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Identification of *Neisseria gonorrhoeae* isolates with a recombinant *porA* gene in Scotland, United Kingdom, 2010 to 2011

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Three isolates of *Neisseria gonorrhoeae* have been identified in Scotland in 2010 and 2011, which lack sequences in the *porA* pseudogene commonly used as the target for confirmatory gonorrhoea polymerase chain reaction assays. Two isolates were clustered temporally and geographically and have the same sequence type and *porA* sequence. A similar strain was reported in Australia during early 2011. The other Scottish isolate was identified separately and is different in sequence type and *porA* sequence.

Introduction

We report three isolates of two different *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST) types in Scotland in 2010–2011 which lack the oligonucleotide binding sites for a *porA* polymerase chain reaction (PCR) in common use as a confirmatory assay for *N. gonorrhoeae* [1].

Nucleic acid amplification tests (NAATs) for *N. gonorrhoeae* are increasingly used in screening and diagnosis of gonorrhoea. They have a number of advantages over culture, particularly increased sensitivity when used on non-invasive and extra-genital specimens and where rapid transport of the specimen to the laboratory is not possible. However, concerns about the specificity of commercially-available NAATs have led to widespread recommendations for the confirmatory testing of reactive specimens [2,3]. This should be performed using a NAAT amplifying a different gene target to the original test.

In Scotland, specimens positive for *N. gonorrhoeae* by NAAT may be referred to the Scottish Bacterial Sexually Transmitted Infections Reference Laboratory (SBSTIRL) for confirmation. In addition, all *N. gonorrhoeae* isolates and those NAAT specimens confirmed locally are referred to SBSTIRL for typing by NG-MAST [4] and antimicrobial susceptibility testing (isolates only). Isolates are stored indefinitely on Microbank beads (Pro-Lab).

Confirmatory *N. gonorrhoeae* NAAT testing at SBSTIRL is performed using a real-time PCR targeting the *porA* pseudogene [1] with an internal inhibition control [5]. Specimens producing indeterminate or negative results are generally tested using Aptima GC (Gen-Probe). However, some referred specimens are insufficient in volume for Aptima GC or are in an incompatible transport medium [6].

In May 2011, two isolates of *N. gonorrhoeae* from the same patient, which harboured a recombinant *porA* gene were reported in Australia [7]. These isolates were NG-MAST type 5377, and were not amplifiable using the PCR primers used also by SBSTIRL.

Patients and isolates

In October 2011, a rectal *N. gonorrhoeae* isolate (GC1) and rectal swab positive by NAAT from the same male patient were referred to SBSTIRL. The NAAT specimen was negative by *porA* PCR, but was insufficient for testing by Aptima GC. A nucleic acid extract of the isolate was tested by the *porA* PCR and was also negative. The identity of the isolate was confirmed as *N. gonorrhoeae* serogroup WII/III by Phadebact Monoclonal GC test (Bactus AB), by carbohydrate utilisation test and by Aptima GC. GC1 was NG-MAST type 5967, and exhibited chromosomal resistance to penicillin, tetracycline and ciprofloxacin, while being sensitive to cefixime, ceftriaxone, azithromycin and spectinomycin. A database search for NG-MAST type 5967, revealed a stored rectal isolate (GC2) from a male patient from the same area of Scotland, diagnosed with gonorrhoea one month previously. The patient reported multiple male partners who remain untraced. There was no NAAT specimen for this patient, and no link was found between him and the previously described patient. GC2 had a similar antimicrobial susceptibility profile to GC1 and also failed to amplify using the *porA* PCR. No further identifications of NG-MAST type 5967 strains have been made in Scotland to date.

A further urethral isolate of *N. gonorrhoeae* (GC3) was identified through a search for *porA*-negative, Aptima GC-positive specimens. The male patient was diagnosed with gonorrhoea in December 2010, by both culture and NAAT, in a different region of Scotland to the previous patients. He reported one male partner who was not traced. GC3 was confirmed to be *N. gonorrhoeae* using the same methods as GC1 and GC2, was serogroup WII/III, NG-MAST type 3149, and exhibited chromosomal resistance to penicillin, tetracycline and ciprofloxacin, while being sensitive to cefixime, ceftriaxone, azithromycin and spectinomycin.

Sequencing of *porA*

The *porA* gene was sequenced bidirectionally using the primers described by Whiley et al. [7] (Figure). Basic Local Alignment Search Tool (BLAST) searches were performed via National Center for Biotechnology Information (NCBI), GenBank. Sequences were aligned using Seqscape software (Applied Biosystems).

Sequences from GC1 and GC2 were identical, and very similar to the sequence previously reported [7]. The sequence from GC3 was quite different from these, but the primer sites for the *porA* PCR were again missing and the sequence aligns most closely with a *porA* sequence from *N. meningitidis*.

Discussion

Similarly to the strain reported in Australia, the *N. gonorrhoeae* strains that we identified in this study have undergone an apparent recombination event with *N. meningitidis* in the *porA* region and therefore lack the sequences targeted by a published PCR assay [1] which may be commonly used in reference laboratories.

In contrast to the *porA* of *N. meningitidis*, the related sequence in *N. gonorrhoeae* is an unexpressed pseudogene. Whilst the consequently low selection pressure appears to have produced a rather conserved sequence, the apparent lack of function may make it vulnerable to mutation, including recombination with *porA* genes of other *Neisseria* species that may coexist with *N. gonorrhoeae*.

The sequences obtained from GC1 and GC2 are identical, and circumstantial evidence suggests that they may have been acquired as part of the same chain of transmission. No further epidemiologically connected cases have been identified and there is no known

Figure

Alignment of *porA* nucleotide sequences derived from Scottish isolates of *Neisseria gonorrhoeae* with the *porA* sequence of *Neisseria gonorrhoeae* FA1090 strain and with *porA* sequences of *Neisseria meningitidis* strains, United Kingdom, 2010–2011

Shaded characters indicate differences to the *Neisseria gonorrhoeae* FA1090 strain *porA* pseudogene sequence.

[a] *Neisseria gonorrhoeae* strain FA1090, *porA* pseudogene; GenBank accession AJ223447. Italicised regions indicate polymerase chain reaction primer and probe binding sites.

[b] *Neisseria meningitidis* strain 278, *porA* gene; GenBank accession GQ173789.

[c] *porA* sequence derived from an isolate *of Neisseria gonorrhoeae* in this study.

[d] *Neisseria meningitidis* strain NGE31, *porA* gene; GenBank accession AF226348.

history of sex abroad or with a person from outside Scotland from either patient. However, the histories supplied by the patients are incomplete.

Isolate GC3 was NG-MAST type 3149, which is not uncommon in Scotland, with sixteen isolates identified by SBSTIRL to date since July 2010, of which GC3 was the fourth to be found. All fifteen other NG-MAST type 3149 isolates are either *porA* PCR-positive or are from patients episodes where there was also a NAAT specimen which was *porA* PCR-positive. It is therefore possible that the *porA* recombination event occurred either in the patient from whom the isolation of GC3 was made, or within a very short chain of transmission. It is very likely, from the history reported by the patient, that this infection was acquired in Scotland from someone resident in Scotland, who has unfortunately not been identified.

NG-MAST type 5967, as represented by isolates GC1 and GC2, comprises alleles por 3558 and transferrin binding protein B (tbp) 4. These alleles are 99.8% and 99.7% similar to alleles por 1297 and tbp 983, respectively (representing in each case one nucleotide difference), which make up NG-MAST type 5377, the sequence type of the *porA*-recombinant strain reported in Australia [7]. In contrast, alleles por 1903 and tbp 110, which make up NG-MAST type 3149 are 92.5% and 79.8% similar to por 3558 and tbp 4, respectively. This represents significant sequence divergence and provides additional evidence that strain GC3 is unrelated to GC1, GC2 and the previously-reported strain.

All patients reported in Scotland and Australia were either men who have sex with men (MSM), or were infected rectally. The most likely site of co-colonisation with *N. gonorrhoeae* and *N. meningitidis*, and therefore of genetic exchange, is the pharynx, which is also the least amenable site to successful eradication of *N. gonorrhoeae* and is a frequent site of infection in MSM. It is notable that we have not so far identified pharyngeal infections with these unusual strains, but important that they are recognised if and when they occur in future.

