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The emergence of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus amongst hospitalised immunocompromised patients in Scotland, November-December, 2009

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To investigate the frequency of oseltamivir resistance in circulating strains of the 2009 influenza A(H1N1) pandemic virus in Scotland, 1,802 samples from 1,608 infected hospitalised patients were screened by the H275Y discriminatory RT-PCR. Among these, we identified 10 patients who developed the H275Y mutation. All of them were immunocompromised and were under treatment or had been treated previously with oseltamivir.

Introduction

The 2009 influenza A(H1N1) pandemic virus emerged in Mexico in March 2009 and spread globally and uncontrollably during the following months. The World Health Organization (WHO) declared a pandemic caused by this virus on 11 June 2009. Since the first case diagnosed in Scotland in May 2009, a total of 6,450 laboratory confirmed 2009 pandemic virus infections leading to 62 deaths had been diagnosed in Scotland by 3 January 2010.

Initial testing of the 2009 pandemic virus found it susceptible to neuraminidase inhibitors, oseltamivir and zanamivir, but resistant to amantadine [1]. Oseltamivir has been used extensively for chemoprophylaxis and treatment for 2009 pandemic virus and the first sporadic cases of oseltamivir-resistant 2009 pandemic virus infection were reported at the end of July 2009 [2]. The emergence of oseltamivir-resistant 2009 pandemic virus remains a major concern, since widespread oseltamivir resistance has been observed in seasonal H1N1 viruses recently [3-5]. The emergence of oseltamivir-resistant seasonal influenza A(H1N1) viruses was first noted in Norway 2007 [3], and these resistant viruses have since evolved into the dominant influenza A(H1N1) seasonal viruses circulating in humans.

Resistance to oseltamivir in influenza A(H1N1) viruses caused by a histidine to tyrosine mutation at residue 275 of the neuraminidase protein (H275Y) was observed both in vitro and in vivo [6,7]. Very recently, this mutation has also been detected in in oseltamivir-resistant 2009 pandemic viruses in China, the United States (US), Vietnam and Canada [8-11]. Although there is no evidence so far that these viruses might have transmitted beyond close contact between cases, two clusters of immunocompromised hospitalised patients infected with the 2009 pandemic virus virus have been detected in Wales, United Kingdom (UK) [12] and North Carolina, US [13]. The possible spread of oseltamivir-resistant 2009 pandemic virus is alarming from the public health point of view because of consequences for treatment and prophylaxis of 2009 pandemic influenza. We report the emergence of oseltamivir-resistant 2009 pandemic virus strains in immunocompromised patients in Scotland.

Study methods and results

To investigate the frequency of oseltamivir resistance in circulating strains of 2009 pandemic virus, the H275Y discriminatory reverse transcription RT-PCR was used to screen 1,802 samples from all 1,608 infected hospitalised patients in Edinburgh and Glasgow between 1 November and 31 December 2009 (Table). In addition, 32 patients with available samples from the time of diagnosis and post treatment with oseltamivir were analysed and clinical features recorded.

The applied method can detect as little as 5% of oseltamivir-resistant 2009 pandemic virus in mixed virus populations [14]. Detection of the H275Y mutation was confirmed by pyrosequencing in the Health Protection Agency's Centre for Infections in London, the United Kingdom. Full-length sequencing of the neuraminidase gene was performed. Patients files with their medical history and virological investigations were reviewed for all patients with evidence for the H275Y mutation.

Sequences amplified from all pandemic influenza virus samples collected pre-treatment were wild type at position 275 in the neuraminidase gene, providing no evidence for circulation of oseltamivir-resistant 2009 pandemic virus in the area. However, 10 patients in our study developed the H275Y mutation during or after oseltamivir therapy.

Full-length sequences of the neuraminidase gene did not reveal any other mutations than the H to Y change at position 275 in our patients. In two of them, resistant virus persisted for at least 25 to 40 days following cessation of treatment, suggesting oseltamivir-resistant 2009 pandemic viruses were not compromised in their replication ability.

