After the detection of the index case, all patients on Ward A were screened for KPC-KP. Screening was repeated weekly until there were no new positive KPC-KP findings on the ward for two consecutive screening rounds, thereafter monthly until all known KPC-KP-carriers were discharged. In addition, the three other wards of Hospital A were screened twice, one ward in Hospital B was screened once and three primary care wards at Hospital B that had frequent patient transfers to Ward A (Hospital A) were screened twice.

All patients who had been on Ward A since 1 May 2013 or been roommates of KPC-KP carriers in January 2013 or later (until 10 June 2013) were defined as exposed and thus screened. Discharged patients were screened as outpatients. Those who had been in other rooms than the KPC-KP carriers but on the same ward between January and April were flagged to be screened at a new admission. Altogether, 101 exposed patients were identified. Two negative sets of screening specimens at least one week after the end of exposure were required to rule out KPC-KP.

All KPC-KP carriers and their family members were interviewed about previous stays in hospitals abroad. The stays and room locations of all KPC-KP carriers at different hospitals and wards during the preceding six months were carefully evaluated to identify potential transmission links. The laboratory results of patients hospitalised concurrently in the same hospital were reviewed for findings of multidrug-resistant *Enterobacteriaceae*.

A screening set included stool or rectal swab, swabs of chronic wounds and catheter urine. Screening for the presence of multidrug-resistant *Enterobacteriaceae* was done by plating specimens on selective chromogenic KPC plates (CHROMagar, France). Isolates were identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (VITEK MS, bioMeriéux), and antimicrobial susceptibilities were determined by disc diffusion method according to standards of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13] or by gradient MIC determination (Etest, bioMeriéux). For isolates with reduced susceptibility to any carbapenem, the presence of carbapenemase genes was analysed by multiplex real-time PCR [14]. The strains possessing carbapenemase genes were subjected to multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) [11].

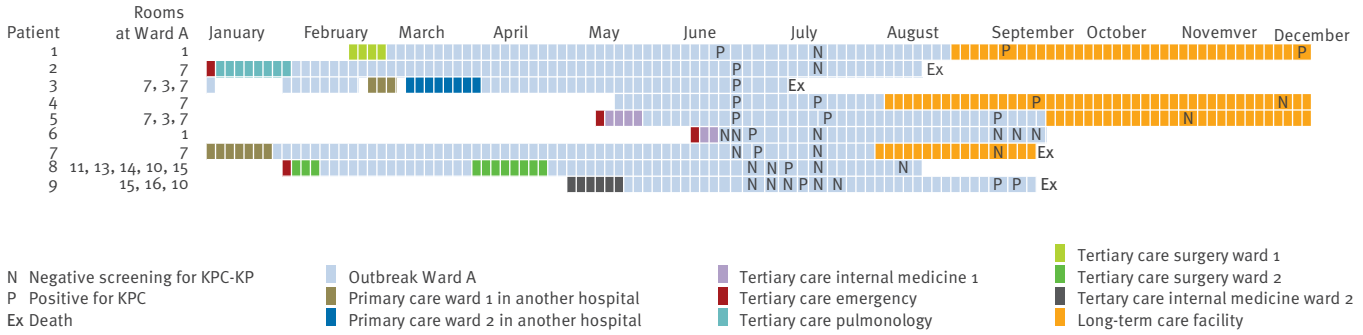
Other infection control measures and environmental cultures

The infection control teams of the primary and tertiary care hospitals worked closely together. KPC-KP carriers and patients exposed to KPC-KP were transferred into single rooms or cohorts, treated in contact isolation and flagged in the electronic patient charts at both hospitals. Ward A was closed for new admissions from 19 June to 26 July 2013.

Infection control measures (hand hygiene, standard precautions and contact isolation) at Ward A were promoted by a lecture given by an infectious diseases specialist and frequent visits by the infection control nurse to the ward meetings up to two or three times a week during the summer. In addition, there were three summarising meetings with the infection control team and the hospital administration. Healthcare workers' hands were inspected for rings, bracelets and chronic wounds, and compliance with hand hygiene and environmental cleaning procedures was monitored by the infection control nurse during the ward visits. The use of the disinfecting washing machine, washing room cleaning and disinfection (especially the bunk and shower chairs) were reviewed and written instructions were provided. Only permanent and trained cleaners were allowed to work at Ward A. Hypochlorite disinfectant was introduced for daily surface cleaning in the rooms of KPC-KP carriers and the washing room. The indications to use urinary catheters and antimicrobial practices were reviewed.

Figure

Duration and location of hospital stays of patients colonised with *Klebsiella pneumoniae* carbapenemase-producing *K. pneumonia*, Finland, 2013 (n = 9)



KPC-KP: *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*.

The date of KPC-KP detection and positive follow-up screenings are marked with P and the dates of negative screening results are marked with N. Colours indicate the various wards a patient was staying on.

Altogether, 10 culture swabs were taken at Ward A from various surfaces frequently touched and thus suspected to be contaminated, e.g. in patient rooms and in bathrooms.

Risk factor analysis

To identify risk factors for KPC-KP carriage, a case–cohort study was performed. The charts of all patients (nine KPC-KP carriers and 133 non-carries) present at the four wards of Hospital A on the day of the first screening round during 14 to 17 June were reviewed by three doctors, MK, KS and KR. Analyses were performed with SPSS (version 20.0 Chicago, IL, US). Differences in the frequency of dichotomic variables between KPC-KP carriers and non-carriers were compared by Fisher's exact test and the means of continuous variables were compared by Mann–Whitney and Kruskal–Wallis tests. Multivariable logistic regression analysis was done with backward selection using Akaike information criteria (AIC).

Results

KPC-KP screening and contact tracing

After the detection of the index case, the first screening round at Ward A revealed four KPC-KP-carriers in a four-bed room that was used for five patients at the time. In each of the four following weekly rounds, one additional KPC-KP carrier was identified (Figure): Patient 6 was a roommate of the index case, Patient 7 was the fifth patient in the overcrowded four-bed room, and the last two patients (number 8 and 9) were in two additional rooms. Patients 6 to 9 had two, one, two and three negative screening results, respectively, before becoming positive. No new KPC-KP were detected in the sixth and seventh screening rounds. The highest prevalence of KPC-KP carriers on Ward A was nine of 38. Specimens obtained during screening as well as clinical specimens obtained at other wards (B, C and D in Hospital A) and at Hospital B) revealed no KPC-KP-carriers. Altogether, 535 screening specimens were collected between 14 June and 2 August in seven screening rounds on Ward A and two screening rounds on Wards B, C and D.

No additional KPC-KP-carriers were found among the 101 exposed patients: Those still present on Ward A (n = 18) were included in the screening rounds there and those transferred to long-term care facilities (n = 27) or discharged home (n = 37) were screened as outpatients. Results for all of them were negative until the end of October 2013. Nineteen of the exposed patients had died before screening.

Apart from two patients with KPC-KP only in catheter urine, all nine positive patients had KPC-KP in stools or rectal swab and one also in a sacral wound. The follow-up screenings showed that four patients spontaneously lost KPC-KP-carriage within one month, while four had persistent or intermittent carriage for at least three months (Figure). One KPC-KP carrier died before any follow-up screening could be done. None of the KPC-KP carriers developed clinical infection during a six-month follow-up period. By the end of September 2013, four of the nine KPC-KP carriers had died due to unrelated underlying diseases, two of them while still on Ward A. Of the nine, four had been admitted to a long-term care facility and three were discharged home.

None of the nine KPC-KP-carriers had been travelling abroad during the year before the outbreak. None of their family members had been hospitalised abroad. All but two of the KPC-KP-carriers had contact to Hospital B, neither at an outpatient visit during their stay in primary care nor at a direct admission. Review of the charts from patients staying at the same time on the same wards in Hospital B as the KPC-KP-carriers did

not provide indications as to the origin of the KPC-KP outbreak either.

Microbiological characterisation of isolates

All nine KPC-KP isolates represented the same MLST type ST512 (a single locus variant of ST258). PFGE analyses showed that all the strains were closely related. Seven strains had identical PFGE profiles (PFGE type A). Two strains, isolated four and 10 days after the first strain, had minor differences compared with the PFGE type A, i.e. PFGE type A1 differed in three bands (of 21 bands seen in the PFGE gel) from type A and PFGE type A2 differed in two bands (of 21 bands seen in the PFGE gel) from type A. In addition, types A1 and A2 differed from each other in four bands.

The isolates were resistant to all beta-lactams (all had a minimum inhibitory concentration (MIC) for meropenem of > 32 mg/L): mecillinam, nitrofurantoin, ciprofloxacin, tobramycin, amikacin and trimethoprim. Their susceptibility varied for gentamicin, fosfomycin, tigecycline, sulfadiazine/trimethoprim and colistin. At the presence of 1 µg/mL rifampicin, the colistin MICs were reduced by a factor of 4 or 8, which rendered isolates in eight patients potentially sensitive to colistin (MIC < 2) (data not shown).

Other infection control measures and environmental cultures

No skin breaks, rings or bracelets were found on the hands of healthcare workers. The ward audits revealed weaknesses in the placement of hand rub dispensers, the placement of clean urinary bottles in the toilets and in the cleaning of bathrooms. During the years 2012 and 2013, the use of alcohol hand rub had increased from 26.5 to 40.2 L per 1,000 patient-days at ward A.
None of the 10 surface swabs was culture-positive for KPC-KP.

Risk factors for KPC-KP

All nine KPC-KP carriers were older than 70 years and had underling illnesses (eight patients with McCabe score ≥ 2 [15]) (Table). Three were receiving antimicrobial treatment on the day the sample for their positive test was taken, one of which was considered inappropriate by the authors when reviewed for this study. KPC-KP carriers were more likely to have received antimicrobial treatment within three months than non-carriers (9/9 vs 90/133). The last date of antibiotic treatment was available for those who had stopped the treatment before screening, namely six of the nine carriers and 63 of the 133 non-carriers. It was more recent among the carriers than among non-carriers, a mean of 40 and 77 days before the first screening, respectively. Several of the carriers had multiple risk factors for contaminating their surroundings (Table): of those, six were not bedridden and four had advanced dementia. Five needed a bathroom chair or bunk for showering. However, none of the differences between carriers and non-carriers or between Ward A and other wards reached statistical significance (Table).

To evaluate risk factors for KPC-KP carriage, we included the following variables in the multivariable model: age, McCabe 3, any antimicrobial at the screening day, admission from tertiary care, immunosuppression, chronic wound, length of stay in the primary care hospital, incontinence in stools, urinary catheter, wheel chair, private toileting, room size 3 or more, and any antimicrobials within three months before the screening day, but none of them was an independent explanatory variable for KPC-KP-carriage.

Discussion

The KPC-KP outbreak described here involved nine patients with asymptomatic colonisation and has been the largest in Finland so far. The outbreak was confined to one geriatric ward. The fact that the index case was found by chance in a clinical culture taken without prescription and that no direct link to travel abroad was detected, suggests the possibility that hidden colonisations may also be present elsewhere. That the outbreak ward was a dedicated cohort ward for patients with carriage of MDROs was only a coincidence, but it may have improved the containment of the outbreak because the staff was familiar with contact precautions. Rapid and extensive screening together with cohorting and isolating carriers and exposed patients were other essential tools in controlling the outbreak.