No partners of any of the patients identified in Scotland are known to have been traced and tested or treated. While important for the interruption of gonorrhoea transmission and a mainstay of the public health response to sexually transmitted infections, partner notification remains a challenge in settings where contacts are frequently anonymous or semi-anonymous.

Due to the isolation of *N. gonorrhoeae*, all three patients were correctly diagnosed and adequately treated despite any difficulty with NAAT confirmation. The antimicrobial susceptibility pattern of all three isolates is typical of gonococci seen regularly in Scottish patients. None of the *N. gonorrhoeae* NAAT tests in use in Scotland for primary diagnosis target the *porA* gene [8] and therefore it appears that false-negative results are unlikely with these strains.

There is a small likelihood that patients exist who have been infected with *N. gonorrhoeae* strains similar to those described, in whom culture was unsuccessful and the original NAAT result was unconfirmed. The SBSTIRL records are currently being reviewed with the help of referring laboratories to attempt to identify such patients, and this work to date suggests that they are very few, if any.

We recommend that laboratories performing *porA*-based PCR to confirm positive *N. gonorrhoeae* NAAT results consider the use of a third NAAT, with an alternative target gene where the confirmatory assay is negative. This third target could alternatively be included as a duplex with the *porA* assay.

Laboratories and clinicians alike should be alert to the propensity of *N. gonorrhoeae* to develop unusual variations in genotype, as well as the well-established phenotypic variations..

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Clinical *Neisseria gonorrhoeae* isolate with a *N. meningitidis porA* gene and no prolyliminopeptidase activity, Sweden, 2011 – danger of false-negative genetic and culture diagnostic results

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We describe a *Neisseria gonorrhoeae* strain, found in Sweden in 2011, that harbours a *N. meningitidis porA* gene causing false-negative results in PCRs targeting the gonococcal *porA* pseudogene. Furthermore, the strain had no prolyliminopeptidase (PIP) activity that many commercial biochemical kits for species verification in culture rely on. Enhanced awareness of the spread of such strains and screening for them can be crucial.

Gonorrhoea remains a global public health threat and the World Health Organization (WHO) estimated that 88 million new gonorrhoea cases occurred in 2005 [1]. In many laboratories worldwide, commercial or in-house nucleic acid amplification tests (NAATs) have rapidly replaced culture of the aetiological agent *Neisseria gonorrhoeae* for the diagnosis of gonorrhoea. The gonococcal *porA* pseudogene is possibly the most common target in in-house PCRs currently used for primary detection and/or verifying detection of *N. gonorrhoeae* globally. This is because the pseudogene is highly conserved and has so far been considered to be present in all gonococcal strains. It is also sufficiently diverse from the meningococcal *porA* gene, and commensal *Neisseria* species are lacking the *porA* gene/pseudogene [2-5]. However, recently the first case of a clinical *N. gonorrhoeae* isolate was found in Australia, in which the gonococcal *porA* pseudogene was replaced with a *N. meningitidis porA* gene sequence, which caused a false-negative result in a gonococcal *porA* pseudogene PCR [6].

This report describes the identification and detailed characterisation of the second case of a *N. gonorrhoeae* isolate harbouring a *N. meningitidis porA* gene that causes false-negative results in PCRs targeting the *N. gonorrhoeae porA* pseudogene.

Case report

In May 2011, a pharyngeal specimen from a woman in her 30s presenting to a dermatovenerological clinic in Sweden was culture-positive for *N. gonorrhoeae*. The patient had recently had oral sex with a man in Sweden who could not be traced. She had no recent trips abroad. She was given therapy with cefixime (400 mg oral dose) and seven days later a test-of-cure using culture was negative, which indicated a successful treatment. However, it is known that culture, especially of pharyngeal specimens, has a suboptimal sensitivity compared to NAATs [7,8].

Characterisation of the *N. gonorrhoeae* strain with a meningococcal *porA* gene

The *N. gonorrhoeae* isolate was initially identified by typical colonies on selective culture medium, rapid oxidase production, presence of Gram-negative diplococci in microscopy, and two phenotypic species-verifying assays, i.e. an in-house sugar utilisation test and Phadebact GC Monoclonal Test (Bactus AB, Sweden).

When screening 200 clinical gonococcal isolates from 2011 with a PCR targeting the gonococcal *porA* pseudogene [2], the isolate obtained from the case above was repeatedly negative. Nevertheless, the phenotypic methods remained positive for *N. gonorrhoeae*, and additional phenotypic methods such as matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS; bioMérieux, France) and API NH (bioMérieux, France) confirmed this species. However, the isolate did not show any prolyliminopeptidase (PIP) activity in the API NH. According to Etest (bioMérieux, Sweden), the isolate was susceptible to cefixime, ceftriaxone, ampicillin, ciprofloxacin and spectinomycin, but resistant to azithromycin (Table). The isolate was also identified as *N. gonorrhoeae* in

APTIMA Combo 2 and APTIMA GC NAATs (Gen-Probe, United States).

For genetic characterisation, DNA was isolated in the robotised NorDiag Bullet (NorDiag ASA Company, Norway) using BUGS n'BEADS STI-*fast* kit (NorDiag ASA Company). The 16S rRNA gene in the isolate showed 100% sequence identity with other *N. gonorrhoeae* strains in a GenBank BLAST search. The strain was assigned to *N. gonorrhoeae* multi-antigen sequencing typing (NG-MAST) ST2382 (*porB* allele 1480 and *tbpB* allele 4) and multilocus sequence typing (MLST) ST7367 (*abcZ* allele 109, *adk* 39, *aroE* 67, *fumC* 111, *gdh* 148, *pdhC* 153, *pgm* 133), performed as previously described [9,10]. However, two gonococcal *porA* pseudogene PCRs [2,4] gave negative results. Sequencing of the full-length gonococcal *porA* pseudogene, performed as previously described [3], identified instead a meningococcal *porA* gene sequence (94% sequence identity with the genome-sequenced meningococcal reference strain MC58 [11]), which was assigned to meningococcal genosubtype P1.21-6,2-48,35-1 (Table). This meningococcal *porA* sequence had multiple mismatches in the target sequences for both the primers and probe used in the two gonococcal *porA* pseudogene PCRs [2,4]. The monoclonal antibody 4BG4-E7 multivalent PorA (which is described and can be obtained at www.nibsc.ac.uk) verified that the meningococcal PorA protein was also expressed.

Discussion

There is one previously published report from Australia on a *N. gonorrhoeae* isolate that lacks the highly conserved gonococcal *porA* pseudogene [6]. We describe here the identification and characteristics of a *N. gonorrhoeae* isolate from Europe lacking the gonococcal *porA* pseudogene. The results from the present study together with the data from the Australian report [6] show that gonococcal strains can harbour a *N. meningitidis porA* sequence instead of the gonococcal *porA* pseudogene that causes false-negative results using *N. gonorrhoeae porA* pseudogene PCRs [2,4], which are commonly used in many laboratories globally. The isolate described in the present study also lacked PIP activity, which might challenge the species verification in culture if commercial biochemical kits such as API NH, RapID NH, Gonocheck II, Bacticard Neisseria and Neisseria Preformed Enzyme Test (PET) are used [12]. These kits are used worldwide and rely entirely or in part on the gonococcal PIP activity. This is of major concern, in particular because global transmission of PIP-negative gonococcal strains has previously been described [12]. The isolate described in the present study was assigned to MLST ST7367 (differing in two of the seven alleles from the previously described strain from Australia [6], i.e. which had *aroE* 170 and *pgm* 65) and to NG-MAST ST2382 (differing from the previously described strain from Australia [6] by 65 bp in a sequence alignment of the *porB* alleles and by 1 bp in the *tbpB* allele). Accordingly, this clone was not identical to the gonococcal clone reported from Australia, which was assigned to MLST ST1901 and NG-MAST ST5377 [6]. Thus it is clear that more than one gonococcal clone has acquired a meningococcal *porA* sequence, most likely through horizontal gene transfer and subsequent recombination.

It is worrying that the sexual contact of the present case could not be traced and this gonococcal strain could therefore be circulating in a larger sexual network. The findings of the present study have prompted us to carry out systematic screening of isolates from the past 10 years, which is currently ongoing.

