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A note from the editors: molecular epidemiology of human pathogens – current use and future prospects

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While it is clear that surveillance and outbreak investigations are increasingly supported by advanced molecular approaches, it is less clear how the future of such tools will evolve. Even if they will most probably not render traditional epidemiological methods superfluous, they will certainly gain importance and a number of questions concerning their use in public health remain to be answered. How will both approaches interact in the future? Will they work hand in hand? How will the current operational constraints be overcome, to allow these advanced techniques to be used in public health practice?

In response to a call for papers [1], a special issue is being published. In the first part, we focus on examples of the value and opportunities of molecular methods in analysing a number of diseases. In the second part, we draw attention to issues related to their wider use in surveillance, prevention and control of infectious diseases. In so doing, we hope to stimulate discussion and add to the debate on the role and potential of modern molecular microbiology to inform public health action.

References

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Within-patient emergence of the influenza A(H1N1) pdm09 HA1 222G variant and clear association with severe disease, Norway

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The association between a particular mutation in the HA1 subunit of the influenza virus haemagglutinin, D222G, and severe and fatal disease in cases of influenza A(H1N1)pdm09 in Norway during the 2009 pandemic was investigated using pyrosequencing. The prevalence of the variant among fatal cases was 8/26 and among severe non-fatal cases 5/52. No D222G mutations were found among the 381 mild cases. This difference could not be attributed to sampling differences, such as body location of sampling, or duration of illness. In cases with mutant virus where clinical specimens from different days of illness were available, transition from wild-type to mutant virus was commonly observed (4/5), indicating that the mutant virus emerged sporadically in individual patients. In patients with paired samples from both the upper and lower respiratory tract (n=8), the same viral genotypes were detected in both locations. In most of the D222G cases (11/13), the mutant virus was found as a quasispecies.

Introduction

Infection with the pandemic influenza A(H1N1) virus that emerged in 2009 led to mild disease in the vast majority of cases; however, there was also an unusual occurrence of viral pneumonia, severe disease and death in younger age groups than commonly observed for seasonal influenza [1]. In a large proportion of severe cases, conditions predisposing for severe disease have been identified [2] and host factors therefore appear to strongly influence the clinical outcome of infection. On the other hand, this novel virus of zoonotic origin differed from the previous seasonal A(H1N1) virus in the resulting disease profile; thus, viral determinants of pathogenicity must also be involved – e.g. it has been shown to be more pneumotropic than seasonal A(H1N1) virus in a ferret model [3]. It is important to understand better what viral and host-related factors determine the observed dichotomous pathogenicity profile.

The first cases of influenza A(H1N1)pdm09 virus infection in Norway were recorded in early May 2009, shortly after emergence in Mexico, but cases were few and scattered across the country until mid-summer. A limited influenza epidemic took place in late July/early August, followed by a comparatively calm period leading up to a major epidemic that surpassed all previous peaks recorded in the current surveillance system. The epidemic reached its highest point in early November and by the end of 2009, it had mostly subsided.

As part of the intensified surveillance carried out during the 2009 influenza pandemic, the national reference laboratory for human influenza at the Norwegian Institute of Public Health received a large number of respiratory specimens from confirmed and possible cases of influenza A(H1N1)pdm09. In late November 2009, we noticed that a particular amino acid substitution – aspartic acid (D) to glycine (G) in the viral haemagglutinin (HA) glycoprotein subunit HA1 at position 222 (D222G) – appeared in fatal cases, while we did not find it in the numerous mild cases analysed. Realising that a similar pattern seemed to be taking place in the Ukraine (R. Daniels, personal communication, November 2009) and given that the mutation had been shown to influence viral receptor specificity in another influenza A(H1N1) virus [4], it was decided to notify international public health authorities and other national authorities about this possible pathogenicity determinant, to expedite assessment of it [5]. A preliminary review in January 2010 of D222G amino acid substitution in the HA of influenza A(H1N1)pdm09 viruses from the World Health Organization (WHO) stated that mutations, including those leading to the D222G substitution in the HA, had appeared sporadically since the first emergence of influenza A(H1N1)pdm09 viruses, and that the substitutions in HA had been reported in viruses obtained from cases of mild to severe to fatal illnesses but that such viruses had neither formed distinct phylogenetic clustering nor been associated with consistent changes in virus antigenicity [6].

Since the first account of our findings in Norway [7], investigations into the occurrence of this mutation resulted in an increasing number of reports [8-28]. While the prevalence of this mutation varied between

the reporting countries, in most studies, the 222G mutation is primarily found in severe and fatal cases. One of the first larger studies came from a group in Hong Kong, who analysed this amino acid position in severe and non-severe cases of influenza A(H1N1) pdm09 [13]. Nine (4.1%) of 219 severe or fatal cases of pandemic influenza had the mutation, in contrast to none of 239 non-severe cases.

Data from these reports indicated that the D222G mutation was absent or uncommon in viruses that were in sustained circulation. However, one case of transmission of a 222G virus was reported [9], but the transmitted virus in this case had acquired an additional mutation that may have influenced receptor binding characteristics.

To further investigate the sporadic occurrence of the 222G mutant influenza virus, we performed a more in-depth analysis of an expanded data set. Our original data set [7] included 266 cases, while the expanded set comprised 462 cases. The present study included assessment of the majority of the fatal cases in Norway, as well as of a larger number of samples from severe non-fatal and mild cases collected throughout the pandemic.

We studied the prevalence of HA1 222 mutations within different clinical outcome groups, in serially collected specimens, in upper versus lower respiratory tract and in early versus late specimens. We also analysed age and sex distribution and examined the characteristics of the fatal cases.

We further compared the mutant viruses phylogenetically, looked for the presence of mutant quasispecies and oseltamivir resistance.

Methods

Data and clinical materials

As part of the intensified surveillance instigated in response to the emergence of influenza A(H1N1)pdm09 in April 2009, virus-containing specimens from all parts of the country were received in the Norwegian Institute of Public Health, which serves as the National Influenza Centre in Norway. A total of 15 medical microbiology laboratories submitted specimens containing influenza virus (original specimens, nucleic acid preparations from original specimens or virus isolates) to the National Influenza Centre for further characterisation. Most of these patient specimens originated from primary care clinics; the remainder were from hospitals.

Each laboratory sent a maximum of five specimens each week plus any specimens from patients with severe disease, suspicion of antiviral resistance or other special circumstances such as suspected vaccine failure. In addition, intensive care and fatal cases remained notifiable to the Norwegian Institute of Public Health throughout the pandemic and we actively solicited materials from microbiology and pathology laboratories that were in possession of specimens from these cases. Together, these collection schemes enabled us to obtain specimens from nearly all the recorded fatal influenza A(H1N1)pdm09 cases in Norway as well as a large number of severe and mild cases. Patient information relevant for this study was obtained primarily from the patient referral forms that came with the specimens, supplemented with information gathered in the notification of severe and fatal cases and, in a few cases, from direct contact with clinicians who cared for the patient. Cases were assigned to clinical outcome groups by a medical specialist, according to WHO guidance criteria [2]. Briefly, the criteria for complicated/severe influenza included: clinical and/or radiological signs of lower respiratory tract disease, central nervous system involvement, severe dehydration, secondary complications (renal failure, multiorgan failure, septic shock, rhabdomyolysis, myocarditis), exacerbation of underlying chronic disease or signs and symptoms of progressive influenza disease. The criteria were modified as follows: hospital admission in itself did not lead to classification as severe influenza, without additional evidence of complicated disease. This modification was necessary due to a low threshold for hospitalisation of patients during parts of the pandemic. Furthermore, sustained virus replication in itself was not regarded as evidence of severe influenza. From the available specimens, we picked all the specimens from fatal and ICU cases as well as a large subset of specimens from the other cases, making sure that viruses from all parts of Norway and from the entire first period of A(H1N1)pdm09 virus circulation in the country (May 2009–January 2010) were well represented. The selected specimens were sequenced with regard to the codon encoding amino acid 222 of the HA1 subunit. Only cases where the HA1 222 genotype could be ascertained in the original specimen were included in the study.

Detection of HA1 222 mutations at nucleic acid level

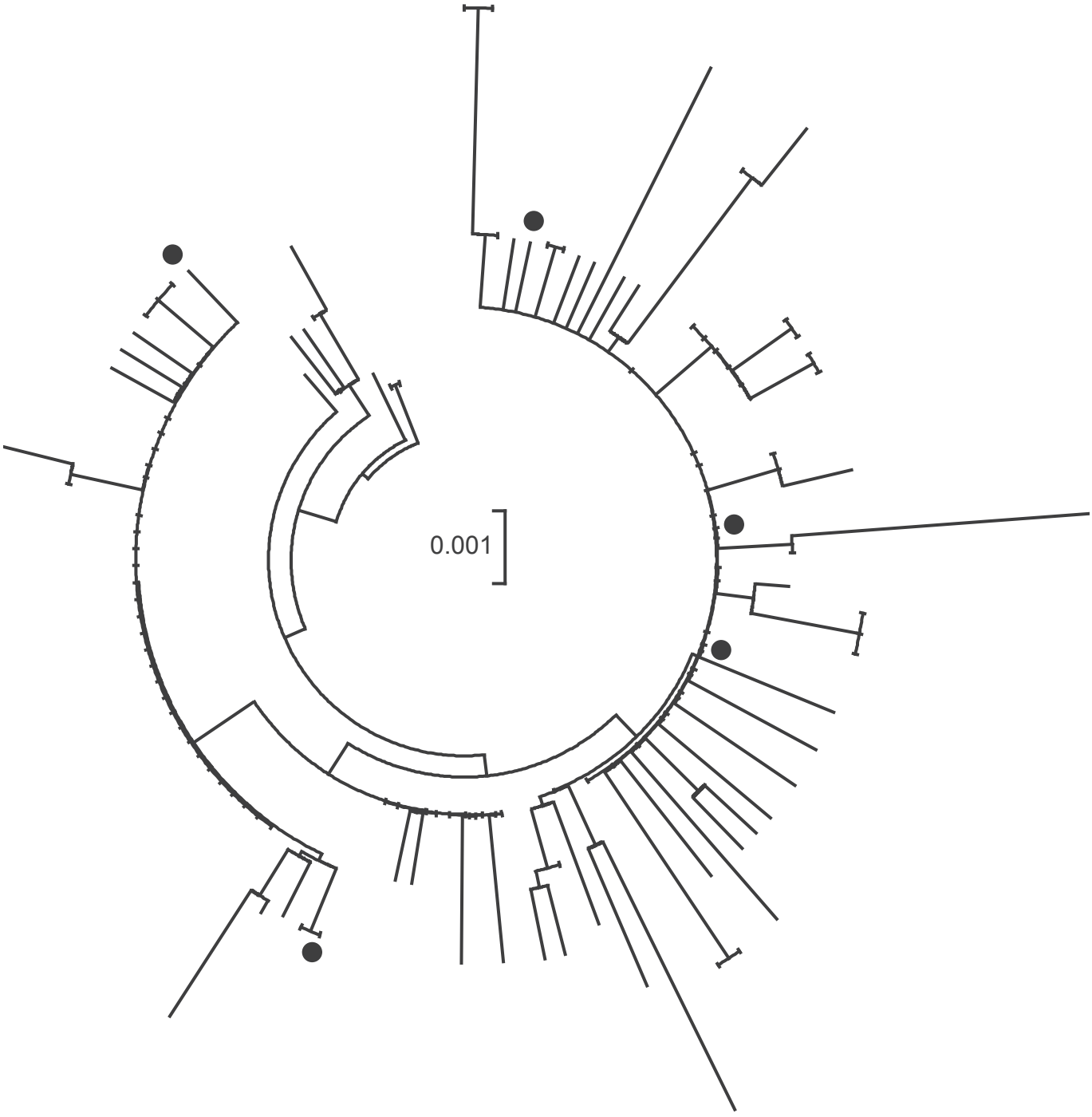
Initially, mutations were detected through conventional (Sanger) cycle sequencing of reverse transcription-polymerase chain reaction (RT-PCR) amplicons obtained from viral RNA from virus isolates and primary specimens.

Viral RNA was extracted using a total nucleic acid extraction kit in the MagNA Pure LC System (Roche Diagnostics, Mannheim, Germany). In general, a modification of the full genome sequencing protocol provided by the United States Centers for Disease Control and Prevention [28] was used for virus isolates, whereas sequencing of the amplicon from a more sensitive RT-PCR [30] was used for many of the primary specimens.

As soon as we became aware of the possible importance of the mutation, a pyrosequencing assay was designed and used. Briefly, a 110-nucleotide amplicon

Figure 1

Phylogenetic reconstruction of influenza A(H1N1)pdm09 haemagglutinin gene (HA1), Norway 2009/10, showing the distribution of 222G viruses across the phylogeny



Viruses harbouring 222G are marked with solid circles. The entire coding region of the HA1 subunit was analysed. The codon for amino acid 222 was excluded for this analysis, in order to investigate how 222G mutants were otherwise related.

Table 1

Risk factors for severe illness in fatal cases of influenza A(H1N1)pdm09 with available haemagglutinin gene (HA1) 222 genotype, Norway, 2009/10 (n=26)

Risk factors for severe disease	Number of cases
Presence of risk factors	
None	4
One or more	21
Data unavailable	1
Individual risk factors[a]	
Chronic respiratory disease	4
Chronic heart disease	7
Chronic renal or hepatic disease	0
Diabetes	2
Pregnancy	1
Obesity[b]	3
Immunosuppression	3
Chronic neurological disease or injury	3
Other	7

[a] Each patient may have several risk factors.

[b] Obesity: for one of these patients, a body mass index (BMI) of >40 was reported. The other two were reported as 'obese' or 'adipose', with no BMI given.

encompassing the region of the HA gene which encodes amino acid 222 was generated from 5 µl specimen RNA combined with each of primers pyro-H1 forward: 5'-AGTTCAAGCCGGAAATAGCA-3' and pyro-H1 reverse: 5'-biotin-TTTCCAGTTGCTTCGAATGTT-3' and reagents from the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity kit (Invitrogen) in a 25 µl reaction and subjected to the following cycling conditions: 30 min. at 50 °C, 2 min. at 94 °C; 45 cycles of 15 sec. denaturation at 94 °C, 30 sec. annealing at 55 °C and 1 min. extension at 68 °C; finally, 5 min. at 68 °C for final extension.

The pyrosequencing reactions were performed as previously described [29] using a residue-specific sequencing primer (5'-AGCAATAAGACCCAAAGTGAGG-3').

The sequenced region begins at nucleotide 747 in the full-length sequence of the viral RNA segment 4, where nucleotides 747–749 encode HA1 amino acid 222. The most common wild-type codon in this position is GAT, which encodes aspartic acid (D). The GAA codon encodes glutamic acid (E), while GGT (or GGA) gives glycine (G). Mutation of G to A in the first position of the GAT codon gives asparagine (N).

The viruses were also analysed for oseltamivir susceptibility by detecting the H274Y mutation by sequence analysis, through either a pyrosequencing assay targeting the relevant single-point mutation [31] or through full- or partial-length cycle sequencing of the coding region for the viral neuraminidase.

Phylogenetic analysis

Phylogenetic analysis was performed on aligned sequences comprising the entire coding region of the HA1 subunit. Kimura 2-parameter corrected pairwise distances between manually aligned sequences were computed using the PHYLIP [32] program DNADIST, and the phylogenetic tree inferred using NEIGHBOR. The resulting tree was visualised using MEGA version 5.0 [33].

Statistical analysis

Statistical analysis was undertaken using PASW 17, version 17.02 (SPSS Inc., Chicago, United States). Descriptive statistics were calculated as medians with upper and lower range or as means with 95% confidence intervals. All hypothesis tests were two-tailed and statistical significance was assessed at the 0.05 level. Comparisons of categorical variables were performed using Fisher's exact test and comparisons of non-normal continuous distributions using the Mann–Whitney U test.

Results

In total, 462 influenza A(H1N1)pdm09 cases where the HA1 222 genotype could be ascertained in the primary specimen were included in the study. A large proportion of the isolated viruses were sent to the WHO Collaborative Centre for Reference and Research on influenza in the National Institute for Medical Research, Mill Hill, United Kingdom, for further characterisation. No changes in virus antigenicity were found (data not shown). We did not find the H274Y mutation, commonly associated with oseltamivir resistance, in any of the viruses in this study. In phylogenetic analysis of the HA1 coding region of Norwegian viruses (Figure 1), the five 222G harbouring viruses for which we could ascertain the full HA1 coding sequence occurred on different branches of the tree, showing that they did not form a distinct genetic cluster. This is in good agreement with the fact that the cases with 222G viruses occurred sporadically across Norway, at different times during the period of widespread virus circulation.

Of the 462 cases included in the analysis, 381 had mild disease, 52 had severe disease but were non-fatal and 26 were fatal (clinical outcome was unknown for three cases).

The median age of the cases with mild disease included was 20 years (range: 0–87), for the non-fatal cases with severe disease 27 years (range: 0–66 years) and for the fatal cases 38 years (range: 0–71). Among all 462 cases, 225 (49%) were men and 237 (51%) were women. The proportion of men versus women was similar for mild cases (48% vs 52%), severe non-fatal cases (52% vs 48%) and fatal cases (46% vs 54%). Of the 13 cases with 222G virus, eight were men and five were women: these proportions (62% vs 38%) were not

Table 2

Prevalence of haemagglutinin gene (HA1) 222 genotypes by clinical outcome (mild, severe non-fatal, fatal disease), influenza A(H1N1)pdm09 cases, Norway, 2009/10 (n=462)

HA1 222 genotype	Clinical outcome[a]					Total n (%)
	Mild n (%)	Severe non-fatal n (%)	Fatal n (%)	Severe including fatal n (%)	Unknown n (%)	
222D wild type	329 (86.4)	40 (76.9)	15 (57.7)	55 (70.5)	2 (66.7)	386 (83.5)
222G	0 (0)	5 (9.6)	8 (30.8)	13 (16.7)	0 (0)	13 (2.8)
222E	51 (13.4)	5 (9.6)	2 (7.7)	7 (9.0)	1 (33.3)	59 (12.8)
222N	1 (0.3)	2 (3.8)	1 (3.8)[b]	3 (3.8)[b]	0 (0)	4 (0.9)[b]
Total	**381 (100)**	**52 (100)**	**26 (100)**	**78 (100)**	**3 (100)**	**462 (100)**

[a] Severe non-fatal and fatal cases are shown separately and jointly.

[b] Additionally, one fatal 222G case harboured 222N virus as a quasispecies.

significantly different from those cases with non-222G viruses (48% vs 52%, n=449) (p=0.41, Fisher's exact test).

In the total data set, 13 cases harboured 222G viruses, 222E occurred in 59 cases and 222N in four cases (one additional case contained both 222G and 222N quasispecies, and was classified as the former). In 386 cases we detected only wild-type 222D viruses (Table 2). The presence of mutants found in the pyrosequencing assay was verified by conventional sequencing in all 10 cases where sequencing of primary samples was possible.