KPC-KP strain ST 512 was probably imported to Finland by an unrecognised infected or colonised patient. All CPE strains (*Escherichia coli* and *K. pneumoniae*) detected in Finland are being typed using MLST, but ST512 had not been detected in Finland before. After the 2013 outbreak, two single ST512 isolates were found in Northern Finland, in August 2013 and January 2014, also without obvious link to abroad, one in a tertiary care and the other in a primary care hospital (data not shown). This is another indication of hidden transmission of ST512 in Finland or of importation by several patients. ST512 is a single-locus variant of the widely spread KPC-2 ST258 clone. It was first described in Israel among isolates producing KPC-3 enzyme [16]. Later it caused outbreaks in the Czech Republic among hospital patients repatriated from Italy [17].

Routine screening for MDROs in Finland applies to patients repatriated from hospitals in other countries and asylum seekers needing hospital care. Screening also covers all patients seeking hospital care with a history of previous hospitalisation in another country in the preceding year. The screening covers MRSA, vancomycin-resistant enterococci (VRE) and multidrug-resistant gram-negative rods, including extended-spectrum beta-lactamase producers (ESBL) and CPE. In addition, patients exposed in Finnish hospitals (roommates of newly identified carriers or patients staying on the affected ward during an outbreak) are also screened. In an outbreak situation, the exposed patients are screened immediately, during their hospital stay. If they have already been discharged home, infection control

nurses either contact and screen the patients as soon as the exposure becomes evident or flag the patients in the electronic patient charts to be screened at the next admission, depending on the available resources and the characteristics of the outbreak. More universal screening for multidrug-resistant gram-negative rods is not routinely performed in Finland.

In our epidemiological situation with no previous domestic CPE outbreaks, we decided to screen the whole ward after detecting a single nosocomial patient with KPC-KP. After it became clear that it was an outbreak, we chose to start multiple follow-up screenings, as recommended by Finnish guidelines, the European Centre for Disease Prevention and Control and the US Centers for Disease Control and Prevention in this type of setting [3,18,19].

KPC-KP carriers were placed in contact isolation and remained there even after negative results in follow-up cultures. Also patients who were considered exposed were pre-emptively isolated until carriage was ruled out. At the outbreak ward, the annual alcohol hand rub use increased by 50%, to more than 40 L per 1,000 patient-days, which is generally considered good, allowing at least six safe visits per patient per day (3 mL before and after a touch). Screening, contact precautions, cohorting and cleaning have been the key factors for successful control in several outbreak settings [6,7,20]. Some hospitals have also used chlorhexidine bathing for patients as well as screening of staff, although no cases were detected on that occasion [7,20]. In endemic settings, for instance in Israel, national interventions including active surveillance, carrier isolation and cohorting of patients and staff have shown to be effective [21].

Despite the control measures, we detected new KPC-KP carriers in consecutive screenings for almost one month after the detection of the index case. In our experience, only one set of specimens, including faeces or rectal swab, wound swab and catheter urine, was not enough to rule out KPC-KP carriage. This may be due to lack of compliance with the control measures and ongoing transmission and/or to low sensitivity of the KPC-KP screening. In addition, most patients did not have any wounds or urinary catheters and therefore submitted only faecal or rectal swabs. However, rectal cultures have been suggested to be the most sensitive single method for detection of CPE colonisation [22]. The follow-up culturing further suggested that carriage can be intermittent or short-lived, which has also been shown elsewhere [21].

Antibiotic selection pressure has been shown to have an impact on the spread of KPC-KP [7]. In our study, one third of all patients in the hospital were on antimicrobial treatment at the time of the chart review and two thirds had received antimicrobials within the preceding three months. Risk factor analysis also revealed a tendency that recent antimicrobial use may be a risk

Table

Characteristics of patients screened for *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*, Finland, 2013 (n = 142)

	KPC-KP (n = 9)	No KPC-KP (n = 133)
	Time (SD)	Time (SD)
Mean age (years)	83 (SD 11.4)	76 (SD 11.1)
LOS primary care hospital (days)	81 (SD 54.2)	53 (SD 74.9)
LOS total hospitalisation (days)	101 (SD 57.8)	88 (SD 96.1)
Days since last antimicrobial treatment (mean)	40 (SD 17.4)	77 (SD 167.0)
	n	n
Male sex	3	60
Previous ESBL carriage	0	9
Admission from tertiary care	6	98
Visit to tertiary care hospital during stay	3	29
Surgery at tertiary care during hospitalisation	1	42
Room with ≥ 3 beds	8	82
Ward change during stay	0	5
Room change during stay	4	62
Any antimicrobial treatment on study day[a]	3	45
Cephalosporin treatment on study day	2	16
Fluoroquinolone treatment on study day	0	13
Carbapenem treatment on study day	0	0
Other antimicrobial treatment on study day	2	21
Antimicrobial treatment within last three months	9	90
Carbapenem treatment within last three months	1	8
McCabe1	1	12
McCabe2	6	99
McCabe3	2	22
Immunosuppression	1	12
Dementia	4	62
Bedridden/wheelchair	3	74
Did not need assistance in the toilet	4	44
Stool incontinence	4	56
Chronic wound	2	31
Urinary catheter	3	24

ESBL: extended-spectrum beta-lactamase producers; KPC-KP: *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*; LOS: length of stay; SD: standard deviation.

None of the characteristics covered in this Table were significantly associated with carriage of KPC-KP.

[a] One patient received two antibiotics.

for KPC-KP carriage. This underlines the importance of prudent antimicrobial use in long-term care. One fifth of the treatments were considered inappropriate by three reviewing doctors in that they were prescribed for colonisation or for unspecific symptoms or for too long.

In Europe, most KPC-KP outbreak reports are from acute care hospitals, e.g. in France, Germany and Greece, where up to 30–70% of case-patients had clinical infections [6,8,9,23]. In our outbreak, the lack of infections may also have been due to early detection of the outbreak and to the level of care: no invasive procedures, mechanical ventilation or dialysis were provided at the primary care hospital. The index case was asymptomatic when the urine culture was taken by the nurse without a physician's prescription. Culturing asymptomatic bacteriuria or uninfected wounds is usually discouraged because it may lead to inappropriate antibiotic use. This recommendation may now need to be seen in a different light if we suspect hidden MDRO-colonisations in Finland: we may even need to encourage staff on long-term care wards to culture uninfected wounds and catheter urine regularly, just to screen for resistance, because true clinical infections caused by these pathogens are not common in long-term care. This, together with continuous training on antibiotic policy, may serve early detection of MDRO. It also conforms with the policy of this particular hospital. Earlier, the hospital had made exceptional efforts in routine universal MRSA screening, with only few cases detected, and now wants to focus on other MDROs. In addition, long-term care facilities can become reservoirs of MDRO due to the presence of several risk factors for spread such as dementia and stool incontinence.

Conflict of interest

None declared.

Authors' contributions

Mari Kanerva: outbreak investigations, manuscript writing. Kirsi Skogberg: outbreak investigations, manuscript writing. Kaisa Ryynänen: outbreak investigations. Anita Pahkamäki: outbreak investigations. Jari Jalava: MLST and PGFE typing, manuscript writing. Jukka Ollgren: statistics. Eveliina Tarkka: detection of bacterial isolates and manuscrip writing. Outi Lyytikäinen: study design, manuscript writing.

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Current prevalence of multidrug-resistant organisms in long-term care facilities in the Rhine-Main district, Germany, 2013

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Multidrug-resistant organisms (MDRO) and in particular multidrug-resistant Gram-negative organisms (MRGN) are an increasing problem in hospital care. However, data on the current prevalence of MDRO in long-term care facilities (LTCFs) are rare. To assess carriage rates of MDRO in LTCF residents in the German Rhine-Main region, we performed a point prevalence survey in 2013. Swabs from nose, throat and perineum were analysed for meticillin-resistant *Staphylococcus aureus* (MRSA), perianal swabs were analysed for extended-spectrum beta-lactamase (ESBL)-producing organisms, MRGN and vancomycin-resistant enterococci (VRE). In 26 LTCFs, 690 residents were enrolled for analysis of MRSA colonisation and 455 for analysis of rectal carriage of ESBL/MRGN and VRE. Prevalences for MRSA, ESBL/MRGN and VRE were 6.5%, 17.8%, and 0.4%, respectively. MRSA carriage was significantly associated with MRSA history, the presence of urinary catheters, percutaneous endoscopic gastrostomy tubes and previous antibiotic therapy, whereas ESBL/MRGN carriage was exclusively associated with urinary catheters. In conclusion, this study revealed no increase in MRSA prevalence in LTCFs since 2007. In contrast, the rate of ESBL/MRGN carriage in German LTCFs was remarkably high. In nearly all positive residents, MDRO carriage had not been known before, indicating a lack of screening efforts and/or a lack of information on hospital discharge.

Introduction

Multidrug-resistant organisms (MDRO) are an increasing problem in hospital care worldwide. In Germany, according to data from the Antibiotic Resistance Surveillance System (ARS) and the European Antimicrobial Resistance Surveillance Network (EARS-Net) system, the meticillin-resistant *Staphylococcus aureus* (MRSA) rates have not increased since 2008, with a slight decrease from 22% in 2010 to 16% in 2012 in the hospital setting [1-3]. However, an increase in the prevalence of multidrug-resistant Gram-negative organisms (MRGN) has been observed in the past decade [3,4], including a sharp increase in carbapenem-resistant organisms (CRO) [3,5,6]. MDRO are regarded as a potentially serious threat to elderly people living in long-term care facilities (LTCFs). Although various studies on the prevalence of MRSA in LTCFs in Germany [7-13] and other European countries [14-25] have been published (‹1% in the Netherlands and Sweden, ›20% in Ireland and the United Kingdom (UK)), studies on the prevalence of MRGN such as extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* and vancomycin-resistant enterococci (VRE) among nursing home residents in German and in European LTCFs are scarce [7,14,24,26,27]. Although an increasing trend in the occurrence of ESBL-producing *Enterobacteriaceae* and even CRO is found in hospitals all over Europe [1,3,28,29], a lack of knowledge on multidrug-resistant organisms (MDRO) in nursing homes has to be stated. Notably, residents of LTCFs may present various risk factors for MDRO carriage and/or transmission (e.g. older age, comorbidities, medical devices or recurrent antibiotic treatments) [30,31]. To issue recommendations for MDRO screening of LTCFs residents, e.g. upon admission to hospital, a better knowledge of current colonisation rates and the most relevant clinical risk factors is needed. The aim of our study was to assess the current prevalence of MDRO, specifically MRSA, ESBL/MRGN and VRE, obtained by case history and by microbiological culture of swabs from nose, throat and perineum.

Methods

The study was approved by the Ethics committee of the Chamber of Physicians, County of Hesse, Germany. Inhabitants of 26 LTCFs in the Rhine-Main region were asked to take part in this study and to agree to having swabs from nose, throat and perineum taken for analysis for MDRO. Data on sex, age, health characteristics such as hospital stay, antibiotic therapy during the previous three months and surgery within the past 30 days, current healthcare-associated infection and/or antibiotic therapy, presence of urinary or vascular catheters, pressure sores and other wounds as well as case history for MDRO were obtained for all participants, using the well-established HALT questionnaire (healthcare-associated infections in LTCFs) in Europe [32]. An identical data set was obtained from non-participants in order to assess potential bias in participation. Information on healthcare-associated infections was obtained according to the McGeer criteria 1990 [33] and adapted by the HALT project, i.e. physician diagnosis of infection had been included as a criterion in all categories of infection in order to avoid underestimation of the infection rate due to lack of on-site diagnostic testing [32].