In conclusion, the identification of a *N. gonorrhoeae* isolate harbouring a *N. meningitidis porA* gene as well as lacking PIP activity highlights the limitations and challenges using NAATs for diagnosis of gonorrhoea as well as in species verification in culture diagnostics for gonorrhoea. The presence of these two genetic changes in the same strain, which allow the strain to escape commonly used diagnostic tests, clearly illustrates how versatile the *N. gonorrhoeae* species is. Enhanced awareness of the spread of such strains is needed, and screening for them can be crucial. The opportunities to use combinations of different diagnostic methods (such as NAAT and culture) and multi-target NAATs in a laboratory remain exceedingly valuable.

Table

Characteristics of a *Neisseria gonorrhoeae* strain harbouring a *N. meningitidis porA* gene that causes false-negative results in gonococcal *porA* pseudogene PCRs, Sweden, 2011

NG-MAST	MLST	PIP activity[a]	Ampicillin MIC (mg/L)	Ciprofloxacin MIC (mg/L)	Spectinomycin MIC (mg/L)	Ceftriaxone MIC (mg/L)	Cefixime MIC (mg/L)	Azithromycin MIC (mg/L)	*porA*[b]	*porA* genosubtype
ST2382	ST7367	Negative	0.064	<0.002	8	0.002	<0.016	8	94% MC58	P1.21-6, 2-48,35-1

MIC: minimum inhibitory concentration (Etest was used); MLST: multilocus sequence typing; NG-MAST: *Neisseria gonorrhoeae* multi-antigen sequence typing; PIP: prolyliminopeptidase.

[a] The *N. gonorrhoeae* strain did not show any prolyliminopeptidase (PIP) activity, which might challenge the species-verification in culture if commercial biochemical kits are used that rely entirely or in part on the gonococcal PIP activity, such as API NH, RapID NH, Gonocheck II, Bacticard Neisseria and Neisseria Preformed Enzyme Test (PET). This is of particular concern because global transmission of PIP-negative gonococcal strains has previously been described [12].

[b] The *porA* gene in the *N. gonorrhoeae* strain showed 94% sequence identity with the *porA* gene in the genome-sequenced *N. meningitidis* reference strain MC58 [11].

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Overrepresentation of influenza A(H1N1)pdm09 virus among severe influenza cases in the 2011/12 season in four European countries

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In France, Ireland, Spain and the United Kingdom, the influenza season 2011/12 started in the final weeks of 2011 and has been dominated by influenza A(H3) viruses with minimal circulation of influenza A(H1N1) pdm09 and B viruses. A relatively greater proportion, however, of influenza A(H1N1)pdm09 viruses were reported in hospitalised laboratory-confirmed influenza cases in four countries. Compared to the season 2010/11, the proportion of subtype A(H3) among hospitalised cases has increased, associated with a larger proportion of cases in the youngest and oldest age groups.

The 2010/11 influenza season in Europe was dominated by influenza A(H1N1)pdm09 viruses, but influenza B viruses also circulated widely, being the dominant type in some countries such as Ireland [1]. In hospitalised cases, the A(H1N1)pdm09 virus was by far the most common virus reported. The 2011/12 influenza season started around week 52 when more than 10% of sentinel samples from the community tested positive for influenza virus [2]. In week 5 of 2012, as influenza activity was increasing throughout Europe, 42% of sentinel specimens tested positive for influenza virus, of which 89% were subtyped as A(H3) [3]. Here we report the distribution of virus strains in hospitalised cases for the 2011/12 season which differed markedly from those seen in primary care.

Influenza surveillance in the European Union

The sentinel surveillance of influenza-like illness (ILI) or acute respiratory infections (ARI) in Europe is carried out by the European Influenza Surveillance Network (EISN) under the coordination of the European Centre for Disease Prevention and Control (ECDC). This surveillance covers the 27 European Union (EU) Member States, Norway and Iceland. The surveillance season lasts from week 40 to week 20 of the following year. On a weekly basis, cases meeting the European definition of ILI or ARI [4] are reported electronically to the European Surveillance System (TESSy) database held at ECDC.

Specimens from a subset of patients in the sentinel population have been collected since 1996 [5]. These specimens (nasal or pharyngeal swabs) are taken by general practitioners from patients with ILI, ARI or both and are sent to influenza-specific reference laboratories for virus detection. The selection of ILI patients to be swabbed is a systematic process that may differ across countries. During the 2009 pandemic, surveillance of hospitalised influenza cases was initiated, relying on the same network, and is still ongoing. Since admission to hospital is a medical decision, it is considered a good proxy for severity. Hence, a severe influenza case was defined as a person admitted to hospital with a laboratory-confirmed influenza infection. The criteria for laboratory confirmation were as described in the European case definition [4].

In the analysis presented here, we included those four EU countries which have been reporting laboratory-confirmed hospitalised influenza cases since the start of the 2011/12 influenza season: Reporting from France and the United Kingdom (UK) included only laboratory-confirmed influenza cases admitted to intensive care units (ICU), while Ireland* reported confirmed influenza cases who were admitted to ICU and confirmed influenza cases who died, and Spain reported all hospitalised laboratory-confirmed influenza cases.

We retrieved sentinel and severe influenza surveillance data for the seasons 2010/11 and 2011/12, restricting our analysis in the second season to the time period from week 40/2011 to week 5/2012 (week 3/2012 for

the UK). Since the UK had not reported severe influenza in season 2010/11 to TESSy, we used data for that season collected by the Health Protection Agency as reported by Bolotin et al. [6]. We compared influenza virus subtype distribution between sentinel specimens and specimens from hospitalised cases as well as the age distribution of hospitalised patients between seasons. Age distributions are presented with their medians and interquartile ranges (IQR) and compared by Mann-Whitney U test. Proportions were compared by chi-square or Fisher's exact tests and a significance level of less than 0.05.

Hospitalised and sentinel influenza cases

From week 40/2011 to week 5/2012, 1,432 sentinel and 199 hospitalised influenza cases were reported by the four countries included in the analysis, France, Ireland, Spain and the UK (Table 1). Of 118 hospitalised cases reported by Spain, 29 (25%) were admitted to ICU. One case of the three reported by Ireland was admitted to ICU. All cases from France and the UK were ICU cases as other cases were not monitored in these countries. In season 2010/11, 6,338 sentinels and 4,059 hospitalised influenza cases were reported by the same countries (Table 2).

Table 1

Distribution of influenza virus subtypes in sentinel specimens and specimens from hospitalised cases, France, Ireland, Spain, and United Kingdom, week 40/2011–week 5/2012 (n=1,631)

Influenza virus subtype	Surveillance level		p value
	Sentinel specimens n (%)	Hospitalised cases n (%)	
A(H1N1)pdm09	14 (1)	20 (10)	<0.01
A(H3)	1,219 (85)	108 (54)	<0.01
A (subtype unknown)	143 (12)	60 (30)	
B	56 (4)	11 (6)	0.28
Total	**1,432 (100)**	**199 (100)**	

Table 2

Distribution of influenza virus subtypes in sentinel specimens and specimens from hospitalised cases, France, Ireland, Spain, and United Kingdom, week 40/2010–week 20/2011 (n=10,397)

Influenza virus subtype	Surveillance level		p value
	Sentinel specimens n (%)	Hospitalised cases n (%)	
A(H1N1)pdm09	3,794 (59.9)	3,076 (75.8)	<0.01
A(H3)	182 (2.9)	24 (0.6)	<0.01
A (subtype unknown)	246 (3.9)	398 (9.8)	
B	2,116 (33.4)	561 (13.8)	<0.01
Total	**6,338 (100)**	**4,059 (100)**	

Virology

Of the 199 laboratory-confirmed hospitalised influenza cases in 2011/12, 20 (10%) were due to influenza A(H1N1)pdm09 virus, of which 19 had been admitted to ICU. Of 1,432 sentinel specimens that tested positive for influenza viruses during the same period, 14 (1.0%) were reported with this subtype (p<0.01) (Table 1). Conversely, 108 (54%) of the 199 hospitalised cases were due to influenza A(H3) virus, compared with 1,219 (85.1%) of the 1,432 sentinel cases (p<0.01). Of the 108 hospitalised influenza A(H3) cases, 33 (30%) had been admitted to ICU. Influenza B viruses were equally distributed between sentinel cases and hospitalised cases.