In order to avoid bias stemming from preferential testing of severe cases in our samples, the prevalence of different genetic variants was recorded within each clinical outcome group, instead of the other way round. Among the 26 fatal cases, eight harboured the 222G virus at some time during the course of infection. The corresponding proportion for severe non-fatal cases was 5/52 (10 %) while none was found in 381 analysed cases with mild disease. The proportions in fatal as well as in severe non-fatal cases were higher than in mild cases (p<0.0005). Furthermore, the higher frequency in fatal cases compared with severe non-fatal cases was also statistically significant (p=0.026, Fisher's exact test).

One specimen from a fatal case contained both 222G and 222N viruses in addition to wild-type 222D. Two of the 222G mutants had adenine in the third codon position and thus are likely to have arisen from 222E viruses. The prevalence of the 222E variant showed no significant difference between the various clinical outcome groups, and this variant represents a circulating clade with no apparent effect on virulence [9.10].

In the majority of 222G cases (11/13), the 222G mutants occurred as quasispecies, typically with wild type 222D sequence being more frequent (exemplified in Figure 2). The proportion of mutant virus in mixed populations as estimated by pyrosequencing tended to be less than 50% (range: 18–59). In all 10 cases analysed by both conventional sequencing and pyrosequencing, these mixtures were also evident as double peaks in conventional sequencing (see example in Figure 2).

In eight cases, paired specimens from upper and lower respiratory tract were available for analysis. Six of these cases had a fatal outcome. These specimen pairs were collected on the same day from the same patients. Upper/lower respiratory tract specimens from the same case but collected on different days were not considered as pairs in this analysis. Both 222D and 222G viruses were found in the samples, but in all eight pairs, the HA1 222 genotype in the upper and lower respiratory tract samples matched (Table 3).

Table 3

Prevalence of haemagglutinin gene (HA1) 222 genotypes in paired[a] upper and lower respiratory tract samples, influenza A(H1N1)pdm09 cases, Norway, 2009/10 (n=8)

HA1 222 genotype		Number of patients
Upper respiratory tract sample[b]	Lower respiratory tract sample[c]	
222D	222D	5
222G	222G	2
222D/G mix	222D/G mix	1

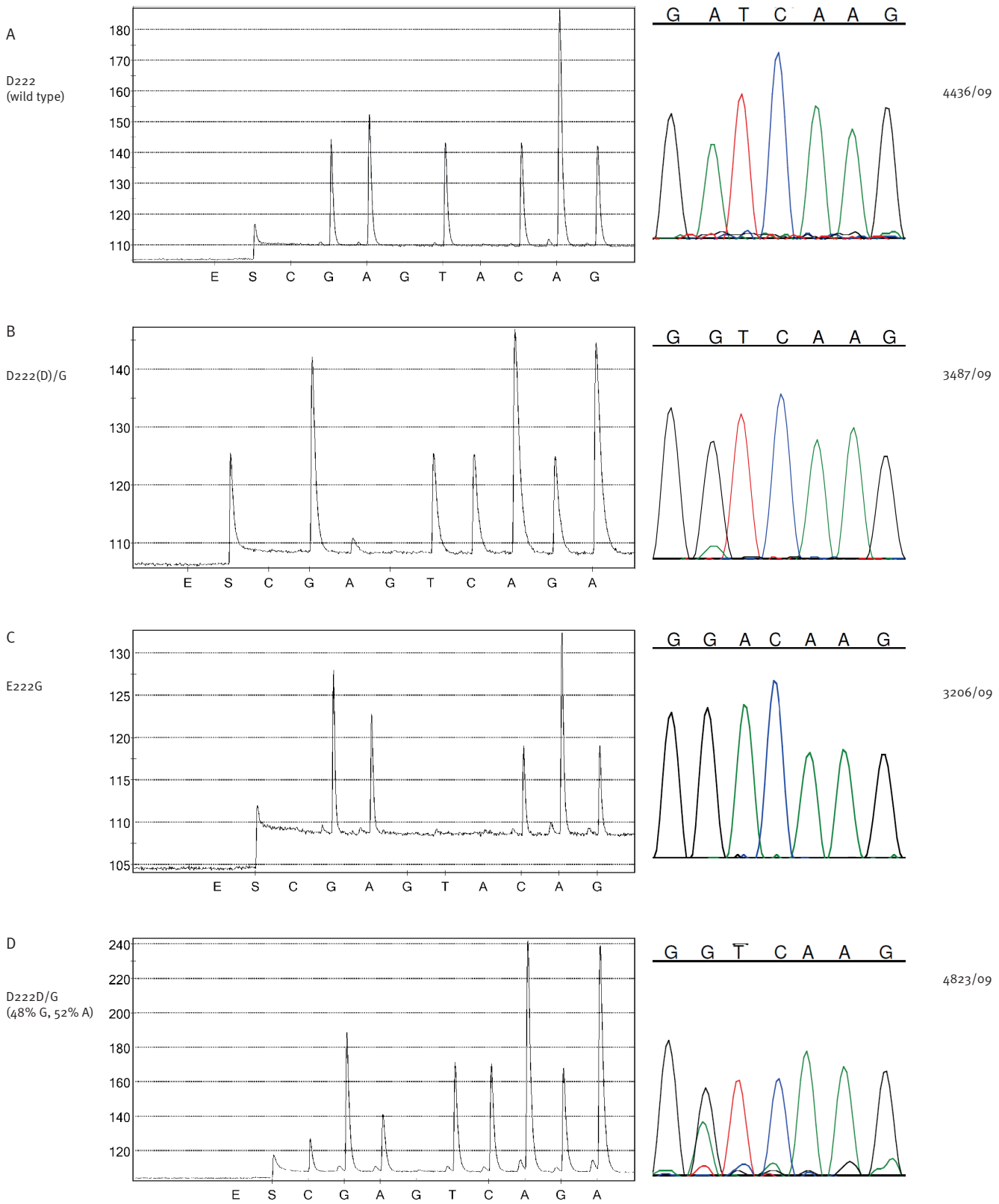
[a] The samples were collected from the same patients on the same day.

[b] Upper respiratory tract samples included nasopharyngeal swabs/aspirates, nasal swabs and throat swabs.

[c] Lower respiratory tract samples included tracheal aspirates and lung autopsy material.

Figure 2

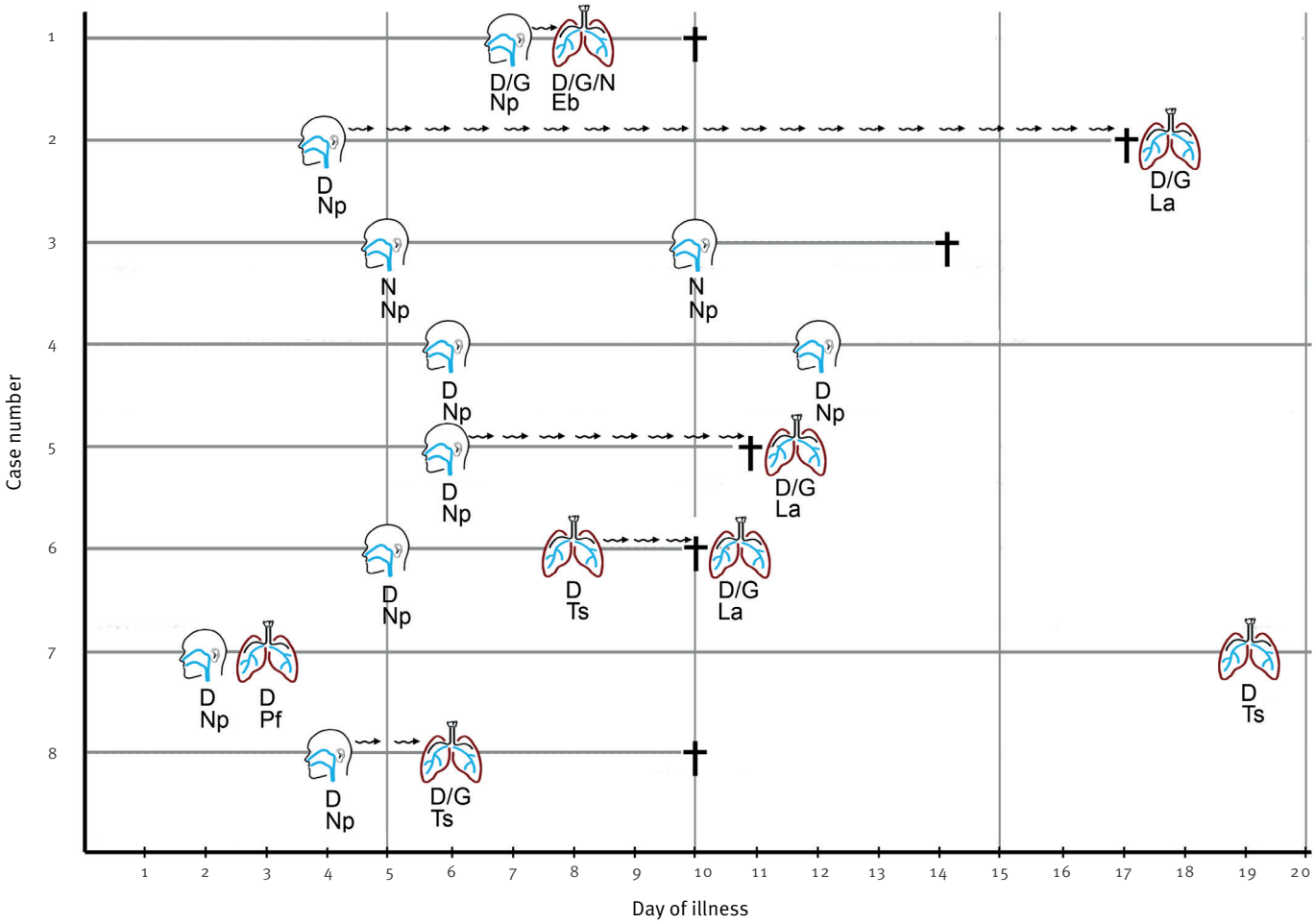
Detection of D222G mutations in the haemagglutinin gene (HA1) using pyro- and Sanger sequencing, influenza A(H1N1) pdm09 cases, Norway 2009/10



Sequence chromatograms at position 222 of influenza A(H1N1)pdm09 virus HA1 gene region derived from four primary clinical samples collected in Norway 2009/10 (4436/09, 3487/09, 3206/09, 4823/09). Chromatograms from pyrosequencing are shown on the left-hand side, those from Sanger sequencing are on the right), illustrating wild-type sequence (panel A), and mutations (panels B–D). In panel D, polymorphism at the 222 codon, with 48% G and 52% A, is illustrated.

Figure 3

Detection of mutations in the haemagglutinin gene (HA1) through the course of illness in influenza A(H1N1)pdm09 cases with serial samples, Norway, 2009/10



Eb	Endobronchial brush
La	Lung autopsy tissue
Np	Nasopharyngeal sample
Pf	Pleural fluid
Ts	Tracheal sample

Upper-airway specimen

Lower-airway specimen

Fatality

Amino acid substitution – D: aspartic acid; G: glycine; N: asparagine.
Genotype changes are shown by arrows.

Of the 13 patients with 222G mutant viruses, we had two or more serial samples available for five (Figure 3). In four of these patients (Cases 2, 5, 6 and 8 in Figure 3), we found only 222D wild type viruses in samples preceding detection of 222G mutant virus. In one patient (Case 1 in Figure 3), both available samples contained 222G mutant virus, but the samples were taken only one day apart, thus reducing the chance of finding differing genotypes. We also had serial samples for three additional non-222G patients (Cases 3, 4 and 7 in Figure 3) who exhibited the same genotype throughout the course of illness (either 222D or 222N).

The date of symptom onset was given for 263 cases: 212 mild, 33 severe non-fatal and 18 fatal. Specimens from the mild cases tended to be collected earlier in the course of illness (mean: 4.3 days after symptom onset; 95% CI: 3.7–4.9) than specimens collected from all severe cases, including fatal cases (mean: 6.5 days; 95% CI: 5.2–7.8) (p=0.001, Mann–Whitney U test). The proportion of 222G mutant viruses was higher in specimens collected late (≥8 days after symptom onset) than in those collected early (<8 days after symptom onset) (p=0.001, Fisher's exact test). Among specimens collected on day 8 or later, more than half were from mild cases (20/39) and there was still a significantly higher proportion of mutant virus in the severe cases, including those that were fatal (6/19 vs 0/20) (p=0.008, Fisher's exact test) (Table 4).

As noted earlier, the median age of the cases included was higher among all the severe cases, including those that were fatal, than the mild cases (p<0.0005, Mann–Whitney U test). The proportion of 222G mutant viruses was higher in specimens collected from patients aged 40 years or older than in younger patients (8.2% (7/85) vs 1.6% (6/371), p=0.004, Fisher's exact test). However, among specimens collected from patients aged 40 years or above, there was still a significantly higher proportion of mutant viruses in the severe cases, including those that were fatal (p<0.0005, Fisher's exact test, data not shown). Thus, the observed difference in 222G prevalence between the different clinical outcome groups is not due to age as a confounding factor.

Discussion

In the present study, we provide further epidemiological evidence of the association between the D222G mutation in HA1 of influenza A(H1N1)pdm09 virus and severe or fatal clinical course. Furthermore, we present evidence that the mutated viruses emerge in individual patients after the onset of illness and demonstrate the presence of mutant virus in both the upper and lower respiratory tract. We also address some potential biases that could conceivably confound the analysis.

The Norwegian cases of infection with HA1 222G genotype viruses have occurred sporadically and do not cluster epidemiologically or in phylogenetic analysis.

As observed by others, the D222G substitution has been observed to occur sporadically in the laboratory when specimens containing predominantly wild-type virus are subjected to virus isolation in eggs or mammalian cell lines [6,13,35].We therefore based our analysis on viral sequences obtained from the primary clinical specimens.

The 222G viruses appear to be rare among circulating strains, but are still quite frequent in patients with severe disease, who are not epidemiologically linked. A likely explanation is that the presence of mutant viruses in these particular individuals experiencing severe disease is due to selective upgrowth of mutant genomes during infection. In all four 222G cases where we could analyse both early and late specimens, we observed a transition from wild-type to mutant virus, which lends support to the hypothesis that the presence of the mutant is due to sporadic emergence rather than widespread circulation.

In our analysis, we could not distinguish between upgrowth from a rare quasispecies, which may be present at inapparent levels in many more cases, and

Table 4

Prevalence of haemagglutinin gene (HA1) 222 genotypes in viruses from influenza A(H1N1)pdm09 cases sampled late in illness (day 8 or later), by clinical outcome, Norway, 2009/10 (n=39)

HA1 222 genotype	Specimens from cases taken on day 8 or later[a]				
	Mild n (%)	Severe n (%)	Fatal n (%)	Severe including fatal n (%)	Total n (%)
222D	17 (85)	8 (73)	3 (38)	11 (58)	28 (72)
222G	0 (0)	3 (27)	3 (38)	6 (32)	6 (15)
222E	3 (15)	0 (0)	1 (13)	1 (5)	4 (10)
222N	0 (0)	0 (0)	1 (13)	1 (5)	1 (3)
Total	**20 (100)**	**11 (100)**	**8 (100)**	**19 (100)**	**39 (100)**

[a] Severe non-fatal and fatal cases are shown separately and jointly.

upgrowth from de novo mutation events. The 222G genotype appears to occur as sporadic mutations with no or little onward transmission, rather than through persistence as a circulating variant. If this is the case, the likelihood of finding mutant viruses is expected to increase during the course of infection. Conceivably, since the severe cases, including those that were fatal, tended to be sampled later in their course of illness than mild cases, the higher occurrence of mutations could alternatively be explained purely as a sampling artefact and not as a marker of virulence. But if the occurrence of the mutation was merely a function of time since infection, there should be no difference in the frequency of 222G mutant in specimens from mild versus severe cases, if taken equally late in infection. Our results show that the significant difference remained even when all early specimens were excluded: thus the difference in sampling day can be ruled out as an alternative explanation for the pattern observed (Table 4).

In several of the cases, simultaneous specimens were available from different body locations. In all of these pairs/sets, the same genotypes were observed, but with differences in the ratio of mutant versus wild type in patients harbouring quasispecies. Other studies have identified D222G quasispecies mainly in endotracheal aspirate or broncheoalveolar lavage (BAL) samples and less frequently in nasopharyngeal aspirate samples [12,14,20]. This discrepancy could be due to the use of different analytical methods. Lower rates of mutant versus wild type in nasal swabs may not have been revealed by conventional sequencing. This was demonstrated by Baldanti et al. [14] in D222G/N cases with available paired nasal swabs and BAL samples, the authors found D222G/N in only one of four cases using direct sequencing, but this proportion increased to three of four cases using clonal analysis. From our study, we cannot exclude the possibility that the occurrence of the mutation as a quasispecies, together with the wild-type, is due to a complementary function of the wild-type. This phenomenon might change if the mutant virus were to develop another fitness-compensatory mutation that permits the mutant to replicate and spread in pure form. This possibility needs to be further studied.

In addition to its possible biological importance the fact that the majority of the cases with 222G mutants carried a mixture of 222D- and 222G-encoding viral genomes, usually with predominance of the wild-type 222D, makes it likely that many influenza A(H1N1) pdm09 cases worldwide carrying D222G mutant viruses could have been missed in analyses that are not sensitive to minor nucleic acid species or where only the majority nucleotide is recorded in the published sequence.

Selleri et al. analysed influenza A(H1N1)pdm09 viral quasispecies and the polymorphism at codon 222 by ultra-deep pyrosequencing [28]. Codon 222 polymorphism was detected in 40.7% of patients by ultra-deep pyrosequencing and in only 7.1% by conventional sequencing. They found that the frequency of polymorphism was significantly higher in samples collected from patients with severe manifestations than in those patients with moderate-mild manifestations. The D222G/N/A mutations were detected as either minor or predominant variants only in severe cases, whereas D222E was equally represented in severe and moderate-mild infections.

The question whether the presence of mutant viruses in lower airways is underestimated due to lack of sampling in the lower respiratory tract was investigated by Baldanti et al. [14] paired nasal swabs and BAL samples from patients admitted to intensive care units for mechanical ventilation or extracorporeal membrane oxygenation were compared with samples from patients with pneumonia not requiring mechanical ventilation and from community patients. By combining data from nasal swabs and BAL samples, the frequency of D222G/N mutants in patients with severe infections increased to 43%, as compared with 7.8% and 0% in patients with moderate and mild infections, respectively [14]. Baldanti et al. also showed that the viral RNA levels were significantly higher in BAL samples than those in nasal swabs [14]. Piralla et al. described the same finding, suggesting higher virus replication in the lower respiratory tract [20].