Anterior bilateral nasal, throat and perianal swabs were collected from April to May 2013 using culture swabs with Amies collection and transport medium (Hain Lifescience, Germany). Swabs from nose, throat and perineum were taken from residents, with their written consent. The swabs from nose and throat were tested for MRSA, while perianal swabs were tested for the presence of VRE and ESBL/MRGN. All specimens were collected by the local nursing personnel. Collected swabs were processed within 24 hours by streaking on Brilliance MRSA 2 agar (Oxoid, Germany), ChromID VRE agar (bioMérieux, Germany) and CHROMagar ESBL (Mast Diagnostics, Germany) for the detection of MRSA, VRE and MRGN including ESBL-producing *Enterobacteriaceae*, respectively. Definite identification of presumptive *S. aureus*, enterobacterial species and enterococci was confirmed by the VITEK MS matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) automated system (bioMérieux, Germany). The determination of the antimicrobial susceptibility profiles and classification as ESBL/MRGN, MRSA and VRE was performed by the VITEK 2 identification system (bioMérieux, Germany), using either VITEK N263 (*Enterobacteriaceae*), P586 (*Enterococcus* spp.) or P580 (*Staphylococcus* spp.) antimicrobial susceptibility testing (AST) cards according to standard laboratory procedures under strict quality-controlled criteria (laboratory accreditation according to DAkkS and DIN15189:2007 standards; certificate number D-ML-13102-01-00, valid through 06.12.2015). MRSA phenotype was confirmed by detection of the *mecA* gene as described [34]. In addition, PCR for the *luk-PV* (encoding Panton-Valentine leukocidin) gene was performed as described [35]. MRSA *spa*-typing was used as first-line typing tool as described previously [36]. We used the BURP algorithm for attribution to clonal complexes (www.ridom.de/staphtype/support). For isolates with *spa*-types which are not very often detected we used multilocus sequence typing (MLST) according to Enright et al. [37] as well as the *S. aureus* MLST database (www.mlst.net) for allelic profile analysis. Finally, prevalence rates, Kruskal–Wallis tests and univariate analysis (odds ratios) were performed with SPSS 15 software, IBM, Stanford University, United States (US).

Results

The total population, i.e. residents present in the 26 homes on the day of the survey, numbered 2,404. Of these residents, 690 (26%) took part in the MRSA analysis, while only 455 of them (19%) consented also in anal swabs for analysis of ESBL and MRGN. Characteristics of the total LTCF population and the final study participants are summarised in Table 1. There were no significant differences between participants and non-participants regarding sex, urinary and vascular catheters, pressure sores, impaired mobility, incontinence and disorientation, hospital stay in the past three months, surgery in the past 30 days, current infection or antibiotic therapy. The participants exhibited more 'other wounds', they were older, had more often a percutaneous endoscopic gastrostomy tube than the non-participants, and they had more often been treated with antimicrobials in the previous three months. Prevalence rates of positive MDRO anamnesis were lower in the group of the participants than in the total group (not significant). Prevalence for MDRO in swab cultures exceeded the prevalence of case history for MDRO by far: MRSA 6.5% vs 0.7%, ESBL 17.8% vs 0.7%, VRE 0.4% vs 0%.

From 45 detected MRSA-isolates, only 36, which all were positive for *mecA* gene but negative for *luk-PV* gene, could be recultured from stored samples and subjected to *spa*-typing. Of these 36, 21 were attributed to clonal lineage ST225, 10 to clonal complex 22, four to clonal complex 5 (other than ST225) and one to clonal complex 45. Antibiotic resistance phenotypes of the isolates were typical of those usually observed for MRSA attributed to ST225 and CC22 (resistance to beta-lactams, erythromycin, clindamycin, fluoroquinolones) [38]. Only one isolate exhibited mupirocin resistance, and three isolates of ST225 were in addition resistant to fusidic acid.

According to the criteria of the German Commission on Hospital Hygiene and Infection Prevention (KRINKO) [39], Gram-negative pathogens are classified as 3MRGN when resistant to three antibiotic groups (ureidopenicillins, third- and/or fourth-generation cephalosporins and fluoroquinolones) represented by piperacillin, cefotaxime and/or ceftazidime and ciprofloxacin as guiding agents to define resistance for each group. ESBL/MRGN were isolated from swabs from 81 residents; 25 of them tested positive for ESBL and 56 were 3MRGN. Among the 25 residents carrying ESBL-producing *Enterobacteriaceae*, we identified 22

Table 1

Characteristics of residents in 26 nursing homes, prevalence of infections, antibiotic therapy, MDRO anamnesis and colonisation status, Rhine-Main district, Germany, April–May 2013 (n = 2,404)

Population characteristics	All residents		Participants		KW test p-value participants vs non-participants
	n = 2,404	%	n = 690	%	
Age › 85 years[a]	1,184	49.3	369	53.5	0.009
Male	679	28.2	200	29.0	0.620
Had urinary catheter	225	9.4	63	9.1	0.800
Had vascular catheter	10	0.4	4	0.6	0.430
Had pressure sores	86	3.6	28	4.1	0.424
Had other wounds	129	5.4	47	6.8	0.047
Were disoriented[a]	1,243	51.7	352	51.0	0.648
Had impaired mobility	1,197	49.8	362	52.5	0.102
Hospital stay in previous 3 months	333	13.9	101	14.6	0.486
Had surgery in the past 30 days	37	1.5	13	1.9	0.385
Were incontinent	1,683	70.0	484	70.1	0.958
Had percutaneous endoscopic gastrostomy tube	134	5.6	55	8.0	0.001
Had antibiotic therapy during previous 3 months	309	12.9	109	15.8	0.006
Prevalence of all infections	64	2.7	18	2.6	0.914
Prevalence of oral antibiotic therapy	33	1.4	6	0.9	0.178
Prevalence MDRO vs anamnesis					
MRSA	32	1.3	5	0.7	0.099
ESBL	18	0.7	5	0.7	0.929
VRE	0	0	0	0	1
Prevalence of MDRO colonisation (analysis)					
MRSA	NA	NA	45	6.5	NA
ESBL	NA	NA	81[b]	17.8	NA
VRE	NA	NA	2[b]	0.4	NA

ESBL: extended-spectrum beta-lactamase; KW: Kruskal–Wallis; MDRO: multidrug-resistant organisms; MRSA: meticillin-resistant *Staphylococcus aureus*; NA: not applicable/not available; VRE: vancomycin-resistant enterococci.

[a] Information missing for one person.

[b] 455 of 690 participants were tested for ESBL and VRE.

Escherichia coli, two *Klebsiella pneumonia*, and one *Klebsiella oxytoca* isolate. Resistance against three antibiotic groups (3MRGN) was detected in 43 *E. coli*, eight *K. pneumoniae*, two *Acinetobacter baumannii*, one *Enterobacter* spp. and two *Pseudomonas aeruginosa* isolates. 4MRGN (Gram-negative *Enterobacteriaceae* resistant against four antibiotic groups, namely those mentioned above for 3MRGN plus resistance to imipenem and/or meropenem) according to the German KRINKO guideline [39] were not detected. Perianal carriage for VRE was observed in two residents (0.4%).

In 43 of 45 of the residents with MRSA colonisation, the colonisation status was previously unknown. Colonisation in both VRE cases and in 79 of 81 residents positive for ESBL-producing *Enterobacteriaceae* had not been known before this study either. In three of five residents with a documented MRSA history, detection of MRSA was not confirmed in our study.

In Table 2, patient numbers and odds ratios for MRSA and ESBL colonisation are shown. Medical history for MRSA (OR = 9.9; 95% CI: 1.6–61.1), urinary catheter (OR = 4.2; 95% CI: 2.1–8.7), percutaneous endoscopic gastrostomy tube (OR = 2.7; 95% CI: 1.2–6.2) and antibiotic therapy during the last three months (OR = 2.6; 95% CI: 1.3–5.1) proved to be significantly associated with MRSA colonisation. The odds ratios for ESBL/MRGN carriage were significantly increased by having a urinary catheter (OR = 1.9; 95% CI: 1.0–3.8). All other characteristics including anamnesis for MDRO (OR › 4) proved not to be significant risk factors for ESBL colonisation. One of the two residents with VRE was bedridden and exhibited pressure sores, but neither of them had a catheter or exhibited other risk factors such as a hospital stay during the previous three months.

Table 2

Numbers and odds ratios of nursing home residents with MRSA and with ESBL/MRGN colonisation, Rhine-Main district, Germany, April–May 2013 (n = 690)

	MRSA-negative n = 645		MRSA-positive n = 45		MRSA OR		ESBL/MRGN-negative n = 374		ESBL/MRGN-positive n = 81		ESBL/MRGN OR	
	n	%	n	%	OR	95% CI	n	%	n	%	OR	95% CI
Aged›85 yearsa	343	53.2	25	55.6	1.097	0.597–2.015	201	53.7	44	54.3	1.024	0.632–1.658
Male	183	28.4	17	37.8	1.529	0.817–2.862	111	29.7	18	22.2	0.677	0.383–1.196
Had urinary catheter	51	7.9	12	26.7	4.228	2.058–8.686	36	9.6	14	17.3	1.962	1.003–3.837
Had vascular catheter	3	0.5	1	2.2	4.856	0.495–47.652	3	0.8	0	0.0	0.821	0.786–0.857
Had pressure sores	24	3.7	4	8.9	2.520	0.835–7.607	18	4.8	3	3.7	0.761	0.219–2.646
Had other wounds	41	6.4	6	13.3	2.263	0.905–5.654	29	7.8	7	8.6	1.125	0.475–2.667
Were disorientedа	323	50.1	28	62.2	1.637	0.879–3.049	199	53.2	48	59.3	1.279	0.786–2.083
Had impaired mobility	332	51.5	30	66.7	1.880	0.992–3.560	197	52.7	52	64.2	1.611	0.980–2.650
Had hospital stay in previous 3 months	95	14.7	6	13.3	0.889	0.366–2.158	61	16.3	9	11.1	0.641	0.304–1.351
Had surgery in the past 30 days	13	2.0	0	0.0	0.933	0.915–0.952	5	1.3	3	3.7	2.838	0.664–12.125
Were incontinent	453	70.2	31	68.9	0.934	0.486–1.794	254	67.9	61	75.3	1.441	0.832–2.497
Had percutaneous endoscopic gastrostomy tube	47	7.3	8	17.8	2.746	1.210–6.235	34	9.1	13	16.0	1.912	0.959–3.812
Had antibiotic therapy during previous 3 months	95	14.7	14	31.1	2.610	1.339–5.088	61	16.3	10	12.3	0.723	0.353–1.480
Prevalence of all infections	15	2.3	3	6.7	2.995	0.834–10.755	12	3.2	3	3.7	1.160	0.320–4.209
Prevalence of oral antibiotic therapy	6	0.9	0	0.0	0.934	0.916–0.953	4	1.1	0	0.0	0.820	0.786–0.857
Prevalence MDRO vs anamnesis												
MRSA	3	0.5	2	4.4	9.938	1.617–61.069	2	0.5	2	2.5	4.709	0.653–33.933
ESBL	5	0.8	0	0.0	0.934	0.916–0.953	1	0.3	1	1.2	4.663	0.289–75.329
VRE	0	0	0	0	NA	NA	0	0.0	0	0.0	NA	NA

CI: confidence interval; ESBL: extended-spectrum beta-lactamase; MDRO: multidrug-resistant organisms; MRSA: meticillin-resistant *Staphylococcus aureus*; NA: not analysed; OR: odds ratio; VRE: vancomycin-resistant enterococci.

[a] Information missing for one person.

Discussion

Our point prevalence study on MDRO such as MRSA, ESBL/MRGN and VRE in residents of LTCFs in the Rhine-Main district in Germany revealed a high MRSA prevalence compared with hospital settings, rehabilitation and dialysis units in Germany [40], and a much higher prevalence for ESBL/MRGN carriage, whereas VRE had a very low prevalence in the studied LTCFs.