In season 2010/11, the proportion of influenza A(H1N1) pdm09 viruses was approximately 1.5 fold higher in reported hospitalised cases compared with sentinel cases, and the proportion of A(H3) viruses in reported hospitalised cases was approximately five times lower than in sentinel cases (Table 2). In 2010/11, the proportion of influenza B virus in hospitalised cases was smaller than in sentinel cases, while in season 2011/12, it was the same in sentinel and hospitalised cases.

Age and sex

Over the last two seasons, the median age of reported hospitalised cases in the four reporting countries was similar with 48 years (IQR 31–60) in season 2010/11 and 54 years (IQR 3–74) in season 2011/12. Nevertheless, the distribution across age groups was very different with young adults (15–44 years) and middle-aged adults (45–64 years) most affected during the 2010/11 season, and the youngest (0–4 years) and oldest (≥65 years) age groups most affected during the 2011/12 season.

Additional stratification by subtype seemed to suggest that changes in age distribution were related to the dominant subtype although this observation relied on small numbers. Thus, influenza A(H3), which is dominating the season 2011/12 seems to cause severe disease mainly in the age groups of 0–4 and ≥65 year-olds, while influenza A(H1N1)pdm09, which dominated the previous season caused severe disease mainly in the age groups of 15–44 and 45–64 year-olds (Figures 1 and 2). Influenza B viruses were more evenly distributed among age groups. The male:female ratio among the severe cases was 1.1 in season 2011/12 which was similar to previous seasons.

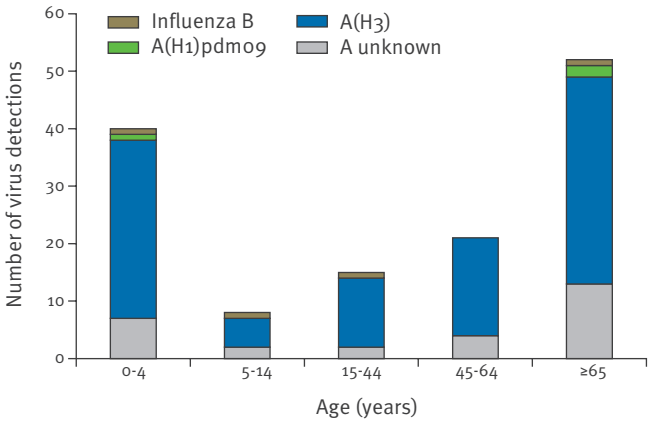
Because a relatively high proportion of influenza A viruses in hospitalised cases were not subtyped in the season 2011/12, we performed a sensitivity analysis in which we assumed that all influenza A viruses of unknown subtype were A(H3) viruses. The higher proportion of influenza A(H1N1)pdm09 in hospitalised cases as compared to sentinel cases remained statistically significant (p<0.01).

Mortality

Of the 97 hospitalised influenza cases with known outcome in season 2011/12, 11 died (11%). Seven of the 11 fatal cases were reported to have influenza A(H3), three with influenza A with unknown subtype and one with influenza B. In season 2010/11, 362 of 1,968 cases with known outcome were fatal (18.4%), which was not significantly higher than in 2011/12 (p=0.08). Of these 362 fatal cases, 286 (79%) were reported with influenza A(H1N1)pdm09, 53 (15%) with influenza A with unknown subtype, 21 (6%) with influenza B and two (1%) with A(H3).

Figure 1

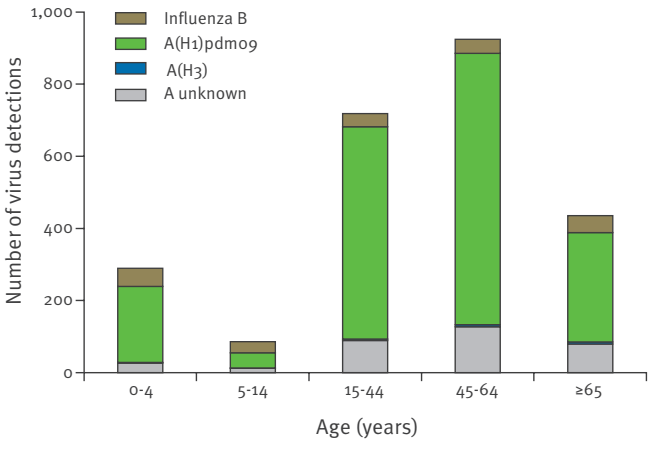
Influenza viruses detected in laboratory-confirmed hospitalised cases, by virus subtype and age group, France, Ireland, Spain and United Kingdom, week 40/2011–week 5/2012 (n=136[a])*



[a] Cases for whom information on age was available.

Figure 2

Influenza viruses detected in laboratory-confirmed hospitalised cases, by virus subtype and age group, France, Ireland, Spain and United Kingdom, week 40/2010–week 20/2011 (n=2,452[a])*



[a] Cases for whom information on age was available.

Discussion

The influenza season 2011/12 is the second season following the influenza A(H1N1)pdm09 pandemic. After the extinction of the former seasonal A(H1) influenza virus, A(H1N1)pdm09 appears to have been replaced by A(H3) viruses in sentinel respiratory specimens. Interestingly, this replacement was not as pronounced in severe cases, in whom A(H1N1)pdm09 influenza virus was found in a higher proportion than in sentinel cases (10% vs. 1%). The reason may be that A(H1N1)pdm09 influenza viruses could be more virulent than A(H3) viruses. The changes in influenza A virus distribution may have had an impact on the age distribution of hospitalised cases. Whilst young and middle-aged adults were more commonly affected during the 2009 influenza pandemic [7], the peaks observed in the youngest and oldest age groups before the 2009 pandemic were seen again in 2011/12.This age shift was also observed in the UK during the 2010-11 season [6]. To our knowledge, there are few publications on this topic because most surveillance systems collecting data on severe influenza cases have been implemented only recently. The main hypothesis explaining this shift assumes changes in demographic patterns and pre-existing immunity to A(H1N1)pdm09 influenza virus in older age groups conferred by prior exposure to viruses circulating in the 1950s [8,9].

This analysis has some limitations. Firstly, we cannot exclude a selection bias with two countries reporting all hospitalised cases and the other two only cases admitted to ICU. The high proportion (19/20) of A(H1N1) pdm09 influenza viruses reported in ICU cases also suggests that our results are biased towards the most severe cases. Secondly, a relatively high proportion of influenza A viruses in hospitalised cases were not subtyped in the season 2011/12 but results of the sensitivity analysis confirmed the observed trend.

Conclusion

The epidemiology of influenza virus types and subtypes may differ between mild and severe cases. Vaccine campaigns targeting populations at risk for severe disease should take this information into account. Our results demonstrate the potential value of collecting data on severe cases to better understand the epidemiology of influenza. Data collection should be harmonised and promoted in more EU countries. It may help to identify more clearly potential biases and to provide decisions makers with more accurate data on severe influenza cases.

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Conflict of interest

R. Snacken did some scientific work and organised meeting for the European Scientific Group on Influenza (ESWI) between 1992 and 2008. ESWI is funded by the pharmaceutical industry.

* Authors' correction

At the request of the authors, the following changes were made on 2 and 7 March 2012: details on reporting of cases from Ireland were corrected. The titles of Figures 1 and 2 and the colours in Figure 2 were corrected.

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Gastroenteritis outbreaks in elderly homes in the east of France during winter 2009/10: aetiology research for a series of 37 outbreaks

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Although acute gastroenteritis is a common cause of morbi-mortality in care homes, there is no national surveillance system in France except for food-borne gastroenteritis. Since 2008, a specific surveillance system has been operating in nursing homes in Alsace, a region in eastern French. In the winter season 2009/10 we had the opportunity to study 37 outbreaks, collecting data on attack rate, duration and aetiology as well as epidemic management in nursing homes. We noticed the responsiveness of the institutions, with a mean period of 1.6 days between the onset of first symptoms and the implementation of management measures (95% confidence interval (CI): 1.0–2.2). One or several stool samples were taken in 27 of the 37 described outbreaks. The only pathogen detected was norovirus, and the positive samples were from outbreaks with a very typical pattern: vomiting in 36 of the outbreaks, a high average attack rate of 36.8% (95% CI: 31.5–42.2) and a short average duration of 8.9 days (95% CI: 5.8–7.0). No severe cases, hospitalisations or deaths were reported. The high frequency of norovirus isolation indicated that systematic bacteriological analysis in local laboratories is not cost-effective. Consequently, Cire Est recommends to test for bacteria and viruses only in cases presenting with fever or atypical symptoms. Nevertheless, Cire Est also recommends to continue sending stool samples to the French National Centre for enteric viruses, more for the benefit of the virological surveillance programme than for diagnostic purposes.