Watanabe et al. have characterised two of the Norwegian virus isolates included in our study, namely A/Norway/3568/2009 (Norway3568) and A/Norway/3487-2/2009 (Norway3487) [34]. The viruses were isolates from a fatal case (Norway3487) and a mild case (Norway3568): the viruses differ by only 10 amino acids and none of the amino acid changes known to affect virulence were found in PB2, PB1-F2, HA, or NS1, except for an amino acid change to 222G in HA1, in Norway3487. More efficient viral replication in cultured cells and delayed virus clearance from ferret respiratory organs was observed for Norway3487 virus (isolated from a severe case), as compared with Norway3568 (isolated from a mild case). To some extent, Norway3487 virus caused more severe lung damage in non-human primates than did Norway3568 virus. Moreover, the authors found that PB2 derived from Norway3487 contributed to higher polymerase activity, possibly leading to more efficient viral replication in vitro and in vivo, which in turn also could play a role in the increased lung damage.

In our analysis, all cases with detectable 222G mutant viruses had severe illness and the prevalence of 222G increased with the degree of severe outcome. If the conditions for virus growth in lower airways are favourable to 222G mutants and the likelihood of upgrowth of mutant virus increases with duration of infection, it follows that individuals who fail to limit infection to upper airways and fail to eliminate the infection rapidly stand at greater risk of acquiring mutant viruses. Regardless

of virulence, such a mechanism may contribute to the observed pattern of mutants being found primarily in severe cases. However, if the mutant viruses are also more virulent, an increased probability of emergence in patients who already have a long-lasting infection involving the lower respiratory tract probably constitutes a vicious circle phenomenon, which underscores the importance of treatment that helps persons with elevated risk to clear the infection rapidly.

The concept of D222G being a determinant of virulence is supported by a growing body of evidence from in vitro and animal studies including non-human primates [36-45]. Increased tropism of D222G mutant virus for type II pneumocytes [44] may result in more efficient infection, reducing the availability of progenitor cells for essential lung functions and regeneration and thus leading to severe pulmonary impairment. Increased viral titres in the lungs also trigger stronger inflammatory responses, augmenting tissue damage and delaying healing in the infected lungs [44].

While one instance of transmission of 222G mutant virus has been documented [9], the transmitted virus in this case had acquired an additional mutation, (G155E) that may have counteracted the putative 222G-induced receptor-binding changes [46] and there are no indications that effective sustained transmission has taken place. In our data set, one of the patients with mutant viruses was the likely source of infection of a healthcare worker. However, this occurred early in the course of illness: virus from an early specimen, as well as the virus collected from the healthcare worker, was wild type.

In light of the fact that 222G mutant viruses are and remain substantially less transmissible than corresponding wild-type viruses, their immediate public health impact is limited to the individual cases in whom such mutants occur and the fact that they may possibly contribute to the severity of illness. Similar to the apparent situation with avian influenza A(H5N1) virus infection in humans, tropism of 222G mutant viruses for lung cells may contribute to both increased virulence and impaired transmissibility, which may thus be linked traits [47]. On the other hand, when the H275Y neuraminidase mutation conferring oseltamivir resistance in seasonal influenza A(H1N1) viruses was first identified, this mutation also correlated with impaired viral fitness [48], a situation that was abruptly changed with the global emergence of high-fitness resistant viruses during the 2007/08 influenza season [49]. Similarly, further adaption of the newly emerged A(H1N1)pdm09 virus to humans as host species may conceivably lead to compensatory mutations that render the 222G mutants more transmissible, or lead to other changes that influence pathogenicity. The evolution of these viruses therefore needs to be closely monitored in a framework that ensures that virological and clinical data are taken into account.

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Molecular epidemiological typing within the European Gonococcal Antimicrobial Resistance Surveillance Programme reveals predominance of a multidrug-resistant clone

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Treatment of gonorrhoea is threatened by antimicrobial resistance, and decreased susceptibility and resistance to recommended therapies is emerging in Europe. Current associations between resistance and molecular type remain poorly understood. Gonococcal isolates (n=1,066) collected for the 2009 and 2010 European Gonococcal Antimicrobial Surveillance Programme were typed by *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST). A total of 406 sequence types (STs) were identified, 125 of which occurred in ≥two isolates. Seven major genogroups of closely related STs (varying by ≤1% at just one of the two target loci) were defined. Genogroup 1407 (G1407), observed in 20/21 countries and predominant in 13/21 countries, accounted for 23% of all isolates and was associated with decreased susceptibility to cefixime and resistance to ciprofloxacin and raised minimum inhibitory concentrations for ceftriaxone and azithromycin. Genogroup 225 (G225), associated with ciprofloxacin resistance, was observed in 10% of isolates from 19/21 countries. None of the other genogroups were associated with antimicrobial resistance. The predominance of a multidrug-resistant clone (G1407) in Europe is worrying given the recent reports of recommended third generation cephalosporins failing to treat infections with this clone. Identifying associations between ST and antimicrobial resistance aids the understanding of the dissemination of resistant clones within a population and could facilitate development of targeted intervention strategies.

Introduction

Gonorrhoea continues to present a public health problem within the European Union/European Economic Area (EU/EEA). In 2010, a total of 32,028 cases of gonorrhoea were reported by 28 European countries, giving a rate of 10.4 per 100,000 population [1]. Given the serious implications of gonococcal infection, which include pelvic inflammatory disease, infertility, ectopic pregnancy, early abortion and a fivefold increase in risk of human immunodeficiency virus (HIV) transmission [2], the need for effective treatment and control of gonorrhoea is evident. A significant challenge to this is that during the antibiotic era, the aetiological agent, *Neisseria gonorrhoeae*, has consistently developed resistance to each antibiotic introduced for widespread empirical treatment [3].

Empirical treatment of gonorrhoea is based on national or regional guidelines, many of which currently recommend third generation cephalosporins as first line therapy. Up until late in 2012, guidelines for Europe recommend 250 mg of ceftriaxone as a single intramuscular dose or 400 mg of cefixime as a single oral dose [4]. These were recently revised to recommend 500mg ceftriaxone combined with 2g azithromycin [5] in response to growing evidence that cephalosporin therapy is becoming compromised by the emergence of isolates exhibiting decreased susceptibility and resistance to the third generation cephalosporins, which have now caused treatment failure in several EU/EEA countries [6-11]. In 2010, the European gonococcal antimicrobial susceptibility programme (Euro-GASP) demonstrated that 9% of isolates from 21 European countries showed decreased susceptibility to cefixime as well as an increasing trend in minimum inhibitory concentrations (MICs) for ceftriaxone, and a high prevalence of resistance to ciprofloxacin (53%) and azithromycin (7%) [12]. In the absence of any alternative antimicrobial treatments, there is clearly an urgent need to develop strategies to limit dissemination of resistant strains to maintain effective treatment.

Molecular epidemiological surveillance has the capacity to provide novel information on the emergence and dissemination of antimicrobial resistant gonococcal

clones which might facilitate appropriate patient management and targeted intervention strategies. *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST) is highly discriminatory, reproducible, objective and transferrable and is well suited to applications aiming to answer short term epidemiological questions. Several studies have applied NG-MAST to characterise gonococci exhibiting decreased susceptibility and resistance to various antimicrobials including the third generation cephalosporins [13-21]. However molecular epidemiological surveillance of consecutive isolates of all antimicrobial phenotypes within a population is necessary to explore associations between genotype and antimicrobial resistance profile and to fully understand how resistant strains emerge and disseminate. While several such surveys have been conducted within individual countries in the EU/EEA [22-25] and elsewhere [26-28], most examined gonococcal populations had been collected in 2008 or earlier and, accordingly, associations between sequence type (ST) and antimicrobial susceptibility may no longer be valid. Furthermore examples of decreased susceptibility to recommended third generation cephalosporins were in most cases not circulating in the gonococcal population examined at the time of sampling.

The current study aimed to assess the public health benefit of NG-MAST molecular epidemiological typing of gonococcal isolates as part of Euro-GASP.

Methods

Isolate collection

N. gonorrhoeae isolates were selected from the most recent sampling period (2010) within Euro-GASP. The sampling strategy for this multi-centre sentinel surveillance scheme has been described previously [12]. Briefly, countries are requested to submit 55 consecutive gonococcal isolates twice annually (in May/June and November/December) aiming as far as possible to provide isolates representative of the national distribution of cases of gonorrhoea. Where available, countries submitted information on date specimen obtained, specimen site, sex of patient, age, sexual orientation, previously diagnosed with gonorrhoea, and concurrent sexually transmitted infection (STI) diagnosed in this episode.

A total of 1,066 isolates were selected for molecular epidemiological typing from 21 EU/EEA countries. The criteria for selection were that isolates were consecutive to ensure no bias in antimicrobial susceptibility, and that a full antimicrobial resistance profile had been determined as part of EURo-GASP.

Most (n=828; 78%) were solely from the first sampling period in May/June 2010 in Austria, Belgium, Denmark, France, Germany, Italy, the Netherlands, Sweden and the United Kingdom (UK). Isolates were selected from this sampling period for all other participating countries but further isolates (n=139; 13%) were additionally selected from the second sampling period (November/December 2010) for countries submitting low numbers (‹50 isolates in the first collection period): Cyprus, Greece, Hungary, Ireland, Romania and Slovakia. The remaining 100 isolates (9%) were additionally selected from 2009 for countries submitting very low numbers (‹50 isolates from both sampling periods in 2010) (Latvia, Malta, Slovenia) or for countries where isolates from the second sampling period in 2010 were unavailable and ‹50 isolates had been submitted in the first sampling period of 2010 (Portugal, Norway and Spain). As Cyprus, Hungary and Romania did not participate in EURO-GASP 2009, only very low numbers (≤17) were available from 2010 for inclusion in the current study.

Antimicrobial susceptibility testing

Gonococcal isolates from each country were submitted on Microbank beads (Pro-Lab Diagnostics, Cheshire, UK) frozen on dry ice to one of three centres (the Health Protection Agency, UK, Örebro University Hospital, Sweden and the Statens Serum Institut, Denmark) for centralised susceptibility testing. All isolates were tested for susceptibility to ciprofloxacin, azithromycin and spectinomycin by a previously decribed breakpoint method [12]. MICs were determined by agar dilution for gentamicin as described [12,29] and by Etest for cefixime and ceftriaxone (AB Biomerieux, Solna, Sweden). The MIC was confirmed by Etest for any isolates resistant to azithromycin by the breakpoint method or showing gentamicin MICs›8 mg/L. All isolates were also tested for penicillinase production using the chromogenic reagent Nitrocefin (Oxoid, Basingstoke, UK).

Determination and analysis of *Neisseria gonorrhoeae* multi-antigen sequence types

Permission to type isolates submitted for susceptibility testing was obtained from all participating laboratories. All typing was performed at either the Health Protection Agency, UK, or at Örebro University Hospital, Sweden. Isolates originally referred to the Statens Serum Institute in Denmark for susceptibility testing were sent frozen on Microbank beads to the Health Protection Agency, London, UK for typing. A total of 1,066 isolates were typed as described [30] by NG-MAST, which differentiates strains on the basis of sequence variation in fragments of two hypervariable genes, the porin PorB (*porB*) gene and subunit B of the transferrin binding protein (*tbpB*) gene. Allele numbers and STs, including any new alleles or STs were assigned via the online NG-MAST database (www.ng-mast.net).

The alleles defining the most frequently observed STs (represented by 10 isolates or more), were compared against all alleles in the dataset to determine if predominant STs were part of a wider collection of closely related types. Similarity of alleles was evaluated in BioNumerics v6.1 by neighbour joining multiple alignment, followed by individual pairwise alignment against the most frequent allele to determine number of base pair (bp) differences. For example, for ST1407 (*porB* 908, *tbpB* 110), all *porB* alleles from isolates

Table 1

Most frequently observed *Neisseria gonorrhoeae* multi-antigen sequence types in each country, and frequency of the three most common among all isolates (n=1,066), EU/EEA countries, 2009–2010

Country	Most frequent ST	STs n	ST1407 n (%)	ST2992 n (%)	ST225 n (%)	Isolates typed n
Austria	ST1407	20	16 (32)	2 (4)	0 (0)	50
Belgium	ST1407	31	7 (14)	4 (8)	1 (2)	50
Cyprus	ST3128	5	0 (0)	0 (0)	0 (0)	12
Denmark	ST225/3158	36	3 (6)	1 (2)	6 (12)	50
France	ST2/2992	27	4 (8)	7 (14)	2 (4)	50
Germany	ST25	18	14 (28)	0 (0)	0 (0)	50
Greece	ST5405[a]/5505[a]	24	6 (12)	0 (0)	1 (2)	50
Hungary	ST5332[a]	10	2 (12)	0 (0)	0 (0)	17
Ireland	ST2992	25	3 (6)	16 (32)	0 (0)	50
Italy	ST1407	25	17 (34)	5 (10)	0 (0)	50
Latvia	ST3227	14	0 (0)	0 (0)	2 (7)	29
Malta	ST225	16	3 (6)	1 (2)	23 (46)	50
Netherlands	ST1407	52	16 (16)	7 (7)	3 (3)	100
Norway	ST2992	26	7 (14)	11 (22)	0 (0)	49
Portugal	ST1407	28	7 (14)	4 (8)	0 (0)	50
Romania	ST1407/4120	7	2 (22)	0 (0)	0 (0)	9
Slovakia	ST437	19	2 (4)	0 (0)	0 (0)	50
Slovenia	ST1407	19	14 (28)	2 (4)	8 (16)	50
Spain	ST1407	43	28 (28)	9 (9)	0 (0)	100
Sweden	ST225	31	1 (2)	3 (6)	4 (8)	50
United Kingdom	ST1407	62	14 (14)	4 (4)	0 (0)	100
Total N (%)			**166 (16)**	**76 (7)**	**50 (5)**	**1,066 (100)**

EEA: European Economic Area; EU: European Union; ST: sequence type.

[a] New ST.

with *tbpB* allele 110 were compared for similarity to *porB* allele 908, and all *tbpB* alleles from isolates containing *porB* allele 908 were compared for similarity to *tbpB* allele 110. Different STs were assigned to a 'genogroup', if one identical allele was shared and the other allele showed ≥99% similarity (≤5 bp difference for *porB* and ≤4 bp for *tbpB*). Genogroups were named using the letter G followed by the number of the predominant ST within each group. For example G1407 is the genogroup in which ST1407 is the predominant ST.

Statistical analyses

Potential associations between genogroups and (i) antimicrobial susceptibilities and (ii) patient characteristics (sex, age and sexual orientation) were explored firstly by univariate analysis and then where appropriate by multivariate analysis using STATA v11.2. For this analysis, patients were subdivided into age groups (0–24 years, 25–34 years, 35–44 years or ≥45 years). Most of the patients in the 0–24 year age group were aged 15–24 years (99%; 360/363), while the remaining three patients were aged 0–7 years.

For the univariate analysis, crude odds ratios (OR) and 95% confidence intervals (CI) were calculated where datasets contained sufficient numbers. A Pearson chi-squared test was used to test if these ORs were significantly different from one (i.e. testing the null hypothesis that there was no difference in odds of resistance/decreased susceptibility between the group in question and the specified baseline group). For datasets where a cell equalled zero, this analysis could not be performed. In these cases of small cell numbers, Fisher's exact test was performed.

The multivariate analysis used logistic regression to model the odds of associations between genogroup and resistance controlling for other variables. The P value produced from the Wald test was used to test the null hypothesis that the odds ratios were not significantly different from one.

Results

Frequency of *Neisseria gonorrhoeae* multi-antigen sequence types

Of the 1,066 isolates typed, 406 different STs were identified, representing 313 *porB* alleles and 113 *tbpB* alleles. One hundred and twenty five clusters (≥2 isolates with the same ST) and 281 STs represented by a single isolate were identified, and 216 new STs defined. The most frequently observed types (represented by ≥10 isolates) were STs 1407 (n=166), 2992 (n=76), 225 (n=50), 25 (n=18), 2 (n=17), 359 (n=16), 387(n=15), 437 (n=14), 3227 (n=12), 5405 (n=10) and 5595 (n=10), with 5405 and 5595 being new STs. Of these, all had unique *porB* alleles but some STs shared the same *tbpB* allele (STs 1407 and 5595, STs 225 and 437, STs 2292 and 359 and STs 387 and 3227 shared, respectively, *tbpB* alleles 110, 4, 29 and 118).

The prevalence of the three most common STs (1407, 2992 and 225) varied between countries (Table 1). ST1407 accounted for >10% of isolates in 13/21 countries, whereas ST2992 and ST225 were observed in >10% of isolates in 3/21 countries in both cases (Table 1). ST1407 was the most frequent ST observed in Austria, Belgium, Italy, the Netherlands, Portugal, Romania, Slovenia, Spain and the UK (Table 1).

Definition and frequency of *Neisseria gonorrhoeae* multi-antigen sequence typing genogroups

Further analysis of the *porB* and *tbpB* alleles of the STs observed in ≥10 isolates showed that several other STs within the total collection were highly related to these, sharing one identical allele and differing by ≤1% at the other allele (in most cases, the *porB* allele). Seven major genogroups, characterised as G1407, G225, G2992, G25, G387, G359 and G2, were defined (Table 2), encompassing 557 (52%) of isolates. The remaining 509 isolates were represented by 313 STs, of which only STs 5405 (n=10), 995 (n=9), 951 (n=8), 4347 (n=8), 5505 (n=8), 292 (n=7), 2400 (n=7), 5402 (n=7), 384 (n=6), 1034 (n=6),1780 (n=6) and 5598 (n=5) were observed in ≥5 isolates. The largest and most diverse genogroups were G1407, comprising ST1407 and 25 STs differing by ≤5 bp in the *porB* allele and two STs by 1 bp in *tbpB*, and G225 comprising ST225 and 28 other STs differing by ≤5 bp at the *porB* locus (Table 2).

Distribution of *Neisseria gonorrhoeae* multi-antigen sequence typing genogroups

G1407 was observed in all countries examined, with the exception of Latvia, and was the most frequent type, observed in 15–50% of isolates from Austria, Belgium, Cyprus, Denmark, England and Wales, Greece, Hungary, Italy, Portugal, Romania, Slovakia, Slovenia, Spain and the Netherlands (Figure). In contrast G1407 was comparatively uncommon (≤8%) in France, Ireland, Malta and Sweden (Figure). G225 was observed in 19/21 countries and was the most frequently observed type in Malta and Sweden and was most common after

Table 2

Neisseria gonorrhoeae multi-antigen sequence types within the seven major genogroups defined based on sequence similarity at *porB* and *tbpB* alleles, EU/EEA countries, 2009–2010

Genogroup n (%)[a]	Predominant ST (n)	STs within each genogroup (n)	
		≥99% similar to *porB* allele x[b]	≥99% similar to *tbpB* allele x[c]
G1407 248 (23)	1407 (166)	5595 (10), 3149 (9), 3158 (8), 3128 (7), 4120 (7), 5570 (5), 5600 (4), 2212 (3), 5594 (3), 3431 (1), 3779 (1), 4275 (1), 4359 (1), 4951 (1), 5588 (1), 5619 (1), 5622 (1), 4974 (1), 5480 (1), 5625 (1), 5581 (1), 5649 (1), 5205 (1), 5599 (1), 5591 (1)	5332 (6), 4741 (4)
G225 107 (10)	225 (50)	437 (14), 5463 (6), 1132 (5), 5017 (2), 5423 (2), 2616 (2), 3150 (2), 5180 (2), 205 (1), 346 (1), 1342 (1), 1365 (1), 1399 (1), 2687 (1), 289 (1), 1340 (1), 2202 (1), 3141 (1), 3153 (1), 3952 (1), 4315 (1), 5703 (1), 2625 (1), 5655 (1), 3056 (1), 4473 (1), 5374 (1), 5330 (1)	
G2992 84 (8)	2992 (76)	5515 (2), 5049 (1) 5194 (1) 5237 (1), 5192 (1) 5385 (1)	5227 (1)
G25 43 (4)	25 (18)	51 (7), 3003 (4), 356 (3), 4589 (2), 5384 (2), 273 (1), 881 (1), 5424 (1), 807 (1), 5341 (1), 4338 (1), 5172 (1)	
G387 37 (3)	387 (15)	3227 (12), 5185 (4), 5191 (1), 5486 (1), 5503 (1), 5186 (1), 5190 (1), 5498 (1)	
G359 20 (2)	359 (16)	1929 (2), 5485 (1), 1313 (1)	
G2 18 (2)	2 (17)	226 (1)	

EEA: European Economic Area; EU: European Union; *porB*: porin B; ST: sequence type; *tbpB*: transferrin binding protein subunit B.