Our study has the following limitations: Of the 214 LTCFs located in in the Rhine-Main district, the 83 members of the MDRO-network Rhine-Main were asked to participate and 26 of them finally participated in this study. With informed consent being necessary for investigation of MDRO colonisation in nursing home residents in Germany, we were able to enrol only 690 (29%) of all residents in the MRSA study and 455 (19%) in the ESBL/MRGN study. Our study has features of cluster sampling, which could lead to wider confidence intervals. Participants had significantly more often reported on antibiotic therapy in the past three months than non-participants and were more often supplied with a percutaneous endoscopic gastrostomy tube. However, no significant differences between participants and non-participants were found regarding sex, impaired mobility, disorientation, faecal or urinary incontinence, urinary and vascular catheter etc. Residents with a positive case history for MRSA, ESBL or VRE were not represented more than other residents in the MDRO analysis. Therefore, the hypothesis that residents with positive MDRO anamnesis may take advantage of the opportunity to receive an MDRO analysis free of charge and thus would be overrepresented in the study did not prove to be true. Thus, although the number of

Table 3

MDRO in residents of long-term care facilities in Frankfurt am Main compared with other studies in Germany and abroad 2000–13

Country	Year of investigation	LTCFs	Residents tested	MRSA	ESBL	VRE	Reference
		n	n	%	%	%	
Germany							
Berlin	1999	NR	NR	NR	NR	4.2	[26]
Different regions	2000	32	1,342	2.4	NR	NR	[8]
Frankfurt am Main	2000	8	159[a]	2.5	NR	NR	[8]
Heidelberg	2000/01	47	3,236	1.1	NR	NR	[13]
North Rhine-Westphalia	2000/01	30	1,057	3.1	NR	NR	[11]
Frankfurt am Main	2001	6	319	0.3	NR	NR	[9]
Frankfurt am Main	2007	8	178	9.0	11.2	0	[7]
Hessen	2010/11	11	240	NR	9.6	NR	[27]
Brunswick	2011	32	1,827	7.6	NR	NR	[12]
Frankfurt am Main	2012	8	184	9.2	26.7	2.7	[10]
Rhine-Main region	2013	26	690[b]	6.5	17.8	0.3	This study
Europe							
France	2004	1	109	37.6	NR	NR	[20]
Slovenia	2005	1	107	9.3	NR	NR	[18]
Belgium	2005	60	2,953	19.9	NR	NR	[19]
Spain	2005	9	1,377	16.8	NR	NR	[23]
Italy	2006	2	551	7.8	NR	NR	[17]
United Kingdom	2007	39	715	22.0	NR	NR	[16]
Ireland	2007	45	1,111	23.3	NR	NR	[15]
Italy	2008	1	120	38.7	64	NR	[24]
Spain	2009/10	17	744	10.6	NR	NR	[21]
Luxembourg	2010	19	954	7.2	NR	NR	[25]
Sweden	2010	9	495	0	3.0	0	[14]
The Netherlands	2011	NR	1,268	0.3	NR	NR	[22]
Other countries							
United States	1998	1	117	24	33	3.5	[45]
Australia	2000	8	292	NR	NR	3.1	[55]
United States	2008	1	84	28	51	4	[43]
United States	NR	1	160	27.5	NR	NR	[41]
United States, California	2008/09	NR	1,000	30.7	NR	NR	[44]
Australia, Melbourne	2010	3	119	NR	12	2	[52]
United States	2006/07	1	161	11.8	22.8	0.6	[42]
China	2011	40	2,020	21.6	NR	NR	[46]

ESBL: extended-spectrum beta-lactamase; LTCF: long-term care facility; MDRO: multidrug-resistant organisms; MRSA: meticillin-resistant *Staphylococcus aureus*; NR: not reported (in the main text or abstract only); VRE: vancomycin-resistant enterococci.

[a] Residents were a subgroup of 1,342 residents tested by Heuck et al. [8] all over Germany, 2000.

[b] 455 of them were tested for both ESBL and VRE.

participating LTCFs and the response rate of 29% (19% for the ESBL study) among residents was rather low, there is no obvious indication for bias in our study, so that the data can be regarded as representative for LTCFs in the Rhine-Main region in Germany in 2013.

The point prevalence of MRSA colonisation was 6.5% and thus much higher than in earlier studies in 2000–01 in Germany [8,9,11,13], but since 2007, the MRSA prevalence in LTCFs in Germany has not increased further and remained between 6.5% and 9.2% [7,10,12]. The MRSA prevalence we observed was lower than in MRSA surveys in recent years in the US [41-45], China [46], the UK [16], France [20], Ireland [15], and Italy [24], but higher than in the Netherlands and Sweden [14,22] (Table 3).

All MRSA isolates were attributed to clonal lineages (ST) and/or clonal complexes (CC) that are prevalent in German hospitals, in particular ST225 is widely disseminated in the west of Germany [47]. These results indicate primary hospital origin. Prevalence of these

clonal lineages was also reported in a study from 2006 in the west of Germany bordering the Netherlands [48]. None of the isolates reported here were attributed to community-associated MRSA (CA-MRSA) or livestock-associated MRSA (LA-MRSA). That CA-MRSA can represent a substantial proportion of MRSA in nursing home residents has been reported from the US [49], and LA-MRSA has been identified among isolates from Dutch nursing homes [50]. In Germany, CA-MRSA is not common so far, nor is LA-MRSA as nasal coloniser and infectious agent in regions of Germany with low density of livestock farming such as the Rhine-Main region [51]. As all our isolates were susceptible to antibiotics that are recommended as treatment alternatives for MRSA infections, e.g. vancomycin, teicoplanin, linezolid, daptomycin, tigecycline, rifampicin and cotrimoxazol, calculated therapy of severe infections should be unproblematic.

Regarding ESBL and VRE carriage, only two other studies in German LTCFs, not done in Frankfurt am Main [7,10], have been published since 1999 [26,27]. ESBL/MRGN prevalence in our studies was 11.2% up to 26.7% [7,10] and therefore much higher than MRSA prevalence. Three studies from LTCFs in the US and one in Italy exhibited higher prevalence rates for ESBL-producing bacteria than our study [24,42,43,45], whereas in Australian and especially in Swedish LTCFs, ESBL prevalence rates were lower than in the Rhine-Main region [14,52] (Table 3). However, in all studies, ESBL rates exceeded those of MRSA by far [7,10,24,42,43,45]. Prevalence rates of MRSA and ESBL/MRGN in the LTCF residents in our study were even higher than those in a survey on 750 ambulatory patients undergoing haemodialysis enrolled in the Rhine-Main area in summer 2012, presenting 2.1% MRSA, 7.5% ESBL and 5.5% VRE prevalence [53].

Compared with studies on MRSA in LTCFs, only few studies on ESBL/MRGN have been published so far, with a maximum of 495 participants per study. Our study encompassing 455 participants was a comparatively large study. In Germany, up to now, MDRO prevalence rates in residents from nursing homes have only been published for the Rhine-Main region [7,10] and the federal state of Hesse [27]. This is striking because of the well-known and published increase in MRGN in the hospital setting in Germany and abroad.

In Germany, guidelines for hygiene and infection prevention in LTCFs have been published in 2005 [51], including recommendations for the care of residents with MRSA colonisation. According to these guidelines, isolation of those persons is recommended for hospitals but does not need to be applied in LTCFs. A single room (no isolation), however, is recommended if the resident with MRSA colonisation or their roommate exhibits risk factors such as medical devices or wounds. In 2012, KRINKO published a guideline on the management of patients carrying 3MRGN and 4MRGN [39]. It recommends that patients with 3MRGN are isolated in risk areas such as intensive care units only, whereas patients with 4MRGN must be cared for in single rooms in combination with barrier nursing in all hospital wards. Although the guideline primarily addresses the hospital setting, the KRINKO expert panel recommends that in other healthcare settings such as LTCFs, hygienic measurements for MRGN should not exceed those defined for MRSA [39]. Therefore, a high standard of hygiene should be applied to residents with ESBL/MRGN, but restriction of their mobility in the home and their contact to other residents is not necessary. Staff, however, need to be well informed about new and emerging antibiotic-resistant organisms and must observe good hygiene for the protection of other residents and themselves. Although 4MRGN have as yet not been detected in the residents in our studies, it can be hypothesised that this may soon be the case as 4MRGN rates are continuously increasing in Germany and Europe [54].

In conclusion, the data suggest that MRSA prevalence in LTCFs in the Rhine-Main region is stable, but a high ESBL/MRGN carriage in LTCFs is recognised. No CRO have been detected yet. In nearly all residents with MDRO, the MDRO carriage had not been known before, indicating a lack of screening and/or a lack of information on hospital discharge.

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Conflict of interest

None declared.

Authors' contributions

PP, DM and UH were responsible for the study design, statistical analysis, and did the literature search. MH, PP, DM, CC, VAK and UH contributed to data collection and analysis as well as writing and review of the manuscript.

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Phylogenetic analysis of highly pathogenic avian influenza A(H5N8) virus outbreak strains provides evidence for four separate introductions and one between-poultry farm transmission in the Netherlands, November 2014

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Phylogenetic analysis of highly pathogenic avian influenza A(H5N8) virus strains causing outbreaks in Dutch poultry farms in 2014 provides evidence for separate introduction of the virus in four outbreaks in farms located 16–112 km from each other and for between-farm transmission between the third and fourth outbreak in farms located 550 m from each other. In addition, the analysis showed that all European and two Japanese H5N8 virus strains are very closely related and seem to originate from a calculated common ancestor, which arose between July and September 2014. Our findings suggest that the Dutch outbreak virus strain 'Ter Aar' and the first German outbreak strain from 2014 shared a common ancestor. In addition, the data indicate that the Dutch outbreak viruses descended from an H5N8 virus that circulated around 2009 in Asia, possibly China, and subsequently spread to South Korea and Japan and finally also to Europe. Evolution of the virus seemed to follow a parallel track in Japan and Europe, which supports the hypothesis that H5N8 virus was exchanged between migratory wild waterfowl at their breeding grounds in Siberia and from there was carried by migrating waterfowl to Europe.

Introduction

pathogenic avian Influenza (HPAI) is a viral disease of poultry causing high mortality [1]. In 2003, HPAI A(H7N7) virus was detected on 241 Dutch farms [2]. After the epidemic was stopped, syndromic surveillance and monitoring programmes were initiated to enable early detection of the introduction of any H5 and H7 avian influenza viruses on poultry farms. Since 2003, only low pathogenic avian Influenza viruses of diverse subtypes were detected. Up to 2014, HPAI H5 viruses had not been detected in the Netherlands.

In China, the H5N8 HP virus had been isolated in 2009–10 as part of a monitoring programme [3]. From January 2014, the HPAI H5N8 virus spread very rapidly in South Korea. Genetic sequence analysis indicated that virus isolates from infected farm ducks in South Korea and dead Baikal teals in the surrounding area strongly resembled the earlier Chinese isolates [4].

Outbreaks in the Netherlands

Outbreak 1

Dead chickens from an indoor farm with 150,000 laying hens (Farm 1), in the municipality of Hekendorp in the province of Utrecht, were submitted for necropsy to the Dutch Animal Health Service in mid-November 2014, with an anamnesis of exponentially increasing mortality in the past days. Swab samples from these chickens were forwarded to the Central Veterinary Institute part of Wageningen UR, Lelystad, which is standard procedure when there is exponentially increasing mortality and inconclusive pathology. The farm was situated next to a river and in the middle of peat land with an abundant presence of wild waterfowl. Exponentially increasing mortality had been observed by the poultry farmer in one of six poultry houses in the days preceding the notification.