Introduction

Gastroenteritis is one of the most frequent causes of infectious disease outbreaks during winter in nursing homes, on a par with acute respiratory infections. Impaired immune function, chronic diseases and communal living are factors that make the elderly particularly vulnerable to these infections. In addition to the individual consequences of a gastroenteritis episode for elderly people [1], the epidemic burden is heavy, especially for viral infections that can reach high attack rates, leading to significant disorganisation in the management of such institutions. Although the aetiology is not investigated very often, it appears that norovirus, a major cause of gastroenteritis worldwide [2,3], is frequently involved [4,5].

There is no surveillance system for gastroenteritis in France except for food-borne outbreaks. Therefore, the available information about gastroenteritis outbreaks in nursing homes is limited, despite their frequency and the different management strategies they required. However, in the eastern French region of Alsace, a surveillance system of gastroenteritis outbreaks during the winter season has been in operation in nursing homes since 2008. This system aims at supporting nursing homes that are experiencing a gastroenteritis outbreak and at improving their responsiveness in implementing management measures to mitigate morbi-mortality associated with the outbreak. Each gastroenteritis outbreak that occurs in a nursing home has to be notified to health authorities and recorded in a database.

This article describes gastroenteritis outbreaks which occurred in Alsace during the winter season 2009/10. Based on our descriptive analysis, we propose recommendations to adapt aetiology research for nursing homes in future gastroenteritis outbreaks.

Methods

Population studied

The region Alsace is divided in two départements, Bas-Rhin (département 67) and Haut-Rhin (département

68). In our study, we included all residents from the 200 nursing homes of Alsace, as well as those members of staff who were in direct contact with residents; we excluded administrative and logistic staff from our data collection.

Gastroenteritis definition

Gastroenteritis was defined as the sudden onset of at least two episodes of vomiting or two episodes of diarrhoea during a 24-hour period. Diarrhoea was defined as at least two thick liquid or watery stools above what is normal for the resident or employee within a 24-hour period [6].

Gastroenteritis outbreak definition

With the first national recommendations report about gastroenteritis outbreaks in nursing homes still under review [6], a quantitative definition does not yet exist in France. The French Institute for Public Health Surveillance (InVS) recommended defining an outbreak of gastroenteritis as an event with more patients (residents or staff) than usual in predetermined places and time periods present gastroenteritis, and there appears to be an epidemiological link relating the patients (same meals and same activities).

In French nursing homes, the medical coordinator or the nursing officer is authorised to report the outbreaks.

Data collection

Data on food-borne outbreaks were not collected, because these are notified in a specific French surveillance system. According to that database, no food-borne outbreak was declared during the winter 2009/10 in nursing homes in Alsace.

The gastroenteritis outbreak surveillance protocol was designed by the Eastern Regional Office of the InVS (Cire Est). Preliminary actions to increase the nursing homes' awareness of the new surveillance programme have been conducted since 2008 in association with the departmental health authorities. Managers of the establishments were invited to local information meetings presenting the subject and to the Regional Day of Hygiene, a well-known local seminar. They received by post or email all tools for the surveillance system (a template for an epidemic curve, notification forms, instructions on how to notify, etc) and advice on how to manage the outbreaks using these tools.

Once the protocol was introduced to the nursing homes, each facility developed an internal procedure to detect and declare gastroenteritis outbreaks and to ensure prompt and appropriate management measures and investigation of the aetiology of the outbreaks.

Cire Est provided a standard form for collecting the following data:

- Place where the outbreak occurred,
- Date of onset of symptoms,
- Date of reporting to public health authorities,
- Diagnosis and clinical signs (predominant symptoms in the outbreak),
- Total number of residents and number of ill residents,
- Number of staff in direct contact with residents and number of sick staff,
- Number of severe cases (deaths or hospitalisations),
- Date of first stool sample taken per outbreak,
- Results of aetiology analysis (laboratory identification of the causal agent),
- Date of implementation of management measures,
- Alerted partners (Regional Agency for Public Health, operational hygiene teams in the establishments where this exists, committees for nosocomial infections, e.g. Agence Régionale de Lutte contre les Infections Nosocomiales)
- Closing date of the folder (recovery date of the last resident).

All data were collected and entered in a single Excel file by a qualified public health officer from the Regional Agency for Public Health of Alsace (representing the local public health authorities), and sent to the Cire Est to be analysed.

Study period

Outbreaks of gastroenteritis were recorded in our database from the beginning of November 2009 (week 45 2009) to the end of May 2010 (week 21 2010). This study period corresponded to the time when gastroenteritis outbreaks usually occur in France [7]. Periods were calculated starting from the date of symptom onset of the first case. The end of the outbreak was the date of recovery of the last case.

Aetiology

Once an outbreak occurred it was recommended that the nursing home should send stool samples (three to five per outbreak if possible) to the local private or public diagnostic laboratory. A bacterial analysis was conducted along with a first level viral analysis (adenovirus and rotavirus). If results were negative, samples had to be sent to the French National Reference Centre for enteric viruses for complementary viral analysis (especially norovirus).

Statistical analysis

A descriptive analysis was performed using EpiData Analysis.

Results

During the study period, 37 outbreaks of gastroenteritis in nursing homes were reported to the Regional Agency for Public Health of Alsace and included in our study. Twenty-two occurred in Bas-Rhin (département 67) and 15 in Haut-Rhin (département 68).

Spatiotemporal distribution of the outbreaks

Figure 1 shows the distribution of the outbreaks by département during the study period.

From week 45 to week 52 of 2009 no outbreaks were reported to the Alsatian health authorities. From the last week of 2009 to the week 8 of 2010, outbreaks were reported at a variable rate of between zero and two outbreaks per week in either département.

In Bas-Rhin, three outbreaks were reported during week 9 2010 and five outbreaks during week 10 2010, which constituted a significant increase. From week 11 2010 to the end of the study period, the number of outbreaks decreased and returned to the level of incidence observed before the epidemic period which began in week 52 2009.

In Haut-Rhin, no significant increase in the number of outbreaks was observed. From week 1 2010 to week 14 2010, a stable level of incidence was observed with no more than two outbreaks a week.

Attack rates

Attack rates were calculated for the residents of all 37 outbreaks. Staff in contact with residents was affected in 30 of the 36 outbreaks for which information was available.

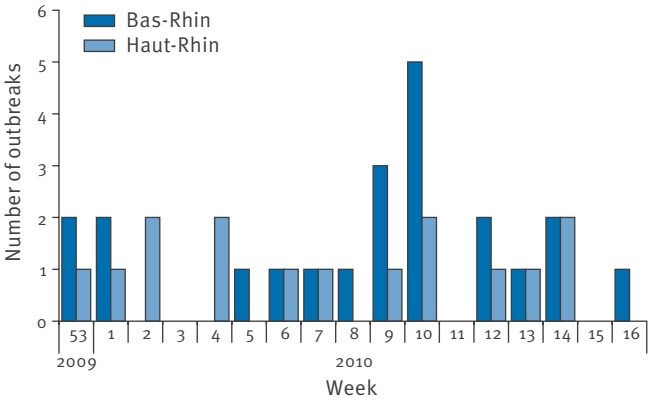
The observed average attack rate among residents was 36.8% (95% confidence interval (CI): 31.5–42.2). It ranged between 4% and 70% for the 37 outbreaks. We also calculated average attack rates for staff in those 30 outbreaks which affected members of staff who were in contact with residents. The observed average staff attack rate was 20.9% (95% CI: 14.5–27.7). It ranged between 3% and 73% for the 30 outbreaks taken into account.

Symptoms

Four types of symptoms were reported in association with the outbreaks. Diarrhoea was described in all 37 registered outbreaks, and vomiting was reported in 36 of them. Nausea and fever were minor symptoms and reported in six and two outbreaks, respectively). No severe cases were reported (hospitalisations or death).

Figure

Distribution of gastroenteritis outbreaks in nursing homes, by week, November 2009–May 2010, Alsace, France (n=37)



Aetiology

No samples were available for 10 outbreaks. In the other 27 outbreaks, between one and five stool samples per outbreak were collected by the nursing homes regardless of the attack rate.