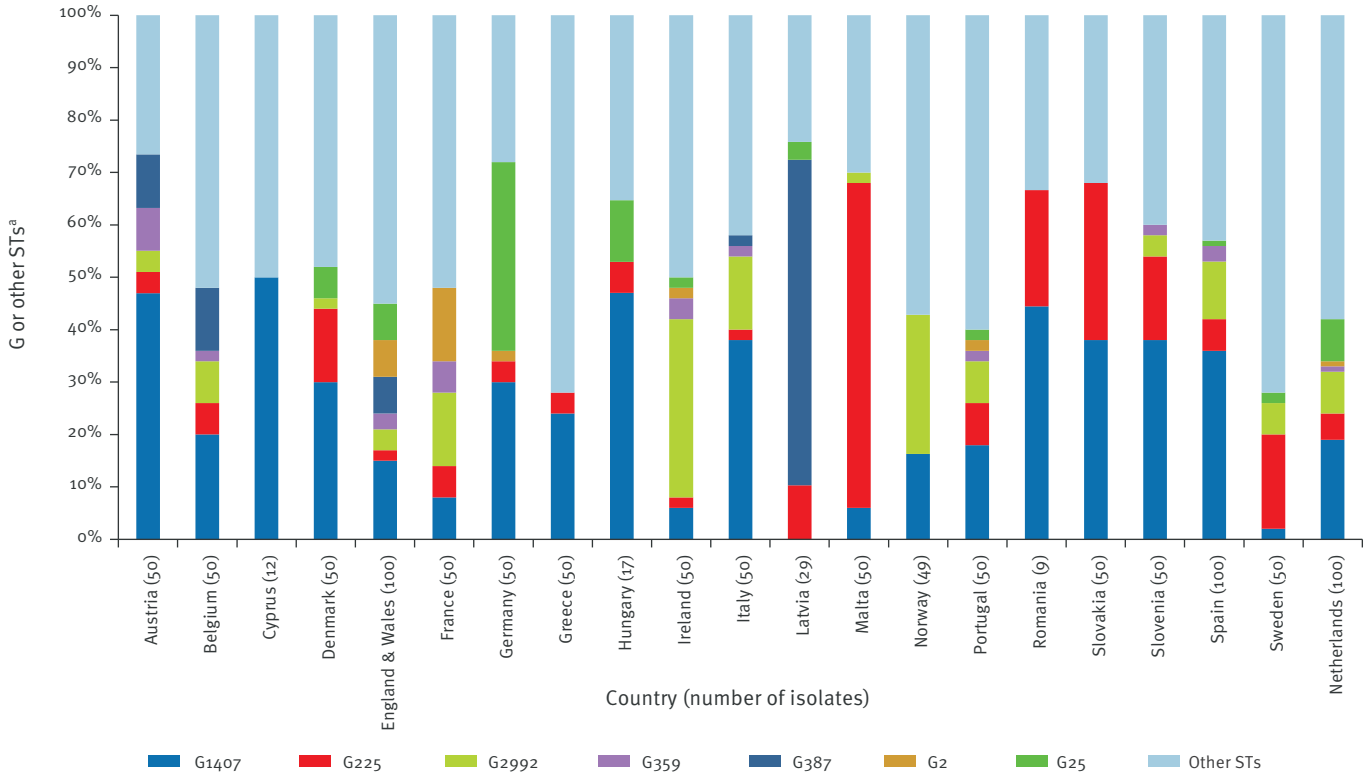
[a] The percentages are calculated from a total of 1,066 isolates.

[b] STs that share an identical *tbpB* allele with the most frequent ST within the genogroup but vary at the *porB* allele.

[c] STs that share an identical *porB* allele with the most frequent ST within the genogroup but vary at the *tbpB* allele.

Figure

Proportion of various *Neisseria gonorrhoeae* multi-antigen sequence typing genogroups within each participating country, EU/EEA countries, 2009–2010



EEA: European Economic Area; EU: European Union; G:genogroup; ST: sequence type.

[a] Other STs refer to the isolates with STs which did not belong to a defined G.

Table 3

Characteristics of patients infected with the most frequently observed *Neisseria gonorrhoeae* multi-antigen sequence typing genogroups/sequence type, EU/EEA countries, 2009–2010

Genogroup (n)	Mean patient age (range in years)	Patients of known sex n	Male patients n (%)	Patients of known sexual orientation[a] n	Males of known sexual orientation n	MSM n (%[b])	Heterosexuals of both sex n (%[b])
G1407 (248)	33.3 (17–69)	244	218 (89)	155	129	72 (46)	83 (54)
G225 (107)	29.1 (7–56)	104	85 (82)	78	59	13 (17)	65 (83)
G2992 (84)	31.7 (18–66)	84	81 (96)	41	38	36 (88)	5 (12)
G25 (43)	24.6 (16–40)	43	18 (42)	35	10	2 (6)	33 (94)
G387 (37)	28.6 (18–69)	36	21 (58)	30	15	0 (0)	30 (100)
G359 (20)	32.9 (18–69)	20	16 (80)	11	7	5 (45)	6 (55)
G2 (18)	22.1 (15–38)	18	12 (67)	11	5	0 (0)	11 (100)
ST5405[c] (10)	30.9 (6–51)	10	8 (80)	9	7	0 (0)	9 (100)
All isolates (1,066)	**31.4 (0–76)**	**1,050**	**868 (83)**	**660**	**478**	**222 (34)**	**438 (66)**

EEA: European Economic Area; EU: European Union; MSM: men who have sex with men.

[a] All female patients were included in the heterosexual patient group.
[b] The percentage is relative to the number of patients of known sexual orientation.
[c] ST5405 was not found to be part of a larger genogroup.

G1407 in Denmark, Romania, Slovakia and Slovenia, accounting for 14–30% of isolates (Figure). G2992 was observed in 14/21 countries and predominated in Ireland and Norway, while G25 predominated in Germany (Figure).

Neisseria gonorrhoeae multi-antigen sequence typing genogroups and epidemiological characteristics of linked patients

The most frequently collected variables were age and sex, known for 1,030 and 1,050 patients, respectively. Sexual orientation was known for only 586 patients (478 males and 108 females) (Table 3).

Statistical analysis showed there was no clear association with age category for most genogroups, with the exception of G1407 which was significantly less common in patients aged <25 years (OR: 0.43; p<0.0001, chi-squared test), whereas G2 was significantly more common in this younger group (OR: 15.63; p=0.0004, chi-squared test). The proportion of males infected was significantly lower for G25 (OR: 0.13; p<0.0001, chi-squared test) and G387 (OR: 0.27; p=0.0001, chi-squared test) but was significantly higher for G2992 (OR: 6.14; p=0.0005, chi-squared test) and G1407 (OR: 2.01; p=0.0017, chi-squared test). Where sexual orientation was known, statistical associations were observed between men who have sex with men (MSM) and infection with G2992 (OR: 15.86; p<0.0001, chi-squared test) and G1407 (OR: 1.75; p=0.003, chi-squared test), although 54% of patients infected with G1407 were heterosexual (Table 3). MSM were less frequently infected with G225 (OR: 0.039; p=0.0015, chi-squared test), G387 (p<0.0001, Fisher's exact test) and G2 (p=0.01, Fisher's exact test).

Neisseria gonorrhoeae multi-antigen sequence typing genogroups or sequence types and antimicrobial resistance

The consensus antimicrobial susceptibility category or modal MIC was calculated for all genogroups/STs represented by ≥10 isolates (Table 4). Most deviation from the consensus was observed for azithromycin, which supplementary testing by Etest showed was due to isolates exhibiting MICs close to the breakpoint for resistance (1.0 mg/L).

The modal cefixime MIC was raised for G1407 compared with other genogroups (Table 4), with 77/248 isolates (31%) showing decreased susceptibility to cefixime (MIC >0.125 mg/L) while 176/248 isolates (71%) had MICs ≥0.125mg/L. Almost all isolates (239/248; 96%) had cefixime MICs ≥0.06mg/L. The association between G1407 and decreased susceptibility to cefixime was highly significant (OR: 17.9; p<0.0001, chi-squared test) by univariate analysis and by multivariate analysis to control for patient age, sex and sexual orientation (OR: 31.2; p<0.0001, chi-squared test). There were no examples of decreased ceftriaxone susceptibility (MIC >0.125 mg/L), but the modal MIC was raised for G1407 compared with other genogroups (Table 4). All isolates of G1407 were ciprofloxacin resistant (p<0.0001, Fisher's exact test). While the consensus azithromycin susceptibility for G1407 was sensitive, the proportion of isolates showing MICs just above the breakpoint and so in the resistant category was significant after controlling for patients age, sex and sexual orientation (OR: 11.5; p<0.0001, chi-squared test). Decreased cefixime susceptibility was observed in a further nine STs representing 10 isolates in the total study population. Of these, seven isolates shared *tbpB* allele 110 but the six *porB* alleles differed from allele 908 by >5 bp. One isolate possessed *porB* allele 908 but differed markedly at the *tbpB* allele from 110. The STs of the remaining two isolates (STs 3168 and 5606) appeared unrelated to ST1407.

All isolates belonging to G225 were resistant to ciprofloxacin, indicating a statistically significant association by Fisher's exact test (p<0.0001). All ten isolates of ST5405 were resistant to ciprofloxacin also (Table 4), significant association by Fisher's exact test (p=0.0032).

Discussion

To our knowledge this is the first study to supplement a regional antimicrobial resistance surveillance programme with molecular typing to provide enhanced surveillance of gonorrhoea. This approach has not only provided novel information on NG-MAST STs circulating in 21 of the 30 EU/EEA countries, but also provides insight into the dissemination of antimicrobial resistant gonococcal clones within the region.

While considerable diversity of gonococcal STs exists both within and between countries, some STs predominate, and this is even more apparent if STs are grouped into genogroups to facilitate robust statistical analyses. Most notably, G1407 accounted for 23% of isolates overall, and predominated in 13/21 countries. Relatedness of NG-MAST STs has been considered in comparatively few studies and multiple approaches are described [14,15,21,25,31,32]. Failure to consider relatedness of STs could lead to the prevalence of clonal groups being underestimated and reduces the power of any statistical analyses exploring associations between organism and/or patient characteristics and STs. Our approach was to consider strains highly related if strains varied at just one allele by ≤1%. Use of such a high cut-off ensures only very closely related strains are clustered, but given the variation in approaches described thus far, there is a need to agree a standard approach in the near future to allow comparability between studies.

Most molecular surveys of consecutive gonococcal isolates from earlier sampling periods ranging between 2003 to 2005 did not demonstrate predominance of ST1407 or related types in individual European countries [24,25,33,34] or elsewhere [26,28]. In contrast ST1407 was documented in more recent surveys in Portugal [25] and Norway [23], and a centre that

Table 4

Consensus antimicrobial susceptibility results for *Neisseria gonorrhoeae* multi-antigen sequence typing genogroups/sequence type represented by 10 or more isolates, EU/EEA countries, 2009–2010

Genogroup or ST	Isolates n	Beta-lactamase	Consensus resistance category (Isolates differing from the consensus)			Modal MIC mg/L (range)		
			Azithromycin	Ciprofloxacin	Spectinomycin	Gentamicin	Cefixime[a]	Ceftriaxone[a]
G1407	248	Negative	S (44[b])	R (0)	S (0)	8 (4–16)	0.125 (0.032–0.25)	0.047 (0.008–0.125)
G225	107	Negative	S (1[c])	R (0)	S (0)	8 (4–16)	0.023 (<0.016–0.064)	0.016 (0.003–0.047)
G2992	84	Negative	S (14[d])	S (1)	S (0)	8 (2–8)	0.023 (<0.016–0.064)	0.006 (0.002–0.016)
G25	43	Negative	S (0)	S (1)	S (0)	8 (4–8)	<0.016 (<0.016–0.064)	0.003 (<0.002–0.016)
G387	37	Negative	S (0)	S (0)	S (0)	8 (4–8)	<0.016 (<0.016–0.016)	<0.002 (<0.002–0.002)
G359	20	Negative	S (1[c])	S (0)	S (0)	8 (4–16)	0.064 (<0.016–0064)	0.008/0.012 (0.004–0.023)
G2	18	Negative	S (0)	S (0)	S (0)	4 (2–8)	<0.016 (<0.016–0.032)	0.003 (<0.002–0.012)
ST5405	10	Negative	S (0)	R (0)	S (0)	8 (4–8)	<0.016 (<0.016–0.023)	0.004/0.006 (0.003–0.006)

EEA: European Economic Area; EU: European Union; MIC: minimum inhibitory concentration; S: susceptible; ST: sequence type; R: resistant.

[a] Cefixime and Ceftriaxone MICs are described according to the Etest scale.
[b] 40 of the 44 with an R category were within one doubling dilution of the breakpoint.
[c] Isolates that differ from the consensus were within one doubling dilution of the breakpoint.
[d] 10 of the 14 with an R category were within one doubling dilution of the breakpoint.

performs molecular typing of all cases of gonorrhoea in Scotland showed that ST1407 first emerged in 2007 and by 2009 accounted for 15.4% of cases [35]. The current study provides further evidence that the wide dissemination of ST1407 in the EU is likely to be a recent phenomenon. ST1407 and related STs are known to be distributed globally, documented in studies specifically investigating decreased susceptibility and resistance to third generation cephalosporins, in Europe [6,9,13,14,19], the United States [20], Canada [21], Australia [18], Hong Kong [17] and Taiwan [36] and in a survey conducted in Japan [27]. ST1407 has also caused most of the treatment failures with third generation cephalosporins in EU/EEA countries [6-11].

Our study demonstrates that there appear to be no isolates of G1407 that are highly sensitive to cefixime, with 96% of isolates showing MICs ≥0.06mg/L, and presents the first clear evidence that all G1407 isolates circulating currently in the general gonococcal population in the EU/EEA are ciprofloxacin resistant, strongly associated with decreased susceptibility/resistance to cefixime and show raised MICs for ceftriaxone and azithromycin. This highlights the potential of molecular epidemiological typing as a tool to predict antimicrobial resistance, as it is evident that ciprofloxacin and cefixime in particular would be inappropriate regimes to treat G1407 infection. Two isolates were identified that exhibited decreased susceptibility to cefixime but were considered unrelated to G1407. The low incidence of these types may suggest they represent less biologically fit gonococcal clones and/or have recently been imported from regions such as the Far East, where greater diversity in STs showing cefixime decreased susceptibility is reported [15-17,36]. ST1407 is known to be part of a major globally predominant clone, multilocus sequence type (MLST) ST1901 which probably originated in Japan [11], and recent analysis of ten additional genomic markers has provided further evidence of the clonal nature of ST1407 and closely related NG-MAST STs [32]. MLST ST7363 is also reported as a major clone associated with decreased susceptibility and resistance to cephalosporins, which also has shown its ability to develop high-level resistance to cefixime and ceftriaxone [37]. MLST was not performed in the current study but is an excellent means of determining longer-term evolutionary relationships between strains [38] and so could help to resolve the lineage of these non-G1407 isolates.

Treatment failure in patients infected with gonococci exhibiting cefixime MICs of the levels in the current study have been documented in the UK [10], Norway [9] and Austria [6] and all belonged to G1407. One ST1407 isolate with high-level resistance to cefixime (MIC=4 mg/L) and ceftriaxone (MIC=1–2 mg/L), also associated with treatment failure using cefixime, has been described from France [11]. This strain demonstrates that the ST1407 clone can by a single additional mutation in its penicillin-binding protein 2 (*penA*) mosaic gene develop high-level resistance to both cefixime and ceftriaxone, which is the last remaining option for single antimicrobial empiric treatment of gonorrhoea [11]. The observed predominance of ST1407 and related STs in many EU/EEA countries is therefore worrying given their potential to be therapeutically challenging. While shown to be associated with MSM, it is evident that G1407 is circulating in the heterosexual population also and, accordingly, the risk of future treatment failure is not restricted to any one patient group. G1407 therefore represents a potential major public health problem if it continues to disseminate without any measures taken to restrict this.

G225 was the most prevalent genogroup after G1407 in the current study. ST225 has been reported as a highly prevalent strain in EU/EEA countries [22,25,33,34], with a confirmed association with ciprofloxacin resistance [22,25,33]. Given that most studies began sampling as early as 2003 to 2005, the continued persistence of G225 by 2010 indicates that it is a highly successful, stable NG-MAST clone. Interestingly, a recent Canadian study of isolates showing decreased susceptibility to cefixime and/or ceftriaxone showed that 19% of isolates were ST225 [21]. All isolates of G225 in the current study were sensitive to cefixime and ceftriaxone, but the potential for this strain to show decreased susceptibility is of concern given its potential to predominate and persist. While strong associations between ST225 and MSM are reported [25,34], these were not confirmed in all studies [39] including this one. This could indicate a shift in the distribution of G225 since the earlier typing studies, with possible bridging between homosexual and heterosexual networks. It is acknowledged however that the completeness of the sexual orientation variable in the current study was low, which may have introduced a bias into this analysis.

None of the other major genogroups defined in the current study showed an association with resistance to the antimicrobials tested, but G2, G25, G359 and G387 would appear to be particularly persistent strains also, all having been reported in a molecular survey in 2004 [34]. Individual STs from these groups have been reported in other studies which confirmed that these were susceptible to the antimicrobials tested [22,25,33]. As observed previously [34], genogroups G2, G25 and G387 were associated with heterosexual patients, demonstrating their persistence within this patient population. In contrast other genogroups (G2992, G1407) were shown to be associated with MSM. While ST1407 has been reported in a high proportion of MSM previously [13], to our knowledge this is the first time ST2992 and related types has been reported as a potentially MSM associated type. None of the molecular epidemiological surveys conducted to date have reported ST2992 as a prevalent ST [24-26,28,33,34]. G2992 accounted for 8% of all isolates in our study and while widely distributed, it was particularly predominant in Ireland and Norway, which may indicate an outbreak within MSM, although further longitudinal surveillance of a sufficiently representative sample would be required to verify this.