Outbreak 2

On 19 November, HPAI suspicion was raised on an indoor farm with 43,000 laying hens (Farm 2), in the municipality of Ter Aar in the province of Zuid-Holland. Three days previously, the poultry farmer

saw exponentially increasing mortality, with birds showing conjunctivitis and ruffled feathers in one of three poultry houses. Egg production started to drop on 18 November and two days later, egg production decreased by 20%. Veterinarians of a specialist team from the Dutch Food and Safety Authority (NVWA) visited the farm on 19 November, took samples from clinically sick birds and the farm was quarantined. Like Farm 1, this poultry farm was also situated in an area with an abundant presence of wild waterfowl. Farm 2 was situated 21 km north-north-west of Farm 1.

Outbreak 3

One day later (20 November), HPAI suspicion was raised on a pullet-rearing farm with 11,100 birds (Farm 3), in the municipality of Kamperveen in the province of Gelderland, housing hens and cocks in two separate houses. This farm was located in a stretch of farmland boarded by a lake on one side and the IJssel river on the other. In the area, large quantities of wild waterfowl were present and it is known to be a congregating place for wild birds before they take off for their journey across the lake to the polder area of the province of Flevoland. Farm 3 was situated 90 km north-east of Farm 2 and 92 km north-east of Farm 1. Increased mortality was first observed on 18 November on Farm 3, as well as a temporary decrease in egg production. On 20 November, during a visit by the competent veterinary authorities after notification of the suspicion, increased mortality was seen in both poultry houses and the birds showed typical signs of HPAI [1].

Outbreak 4

In the evening of 19 November, a poultry farmer submitted several dead meat ducks to his veterinarian. The poultry farm, which housed 15,000 meat ducks each in two poultry houses indoors, was situated 550 m from Farm 3. The carcasses of the meat ducks were submitted the following day for necropsy to the Dutch Animal Health Service, who notified the Dutch Food and Safety Authority. Swab samples from the ducks at necropsy were forwarded, in the framework of the Dutch early-warning system for avian influenza, to the Central Veterinary Institute part of Wageningen-UR.

Outbreak 5

The fifth outbreak was reported on 29 November following exponentially increasing mortality in the four preceding days in an indoor farm housing 29,000 layer hens. This farm was located in Zoeterwoude about 30 km west of Farm 1 and 20 km south-west of Farm 2. The five outbreak locations are shown in Figure 1.

At the Central Veterinary Institute part of Wageningen UR, swab samples of sick chickens and ducks from the five outbreak farms were tested following our accredited procedures. All five farms were confirmed to be positive for HPAI H5N8.

Backward and forward tracing of possible dangerous contacts in the framework of the standard epidemiological investigation by the Dutch Food and Consumer Product Safety Authority (i.e. transport, professional visitors such as advisors, veterinary practitioner, etc.; possibly contaminated materials delivered to the farm such as feed, bedding etc.; possibly contaminated transport vehicles, etc.) by the Dutch Food and Safety Authority revealed no indication for dangerous contacts of such connections between HPAI H5N8 outbreaks in Asia, Germany, the United Kingdom and the Netherlands occurring between October and December 2014 [5]. Moreover, no links were discovered between the outbreaks in the Netherlands. In addition, recent analysis from our group showed that the Dutch virus (A/Ch/Netherlands/14015526) has high similarity to two South Korean and one Japanese strains [6].

The question arose as to whether the outbreaks on the five Dutch poultry farms were caused by separate virus introduction or by transmission between farms. In an attempt to answer this question, we sequenced the complete virus RNA genome obtained from several animals from each farm. The aim of our study was to assess possible routes of transmission of the virus by sequence and temporal phylogenetic analysis.

Figure 1

Location of five highly pathogenic avian influenza A(H5N8) outbreaks in the Netherlands, November 2014

The red dots represent the location of the outbreaks, based on postal code. The internal lines represent the boundaries of the 12 provinces.

Methods

Genome sequence analysis

RNA was extracted from cloacal and oropharyngeal pools of five samples originating from clinically affected hens positive in a screening matrix-gene real-time PCR [7,8], which detects all avian influenza virus subtypes. The positive samples were then checked for the presence of notifiable subtypes (H5 and H7) by real-time PCR as recommended by the European Union reference laboratory in Weybridge, United Kingdom [9,10]. Haemagglutinin (HA) and neuraminidase (NA) sequence analysis was based on PCR fragments that were generated according to the so-called KHA PCR [11] and PanHA [12] and PanNA [13] protocols. The sequence of the HA gene revealed polybasic amino acids – RRRKR*GLF – at the HA cleavage site, a motif typical for HPAI viruses. In addition, HA and NA sequence results showed that the virus was of the H5N8 subtype.

The sequencing results of the cleavage site also revealed high similarity to the German outbreak strain (November, 2014) [14]. However, as complete genome sequencing is necessary to have more insight into the origin and emergence of this virus into Europe and specifically into the Netherlands, we amplified all eight RNA genome segments of the outbreak viruses using universal eight-segment primers and directly sequenced [15]. Purified amplicons were sequenced at high coverage (average›1,000 per nucleotide position) using the Nextera library preparation method and subsequently sequenced using Illumina MiSeq paired-end 150 base pairs sequencing (Illumina, San Diego, CA, United States). Quality control-passed sequence reads of high quality were iteratively mapped on resulting consensus sequences using Bowtie2 [16] starting against the South Korean H5N8 genome sequence (GenBank accession numbers KJ511809–KJ511816) to generate a majority (›80% evidence) consensus sequence of all segments. The consensus sequences were compared with de novo assembled sequences reads using SPAdes-v3 [17] and no significant differences were detected. The majority consensus sequences were submitted to the Global Initiative on Sharing All Influenza Data (GISAID) (EPI_ISL_167905, EPI_ISL_174349, EPI_ISL_174350, EPI_ISL_174351, EPI_ISL_174352) and subsequently for all nucleic acid sequences, a basic molecular phylogenetic analysis was performed using the maximum likelihood method based on the Tamura–Nei model using a gamma distributed nucleotide substitution rate [18]. Between two and four pools of samples per farm were sequenced.

Table

Origin of the sequences of highly pathogenic diverged avian influenza A(H5N8) viruses used for the comparative analysis

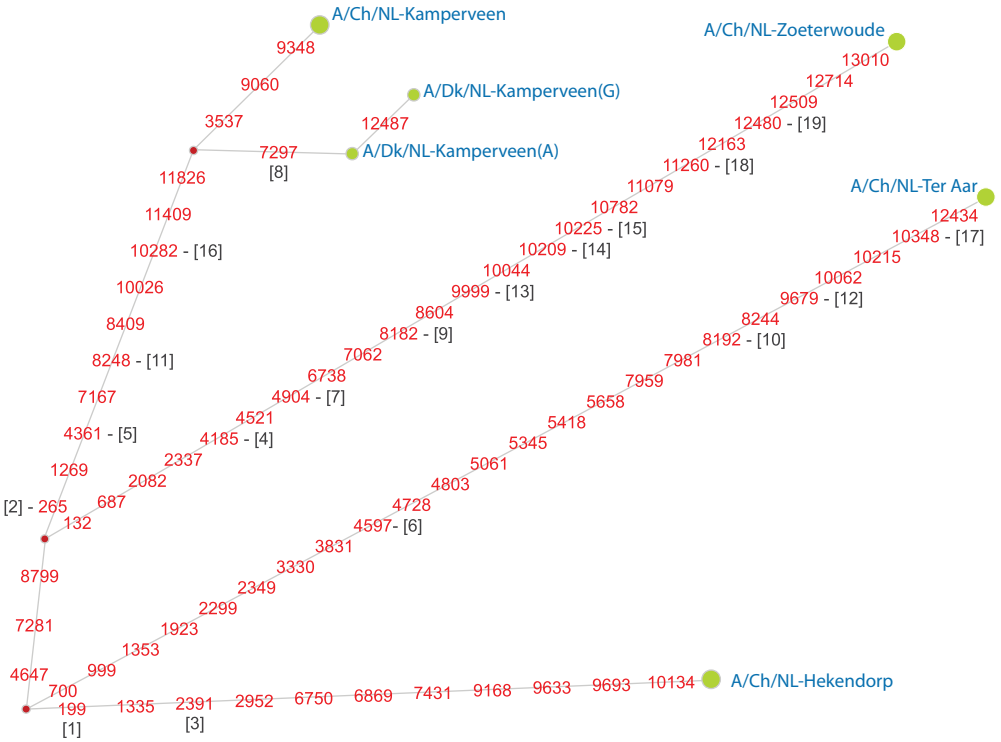
Isolate ID	Isolate name	Submitting laboratory	Authors
EPI_ISL_167140	A/turkey/Germany-MV/R2472/2014	Friedrich-Loeffler-Institut	Starick et al.
EPI_ISL_169427	A/wigeon/Sakha/1/2014	State Research Center of Virology and Biotechnology Vector	Susloparov et al.
EPI_ISL_171705	A/breeder chicken/Korea/H122/2014	Other Database Import	Jeong et al.
EPI_ISL_159719	A/Chicken/Kumamoto/1–7/2014	National Agriculture and Food Research Organization	Kanehira et al.
EPI_ISL_167904	A/duck/England/36254/14	Animal and Plant Health Agency (APHA)	Hanna et al.
EPI_ISL_166694	A/duck/Beijing/CT01/2014	Institute of Microbiology	
EPI_ISL_166693	A/duck/Beijing/FS01/2014	Institute of Microbiology	
EPI_ISL_166584	A/duck/Beijing/FS01/2013	Institute of Microbiology	
EPI_ISL_162301	A/duck/Zhejiang/6D18/2013	Other Database Import	Wu et al.
EPI_ISL_162300	A/duck/Zhejiang/W24/2013	Other Database Import	Wu et al.
EPI_ISL_139385	A/duck/Jiangsu/k1203/2010	Other Database Import	Zhao et al.
EPI_ISL_171709	A/breeder duck/Korea/H158/2014	Other Database Import	Jeong et al.
EPI_ISL_167178	A/duck/Shandong/Q1/2013	Other Database Import	Li et al.
EPI_ISL_157610	A/broiler duck/Korea/Buan2/2014	Other Database Import	Lee et al.
EPI_ISL_157609	A/breeder duck/Korea/Gochang1/2014	Other Database Import	Lee et al.
EPI_ISL_157611	A/baikal teal/Korea/Donglim3/2014	Other Database Import	Lee et al.
EPI_ISL_169280	A/eurasian wigeon/Netherlands/emc-2/2014	Erasmus Medical Center	Fouchier et al.
EPI_ISL_169281	A/eurasian wigeon/Netherlands/emc-1/2014	Erasmus Medical Center	Fouchier et al.
EPI_ISL_156815	A/mallard duck/Shanghai/SH-9/2013	Institute of Laboratory Animal Sciences, Chinese Academy	Fan et al.
EPI_ISL_171711	A/mallard/Korea/H207/2014	Other Database Import	Jeong et al.
EPI_ISL_166630	A/mallard/Korea/W452/2014	Other Database Import	Choi et al.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database on which this research is based. All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

Figure 2

Median-joining network analyses of five highly pathogenic avian influenza A(H5N8) sequences, the Netherlands, November 2014

Number	Gene	Nucleotide position	Amino acid mutation
1	PB2	199	I -> V
2		265	V -> M
3	PB1-F2	2,391	D -> G
4	PB1	4,185	K -> N
5		4,361	S -> N
6	PA	4,597	V -> I
7		4,904	R -> Q
8	HA	7,297	P -> S
9		8,182	E -> K
10		8,192	E -> G
11		8,248	K -> E
12	NP	9,679	T -> A
13	NA	9,999	I -> M
14		10,209	K -> N
15		10,225	V -> I
16		10,282	V -> L
17		10,248	V -> M
18		11,260	E -> K
19	NS	12,480	R -> H



The median-joining network was constructed from the combined sequence of eight gene segments data. This network included all the most parsimonious trees linking the sequences.