All patients who developed oseltamivir resistance were immunocompromised, eight of them with haematological malignancy The frequency of resistance-development in patients with 2009 pandemic influenza treated with oseltamivir was assessed further by analysing data for 32 patients from Edinburgh of whom 10 were immunocompromised and 22 non-immunocompromised. Antiviral-resistant viruses were detected in five of the 10 immunocompromised patients, all of whom had been treated with oseltamivir. In comparison, none of the non-immunocompromised patients developed resistance during or after oseltamivir-treatment (0/22 versus 5/10, p=0.0012, Fisher's exact test).

Conclusions

Systematic follow-up of patients hospitalised with 2009 pandemic influenza and treated with oseltamivir and large-scale screening of untreated hospitalised 2009 pandemic influenza patients showed an association between the appearance of the H275Y mutation and oseltamivir treatment. In our study population, we found no evidence for the spontaneous emergence of resistance in untreated patients. This contrasts with very recent findings of resistance in a small number of Chinese and Vietnamese subjects who had not received oseltamivir prophylaxis or treatment [8,10]. These results have been interpreted as showing that spontaneous mutation can occur either before or during infection, or that there is transmission of oseltamivir-resistant virus in some geographical regions.

In our study, oseltamivir resistance development was restricted to immunocompromised subjects, consistent with the previous descriptive study of the isolation of oseltamivir-resistant 2009 pandemic virus from two severely immunosuppressed patients with haematologic malignancy [13]. In both of them, persistent viral shedding led to prolonged use of oseltamivir and the subsequent development of oseltamivir-resistant 2009 pandemic virus variants. The prolonged period of infection (up to 61 days in our study) and large population sizes associated with poorly controlled virus replication favoured the development of resistance. This could provide evidence that emergence of resistance can result from selection of mutants from genetically diverse quasispecies within the infected individual. Despite the small study numbers, the result that 5 of 10 of immunocompromised patients treated with oseltamivir developed drug resistance could have implications for further management of 2009 pandemic virus infections.

An additional factor influencing resistance development is the dosage of antivirals administered. In previous studies, oseltamivir-resistant 2009 pandemic virus strains were detected in patients who became infected during oseltamivir chemoprophylaxis [2,9,11]. In these cases, subtherapeutic levels of oseltamivir may have only partially inhibited viral replication, facilitating the emergence of resistance. All our study subjects received the adequate and recommended treatment doses of oseltamivir. However, the efficacy of recommended oseltamivir treatment in immunocompromised

Table

H275Y mutation resistance testing of confirmed 2009 pandemic influenza A(H1N1) virus samples from hospitalised patients, Scotland, November 2009-December 2009 (n=1,802 samples)

	Edinburgh	Glasgow	Total
Number of samples tested for 2009 pandemic virus	2,507	6,300	**8,807**
Number of 2009 pandemic virus positive samples	423	1,379	**1,802**
Number of 2009 pandemic virus positive patients	352	1,256	**1,608**
Number of H275Y positive patients	5	5	**10**
Time period	1.11-31.12.2009	6.11-31.12.2009	
Method for H275Y screening	All 2009 pandemic virus positive samples tested with H275Y RT-PCR	All respiratory samples tested with multiplex RT-PCR (2009 pandemic virus, FluA and H275Y)	

FluA: influenza virus type A; RT-PCR: reverse transcription-polymerase chain reaction

patients has not been established and further studies are needed in this respect.

Finally, the emergence of resistant forms of influenza viruses depends on the relative fitness of drug-resistant strains compared to wild type virus. A recent study showed that a seasonal influenza A (H1N1) virus isolated in Canada with H275Y mutation had at least comparable viral fitness both in vitro and in ferrets relative to that of a closely related wild type strain [15]. Although we were unable to assess the replication fitness or infectivity directly, the appearance of oseltamivir-resistant variants and replacement of the original wild type strains eight to 12 days after the end of treatment in two individuals could hint towards a possible fitness advantage of oseltamivir-resistant viruses.