Stool samples from three outbreaks were directly analysed and found positive by the reporting nursing home, using a norovirus rapid diagnosis test; the genogroup was not specified. All collected samples of the 24 other outbreaks were sent to local laboratories for bacterial analysis and first level viral analysis (adenovirus and rotavirus). All of them were negative. Our recommendation was to send all negative samples to the French National Reference Centre for complementary viral analysis. Samples from 17 outbreaks were sent: one sample was negative, three were positive for norovirus genogroup I (GI), and 13 were positive for norovirus genogroup II (GII). The results are summarised in Table 1.

Genotyping was performed for the 16 outbreaks for which samples were sent to the French National Reference Centre for Enteric Viruses. The three GI norovirus isolates were of genotype 4. Among the 13 GII norovirus isolates, nine were genotype 4 variant 2010, three were genotype IIb/ II.1, and one was genotype 4 variant 2006b.

Outbreak management

Table 2 summarises the reactivity of the nursing homes (notification period, period before the implementation of management measures), and the duration of the outbreaks.

We noticed that the management measures were implemented rapidly, with an average of 1.6 days after the start of the outbreak (95% confidence interval (CI):

Table 1

Microbiological analyses, gastroenteritis outbreaks in nursing homes, November 2009–May 2010, Alsace, France (n=37)

	Number of outbreaks
No stool sample taken	10
Negative bacteriology and negative first level viral research[a]	7
Negative virology (French National Reference Center for Enteric Viruses)[b]	1
Positive for norovirus, no genotyping (rapid diagnosis test at nursing home)	3
Positive for norovirus G II (French National Reference Center for Enteric Viruses)[b]	13
Positive for norovirus G I (French National Reference Center for Enteric Viruses)[b]	3

[a] The stool samples were not sent to the French National Reference Centre for enteric viruses.

[b] The samples were negative in bacterial and the first level viral diagnostics and therefore sent to the French National Reference Centre for Enteric Viruses for complementary viral analysis.

1.0–2.2), and that half of the outbreaks were managed within one day or less. The longest period before implementation of measures was seven days. The outbreaks were reported to the health authorities after an average of 7.9 days (95% CI: 4.9–10.6) and lasted an average of 8.9 days (95% CI: 5.8–7.0).

Those nursing homes that observed the recommendations more closely and had shorter delays of outbreak notification and implementation of measures were more likely (Student's t-test, $p<0.05$) to collect stool samples. All these parameters were independent of the attack rates (chi-square test with Yates correction $p<0.05$).

Discussion

From November 2009 to May 2010, 37 gastroenteritis outbreaks were reported to the health authorities in Alsace. Only in Bas-Rhin did the number of outbreaks increase significantly during the first half of March. No bacterial agent was found in the 27 outbreaks for which aetiology research was conducted. Only noroviruses were isolated. The attack rates in residents and staff were high with an average of 36.8% (95% CI: 31.5–42.2) in residents and of 20.9% (95% CI: 14.5–27.7) in staff. At least one member of staff was affected in the majority of the reported outbreaks. The most frequent outbreak-associated symptoms were diarrhoea and vomiting.

Late start of gastroenteritis season

Usually, gastroenteritis winter outbreaks in elderly communities are first noticed in November, at the same time as transmission in the general population begins [7]. In our study, the first outbreak was reported only at the end of December 2009. The same late start was observed among the general population in France by the general practitioner network of the National Institute for Health and Medical Research (Institut National de la Santé et de la Recherche Médicale, INSERM) sentinels.

Seeing as the winter 2009/10 was characterised by the 2009 influenza A(H1N1) pandemic, which began to decrease during the second half of December [8], we propose two hypotheses that could explain the late start of the norovirus season: (i) Strict hygiene measures taken to control the spread of influenza A(H1N1) pdm09 could at the same time have limited gastroenteritis transmission inside nursing homes; (ii) Medical staff focusing on controlling the influenza A(H1N1) pdm09 burden may have been less attentive to gastroenteritis outbreaks notification.

Table 2

Outbreak management parameters, gastroenteritis outbreaks in nursing homes, November 2009–May 2010, Alsace, France (n=37)

	Median	Mean	95% CI	Min	Max
Notification period (days)	5	7.92	[4.93–10.6]	0	37
Period for implementing management measures (days)	1	1.63	[0.99–2.22]	0	7
Outbreak duration (days)	7	8.94	[5.84–6.97]	2	26

CI: confidence interval.

Epidemiological and clinical pattern and outbreak management

The epidemiological and clinical pattern with high attack rates in residents and staff of 37% and 21%, respectively, and a high frequency of vomiting was indicative of norovirus infection [9]. This was confirmed by the microbiological tests, which identified norovirus in 19 of the 27 outbreaks for which stool samples were collected. No other bacterial or viral causal agent was found. There was no statistically significant difference in attack rates and clinical features between outbreaks for which aetiology research was or was not done (Student's t-test, $p<0.05$). We conclude that most of the 37 outbreaks could have been be due to norovirus.

The nursing homes participating in our surveillance system adapted quickly to the task of reporting and managing gastroenteritis outbreaks. The median period for implementing management measures was short (one day). Consequently, the median duration of the outbreaks was short (seven days), in spite of the high attack rates. Norovirus epidemics are usually difficult to control and last longer than other viral or bacterial epidemics. This is due to the contagiousness of these viruses, their persistence in the environment [10], and the fact that they can be excreted after the symptomatic period [4,9]. Furthermore, aetiology research was conducted for more than two thirds of the outbreaks, a sign of the readiness of the reporting structures to follow the protocol and to manage the situation correctly.

Aetiology research

In 16 of the 17 outbreaks for which stool samples were sent to the French National Reference Centre for enteric viruses, norovirus was found. Norovirus is the most common cause of epidemic non-bacterial gastroenteritis worldwide [11-13] and an important cause of gastroenteritis in care facilities for the elderly [5,9]. It was the cause of 82.6% of all viral gastroenteritis outbreaks covered in a review of enteric outbreaks in long-term care facilities from January 1997 to June 2007 [4]. The 2010 variant of norovirus GII.4 was the most frequent causal agent found in our study. Variants of this genotype (GII.4) have emerged in the past decade as the predominant strain worldwide and cause regular outbreaks [14-16].

The clinical symptomatology (high frequency of vomiting) and epidemiology (high attack rates from the very first days) of norovirus outbreaks allow clinical diagnosis without aetiology research. We can question the cost-effectiveness of a systematic stool analysis for diagnostic purposes and especially the necessity of the bacteriological analysis in local laboratories.

Analysis of this series of gastroenteritis outbreaks made Cire Est change its recommendations on aetiology research. In agreement with the InVS, Cire Est recommend bacterial and first level viral analyses of stool samples in local laboratories exclusively in outbreaks where patients present fever or an atypical clinical picture. When the clinical picture suggests norovirus, stool samples from chosen outbreaks should be sent directly to the French National Reference Centre for enteric viruses as a contribution to virological surveillance. These recommendations refer to the entire year, not only the winter, given that the establishments perform surveillance all year round.

Limitations of the study

We identified limitations inherent in the data collection. Firstly, symptoms were not collected at the individual level but at the outbreak level. In our database, we were not able to determine the frequency of each symptom among residents or staff, nor their duration. Secondly, staff affected by digestive disorders may not have systematically notified them to employers, possibly because they did not realise they were a potential source of contamination, or because they were worried that sick leave would not be paid [17]. If that was the case, we may have underestimated attack rates among staff. Finally, we cannot exclude a selection bias in our outbreak series. The largest outbreaks or those with more serious symptoms may have been more likely to be notified.

Conclusion

We can conclude that the nursing homes aware of the surveillance protocol collaborated well with health authorities and respected instructions. Indeed, the outbreaks were rapidly controlled and we collected a sizeable and valuable set of data, including results of the aetiological research for a large proportion of them.

Given the frequency of norovirus implicated as the causative agent in our outbreak series and the obvious clinical pattern, we recommend, as an evolution of the surveillance system, to give up systematic stool collection, and to reserve it for occasions involving fever or atypical clinical pattern. Samples from a small selection of outbreaks would however be sent directly to the National Reference Centre for enteric viruses in order to supply the virological surveillance which aims to describe the national circulation of enteric viruses. .