Each unique sequence genotype is represented by a yellow circle sized relative to its frequency in the dataset. Numbers refer to the position of the mutation within the combined sequences. Red circles represent median vectors. The sequence detected in samples from the duck farm in Kamperveen (Outbreak 4) was heterogeneous at position 12,486 (A or G).

Temporal phylogenetic analysis

The five fully sequenced Dutch H5N8 sequences were each aligned with 22 H5N8 sequences obtained from GISAID using Muscle in MEGA6 [19]. For each of the eight segment alignments, the simplest evolutionary model fitting the dataset was the Hasegaw–Kishino–Yano model with gamma distributed rates [20]. Nucleotide substitution rates were estimated using Bayesian Markov Chain Monte Carlo methods [21]. Analysis was performed using the programme BEAST v1.8.1 [22] using strict or relaxed uncorrelated molecular clocks that were calibrated using the sample isolation dates. All genome segments were treated as virus partitions with individual substitution and clock models and analysed for 30 million generations, sampling every 3,000 generations. Effective sample sizes were checked using Tracer 1.6 [23]: the values were far above the minimum threshold of 200. A maximum clade credibility tree was constructed to summarise all 10,000 trees after 10% burn-in using TreeAnnotator [21]. The final time-scaled phylogenetic tree was visualised and annotated using FigTree 1.4.2 [24]. Three or eight gene segment alignments were manually concatenated to generate a single alignment that was used to construct phylogenetic networks using the median-joining method implemented in the programme NETWORK as described by Bataille et al. [25]. This model-free method uses a parsimony approach, based on pairwise differences, to connect each sequence to its closest neighbour, and allows the creation of internal nodes ('median vectors'), which could be interpreted as unsampled or extinct ancestral genotypes to link the existing genotypes in the most parsimonious way.

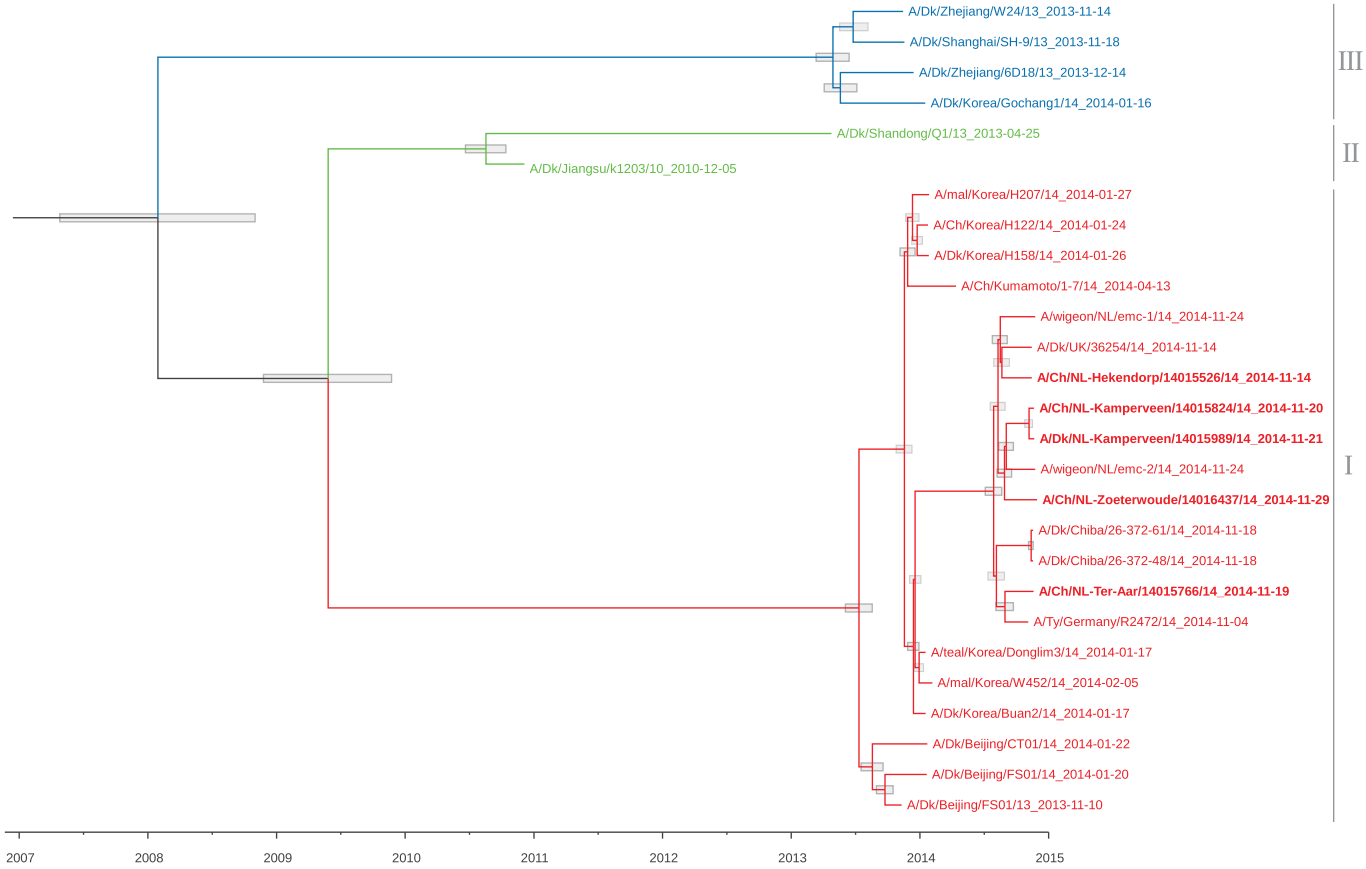
Results

The number of nucleotide differences between sequences of all eight segments of viruses detected at the five Dutch outbreak farms varied from four for the viruses from the two farms in Kamperveen to 51 nucleotides resulting in 12 amino acid differences between the viruses from Ter Aar and Zoeterwoude (Figure 2).

The median-joining network shows that all five sequences were derived from one or more calculated ancestors and were not descendants of each other. To determine the relationship with other European isolates, we aligned the five Dutch sequences with 22 sequences obtained from the EpiFlu database. Isolate ID numbers and providers are listed in the Table.

Figure 3

Phylogenetic trees derived from complete genome sequences of highly pathogenetic avian influenza A(H5N8) viruses



Time-scaled phylogenies (dates shown on the horizontal axis) were inferred using strict-clock Bayesian Markov Chain Monte Carlo analysis including all genome segments. Times of most recent common ancestors with 95% highest posterior density intervals are shown by the horizontal bars at each node. The three distinct evolutionary lineages are indicated in different colours and the symbols I, II and III. Indicated dates are the dates of sampling. A/Ch/NL-Kamperveen was detected at Farm 3 and A/Dk/NL-Kamperveen on Farm 4. Viruses detected at the Dutch farms are shown in bold.

Analyses of the 27 H5N8 isolates showed diversification into three lineages that diverged between 2009 and 2010 (Figure 3).

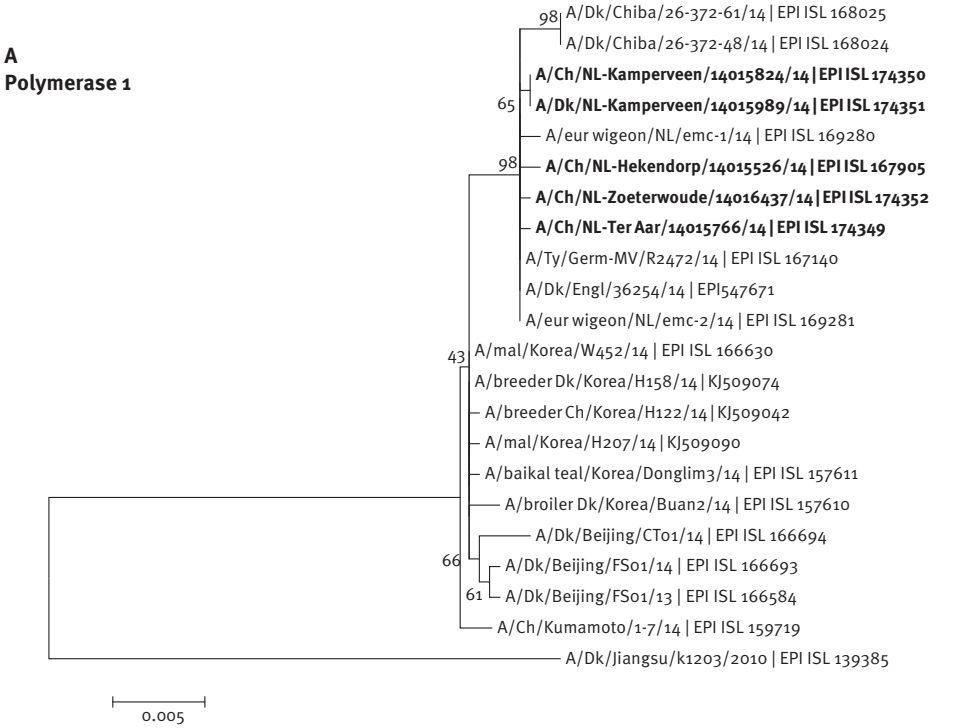
Calculation of the time of most recent common ancestor reveals that the origin of the H5N8 viruses occurred between 9 September 2007 and 20 June 2008 (Supplementary Table 1 [26]). Calculations were also performed using a strict clock or uncorrelated relaxed clocks, with essentially similar outcomes (Supplementary Tables 2 and 3 [26]). The viruses isolated in Europe are located within lineage I, forming a separate sublineage. Lineage I diversified between the end of June and end of December 2013 into three sublineages of viruses that caused outbreaks in poultry and wild birds in South Korea and China in the beginning of 2014. Viruses isolated in Europe and Japan (Chiba) in the autumn of 2014 seem to have evolved from viruses that circulated in wild birds, including Baikal teals, in South Korea in beginning of 2014 The common ancestor of the isolates from Europe and Chiba was estimated to have emerged somewhere between 15 July and 8 August 2014 (mean: 28 July).