In our study the selection criteria for isolates were based on accepting from each country consecutive isolates with a full antimicrobial profile. There were no exclusion criteria based on epidemiological characteristics and all patient ages were included. One of the age groups used for the analyses spanned 0 to 24 year-olds however only three of the 363 patients in this group were less than 15 years-old. The inclusion of isolates from the latter patients was valid for the antimicrobial resistance analyses. Only one of the three patients was related to a common genogroup (G225) therefore including these three patients should have had little effect on the overall statistical analyses.

In conclusion, this study shows clear associations between antimicrobial resistance and molecular type. The use of molecular epidemiological typing to predict antimicrobial resistance and follow the spread of antimicrobial resistant gonococcal clones has added public health benefits as it can aid understanding of the dissemination of resistance within a population and facilitate development of targeted intervention strategies, for example by ensuring patients infected with strains likely to be therapeutically challenging are managed with aggressive therapy and test of cure. Additionally if the prediction of antimicrobial resistance was sufficiently reliable this approach could directly impact on appropriate management of patients for whom culture and associated antimicrobial susceptibility testing has not been performed. While it is acknowledged that sample representativeness is a limitation of the current study [12], additional molecular epidemiological typing has successfully demonstrated a 'proof of principle' that this approach could be valid and have real public health value. However for this to be effective, ongoing, frequent molecular typing surveillance will be essential, as there is continual potential for novel STs and novel antimicrobial susceptibility profiles to emerge. Future work should focus on longitudinal typing of a representative sample to monitor stability of associations between ST and antimicrobial resistance and/or epidemiological characteristics, and to identify temporal changes and emergence of novel STs.

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A snapshot of genetic lineages of *Mycobacterium tuberculosis* in Ireland over a two-year period, 2010 and 2011

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Mycobacterial interspersed repetitive-unit-variable-number tandem repeat typing alone was used to investigate the genetic lineages among 361 *Mycobacterium tuberculosis* strains circulating in Ireland over a two-year period, 2010 and 2011. The majority of isolates, 63% (229/361), belonged to lineage 4 (Euro-American), while lineages 1 (Indo-Oceanic), 2 (East-Asian) and 3 (East-African–Indian) represented 12% of isolates each (42/361, 45/361, and 45/361, respectively). Sub-lineages Beijing (lineage 2), East-African–Indian (lineage 1) and Delhi/central-Asian (lineage 3) predominated among foreign-born cases, while a higher proportion of Euro-American lineages were identified among cases born in Ireland. Eighteen molecular clusters involving 63 tuberculosis (TB) cases were identified across four sub-lineages of lineage 4. While the mean cluster size was 3.5 TB cases, the largest cluster (involving 12 Irish-born cases) was identified in the Latin American–Mediterranean sub-lineage. Clustering of isolates was higher among Irish-born TB cases (47 of 63 clustered cases), whereas only one cluster (3/63) involved solely foreign-born individuals. Four multidrug-resistant cases identified during this period represented lineages 2 and 4. This study provides the first insight into the structure of the *M. tuberculosis* population in Ireland.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains a serious challenge to public health worldwide. Despite an overall decline in case notification rates for TB across Europe, rates vary significantly, with the highest rates reported from eastern Europe and the Baltic States [1]. Multidrug-resistant (MDR) TB continues to be a major problem and an added burden in high-incidence countries such as Romania (108.2/100,000) and the Baltic states Lithuania (62.1/100,000), Latvia (43.2/100,000) and Estonia (30.7/100,000) [1]. Ireland has a low incidence, with notification rates that ranged between 9.7 and 11.3 per 100,000 population between 2001 and 2010 although to some extent this may have been influenced by migrants arriving from high-burden countries [1-2]. In Ireland, TB is a statutorily notifiable disease, and in a recent report on the epidemiology of TB in the country, the proportion of culture-confirmed TB cases was 71.2% in 2009 and 63.2% in 2010 (data from 2010 were not finalised at the time of submission) [3]. These proportions are similar to those reported for previous years [3].

Disruption of transmission chains is a key factor in controlling TB both at a national and international level [4]. In recent years, there have been significant advances in developing the molecular tools required for rapid diagnosis of TB [4]. Analysis of variable-number tandem repeat (VNTR) sequences at mycobacterial interspersed repetitive units (MIRU) has emerged as a valuable marker for genotyping strains of the *M. tuberculosis* complex [5]. In large population-based studies, MIRU-VNTR typing has been shown to have similar discriminatory power when compared to IS*6110* restriction fragment length polymorphism (RFLP) typing [5-7]. An optimised set of 24 MIRU-VNTR markers has become the gold standard for genotyping *M. tuberculosis* complex strains worldwide [5,7]. MIRU-VNTR typing is a PCR-based method that yields rapid, reproducible results that are expressed as a 24-digit numerical code which allows for easy exchange of data [5-8]. This method can be applied to early mycobacterial cultures and more recently has been successfully applied directly to smear-positive specimens [5,9]. Previous studies have shown MIRU-VNTR typing to be useful in comparing strains (i) at national and international level, (ii) among household contacts, (iii) associated with drug resistance and (iv) to determine the evolutionary pathway of TB [5-6,10-16]. At European level, MIRU-VNTR typing has been adopted for the molecular surveillance of the international transmission of MDR-TB and extensively drug-resistant TB [7].

In November 2009, molecular genotyping in the form of 24-locus MIRU-VNTR typing was introduced at the

Irish Mycobacteria Reference Laboratory (IMRL) which both cultures and receives isolates from microbiology laboratories around the country. To allow for rapid, high-throughput genotyping of *M. tuberculosis*, the commercial MIRU-VNTR typing kit (GenoScreen, Lille, France) was introduced in 2010 [5,7]. As 24-locus MIRU-VNTR typing is considered the gold standard genotyping method, all *M. tuberculosis* isolates identified at the IMRL are currently typed prospectively with this method, and it is envisaged that all *M. tuberculosis* isolates recovered since 2000 will be typed on a retrospective basis.

Here we report the first analysis of the structure of the *M. tuberculosis* population in Ireland for isolates recovered during 2010 and 2011 following the introduction of 24-locus MIRU-VNTR typing to the diagnostic laboratory. It needs to be noted that at the time there was an under-representation of isolates from the southern region of Ireland.

Methods

MIRU-VNTR typing

M. tuberculosis isolates (n=361) recovered in or referred to the IMRL over a two-year period (2010–11) were typed with the MIRU-VNTR typing kit (GenoScreen) [5]. Validation of the MIRU-VNTR technique was performed using the MIRU-VNTR Calibration Kit (GenoScreen). PCR products were subjected to electrophoresis using a 3130 genetic analyser (Applied Biosystems). Sizing of fragments and MIRU-VNTR allele assignation was performed using GeneMapper software (Applied Biosystems). Phylogenetic lineages were assigned to each isolate using the MIRU-VNTR*plus* online tool [17-18].

The 24-locus MIRU-VNTR panel comprised the following loci: MIRU 02, VNTR 42, VNTR 43, MIRU 04, MIRU 40, MIRU 10, MIRU 16, VNTR 1955, MIRU 20, QUB 11b, ETR A, VNTR 46, VNTR 47, VNTR 48, MIRU 23, MIRU 24, MIRU 26, MIRU 27, VNTR 49, MIRU 31, VNTR 52, QUB 26, VNTR 53, and MIRU 39. The MIRU-VNTR profiles are reported as a series of 24 numbers that correspond to the number of alleles at each of the loci described above.

Clusters of isolates were defined as two or more isolates with indistinguishable MIRU-VNTR patterns. The strain clustering rate was calculated as $(n_c\text{-}c)/n$, where n_c was the total number of strain-clustered cases, c was the number of clusters and n was the total number of isolates [5].

Epidemiological analysis

Enhanced surveillance of TB was implemented in Ireland in 1998. Enhanced TB notification forms are completed by public health doctors, summarising all available clinical, microbiological, histological and epidemiological information. These data are collated in the regional public health departments. Anonymised data are then submitted electronically to the Health Protection Surveillance Centre (HPSC) for the production of reports on a weekly, quarterly and annual basis.

Since January 2011, cases of TB have been reported through the Computerised Infectious Disease Reporting system (CIDR). CIDR is a web-based system developed to integrate case-based clinical and laboratory data in order to manage the surveillance and control of notifiable infectious diseases in Ireland. Prior to using CIDR for TB surveillance, MIRU-VNTR typing results were not linked to case-based epidemiological data. In addition to recording sporadic case-based data, CIDR also facilitates the reporting of clustered cases, according to Irish outbreak case definitions [2]. Clustered cases can be reported via a summary aggregate outbreak data module to which the relevant disaggregate case-based surveillance data can also be linked.

Data analysis

The TB enhanced surveillance data for 2011 (epidemiological and linked laboratory data) used in this publication were extracted from CIDR on 17 April 2012 using Business Objects XI software and were analysed using Microsoft Excel. Data for 2011 were provisional at the time of extraction and subject to ongoing validation and revision.

Results

Results are presented in two separate sections. In the first part, genotyping results for isolates recovered in 2010–11 are presented. As epidemiological data linking was available from 2011 onwards, the second part of the results section (enhanced surveillance) refers to genotyping results linked to epidemiological data for 2011 isolates only.

Mycobacterium tuberculosis genotyping, 2010–11

Some 361 *M. tuberculosis* isolates were recovered in or referred to the IMRL during 2010–11, representing 63.6% of culture-positive cases identified through the national TB surveillance system in that period. Genotyping of *M. tuberculosis* isolates recovered during the study period yielded four global lineages (Table 1). The majority (63%) belonged to lineage 4 (Euro-American), while lineages 1 (Indo-Oceanic), 2 (East-Asian) and 3 (East-African–Indian) represented 12% each. Among the 229 Euro-American strains, sub-lineages Latin American–Mediterranean (LAM) (23%), Haarlem (21%), H37Rv (19%) and Haarlem/X (13%) were most prevalent (Table 1).

Within lineage 4, 18 clusters were identified involving 63 TB cases (Table 2). The strain clustering rate varied between different sub-lineages, but was highest for the LAM sub-lineage (6.9%). While the mean cluster size was 3.5 TB cases, the largest cluster (involving 12 Irish-born cases, representing 19% of all clustered isolates) was identified within the LAM sub-lineage (Table

Table 1

Distribution of lineages among *Mycobacterium tuberculosis* isolates, Ireland, 2010–11 (n=361)

Global lineage	Sub-lineage	No. of isolates	% of isolates
1 Indo-Oceanic	East-African–Indian	42	12
2 East-Asian	Beijing	45	12
3 East–African–Indian	Delhi/central-Asian	45	12
4 Euro-American	Lineage 4 total	229	63
	Latin American–Mediterranean	52	
	Haarlem	47	
	H37Rv	44	
	Haarlem/X	29	
	Cameroon	13	
	S	6	
	TUR	8	
	X	5	
	Ghana	3	
	URAL	3	
	Uganda I & II	6	
	NEW-1	2	
	Others[a]	11	
Total		**361**	**100**

[a] The category Others includes isolates for which the sub-lineages were not clearly defined

2). Four other clusters within the LAM sub-lineage contained between 4.7% (3/63) and 12.7% (8/63) of clustered cases. Among the clustered cases of Haarlem, H37Rv and Haarlem/X, cluster sizes ranged from 3.2 to 9.5%, at 3.2% and from 3.2 to 4.7% of isolates, respectively.

Only one cluster (3/63) contained exclusively foreign-born individuals, 12 clusters (47/63) involved Irish-born cases only, while five clusters (13/63) were mixed. In addition, one small cluster was observed among the isolates from lineage 2.

The four MDR-TB cases identified during this period represented lineages 2 (Beijing) and 4 (Ural, H37Rv and LAM). None of the MDR-TB cases were clustered.

Tuberculosis enhanced surveillance data for 2011 isolates (epidemiological and laboratory)

In 2011, 432 TB cases were provisionally reported on CIDR, of which approximately 166 (38%) were typed. At the time of data extraction, 136 TB cases were updated to include MIRU typing results (representing 81.9% of 166 typed isolates). Of the 136 TB cases with a MIRU typing result, 34 were clustered in 11 clusters with different MIRU types. Clusters ranged in size from eight to two TB cases. Of the 11 MIRU type clusters, five, comprising 18 TB cases, were confirmed by public health departments as outbreaks meeting the Irish case definition.

The Beijing sub-lineage was most prevalent (15.4%) and associated with a small cluster. Sub-lineages Haarlem, LAM, and H37Rv were most prevalent among lineage 4 strains, while lineage 1 and lineage 3 represented 11.8% and 10.3% of typed isolates, respectively. Interestingly, isolates recovered from pulmonary specimens were mostly correlated with lineage 4 strains, while the majority of isolates recovered from extra-pulmonary specimens belonged to lineages 1 and 3 (Figure 1). In lineage 3, nine of 14 isolates were recovered from patients born in Pakistan, while the remaining five isolates were recovered from patients born in India (n=2), Kenya (n=1), Nepal (n=1) and Nigeria (n=1). Only one lineage 1 isolate was recovered from an Irish-born patient, while six were recovered from patients born in the Philippines. Other countries represented among lineage 1 isolates were Bangladesh, India, Mozambique, Pakistan, Somalia and Vietnam.

The distribution of lineages among Irish-born and foreign-born TB cases is shown in Figure 2. Of the 127 TB cases for whom MIRU-VNTR and country of birth were known, 51.5% were foreign-born and 41% were Irish-born. Lineages 1, 2 and 3 predominated among foreign-born TB cases, while a higher proportion of lineage 4 isolates were identified among Irish-born cases.

Discussion

This study has provided a snapshot of the genetic diversity of *M. tuberculosis* in Ireland. Due to the small numbers of isolates in our study, statistical analysis would not be significant and was not performed. Although data on sub-lineages were analysed by age and sex, the resulting frequencies were too small to draw firm conclusions from. However, when age and sex analyses were further stratified by country of birth, these data were broadly similar to the age and sex profile of the Irish TB notification data.

A large diverse group of isolates has been identified, suggesting a low degree of active transmission among TB patients. The distribution of genetic lineages is similar to other recent studies that used different typing techniques and in which lineage 4 (Euro-American) predominated among circulating *M. tuberculosis* strains [12,19-21]. In previous work conducted in the south-west region of Ireland, lineage 4 predominated, and clustering of isolates was associated with Irish nationals and lineage 4 isolates only [22]. In our study, the distribution of genetic lineages among extra-pulmonary specimens (where lineages 1 and 3 predominated) was similar to a recently published large-scale study conducted in the United States (US) investigating the relationship between genetic lineages and clinical sites of infection [23]. In the US study, the highest percentage of isolates recovered from extra-pulmonary

Table 2

Clusters of *Mycobacterium tuberculosis* isolates within lineage 4 (Euro-American), Ireland, 2010–11 (n=172 isolates)

Sub-lineage	Total no. of cases	No. of clustered cases (%)	No. of clusters	Strain clustering rate (%)	No. of isolates/ cluster (% clustered cases)	MIRU-VNTR profile[a]
Latin American–Mediterranean	52	30 (8.3)	5	6.9	3 (4.7)	12424433222412615333 2832
					8 (12.7)	14224433222412615332 2622
					12 (19)	14224433222412614332 2622
					3 (4.7)	13224433222412515332 2222
					4 (6.3)	13224433222412613332 2622
Haarlem	47	18 (5)	6	3.3	2 (3.2)	22322534233442514332 3332
					6 (9.5)	2232253423344251533 23_32
					2 (3.2)	12323533263442514342 3332
					4 (6.3)	2232253423344251433 23_32
					2 (3.2)	22323533153242315333 3632
					2 (3.2)	22321533233442515332 3632
H37Rv	44	8 (2.2)	4	1.1	2 (3.2)	22424312223422515323 4422
					2 (3.2)	22424312243422515333 5512
					2 (3.2)	22421322253422615333 4522
					2 (3.2)	22421332233422615333 5522
Haarlem/X	29	7 (1.9)	3	1.1	2 (3.2)	22423434233442515413 5832
					3 (4.7)	24324433243442515334 3a32
					2 (3.2)	24323433223442514333 1832
Total clustered lineage 4 cases	**172**	**63**	**18**	-	-	-

[a] The numbers in this 24-digit profile correspond to the number of alleles at each of the following loci: MIRU 02, VNTR 42, VNTR 43, MIRU 04, MIRU 40, MIRU 10, MIRU 16, VNTR 1955, MIRU 20, QUB 11b, ETR A, VNTR 46, VNTR 47, VNTR 48, MIRU 23, MIRU 24, MIRU 26, MIRU 27, VNTR 49, MIRU 31, VNTR 52, QUB 26, VNTR 53, MIRU 39.

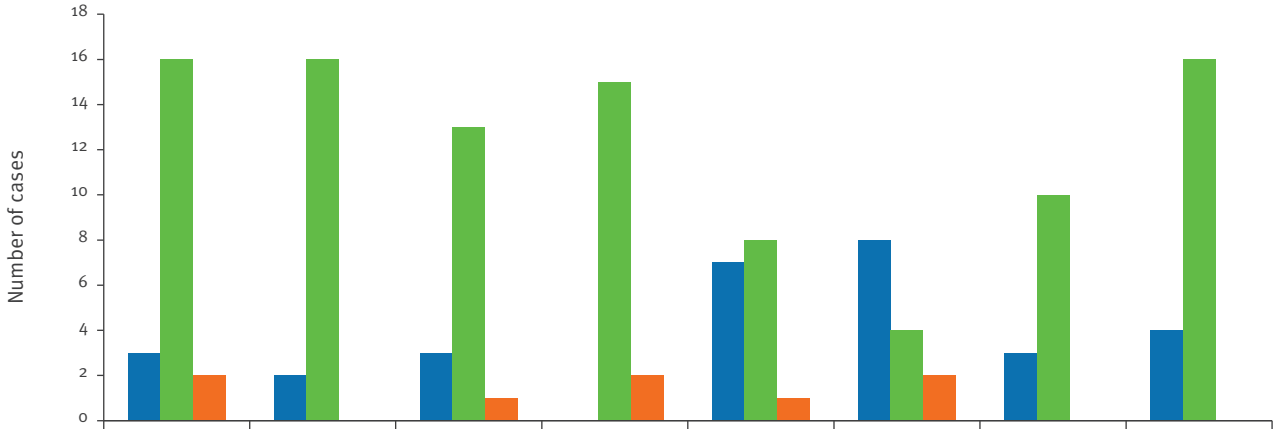
specimens was from lineages 1 (22.6%) and 3 (34.3%) [23]. However, due to the small numbers of exclusive extra-pulmonary specimens (n=30) and limited epidemiological data, statistical analysis of the relationship between lineage and clinical site of infection was not possible in our report.