The majority of circulating 2009 pandemic virus strains worldwide has remained susceptible to oseltamivir [2]. However, the recent spontaneous emergence of oseltamivir-resistant seasonal influenza A(H1N1) virus [4,5] shows that in theory there could be the possibility that oseltamivir-resistant 2009 pandemic virus may also become dominant during the next influenza season.

The induction of resistance in immunosuppressed patients seen in our study highlights the importance of close monitoring and containment of this group during therapy. All possible interventions should be adopted to prevent the emergence of oseltamivir-resistant 2009 pandemic virus strains, including the vaccination of immunocompromised individuals and their household contacts with the pandemic influenza vaccine.

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Cluster of botulism among Dutch tourists in Turkey, June 2008

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In June 2008, three Dutch tourists participating in a mini-cruise in Turkey needed urgent repatriation for antitoxin treatment because of symptoms of botulism. Because there was a shortage of antitoxin in the Netherlands, an emergency delivery was requested from the manufacturer in Germany. An outbreak investigation was initiated into all nine cruise members, eight of whom developed symptoms. *C. botulinum* type B was isolated in stool culture from four of them. No other patients were notified locally. Food histories revealed locally purchased unprocessed black olives, consumed on board of the ship, as most likely source, but no leftovers were available for investigation. *C. botulinum* type D was detected in locally purchased canned peas, and whilst type D is not known to be a cause of human intoxication, its presence in a canned food product indicates an inadequate preserving process. With increasing tourism to areas where food-borne botulism is reported regularly special requests for botulism antitoxin may become necessary. Preparing an inventory of available reserve stock in Europe would appear to be a necessary and valuable undertaking.

Introduction

Botulism is a disease caused by the neurotoxin of *Clostridium botulinum*. Seven serotypes of botulinum neurotoxin have been identified, A to G. Serotypes A, B, E and rarely F, can affect humans. Types A and B are related to food-borne botulism, of which type B seems to predominate in Europe [1]. Type E is associated with consumption of seafood products [2]. Serotypes C and D cause botulism in animals (birds, mammals), but not in humans. With increased standards of food processing and preservation, food-borne botulism has become a rare disease in the Netherlands. According to the Dutch law on public health, botulism is a notifiable disease, and each year on average one case is reported [3]. These cases presumably result either from contaminated honey (infant botulism), injection of contaminated heroin, or consumption of unprocessed food which has been purchased abroad and consumed abroad or in the Netherlands after returning.

Outbreak description

On 24 June 2008, the Centre for Infectious Disease Control (CIb) of the Dutch National Institute for Public Health and the Environment (RIVM) was contacted by a medical repatriation organisation about four Dutch patients with clinical signs of botulism, hospitalised in Fethiye, Turkey. The first case had been admitted on 21 June with signs of botulism and treated with botulism antitoxin. Two days later, three more Dutch patients from the same tourist group were admitted and considered epidemiologically related cases. Since all locally available antitoxin had been used for treatment of the first patient, urgent repatriation was requested for the other three patients for treatment in the Netherlands.

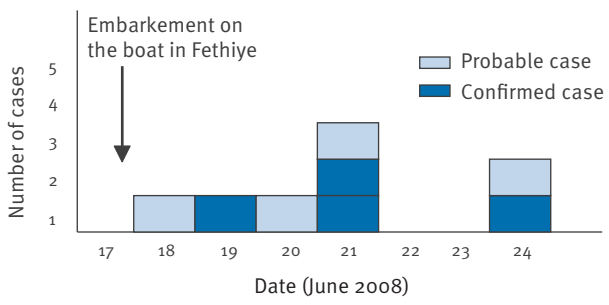
The patients belonged to a group of nine participants of a mini cruise, including seven tourists, a cook and the ship owner. All nine were Dutch and had started a sailing trip on 17 June for the duration of one week.

In the Netherlands, an outbreak investigation was initiated into the nine participants of the cruise. The onset of symptoms was between the afternoon of 18 June and 24 June (Figure 1).