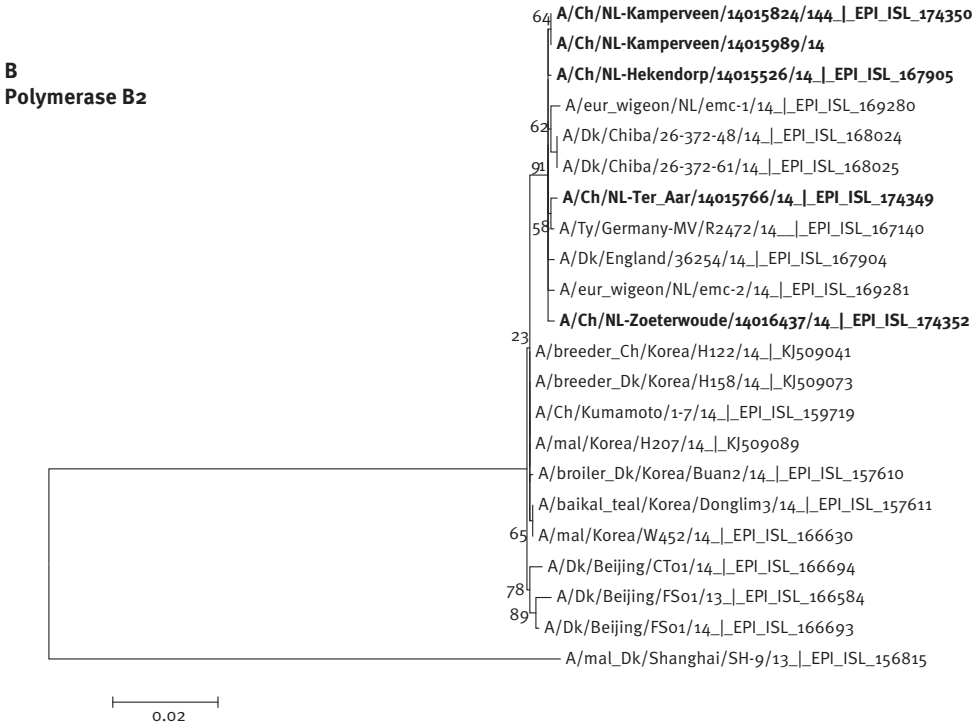
Four of the five Dutch strains were differently located in lineage I: A/Ch/Nl/Ter_Aar and A/Ty /Germ-MV are closely related and diverted around the end of August 2014. In contrast, A/Ch/NL-Hekendorp, A/Dk/Eng, A/Ch/NL-Kamperveen and A/Ch/NLZoeterwoude diverged slightly earlier, around 8 August. Both viruses isolated from Kamperveen diverged only in the first week of November, consistent with the possible transmission between the two farms (Figure 3).

Trees of each eight gene segments of the 27 H5N8 viruses were constructed using the minimal likelihood method using a Tamura–Nei substitution model, gamma-distributed substitution rate and 1,000 bootstraps. The five Dutch sequences, (A/Ch/NL-Hekendorp, A/Ch/NL-Ter Aar, A/Ch/NL-Kamperveen, A/Dk/NL-Kamperveen, A/Ch/NL-Zoeterwoude), A/Dk/Eng, A/Germ/-MV, A/Ty/Engl and A/Dk/Chiba, were located in the same subgroup of lineage I and clustered in a similar fashion to each other (Figure 4).

Figure 4A

Phylogenetic trees of nucleotide sequences of influenza A(H5N8) viruses

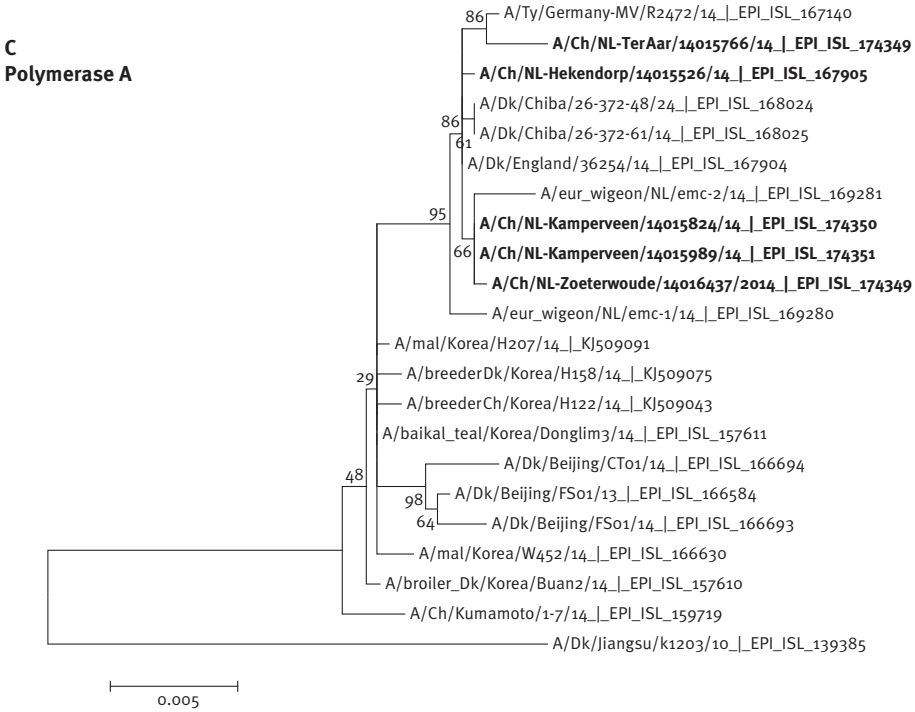


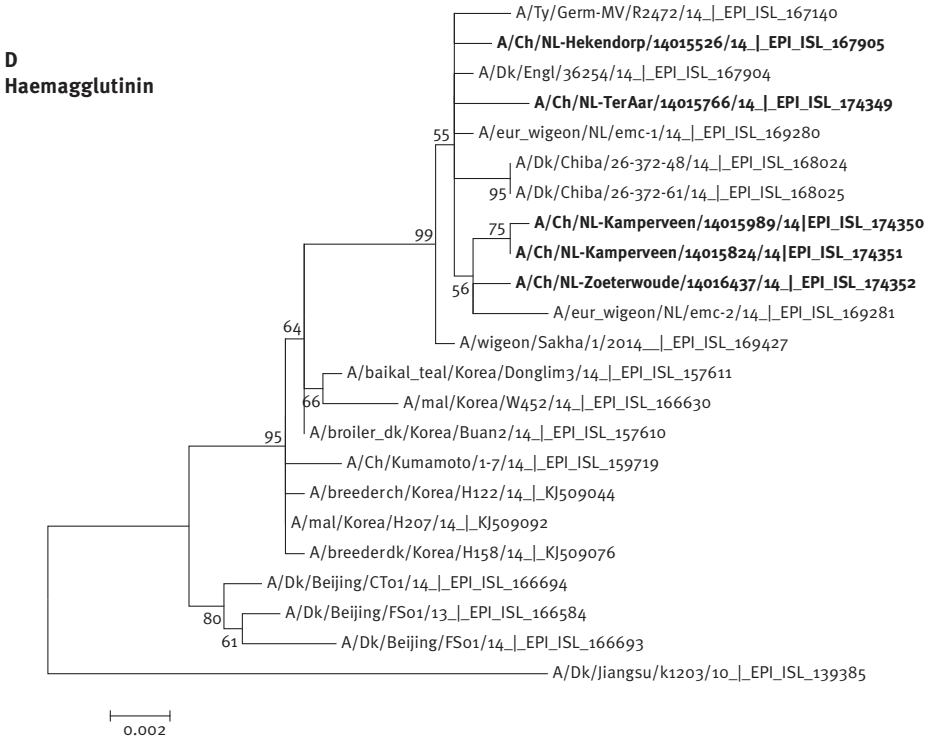


Evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model [18]. The trees of polymerase 1 (panel A), polymerase B2 (panel B), polymerase A (panel C), haemagglutinin (panel D), nucleoprotein (panel E), neuraminidase (panel F), matrix protein (panel G) and non-structural protein (panel H), with the highest log likelihood shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 [19]. Viruses detected at the Dutch farms are shown in bold.

Figure 4b

Phylogenetic trees of nucleotide sequences of influenza A(H5N8) viruses

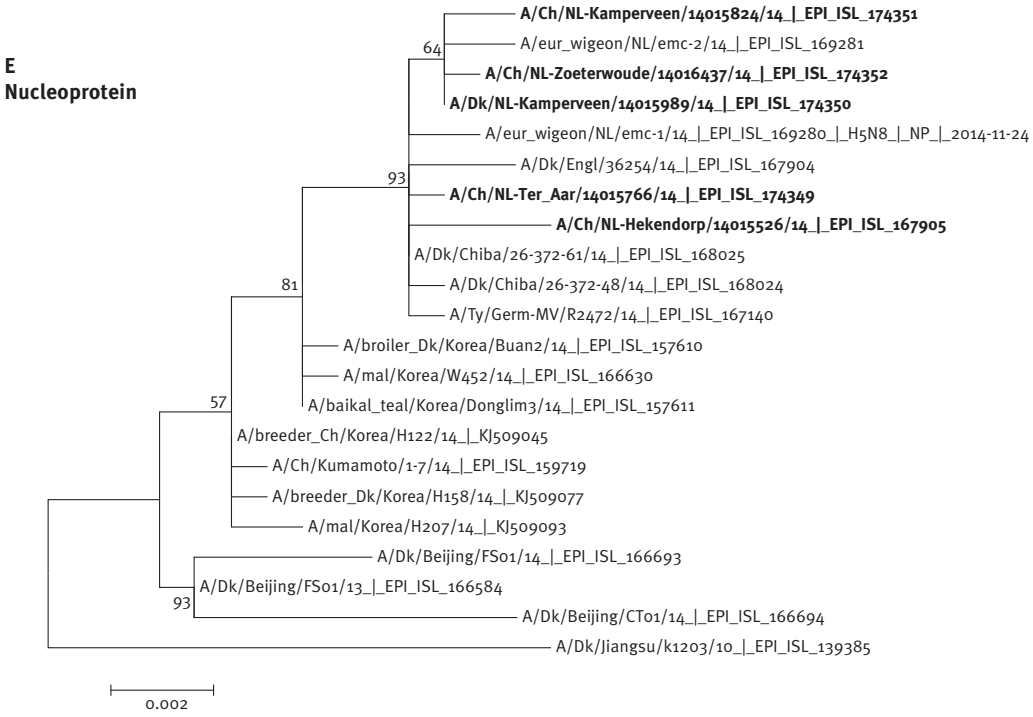


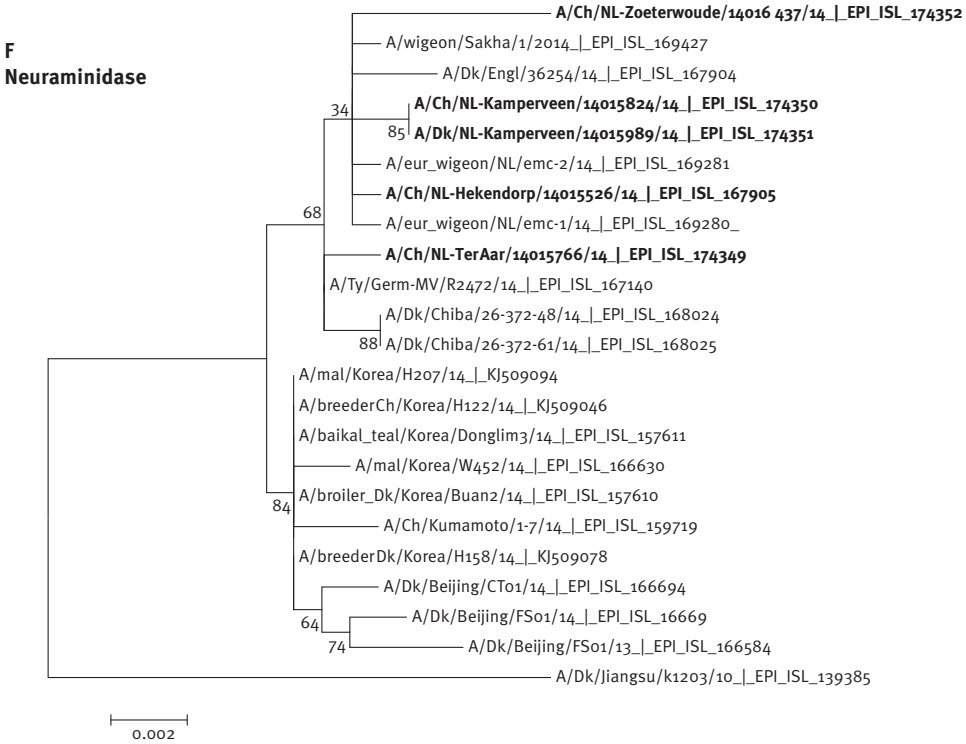


Evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model [18]. The trees of polymerase 1 (panel A), polymerase B2 (panel B), polymerase A (panel C), haemagglutinin (panel D), nucleoprotein (panel E), neuraminidase (panel F), matrix protein (panel G) and non-structural protein (panel H), with the highest log likelihood shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 [19]. Viruses detected at the Dutch farms are shown in bold.