Molecular clustering of isolates in our study was more common among Irish-born individuals. These findings were similar to a previous Irish study conducted by Ojo et al. in the south-west region of Ireland, but unlike a recent study conducted in Switzerland [12,22]. We identified 18 clusters in lineage 4, and the mean cluster size was 3.5 TB cases. The largest cluster, involving 12 TB cases, belonged to the LAM lineage and spanned a period of 18 months. A second cluster identified in the LAM lineage differed by a single locus variant (SLV) at locus 2996. Similarly, in the Haarlem lineage, the two largest clusters differed by a SLV at locus 2996 also. MIRU 26 (or locus 2996) has yielded stable comparable results in a large-scale study investigating 824 *M. tuberculosis* isolates conducted at the Institut Pasteur de Lille, France, in 2006 [5]. Molecular typing played a key role in identifying a dominant *M. tuberculosis* strain (known as the Mercian strain) circulating in the West Midlands region in the United Kingdom (UK) over a five-year period, highlighting the importance of cluster analysis [14]. Prospective molecular typing can identify rapidly expanding clusters of *M. tuberculosis* before they spread further into the community. A single dominant MIRU-VNTR type was not observed in this study, however, this could be due to the study period being short. In contrast, prospective molecular typing of *M. tuberculosis* by RFLP, performed since 1993 in the Netherlands, has proven to be effective. DNA fingerprinting data has been shown to be a powerful tool in defining epidemiological links and guiding TB control programmes in the Netherlands [24-25].

Another limitation of this study is that only one typing method was used to investigate the *M. tuberculosis* population structure in Ireland. Previous studies have shown that a combination of MIRU-VNTR typing and spoligotyping can differentiate more readily between *M. tuberculosis* strains [26-27]. However, in a previous Irish study using both spoligotyping and MIRU-VNTR typing, MIRU-VNTR typing identified clusters among spoligotype groups, thus providing supporting evidence that MIRU-VNTR typing is a more discriminatory typing method [22]. The discriminatory power of the 24-locus MIRU-VNTR panel used in this study has shown to be similar to IS*6110* RFLP analysis [5]. However, the discriminatory power of 24-locus MIRU-VNTR typing differs among genetic lineages, and the inclusion of

Figure 1

Distribution of *Mycobacterium tuberculosis* lineages by site of infection, Ireland, 2011 (n=136)

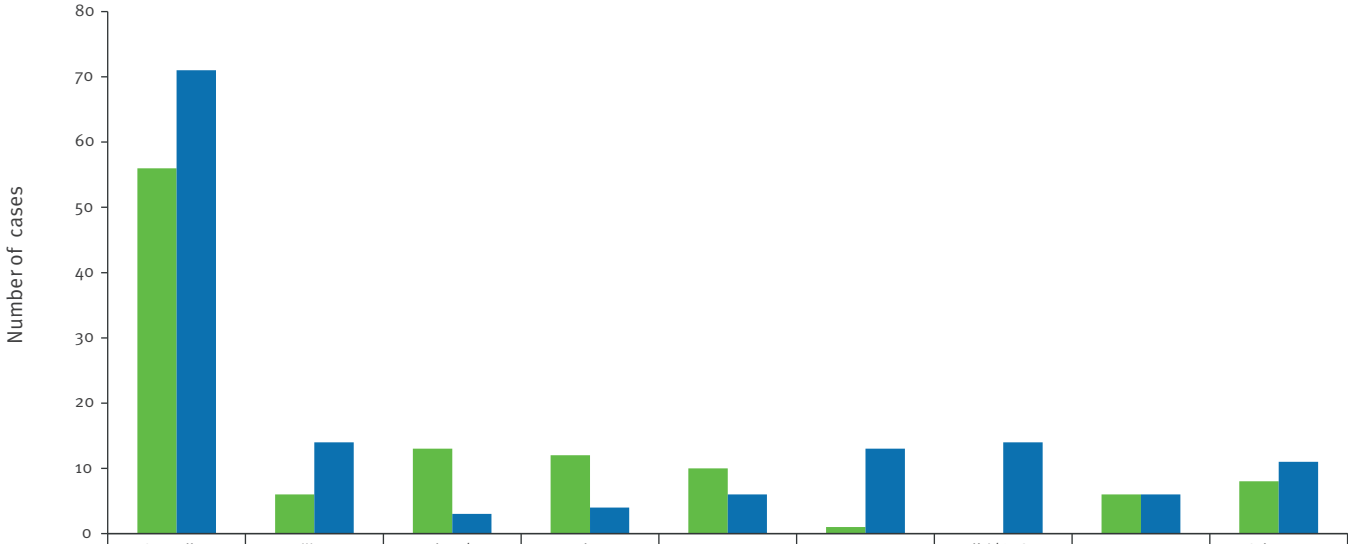


	Beijing n=21	Haarlem/X n=18	Haarlem n=17	LAM n=17	EAI n=16	Delhi/CAS n=14	H37Rv n=13	Others n=20
Extra-pulmonary	3	2	3	0	7	8	3	4
Pulmonary	16	16	13	15	8	4	10	16
Both	2	0	1	2	1	2	0	0

CAS: central-Asian; EAI: east-African–Indian; LAM: Latin American–Mediterranean.

Figure 2

Distribution of lineages among Irish and non-Irish typed *Mycobacterium tuberculosis* cases, Ireland, 2011 (n=127)



	Overall (n=127)	Beijing n=20	Haarlem/X n=16	Haarlem n=16	LAM n=16	EAI n=14	Delhi/CAS n=14	H37Rv n=12	Others n=19
Irish	56	6	13	12	10	1	0	6	8
Non-Irish	71	14	3	4	6	13	14	6	11

CAS: central-Asian; EAI: east-African–Indian; LAM: Latin American–Mediterranean.

additional hypervariable loci may be required to differentiate among strains of lineages 2 (Beijing) and 3 (Delhi/central-Asian). For enhanced cluster or outbreak analysis, whole-genome sequencing has been shown to differentiate among strains with identical 24-locus MIRU-VNTR patterns [28-29]. The role of whole-genome sequencing in investigating community outbreaks in the UK was reported recently [29]. Walker et al. estimated that the rate of genetic changes was 0.5 single nucleotide polymorphisms (SNPs) per genome per year. Furthermore, the maximum number of genetic changes over three years would be five SNPs and 10 SNPs over 10 years [29]. It has also been proposed that clustering of isolates increases over longer periods as transmission chains are more efficiently analysed and reported [30]. But the *M. tuberculosis* genotype involved in the cluster must be considered as for example the Beijing lineage has increased ability to spread and cause disease. While clustering was limited in our study, the study period was too short to draw clear conclusions.

Although the reproducibility of MIRU-VNTR typing has been well documented, results from the first worldwide proficiency study on this method were surprising [7]. Intra- and inter- laboratory reproducibility varied depending on the typing methods employed in each laboratory. In our setting, when the commercial MIRU-VNTR typing kit was used to analyse the quality control panel, 100% concordance was achieved with the reference data (30/30 tested strains) and 100% intra-laboratory reproducibility was achieved. These findings are important to consider when typing data is exchanged between laboratories.

Although six of the 11 MIRU typing clusters identified during 2011 were not confirmed as outbreaks by public health departments, it is possible that the reason why four of these clusters did not meet the Irish TB outbreak case definitions was the small number (n=2) of involved cases [2].

In summary, this study has provided the first insights into the structure of the *M. tuberculosis* population in Ireland. Although the incidence of TB has remained static in Ireland over the last decade, there has been mass immigration to this island nation. Not surprisingly, lineage 4 predominated among circulating strains of *M. tuberculosis* in the present study. But the degree of diversity among *M. tuberculosis* was unexpected. Future studies in the IMRL involving retrospective genotyping analysis of *M. tuberculosis* isolates collected since 2000 may provide an interesting epidemiological picture. Continued molecular surveillance is important as it has been suggested that the transmissibility profile of *M. tuberculosis* strains may be influenced by their genetic and evolutionary background. This understanding of the dynamics of *M. tuberculosis* strains will provide novel insights into the *M. tuberculosis* population structure and how it relates to the epidemiology of TB in Europe and beyond.

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Imported diphyllobothriasis in Switzerland: molecular methods to define a clinical case of *Diphyllobothrium* infection as *Diphyllobothrium dendriticum*, August 2010

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Following a first clinical case of infection by *Diphyllobothrium dendriticum* in Switzerland in 2006, we report a second case in the country. The species was identified by molecular methods. In the Swiss, French and Italian subalpine regions, human diphyllobothriasis has seen a comeback since the late 1980's, and *Diphyllobothrium latum* is usually considered the causative agent of the disease. In addition, several locally acquired and imported clinical infections due to allochthonous *Diphyllobothrium* species have been documented in the last years. Due to the colonisation potential of these parasites and their probably underestimated presence in the human population, there is a need for discriminating them at the medical laboratory level. Because the morphological characters are very similar among the different taxa, a correct identification requires the use of molecular methods. Molecular identification would improve diagnosis and help monitor the distribution of *Diphyllobothrium* species in Europe.

Introduction

Diphyllobothriasis is due to tapeworms of the genus *Diphyllobothrium* and is acquired through the consumption of raw or poorly cooked freshwater fish. In distinct stages of their life cycle, *Diphyllobothrium* spp. occupy different host species. The eggs present in the water are ingested by small crustaceans, such as copepods, constituting the first intermediate hosts in which they develop to a larval stage. As crustaceans are predated by fish, fish become the second intermediate hosts where *Diphyllobothrium* larvae further develop. The definite hosts are fish eating mammals (including humans) or birds, where *Diphyllobothrium* matures into an adult stage.

Human diphyllobothriasis is often a mild illness: about half of the patients are asymptomatic, while the others mostly suffer from minor discomforts, such as diarrhoea, abdominal pain and digestive troubles. Rare cases of megaloblastic anaemia associated with vitamin B12 deficiency have been reported in the literature [1].

Globally, the incidence of human diphyllobothriasis has decreased in the last 20 years, particularly in northern and eastern Europe [2]. Nevertheless, in some Swiss, French and Italian subalpine regions the disease has seen a comeback, as shown by the more than 530 cases reported since 1987 from around lakes Leman (Geneva), Morat, Bienne, Maggiore, Lario (Como), Iseo and Garda [2]. In these areas, the species *Diphyllobothrium latum* is considered the causative agent of diphyllobothriasis. However, infections due to allochthonous species (*D. nihonkaiense, D. dendriticum*) have been recently documented [3-7]. A first clinical case due to *D. dendriticum* was diagnosed in 2006 in Switzerland, raising the question of potential transmission to susceptible intermediate hosts present in the local environment [4]. In this report, we describe a second case of symptomatic infection by *D. dendriticum* in Switzerland. Confirmation of the species was done by molecular identification.

A four year-old boy expelled tapeworm segments in stool, 12 days after returning from a 15-day holiday travel in Singapore and Bali in August 2010. He had been suffering from abdominal pains (cramps) and loose stools since his return to Switzerland. Standard laboratory procedures held in the clinical laboratory (Dianalabs) led to the identification of *Diphyllobothrium* spp., based upon the presence of typical operculated eggs in segments. However, because of the unusual shape of a tapeworm proglottid (longer than wide as if stretched, with an excentred uterus) and the possible Asian origin of infection, the specimen was preserved in 70° ethanol and sent to the Institute of Parasitology in Bern and to the Cantonal Institute of Microbiology in Bellinzona, where it was identified as *D. dendriticum* by molecular methods.

Mebendazole was first administered to the patient, with no curative effect, as confirmed by the persisting presence of eggs in stool after three weeks. The patient was then re-medicated with praziquantel and recovered promptly. No parasites were found upon stool testing six weeks after praziquantel therapy.

The patient's family did not present with symptoms and underwent no further investigation, except for the seven year-old patient's sister who was checked for intestinal parasites but found negative.

Methods

The faecal specimen was processed by standard sedimentation technique [8] to concentrate putatively present *Diphyllobothrium* eggs and subsequently assess these by light microscopy. A segment of proglottids of approximately 5 cm length was processed for staining with lacto-acetic carmine according to Rukhadze and Blajin [9].

Genomic DNA from about 50 mg of proglottid tissue was extracted with the DNeasy Blood and Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was performed using the Taq PCR Master Mix Kit (Qiagen) with primers targeting a region of the 5.8S ribosomal RNA (5.8S rRNA) comprising internal transcribed spacers (ITS) 1 and 2 [10], the 18S ribosomal RNA (18S rRNA) [11] and the cytochrome c oxidase subunit 1 gene (cox1) [3,12] sequences. The amplification of all targets was carried out under the following conditions: 5 min at 94 °C, 35 cycles consisting of 30 s at 94 °C, 40 s at 45 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Amplicons were visualised by electrophoresis in a 0.8% agarose gel containing ethidium bromide, and purified through Sephadex G-50 columns (GE Healthcare). DNA was quantified with a ND-100 Spectrophotometer (NanoDrop Technologies Inc.). Sequencing was performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the provider's recommendations. Samples were purified by osmosis with 0.025 µm nitrocellulose filters (Millipore) in tris ethylenediaminetetraacetic acid (TE) buffer pH 8 for two hours. Eight µl of purified solution were placed in 0.5 ml Genetic Analyzer Sample Tubes with 12 µl Hi-Di Formamide (Applied Biosystems). Samples were then loaded in an automated sequencing system (ABI PRISM 310 Genetic Analyzer; Perkin Elmer).

Sequence electropherograms were corrected by using the software EditSeq (DNASTAR Inc.). Their identity was first checked by basic local alignment search tool (BLAST) [13]. Sequence fragments of 657 and 375 nucleotides in length, derived from the PCRs targeting the ITS1-5.8SrRNA and cox1 genes were then respectively compared to representative ITS1-5.8SrRNA or cox1 sequences from different *Diphyllobothrium* spp. by pairwise and multiple alignments using ClustalW [14] with the software Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 [15]. Phylogenetic trees (neighbour-joining method; Kimura-2 parameters; bootstrap test for 500 replicates) were subsequently inferred from the alignments.

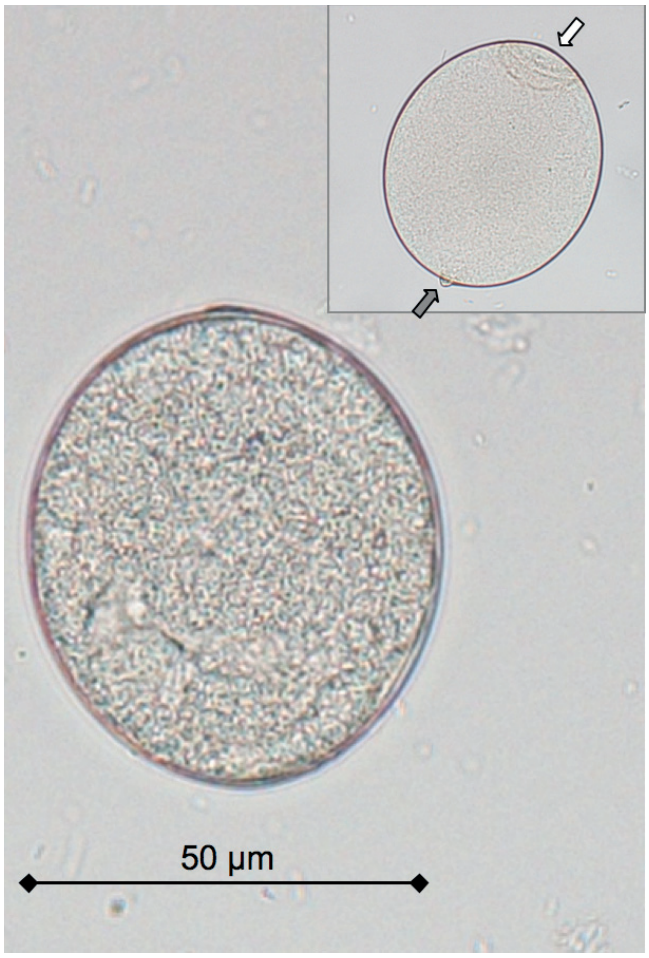
Results

Microscopical analyses of the coprological sediment revealed the presence of oval-shape unembryonated eggs (mean size: 49 x 64 µm; range: 48.5–52.5 x 62.5–70 µm), characterised by the presence of a hardly visible operculum and a small knob at the abopercular end (Figure 1).

Microscopical analyses of the stained proglottids revealed the presence of only one set of reproductive organs per proglottid (Figure 2). The central uterine structure showed several rosette-shaped loops. Morphological criteria matched to those described

Figure 1

Diphyllobothrium dendriticum eggs recovered from a patient stool, Switzerland, 2010

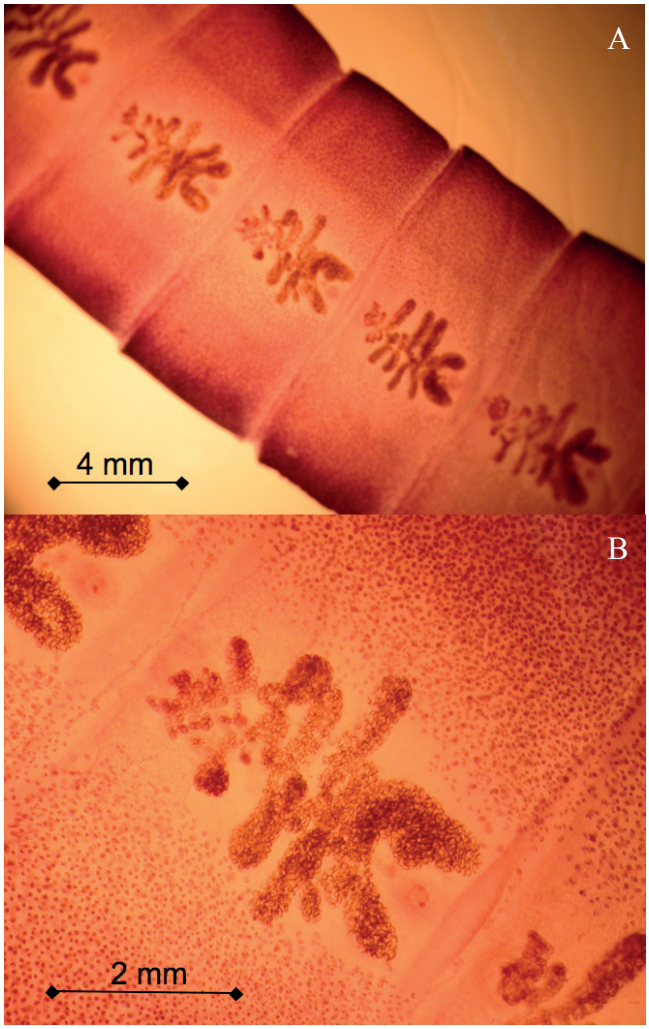


White arrow: operculum; grey arrow: knob.

The pictures were taken under 400x magnification and the mean size of the eggs was 49 x 64 µm.

Figure 2

Diphyllobothrium dendriticum segment recovered from a patient stool, Switzerland, 2010



A. *Diphyllobothrium dendriticum* segment.
B. Enlargement of an individual proglottid where the central uterine structure forms several rosette-shaped loops.

for *Diphyllobothrium* spp. [16], while a species-specific identification was not possible based upon morphology.