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A norovirus outbreak triggered by copper intoxication on a coach trip from the Netherlands to Germany, April 2010

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We report an unusual outbreak of norovirus infection on a coach trip. Overall, 30 of 40 people (including drivers and crew) developed nausea, vomiting and/or diarrhoea, 11 of them on the first day of the trip. The incidence epidemic curve showed a first peak on Day 1 and a second on Day 4. Nine passengers were hospitalised with gastrointestinal symptoms. Norovirus was found in stool samples from two patients, but the infection could not explain the first peak in the epidemic curve only a few hours after departure. Interviews with the passengers and an inspection of the coach and its water supply implicated the water used for coffee and tea as the potential source. Microbiological investigations of the water were negative, but chemical analysis showed a toxic concentration of copper. Blood copper levels as well as renal and liver function were determined in 28 of the 32 passengers who had been exposed to the water. One passenger who did not have gastrointestinal symptoms had an elevated copper level of 25.9 µmol/L, without loss of liver or renal function. It is likely that the spread of norovirus was enhanced because of vomiting of one of the passengers due to copper intoxication.

Introduction

Noroviruses are RNA viruses that belong to the family of *Caliciviridae*. The viruses are highly infectious and cause gastroenteritis in humans. Transmission can easily occur through consumption of contaminated food or water, through contact with people shedding the virus or through contaminated aerosols resulting from vomiting [1]. Attack rates are high, and outbreaks are seen among large groups of people, as in nursing homes for the elderly, day nurseries, hospitals and restaurants. Noroviruses are also a well known cause of acute gastroenteritis on cruise ships [2] and among coach passengers [3].

In April 2010, the Dutch Public Health Service (PHS) of Nijmegen was informed by the German PHS of Hochsauerlandkreis that an outbreak of vomiting and diarrhoea was occurring among elderly people on a five-day coach trip from the Netherlands to Sauerland, Germany. The symptoms started a few hours after departure. Stool samples of two patients who were admitted to hospital were found positive for norovirus. However, the incubation period of a norovirus infection is 10 to 72 hours [4], while the symptoms in these passengers started only a few hours after departure. Therefore, norovirus alone did not explain this outbreak, and further investigation was warranted.

Outbreak description

On Day 1, the coach with 34 passengers, four crew members and one driver, departed at 10.15 am. The majority of the passengers were female and 70 to 95 years-old, with an average age of 80 years. Except for married couples (twelve passengers), none of the passengers had had contact with other passengers before the trip.

At 12.15 am, coffee and tea was made using a hot water boiler in the coach. Someone noticed that the water had a blue colour. The first pot of hot water was shared by six persons who did not get sick on Day 1. The second pot was shared by 26 persons, one of whom started vomiting 10 minutes later. By 1.00 pm, 10 people were sick and had vomited; one person became sick later that day. Two hours later the coach arrived at the hotel, where people started having diarrhoea. Of the 11 passengers who had symptoms on the first day, only six had diarrhoea. The other five reported nausea and/or vomiting. From Day 2 to Day 7, 19 more people got sick, including a second driver who replaced the first driver to drive the coach back from Hochsauerland. 16 of them also had diarrhoea.

All nine passengers admitted to a hospital were treated for norovirus infection and/or dehydration, mostly without laboratory confirmation of norovirus. One of them

was admitted to the intensive care unit for a few days, because of (pre-existing) renal failure and hypokalaemia, probably induced by the diarrhoea.

On Day 4, all 24 passengers who were recovered or healthy returned to the Netherlands with the same coach and a new driver. This driver reported symptoms one day later. The remaining 14 sick passengers were repatriated on Day 6.

Methods

We conducted a cohort study among a defined and closed population, consisting of all bus passengers, crew members and the drivers. A probable case was defined as a passenger of the coach trip to Germany, including crew and driver, who developed one or more of the following symptoms within eight days after the start of the trip: nausea, stomach pain, vomiting and/or diarrhoea. A confirmed case was defined as a probable case with laboratory confirmation of norovirus. We interviewed the passengers and performed microbiological analyses of stool samples of three passengers, chemical blood analyses of passengers exposed to boiler water, and chemical environmental analysis on boiler water of the coach.

Interviews with cases

On Day 4, the PHS of Hochsauerlandkreis was notified about the outbreak by the local hospital. The environmental health officers of the German PHS interviewed the members of the crew and three patients at the hotel where they were staying. To further investigate the outbreak, public health nurses of the PHS of Nijmegen interviewed all passengers and the crew by telephone questionnaire [5], from Day 5 until Day 13 of the outbreak. They were asked about the consumption of food and drinks during the trip, how much coffee they had had, where they were seated in the coach, which symptoms they developed and when these started, whether they had any underlying illnesses, and if they were admitted to a hospital during the trip.

Inspection of the coach

The PHS of Hochsauerlandkreis inspected the coach and interviewed the driver after they were informed about the blue colour of the water during the initial interviews. A further thorough inspection of the coach's water supply was carried out after it had returned to Nijmegen by a medical officer of the PHS of Nijmegen.

Chemical analysis and microbiological testing of boiler water

A sample of boiler water from the coach was subjected to chemical and microbiological tests. The pH was determined electrochemically. Chemical tests for organic and inorganic compounds were performed by the Environmental Accident Service (MOD) of the National institute for public health and the environment (RIVM) using gas chromatography-mass spectrophotometry and X-ray fluorescent (XRF) analysis. The sample was cultured on blood agar for *Staphylococcus aureus* and *Bacillus* spp., since these bacteria are known to produce toxins that can cause abdominal symptoms a short time after exposure [6,7].

Virological testing of stool samples

The German hospital tested eight patients (Day 4) and the Dutch PHS tested three patients (Day 6) for norovirus by PCR [8,9]. The Dutch PHS also tested for adenovirus and rotavirus by antigen detection (enzyme immunoassay RIDA QUICK Rotavirus/Adenovirus combi, R-Biopharm) and PCR.

Results

Interviews of the cases

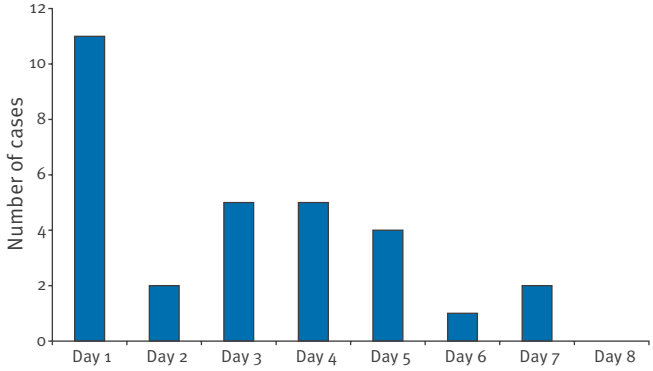
The PHS of Nijmegen completed questionnaires by telephone, with a response rate of 29 of the 30 cases. Only one passenger with symptoms could not be reached, and their questions were answered by one of the other participants of the trip. These data were used to produce an epidemic curve of the dates of symptom onset (Figure). It showed a biphasic pattern with a peak on Day 1 (11 passengers) and a second peak on Day 4 (five passengers). Two patients had two episodes of illness (in the figure only the first onset is shown).

In total, 30 of 40 people became ill during this trip, including one of two drivers and three of four crew members. Those who became ill on Day 1 reported less diarrhoea compared to those who became ill on Day 2 or later. Nine passengers were admitted to a hospital, eight in Germany, and one in the Netherlands on Day 5 of the outbreak.

Norovirus was found in stool samples of two of the eight patients hospitalised in Germany. This diagnosis was later confirmed by the Dutch PHS of Nijmegen, who found norovirus in all of three other tested non-hospitalised passengers (who had not been tested in Germany). All other patients were classified as clinical-epidemiological cases, without further microbiological testing. 25 of 32 people exposed to the water from the boiler in the coach developed symptoms, while four

Figure

Gastroenteritis cases by day of onset of symptoms, coach trip Netherlands–Germany, April 2010 (n=30)



of seven unexposed people became ill, leading to an odds ratio of (25*3)/(7*4)=2,68.