Figure 4C

Phylogenetic trees of nucleotide sequences of influenza A(H5N8) viruses

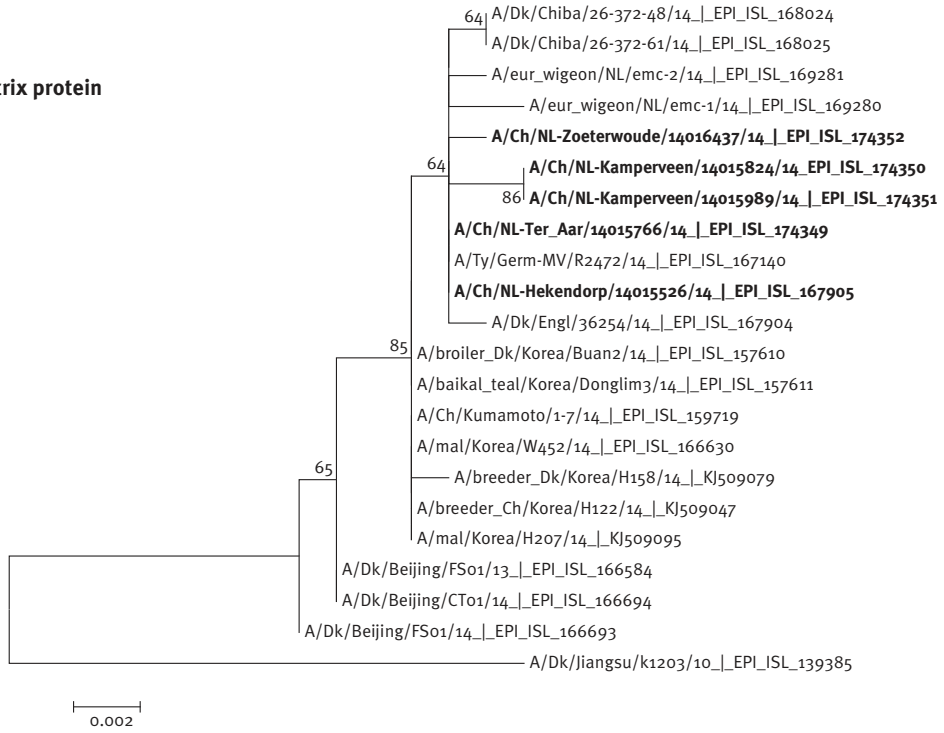


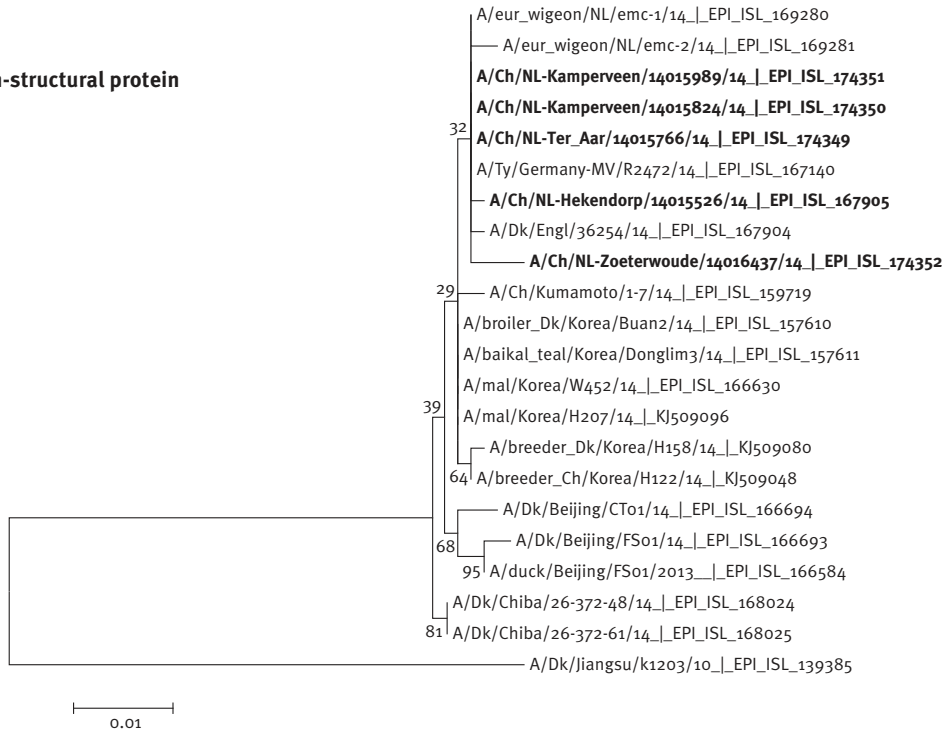


Evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model [18]. The trees of polymerase 1 (panel A), polymerase B2 (panel B), polymerase A (panel C), haemagglutinin (panel D), nucleoprotein (panel E), neuraminidase (panel F), matrix protein (panel G) and non-structural protein (panel H), with the highest log likelihood shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 [19]. Viruses detected at the Dutch farms are shown in bold.

Figure 4D

Phylogenetic trees of nucleotide sequences of influenza A(H5N8) viruses





Evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model [18]. The trees of polymerase 1 (panel A), polymerase B2 (panel B), polymerase A (panel C), haemagglutinin (panel D), nucleoprotein (panel E), neuraminidase (panel F), matrix protein (panel G) and non-structural protein (panel H), with the highest log likelihood shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 [19]. Viruses detected at the Dutch farms are shown in bold.

Discussion

Comparative analyses of all European and Asian H5N8 viruses using Bayesian Markov Chain Monte Carlo and median-joining network analyses suggest that four of the five outbreaks of HPAI H5N8 virus in the Netherlands were caused by separate introduction and not by farm-to-farm spread. In addition, the analyses suggest between-farm transmission between the third and the fourth outbreaks, both located in Kamperveen, at a distance of 550 m from each other, although we cannot entirely exclude that both outbreaks resulted from two separate introductions from the same source. If the virus had spread between farms after a single introduction, it is expected that the Dutch viruses would have diversified from a single node in the tree [25]. Moreover, the five Dutch viruses had a maximum of 20 nucleotide substitutions in the PB2, HA and NA gene segments (Supplementary Figure 1 [26]) that must have been generated during circulation in poultry during nine days, if we assume that between-farm spread caused all outbreaks. However, during the HPAI H7N7 outbreak in the Netherlands, a maximum of 25 substitutions in the same three genes were generated in nine weeks and in Italy, 66 substitutions in nine months [27]. On the basis of these numbers, we would have expected between three and six mutations between the five H5N8 outbreak virus sequences. In addition, the fact that H5-specific antibodies were not detected in animals of the outbreak farms (data not shown) excluded the possibility that the virus had circulated for some time unnoticed. Moreover, tracing of contacts by the Dutch Food and Safety Authority revealed no indication of epidemiological connections between the Dutch outbreaks and the farms in outbreaks 1, 2, 3 and 5 were located far from each other (16 to 112 km).

Remarkably, H5N8 viruses isolated from non-specified species of ducks (Anatidae) in Chiba, Japan, in November 2014 [28] most likely derived from the same precursor as that of viruses isolated in Europe in the same period. Rates of nucleotide substitution and time of most recent common ancestor analyses showed that the origin of the H5N8 Japanese and European H5N8 viruses dated back to late summer of 2014 based on the combined PB1, PB2, PA, HA, NP, NA, MP and N genome components in the dataset (Figure 3). Values for the time of most recent common ancestor and highest posterior density were robust whether a strict or relaxed clock (Supplementary Tables 1, 2 and 3 [26]) or different viruses (Supplementary Figures 2 and 3 [26]) were used in the analyses. The results are consistent with the hypothesis that the precursor virus arose in Siberia on breeding grounds where migratory birds from the East Atlantic Flyway and Asia Australia Flyways [29] may have mingled during the breeding season in 2014. Recently published HA and NA sequences of virus RNA detected in a wigeon that was shot in September 2014 in north-east Russia show that the HA and NA sequences of this virus are phylogenetically located near the node of the Eurasian and Chiba viruses [6] (Figure 4 panels D and F).

It is known that viruses that pass species barriers will show adaptation mutations [30]. These adaptations become visible in phylogenetic analysis. The fact that European viruses diverged from the same ancestor as that of two Japanese viruses might indicate that the virus travelled from Asia to Europe in just a single or a few bird species. If a lot of bird species were involved in the transport of H5N8 from Asia to Europe, you would expect no Asian viruses in lineage I. In addition, none of the amino acid mutations in the five Dutch viruses (Figure 2) are known to affect virulence of the virus in mammals.

Pathogenicity studies showed that the virus was highly virulent for chickens, but mildly to moderately virulent for wild ducks [31], suggesting the potential for transport of the virus over large distances. From January 2014, HPAI H5N8 virus spread very rapidly in South Korea, initially mainly among farm ducks. At the time of the first outbreaks among farm ducks, a large number of dead Baikal teals (Anas formosa) – a species of migratory ducks – were found near the affected farms, leading to the hypothesis that the infection may have been carried by these migratory ducks]. Genetic sequence analysis indicated that isolates of infected farm ducks in South Korea and dead Baikal teals in the surrounding area strongly resembled Chinese isolates from 2010 to 2013 [4], while it was also noted that isolates of HPAI H5N8 virus in South Korea was a product of reassortment of A/duck/Jiangsu/k1203/2010 (H5N8) and other avian influenza virus subtypes that co-circulated in birds in east Asia from 2009 to 2012 [32]. Kang et al. very recently demonstrated in a pathogenicity study of various HPAI H5N8 virus isolates that the virus was moderately virulent in experimental infection trials with wild ducks (Anas platyrhynchos) and Baikal teals and did not result in serious disease and/or mortality [31].

These findings emphasise the clear need for the utmost attention concerning hygienic measures and biosecurity by poultry farmers to prevent introduction of disease agents into poultry houses. Somehow, the virus was brought into indoor poultry farms. For example, via persons with contaminated clothing/boots/materials/feed, or by vermin and flies. In addition, it would be wise at this moment in the epidemic to house poultry that normally use outdoor facilities in order to prevent exposure to possibly infected wild waterfowl and their excrement [33].

In conclusion, phylogenetic analysis of the Dutch HPAI H5N8 outbreak strains helped to unravel possible routes of transmission. These kinds of analyses, combined with other epidemiological and laboratory data, might provide tools to support specific preventive measures.

Acknowledgments

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Conflict of interest

None declared.

Authors' contributions

Ruth Bouwstra, Guus Koch and Alex Bossers were mainly responsible for the design and supervision of the study. René Heutink, Alex Bossers and Frank Harders performed the laboratory work. Arco van der Spek was responsible for the epidemiological field investigations at the outbreak flocks. Guus Koch and Alex Bossers analysed the results and all authors were involved in the discussion of the results. Ruth Bouwstra, Armin Elbers, Alex Bossers and Guus Koch drafted the paper.

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