The nucleotide sequences of the ITS1-5.8S rRNA, 18S rRNA and cox1 derived from the *Diphyllobothrium* affecting the patient were deposited in public databases and are available in the European Molecular Biology Laboratory (EMBL), GenBank and DNA Data Bank of Japan (DDJB) databases under accession numbers HQ682065, HQ682066 and HQ682067. Due to technical problems, the sequence of ITS2 was not fully obtained and therefore not used in the analysis.

The results of BLAST search showed that all the sequenced targets respectively reached ≥99% identity (highest scores) at the nucleotide level with *D. dendriticum* homologous reference sequences of respective GenBank accession numbers FM204787 (ITS1-5.8S rRNA), DQ768164, DQ181945 (18S rRNA) and AM412738 (cox1). The 18S rRNA sequence also showed 99% identity with those of *D. ditremum* (GenBank accession numbers: DQ768165, DQ181944) and *D. latum* (GenBank accession number: DQ316795).

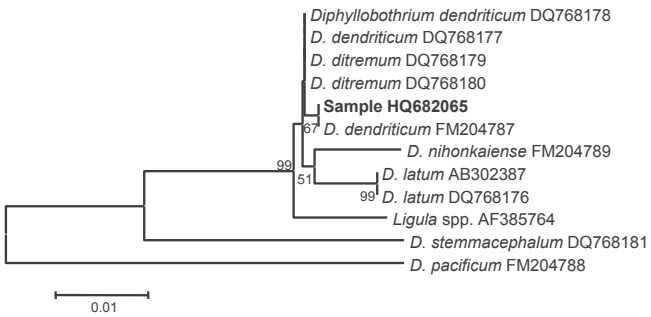
The position of the sample sequences of ITS1-5.8S rRNA and cox1 regions in the phylogenetic trees confirmed the *Diphyllobothrium* spp.affecting the patient as *D. dendriticum* (Nitsch, 1824) (Figures 3 and 4). The phylogenetic tree built on the basis of 18S rRNA sequences is not shown, because this target is not useful for the discrimination of *D. dendriticum* from other species [2].

Discussion

At the time of tapeworm evacuation, the patient was known to have had a meal in a Japanese restaurant in Singapore (on day 4 of his journey) where the family had consumed various dishes (fish, chicken). According to the recollection of the family members, the meal did not contain raw fish. Retrospective investigations revealed that the patient regularly consumed fish, e.g. smoked salmon with pasta at home. This salmon was always bought in the same department store in France and was of the same brand. According to the product information, it belonged to the species *Salmo salar,* was farmed in Norway, smoked in France and guaranteed 'never frozen'. The homemade sauce was made by dropping slices of smoked salmon into boiling cream

Figure 3

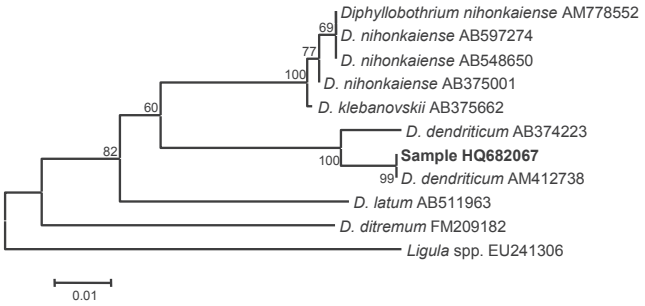
Neighbour-joining tree based on 5.8S ribosomal RNA internal transcribed spacer 1 sequences, to determine the *Diphyllobothrium* species affecting a patient, Switzerland, 2010



The tree is based on 5.8S ribosomal RNA internal transcribed spacer 1 (5.8S rRNA-ITS1) sequence fragments of 657 bp. The 'sample' refers to the sequence of the unknown *Diphyllobothrium* spp. affecting the patient. GenBank accession numbers of all the sequences used to construct the tree are indicated. On the tree nodes, Kimura-2 parameters bootstrap values >50 for 500 replicates are shown.

Figure 4

Neighbour-joining tree based on cytochrome c oxidase subunit 1 sequences, to determine the *Diphyllobothrium* species affecting a patient, Switzerland, 2010



The tree is based on cytochrome c oxidase subunit 1 (cox1) sequence fragments of 375 bp. The 'sample' refers to the sequence of the unknown *Diphyllobothrium* spp. affecting the patient. GenBank accession numbers of all the sequences used to construct the tree are indicated. On the tree nodes, Kimura-2 parameters bootstrap values >50 for 500 replicates are shown.

and leaving to cook for one minute before serving. According to his mother, the patient enjoyed picking pieces of uncooked fish during the preparation of the meal. Short before travelling to south-east Asia, the patient consumed poorly cooked perch fillets (*Perca fluviatilis*) fished in the Leman lake in a restaurant near home. He also ate sushi in Switzerland one week before tapeworm segments were noticed.

Aquatic birds (especially Laridae) are usually the definitive hosts of *D. dendriticum*, while humans are only occasionally infected. According to literature, the incubation period of *D. dendriticum* in herring gulls (*Larus argentatus*) lasts from five to 20 days [17]. In humans, *Diphyllobothrium* plerocercoids generally develop into mature adults in two to six weeks [2].

Based on these observations, the source of the patient's infection might be either salmon from Norway, unknown fish from Asia or perch from Switzerland. However, because the presence of *D. dendriticum* in perch has not been documented so far, it is unlikely that the perch meal was the source of infection. The sushi meal in Switzerland can be excluded because the symptoms were already present at that time.

In the last two decades, diphyllobothriasis has shown a recrudescence in a number of European countries, especially in the subalpine lakes region [2]. The use of molecular methods also showed the presence of allochthonous *D. nihonkaiense* and *D. dendriticum* in France, Switzerland, Finland and the Czech Republic (Table). Except for two cases of *D. dendriticum* diagnosed in Switzerland [4] and the Czech Republic [18] that were probably acquired abroad (Norway, Alaska or Canada), most of the documented infections were locally acquired, attributed to imported salmons.

Susceptible intermediate hosts for *D. dendriticum* such as copepods (*Eudiaptomus* and *Cyclops species* [19]) and fish (*Oncorhynchus mykiss, Salmo trutta, Coregonus clupeaformis, C. albula, C. lavaterus* [19-21]) are known to be present in Switzerland. This would theoretically allow the introduction and the autochtonous transmission of the parasite. This colonisation potential emphasises the need for correct identification of *Diphyllobothrium* species involved in clinical cases of infection.

Interestingly, in the two Swiss cases due to *D. dendriticum*, the molecular investigation was undertaken because of the unusual shape of some segments.

The transmission of *Diphyllobothrium* plerocercoids may be prevented by freezing fish at -20 °C for one to seven days, depending on its thickness. The Swiss law [22] provides that it is forbidden to sell any kind of fresh fish products (both local and imported) intended to be consumed raw or semi-raw, unless they have been stored at a temperature not higher than -20 °C for a least 24 hours. However, it has been demonstrated that this rule is not always followed properly [23], and of course there is no control of food bought abroad by individuals for personal use. Therefore cooking at 55 °C or more, for at least 5 min, remains the most reliable way to prevent transmission of parasitic worms possibly present in fresh fish [2].

Conclusion

A correct diagnosis has become crucial to evaluate the distribution of human-infecting *Diphyllobothrium* species, as well as their fish hosts, and to prevent the spread of allochthonous parasites in aquatic environments. Due to the difficulties in discriminating the different *Diphyllobothrium* taxa by morphological characters, molecular analysis has proven to be fundamental to identify these helminths at the species level. A cheap and rapid molecular test based on multiplex PCR with partial cytochrome *c* oxidase subunit 1 (cox1) gene, without the need of sequencing, was recently developed for the differential identification of the most common species infecting humans [24]. In case of atypical specimens of proglottids and eggs and/or specimens derived from patients who have been abroad, this test could be used to verify the parasite's identity. Improving the diagnosis of *Diphyllobothrium* parasites would help to monitor the distribution of species in Europe and trace the source of infections, an important goal at a time when eating habits are changing, fish markets are globalising and climate is changing [25].

Table

Case reports of allochthonous *Diphyllobothrium* infections in Europe, identified by molecular methods, 2005–2011

Year of parasite recovery	Country	Patient (age in years)	*Diphyllobotrhium* species	Parasite characteristics	Clinical features	Probable source of infection	Molecular identification	Therapy	Reference
2005	Finland	Man (60)	*D. nihonkaiense*	ND	ND	ND	Cox1	ND	Data not shown
	France	Woman (44)	*D. nihonkaiense*	Egg size 41 × 60 µm	Nausea, epigastric pain, diarrhoea	Wild salmon carpaccio (*Oncorhynchus keta*) imported from Canada (Gulf of Alaska, Pacific Ocean), purchased in France	Cox1, MT-ND3	Single dose of praziquantel at 10 mg/kg.	Yera et al., 2006 [6]
	Switzerland	Woman (55)	*D. nihonkaiense*	Egg size 56.68–57.82 × 43.58–45.42 µm	Diarrhoea (probably due to another cause); proglottids passed in faeces	Raw salmon and sushi purchased in Switzerland	18S rRNA, cox1, ITS1 and 2	Praziquantel	Wicht et al., 2007 [3]
2006	Switzerland	Woman (52)	*D. nihonkaiense*	ND	None	Raw Pacific salmon (*O. keta*) imported from Canada or North America (Pacific Ocean), purchased in France	18S rRNA, cox1, ITS1 and 2	Single dose of praziquantel	Wicht et al., 2007 [3]
	Switzerland	Boy (5)	*D. nihonkaiense*	Egg size 57.5–65.0 × 40.0–42.5 µm	Mild eosinophily (7.1%)	Pacific salmon purchased in Switzerland	ITS1, cox1, MT-ND3	Single dose of praziquantel at 12 mg/kg	Shimizu et al., 2008 [5]
	Switzerland	Woman (59)	*D. dendriticum*	Egg size 49.62–63.86 × 35.75–43.41 µm; some proglottids longer than wide, with excentred utera	Chronically relapsing courses of diarrhoea	Regular consumption of wild salmon, Japanese sushi or fish carpaccio; journeys in Canada, Alaska and Norway	18S rRNA, cox1	Single dose of praziquantel at 10 mg/kg	Wicht et al., 2008 [4]
2008	France	Woman (33)	*D. nihonkaiense*	Egg size 57 × 44 µm	Persistant, mild eosinophily	Wild salmon carpaccio or marinade purchased in France	Cox1	Single dose of praziquantel at 15 mg/kg	Paugam et al., 2009 [7]
2010	Switzerland	Boy (4)	*D. dendriticum*	Egg size 48.5–52.5 × 62.5–70 µm; one of the proglottids longer than wide, with excentred uterus	Abdominal cramps, loose stools	Smoked salmon (*Salmo salar*) imported from Norway, purchased in France; poorly cooked perch from Switzerland; fish eaten in Asia	ITS1 and 2, 18S rRNA, cox1	Praziquantel	Present paper
2011	Czech Republic	Woman (28)	*D. dendriticum*	Egg size 49.5 × 64 µm	No symptoms reported; proglottids passed in faeces	Salmons (*O. tshawytscha, O. keta, O. kisutch, O. nerka, O. gorbuscha*) and *Coregonus autumnalis* eaten in Alaska	Cox1	Single dose of praziquantel (750 mg)	Kuchta et al., 2012 [18]

Cox1: cytochrome c oxidase subunit 1; ITS: internal transcribed spacer of the 5.8S ribosomal ribonucleic acid; MT-ND3: mitochondrially encoded NADH dehydrogenase 3; NADH: reduced nicotinamide adenine dinucleotide; ND: not determined; rRNA: ribosomal ribonucleic acid.

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Molecular-based surveillance of campylobacteriosis in New Zealand – from source attribution to genomic epidemiology

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Molecular-based surveillance of campylobacteriosis in New Zealand contributed to the implementation of interventions that led to a 50% reduction in notified and hospitalised cases of the country's most important zoonosis. From a pre-intervention high of 384 per 100,000 population in 2006, incidence dropped by 50% in 2008; a reduction that has been sustained since. This article illustrates many aspects of the successful use of molecular-based surveillance, including the distinction between control-focused and strategy-focused surveillance and advances in source attribution. We discuss how microbial genetic data can enhance the understanding of epidemiological explanatory and response variables and thereby enrich the epidemiological analysis. Sequence data can be fitted to evolutionary and epidemiological models to gain new insights into pathogen evolution, the nature of associations between strains of pathogens and host species, and aspects of between-host transmission. With the advent of newer sequencing technologies and the availability of rapid, high-coverage genome sequence data, such techniques may be extended and refined within the emerging discipline of genomic epidemiology. The aim of this article is to summarise the experience gained in New Zealand with molecular-based surveillance of campylobacteriosis and to discuss how this experience could be used to further advance the use of molecular tools in surveillance.

Controlling campylobacteriosis – recent successes

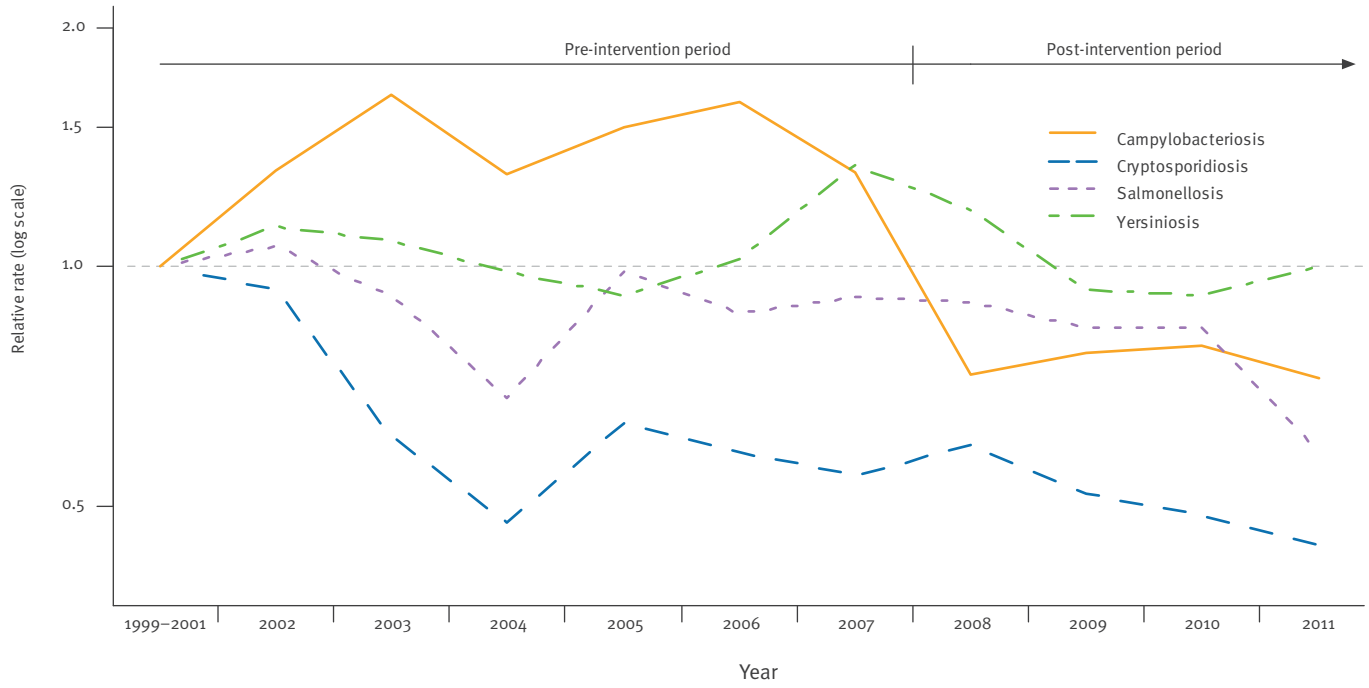
Molecular tools are being used increasingly to inform the control of enteric zoonosis worldwide [1] and to meet a wide range of public health aims and objectives [2-4]. In New Zealand, a country with a historically high rate of campylobacteriosis notifications [5,6], results from molecular-based surveillance in a sentinel site founded in 2005 – where human cases and potential sources were sampled and typed by multilocus sequence typing (MLST) simultaneously over consecutive years [7,8] – provided strong evidence that a large proportion of human cases were linked poultry meat consumption. These findings contributed to a mounting body of evidence [5,9] and stimulated the implementation of regulatory and voluntary control strategies along the poultry supply chain. They were announced in 2007 and fully implemented in 2008 (when they became mandatory) [10], resulting in a 50% reduction in disease incidence of cases in 2008 compared with the previous high level during 2002 to 2006 [10,11].

Campylobacteriosis notifications in humans were markedly above the reference value until 2008, when the incidence dropped considerably (Figure 1); a likely effect of a reduction in poultry-associated cases due to the implementation of the control strategies in the poultry supply chain [10,11]. No comparable changes in the annual incidence of other enteric notifiable diseases were observed over the same time period (2002–2011) (Figure 1). Sustained decline in campylobacteriosis case numbers has been shown to have additional health and economic benefits by, for example reducing the incidence of Guillain–Barré syndrome, an autoimmune condition associated with prior *Campylobacter* spp. infection [12].

Furthermore, in the New Zealand sentinel surveillance site, a dominant poultry-associated MLST sequence type of *C. jejuni* (ST-474) was identified that, to date, has been reported rarely from other countries. Before the implementation of the poultry interventions, ST-474 accounted for 30% of human cases in the sentinel site [14,15], but in 2010–11, it was isolated from less than 5% of cases [16]. Figure 2 shows the dramatic reduction in two major poultry-associated genotypes, ST-474 and ST-48 (Figure 2, panel A), and provides a comparison with other STs over the same time period (Figure 2, panels B and C).

Figure 1

Relative rates[a] of notification of campylobacteriosis, cryptosporidiosis, salmonellosis and yersiniosis, New Zealand, 2002–2011 compared with 1999–2001



[a] Rates were calculated using a negative binomial model, which was used to estimate the change in incidence between each year from 2002 to 2011 and the reference period of interest, 1999–2001, as previously described by Henao et al. [13]. Values above the reference line indicate increases in notification incidence and points below the line show decreases, relative to the 1999–2001 reference period.

The pre- and post-intervention periods refer to the implementation of a number of control measures in the poultry supply chain by the regulatory authority. The annual incidence of other enteric notifiable diseases (cryptosporidiosis, salmonellosis and yersiniosis) over the same time period is displayed to show that notification rates were stable for other comparable disease and that the drop in campylobacteriosis notifications was not a surveillance artefact.

Focused molecular epidemiological studies have been contributing to our understanding of the epidemiology of this widespread disease both in New Zealand and elsewhere [7,14,15,17]. For example, the association between ruminant-associated genotypes and preschool-age children (0–5 years of age) in rural areas has provided evidence for direct contact with faecal material being the foremost infection route in this high-incidence group [14].