The Table summarises the symptoms, hospital admittance and exposure to boiler water in relation to the day of onset of symptoms of the cases. All people that became ill on Day 1 had taken coffee or tea. Not all the people that became ill later had taken coffee or tea on the first day. Four of the 30 cases experienced nausea only (three of those on Day 1 of the trip), four people had abdominal discomfort and/or vomiting without diarrhoea (two of those on Day 1), and 22 people developed diarrhoea (six of them on Day 1). The symptoms lasted on average three days in the 24 persons for whom the date of recovery was known.

Inspection of the coach

The first inspection of the water system of the coach by the PHS in Germany revealed nothing unusual. During the second inspection by PHS of Nijmegen a closed hot water boiler was identified as potential source of infection. The driver had put aside a sample of the suspect boiler water that had a faint blue colour. When investigated by the medical officer of the PHS of Nijmegen, the pH was estimated to be around 4.0 by pH-indicator strip (Merck Acilit). The coach company mentioned that the boiler had recently been treated for calcification with acetic acid, and rinsed afterwards. This could explain the acidic pH, but not the blue colour.

Electrochemical pH measurement of the water from the boiler revealed a pH of 4.4. Microbiological investigations did not detect *Bacillus* or *Staphylococcus* spp. Standard chemical tests for organic compounds were negative. XRF analysis showed a high copper level of 87 mg/L.

Chemical analysis of blood from exposed passengers

Immediately after the high copper level was detected on Day 11 of the outbreak, the PHS of Nijmegen informed the coach company and the passengers by telephone.

Table

Symptoms, hospital admittance and exposure of gastroenteritis cases, by day of onset of symptoms, coach trip Netherlands–Germany, April 2010 (n=30)

	Symptoms on Day 1	Symptoms on Day 2 or later	Total
Symptoms	11	19	**30**
Nausea or abdominal discomfort only	3	1	**4**
Vomiting only	2	2	**4**
Diarrhoea only	0	6	**6**
Vomiting and diarrhoea	6	10	**16**
Admitted to hospital	4	5	**9**
Patients exposed to boiler water/patients with symptoms	11/11	14/19	**25/30**

All 32 passengers who had taken coffee or tea in the coach received a letter in which they were asked to see their General Practitioner (GP) for precautionary blood tests. The GPs were informed and instructed to test liver function by alanine aminotransferase (ALAT), renal function by creatinine and the copper level in the blood [10,11]. Twenty-eight of the exposed passengers were tested.

In one person, tested 19 days after the incident, the blood copper level was found to be slightly elevated (25.9 µmol/L over reference 13.0–24.0 µmol/L). Liver and renal functions were normal. This person had consumed one cup of coffee on the coach, but did not report any gastrointestinal symptoms during the trip. In four other patients, liver and/or renal functions were abnormal. One patient was known to have a pre-existing renal condition, the other three only had mildly decreased liver or renal function. As previous values of those patients were not known, interpretation was difficult.

Virological testing of stool samples

In Germany, stool samples taken from two hospitalised cases on Day 3 and 4 were found to be PCR-positive for norovirus. On Day 6 of the outbreak, the Dutch PHS tested stool samples from three other patients with diarrhoea. All three samples showed positive PCR results for Norovirus, and negative results for rotavirus and adenovirus.

Control measures

The PHS of Hochsauerlandkreis was notified on Day 4 of the outbreak based on the laboratory diagnosis and immediately implemented standard outbreak control measures for norovirus infections in the hotel where the passengers stayed [12,13]. These measures included advice on personal hygiene for cases and contacts as well as advice on cleaning and disinfection of the rooms. Among the hotel staff and other guests, none developed symptoms of norovirus infection. The coach driver was advised not to serve drinks prepared from the water supply of the coach during the return journey and to have the system checked after returning home.

The coach company cleaned the bus. To prevent re-occurrence of these problems in their coaches, the PHS advised to adjust the cleaning procedures, replace materials and implement a code of conduct in relation to water refreshment. One month after the outbreak, the PHS of Nijmegen organised an information meeting for all passengers and their relatives. Professionals of the environmental health unit and of the infectious diseases department of the PHS Nijmegen provided information and answered questions.

Discussion and conclusion

The initial interviews of the cases, performed by the PHS of Hochsauerlandkreis indicated viral gastroenteritis as the likely explanation for the outbreak, which

was confirmed by the finding of norovirus in two stool samples. Norovirus is a well known cause of gastroenteritis outbreaks during coach trips [3]. A common factor like a common meal before departure was expected [14], but no such source was identified in the interviews. Furthermore, the early onset of symptoms only a few hours after departure, the biphasic pattern of the epidemic curve, and the fact that two people had two episodes of illness made it unlikely that norovirus was the sole explanation for this outbreak [4]. An additional intoxication was suspected.

The National Poisons Information Centre (NVIC) of the RIVM was contacted. Because nausea, vomiting and diarrhoea are very unspecific symptoms and can have many causes, particular toxins could not be identified as possible causes. Because the outbreak seemed food-borne, the Food and Consumer Product Safety Authority (VWA) was contacted. According to the VWA, coffee was unlikely to be the toxic source. They were unable to suggest other possible causes of intoxication. Fortunately, the boiler tank in the coach still contained water, so a sample could be taken for analysis.

The low pH value of the water and the blue colour were reason to insist on further investigations. Surprisingly, the analyses revealed a high copper level in the boiler water (87 mg/L). This explained the blue colour but not the acidity of the water. A possible explanation was that the acetic acid, used for decalcification of the boiler, resulted in dissolution of copper from the inside of the boilers' tap system. The acid had probably remained in the boiler for a longer period. The coach company claimed that the boiler was rinsed after the treatment. Apparently, this rinsing was either not performed or not done properly.

Although copper is an essential nutrient involved in haemoglobin formation, excess copper intake can result in adverse health effects [15-17]. Water can be an important source of excess copper intake [16,17]. A commonly reported acute adverse health effect after a single oral intake of excess copper is gastrointestinal distress with nausea, vomiting, abdominal pain and diarrhoea as the main symptoms [11,18]. These effects occur shortly after exposure and are usually not persistent. Acute gastrointestinal disturbances following a single oral dose of copper in drinking water are reported for copper levels of 30 mg/L and higher [19].

The copper content in the water supply was high enough to cause single-dose toxicity with the gastrointestinal tract as the primary target organ. The Agency for Toxic Substances and Disease Registry (ATSDR) in Atlanta, United States, has derived a minimal risk level for acute-duration oral exposure (1–14 days) for copper of 0.01 mg/kg/day [11]. An adult of 70 kg bodyweight drinking one cup of coffee (0.25 L) with a copper content of 87 mg/L by will ingest a single dose of 0.30 mg/kg, 30 times the acute minimum risk level.

The copper intoxication explains the early and acute onset of illness on the first day of the trip, while norovirus infection explains the cases on the later days. We assume that the index patient of the norovirus outbreak vomited as a result of copper intoxication, and thereby accelerated the spread of norovirus. Norovirus is well-known for its transmission by aerosol after vomiting. As far as we know, a norovirus outbreak triggered by vomiting as a result of intoxication has not been described in the literature.

Oral intake of a very high single dose or a high repeated dose of copper is associated with liver and kidney damage, anaemia, immunotoxicity and developmental toxicity. Damage to organs is dependent on genetics, age and copper intake. All exposed passengers were tested for serum copper levels and renal and liver function because of the risk of decreased renal and liver function after copper intoxication at old age [10,11]. However, the tests were done at least ten days after the incident, when elevated copper levels could have normalised by then. Nevertheless, a slightly elevated copper level was found in one passenger. Since treatment by chelating therapy is only indicated in acute and severe cases (i.e. complications as haemolytic anaemia, anuria or hepatotoxicity), the patient was only monitored [20]. This person did not report any symptoms at the time of the incident, the liver and renal functions were repeatedly normal and the copper levels returned to normal. Of the other exposed passengers, four people had abnormal liver or renal function; one of them was known to have pre-existing renal dysfunction. The interpretation of liver and renal functions was quite complicated, because they tend to decrease at old age, and baseline values of most of the patients were not available.

This is as far as we know the first report of a combined outbreak of norovirus and intoxication, in our case copper intoxication. This outbreak has demanded a high level of cooperation between the Environmental Health Unit and the Infectious Disease Department of the Dutch PHS, which was a very informative experience. None of the passengers have become critically ill as a result of copper intoxication.

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