This is of high relevance for the development and evaluation of appropriate, country-specific control strategies to decrease the human disease burden. Since the number of human cases linked to poultry has fallen in New Zealand, there has been a relative increase in importance of ruminant strains of *C. jejuni*, and ongoing work is investigating the complex epidemiology of *Campylobacter* in ruminant [18] and wildlife sources [19]. While this article describes the MLST-supported *Campylobacter* surveillance conducted at the sentinel site, other typing approaches are used to increase resolution of the molecular analysis. For example, research is currently underway to further differentiate between exposure to ruminant-associated *Campylobacter* subtypes of food and non-food origin to refine attribution estimates using antigen gene sequence typing [20], ribosomal MLST [21] and targeted genes identified by whole genome analysis [22]. In this article, we summarise the experience gained in New Zealand and discuss how this experience could be used to further advance the use of molecular tools in surveillance.

What have we learned?

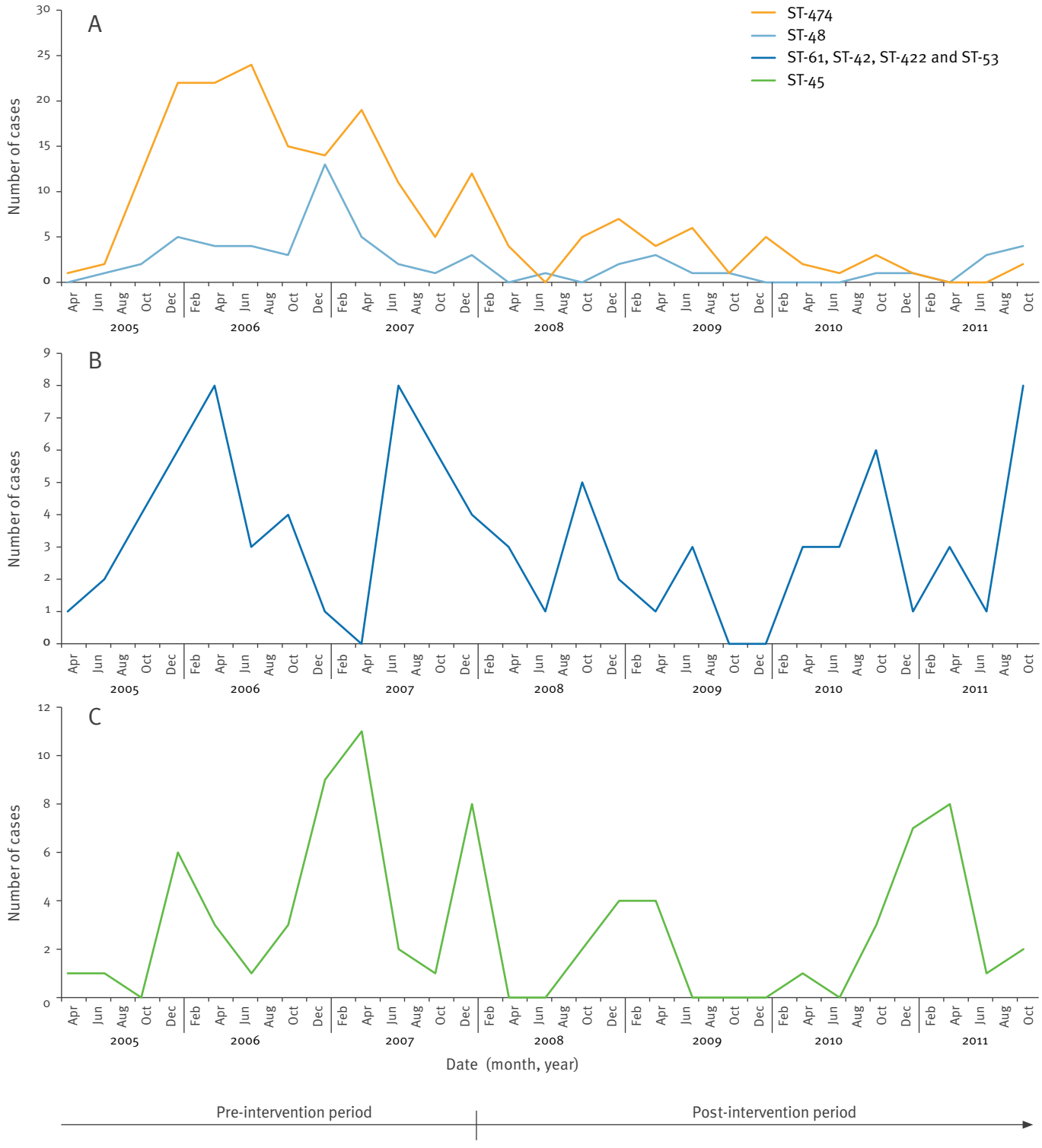
Experience from New Zealand and elsewhere has provided insight into key aspects of molecular-based surveillance. These include the following: (i) its application to both control-focused and strategy-focussed surveillance; (ii) a change in our definition of epidemiological response variables; and (iii) the emergence of genomic epidemiology.

Application of molecular tools to disease surveillance

The framework developed by Baker et al. [23], which differentiates between control-focused and strategy-focused surveillance, provides a meaningful way to

Figure 2

Human cases of campylobacteriosis caused by poultry- and ruminant-associated *Campylobacter jejuni* MLST types, as well as a ubiquitous ST in a sentinel surveillance site, New Zealand, 2005–2011



MLST: multilocus sequence typing; ST: sequence type.

Panel A shows the time series of human campylobacteriosis cases with two poultry-associated genotypes, ST-474 and ST-48 and illustrates the drop in the number of cases following interventions in the poultry production chain.

Panrel B shows the trend in human campylobacteriosis cases with ruminant-associated genotypes ST-61, ST-42, ST-422 and ST-53.

Panel C shows the time series of human campylobacteriosis cases with the ubiquitous ST-45.

categorise molecular approaches to disease surveillance. Approaches that are suitable for control-focused surveillance, such as those used in an outbreak setting, are potentially of lesser value for strategy-focused surveillance, where the aim is often to monitor long-term changes in epidemiology [24,25], and vice versa.

The purpose of control-focused surveillance is 'to identify each occurrence of a particular disease, hazard, or other health-related event that requires a specific response, and to support the delivery of an effective intervention' [23]. Such surveillance requires methods that have a high degree of timeliness, sensitivity and security (i.e. that can be maintained on an ongoing basis) [23]. The molecular typing tools and associated modelling approaches required for this objective need to be capable of identifying genotypes that indicate a common source of disease or highlight a particular transmission pathway. Often, but not always, this is achieved through highly discriminatory typing tools.

Using a recently developed model-based tool for identifying clusters of campylobacteriosis cases related in space and time [26], eight cases in a small area of New Zealand's North Island were identified as having a high probability (>0.8) of being part of an anomalous cluster (i.e. they were more spatially and temporally localised than would be expected given the average temporal and spatial patterns). Inspection of the epidemiological information linked to each case revealed that they were reported within a single two-week period and typing data showed that they were all the same MLST sequence type (ST-520). When compared with a larger database of over 3,000 sequence types isolated from humans, animals and food in New Zealand it was shown that this type was associated with ruminants in New Zealand, but was a relatively uncommon cause of human infection. This finding triggered a more detailed investigation into the cases, requiring further contact with some affected individuals, which revealed that all cases had consumed unpasteurised (raw) milk – a relatively rare risk factor – and that 7/8 cases reported purchasing the milk from the same source farm. This combination of epidemiological information and typing data lead to a local response and also informed the ongoing debate on the national policy concerning the sale of raw milk.

The purpose of strategy-focused surveillance is 'to provide information to support prevention strategies to reduce population health risk, such as describing the epidemiology of the annual influenza season and the characteristics of the seasonal influenza viruses' [23]. Such surveillance requires methods that have a high degree of representativeness, completeness (referring to the data recorded with each event) and validity [23]. Different molecular and modelling approaches are required in this instance, with the optimal tools providing information on the long-term epidemiology of a pathogen rather than short-term changes. An example is the recent emergence of new approaches to source attribution using molecular subtyping, which has been used successfully in several countries to understand the relative contribution of different sources to the burden of human campylobacteriosis and salmonellosis [27-30]. Source attribution models based on microbial subtyping were initially developed in Denmark as a tool for salmonellosis risk management [31]; they provide estimates of the number of human cases originating from different sources or reservoirs based on a comparison of genotypes [31,32].

In New Zealand, attribution models were adapted to data from the MLST surveillance site. Two models were used, a population genetics-based attribution model [32] and the microbial subtyping-based model by Hald et al. [31], to quantify the contribution of selected sources to the human disease burden. These studies revealed that between 2005 and 2008, poultry was the leading source of human campylobacteriosis, causing an estimated 58–76%, of notified cases [8]. Contributions by individual poultry suppliers showed wide variation and supplier specific strains were detected [15]. The use of these models to monitor changes over time and to assess the effectiveness of interventions is ongoing [10,11].

Re-defining response and explanatory variables using molecular tools – a new epidemiological approach to inform surveillance?

A common starting point of epidemiology is seeking non-random associations between response variables and potential explanatory variables. Regression modelling, for example, may be used to identify statistically significant predictors of increased risk of adverse health effects [33]. However, the use of such traditional methods for quantifying the contribution of different sources of campylobacteriosis to the disease burden in New Zealand (notably case–control studies [9,34], which identified poultry as the major source of human infection) had not provided sufficient compelling evidence for decision-makers to invest in controlling the poultry source. The epidemiology of campylobacteriosis is challenging: as a multi-host pathogen, infection with *C. jejuni* is associated with a large number of risk factors [35] and human cases arising from exposure to different sources may have very different risk factors, some of which may even be protective for some sources and increase the risk for others.

Using molecular tools, pathogen genetics and evolution can be incorporated into epidemiological modelling to make inferences about disease or transmission risks rather than simply relying on the association of response and explanatory variables. Such tools can be used to refine outcome variables, for example by using case–case comparison of poultry- and ruminant-associated cases of campylobacteriosis to identify more subtle associations [14,17] or to investigate the cause of a disease outbreak [24]. However, greater epidemiological gains are likely to be made when models combine pathogen evolution and transmission in

an integrated way [36,37]. This may be best achieved by modelling a relatively low number of isolates with high-coverage sequence data, such as increasingly available full genome data [38] or a larger number of isolates with low coverage such as a 7-locus MLST scheme. The additional information provided even by routinely applied molecular tools such as pulsed-field gel electrophoresis (PFGE) adds to our understanding of epidemiological variables. For example, the level of similarity and relatedness of restriction-enzyme profiles in the analysis of a food-borne outbreak can be directly used to refine epidemiological investigations. It is the synergy between the epidemiological and typing information that makes molecular tools so powerful and novel modelling approaches are constantly being developed to advance research at this interface [39,40].

Into the future: genomic epidemiology

New modelling approaches are being adopted to utilise the abundance of molecular data available [24,39,40]. Bell et al. [41] argue that the enormous volumes of data that can be provided by new technology provides many challenges for data management and analysis, and that we have entered a new area of data-intensive science that requires specialised skills and analytical tools. This argument holds true in the area of molecular epidemiology: next generation high throughput sequencing has vastly increased the availability of pathogen genome sequence data [38] and as the costs decrease, these tools will be more frequently incorporated into epidemiological studies and surveillance. By fitting statistical genetics and epidemiological models to sequence data, and combining these within a single framework [42], new insights can be gained into pathogen evolution, the nature of associations between strains of pathogens and host species, the timing of emergence, origin and geographical spread of pathogens, and aspects of between-host transmission [43]. Furthermore, advances in statistical methods for modelling evolutionary ancestry are resulting in better reconstructions of pathogen genealogies and improved estimates of evolutionary parameters. Although complex in nature, these models can be extremely valuable – for example, they can be used to enable the contribution of different sources and transmission pathways to the human disease burden to be determined [32].

In New Zealand, whole genome sequencing is being used to understand the evolution of epidemiologically important strains of *C. jejuni* and identify potential markers for host association [44]. This may help to improve the discrimination of sources of human infection, such as between cattle and sheep, and result in more precise source attribution estimates. Similarly, full genome sequence data from multiple *Campylobacter* isolates and *Escherichia coli* O157 are being combined with phenotypic microarray data to improve the understanding of the relationship between phenotype and genotype. The identification of genetic markers for stress resistance, such as pH, temperature, oxidative stress, and freeze-thaw [45], could help to determine which sources and transmission pathways strains isolated from humans have been acquired from, further refining attribution studies and strategy-focused surveillance.

By furthering our understanding of host associations with particular strains of pathogens, and the relative rates of transmission between animals and humans, the melding of statistical genetics and epidemiology with partial and full genome sequence data will further inform and refine control strategies for enteric pathogens in New Zealand and elsewhere.

Conclusion

New Zealand provides a distinctive island ecosystem in which to study infectious diseases [46]. The relative isolation and management of farmed livestock has contributed to the epidemiology and population structure of microbial pathogens. For example, the country's poultry industry is structured in a way that is different to most countries, with no importation of untreated poultry products and freedom from several important poultry diseases such as Newcastle disease and *Salmonella enterica* serovar Enteritidis PT4. Furthermore, the production of poultry meat is highly integrated, with three companies supplying about 90% of all chicken meat [15]. In addition a risk management strategy developed by the regulator supports a strong collaboration with researchers and science-based decision-making [47]. While the situation in other countries is likely to be more complex, for example through the presence of federal regulations or the risks associated with poultry importations, lessons learned from New Zealand can be applied elsewhere.

The New Zealand approach, which includes the first large scale implementation of effective regulatory *Campylobacter* control measures in broilers, is of high relevance internationally, including Europe. Findings have been incorporated in scientific opinions of the European Food Safety Authority. In 2008, it was acknowledged that the MLST approach to source attribution developed in New Zealand may be the way forward [48] and the approach is being used in several European countries, including the Netherlands and Scotland [2,49]. The New Zealand experience was also included in an assessment of the extent to which meat derived from broilers contributes to human campylobacteriosis at the European Union level [50].

The molecular tools deployed in epidemiological and evolutionary analyses clearly need to be fit-for-purpose. Ideally, during their development phase, measures of their utility in specific settings, such as discriminatory power and the strength of association between genotype and host, should be considered and attempts made to optimise their performance for the outcome in mind. In the case of 7-locus MLST, for example, retrospective analyses have shown this to be a valuable approach for certain types of surveillance,

including reservoir attribution, but the method was not designed for this purpose and an alternative approach based on a different set of gene targets may perform better and be more cost-effective. Equally important are rigorous sampling size considerations and guidance on the number of samples from different sources to acquire a desired level of precision – for example, in source attribution estimates. Further work will be necessary to develop expert agreement and sound working principles on these matters.

The field of molecular epidemiology is continually evolving and its role in advancing our ability to understand and control infectious diseases will also keep increasing. Its interdisciplinary nature will provide key support to One Health approaches to disease control, by supplementing medical and veterinary expertise with an in-depth understanding of the molecular biology of pathogens. As genotyping approaches and analytical models continue to evolve, an understanding of the complex interface of both disciplines becomes a crucial element of molecular-based disease surveillance. In New Zealand, we have learned that close collaboration between laboratories and epidemiologists is extremely important for the success of molecular-based surveillance: in our example, this started when the sentinel surveillance site was first set up. In a small and geographically isolated country, such early collaboration is likely to be more easily achieved; nevertheless, the general principle still applies and could add value to molecular surveillance in other countries.

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ECDC starts pilot phase for collection of molecular typing data

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The European Surveillance System (TESSy) molecular surveillance service (MSS) was launched in late November 2012. This new service enables European Union and European Economic Area countries to upload detailed molecular typing data for *Salmonella*, *Listeria*, *E. coli* (VTEC/STEC) and multidrug-resistant *M. tuberculosis* (MDR-TB) to the MSS database, hosted by the European Centre for Disease Prevention and Control (ECDC). So far, 13 countries are providing data for the three food and waterborne pathogens, and it is likely that more countries will join the system in 2013. For *M. tuberculosis*, 23 countries are currently providing molecular typing data. It is likely that in the future, more pathogens will be added to the MSS.

The objective of the data is to (i) improve the speed of detection of dispersed international outbreaks, (ii) improve trace-back of the source of an outbreak and identify risk factors, and (iii) improve investigation of transmission chains across the EU and globally, and (iv) improve Member State response to outbreaks.

Nominated users in countries can query ECDC databases to see if isolates that are genetically similar to isolates found in their country have been detected in other countries. A team of curators guarantees the quality of the data and also performs routine cluster detection. If a cluster is found, countries can then use this cluster information for possible public health action. In such cases, the ECDC Epidemic Intelligence Information System will be used as before to help coordinate action across countries for *Salmonella*, *Listeria* and *E. coli* whereas the European Reference Laboratory Network for TB would be used in the case of MDR-TB. An evaluation of the pilot phase is planned for the end of 2013.

Call for applications for EPIET and EUPHEM fellows

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Applications are invited for fellow positions in the European Programme for Intervention Epidemiology Training (EPIET) and the European Public Health Microbiology Training (EUPHEM) programme which are coordinated and funded by the European Centre for Disease Prevention and Control (ECDC).

Closing date for the applications is 3 February 2013. The fellowship programmes will start in September 2013.

The European Programme for Intervention Epidemiology Training (EPIET) is a two-year fellowship programme, which provides training and practical experience in intervention epidemiology at the national and regional centres for surveillance and control of communicable diseases in the European Union (EU) and European Economic Area (EEA). The programme is aimed at medical practitioners, public-health nurses, microbiologists, veterinarians and other health professionals with previous experience in public health. Applicants should have a keen interest in epidemiology and be interested to learn how to control infectious diseases. The EUPHEM is a two-year training programme and will include training and practical experience in the public health microbiology area such as public health microbiology management, laboratory investigations, applied public health microbiology, bio risk management, outbreak investigation and research.

For more information and the application form see the ECDC home page and the EPIET and EUPHEM websites.

New issue of EpiNorth journal is online

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The latest issue of EpiNorth Journal (No. 3 Vol 13 – 2013) is available online at:

http://www.epinorth.org/eway/default.aspx?pid=230&oid=3403&trg=__new_5356&MainArea_5260=5299:0:15,2940:1:0:0:::0:0&__new_5356=3403:48080

It contains:

- Editorial - The EpiNorth Journal Will be History
- Public Health Preparedness in Mass Gatherings
- Emergence of Cases of Dirofilariasis in Novgorod Oblast, Russia, in 2010-2012
- Drug Addiction Influence on Incidence of Infections with Parenteral Transmission in St. Petersburg in 1999-2011
- Epidemiological Situation for Tick-Borne Encephalitis and Lyme Disease in the Republic of Karelia, Russia, in 2002-2011
- Activities Performed by the State Sanitary and Epidemiological Service of Ukraine in Control over Sanitary and Epidemiological Situation During EURO-2012
- Cholera Outbreak in Donetsk Oblast, Ukraine, in 2011
- EpiNorth Network - a New Project on Communicable Disease Cooperation in Northeastern Europe

This issue is the last issue of EpiNorth Journal. It has been published in English and Russian, online and in print EpiNorth Journal has been a non-profit publication, funded by the European Centre for Disease Prevention and Control (ECDC), the Norwegian Barents Health Programme and national institutes for infectious disease control in the EpiNorth region and been a part of the EpiNorth project.

At the end of 2013 the present form of the EpiNorth project will come to an end. As a consequence, the latest issue is the last published issue of the EpiNorth Journal. The new EpiNorth Network project is further described in this final issue.