In this cluster, a confirmed case was defined as a person who participated in the sailing trip from 17 June onwards, who met the clinical criteria for botulism (at least signs of bilateral cranial nerve neuropathy as

Figure

Cases of botulism by day of onset of disease among cruise participants, Turkey, 17-24 June 2008 (n=8)



diplopia, blurred vision, dysphagia, bulbar paralysis and/or peripheral symmetrical muscle weakness), with laboratory confirmation (toxin in serum or faeces, or *C. botulinum* cultured in faeces). A probable case was defined as a person who participated in the sailing trip starting on 17 June and who met the clinical criteria for botulism.

The RIVM informed the Turkish National Focal Point (NFP) about the situation because the Dutch patients might have been part of a larger cluster. The Turkish NFP reported to the RIVM that no additional cases of botulism were reported in Fethiye.

Clinical characteristics and treatment

The first patient was admitted to hospital with symptoms of blurred vision, diplopia, dry mouth, dysphasia, dysarthria and muscle weakness. The diagnosis botulism was supported by electromyogram and botulism antitoxin was administrated. The three following patients, including the cook, returned to the Netherlands for treatment on 25 June and were admitted to different hospitals [4]. As their clinical symptoms matched with botulism, botulism antitoxin type A, B, E was administered to all of them. Three other group members were repatriated one day later and were also hospitalised with signs of botulism.

Eight of the nine group members developed clinical symptoms of botulism. Seven of the eight patients were admitted to hospital, in Turkey and/or the Netherlands. The symptoms reported most by the seven patients were dysphagia (n=7), dry mouth (n=7), dysarthria (n=6), feeling of 'thick tongue' (n=6) and tiredness (n=5). Also change in voice (n=4), subjective weakness of muscle (n=4), hoarseness (n=3), dizziness (n=4), diplopia (n=3) and blurred vision (n=3) were reported.

Five patients developed symptoms to such an extent that botulism antitoxin was administered in Turkey (one person) or the Netherlands (four persons). In the Netherlands antitoxin administration was based on the patients' clinical signs in combination with the epidemiological connection; laboratory confirmation was not waited for. As botulism is a rare disease in the Netherlands, the available stock of antitoxin is limited.

Table 1

Food items consumed by cruise participants in Turkey on 17 and 18 June 2008

Case number	1	2	3	4	5	6	7	8	9
Date of onset of disease	18.6	19.6	20.6	21.6	21.6	21.6	24.6	24.6	-
	Probable case	Confirmed case	Probable case	Confirmed case	Confirmed case	Probable case	Probable case	Confirmed case	Non-case
17 June 2008									
Lunch: Pasta salad: spicy sausage, pesto, capers, pickles, garlic at water, black olives	Yes	Yes	Yes	Yes	Yes	Yes (no pickles)	Yes	Yes	Yes (no olives
Dinner in restaurant on the mainland:									
Starters: Meze: Eggplant, cheese rolls, tzatziki, salad (tomatoes, green beans, cucumber, feta cheese and yoghurt) and rice salad	Yes (no tzatziki)	Yes (no eggplant, no salad)	Only one cheese roll	Yes (no tzatziki)	Yes (no tzatziki)	Yes	Yes (no tzatziki)	Yes	Only salad
Main course:									
Chicken	NA	No	NA	NA	Yes	No	NA	No	No
Grilled fish with onion and tomatoes	Yes	Yes	Yes (a bit)	Yes	NA	NA	Yes	Yes	No
Fried calamari	NA	No	NA	NA	NA	Yes	NA	No	Yes
Meat	NA	No	NA	NA	NA	No	NA	No	No
18 June 2008									
Breakfast: Bread with Dutch cheese (from the Netherlands)	NA	Yes	NA	NA	NA	Yes	NA	Yes	NA
Lunch: Chicken soup (with canned coconut milk and canned corn)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Dinner: Meat roll (with canned green peas, canned tomato puree and canned minced meat)	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes

NA: not able to remember.

With this sudden request, additional antitoxin had to be delivered by courier from the emergency stock of the manufacturer in Marburg, Germany. All patients recovered.

Food questionnaire

All group members were asked to complete a questionnaire on food items consumed since the onset of the trip. As the average incubation time for food-borne botulism is 12 to 72 hours (range: two hours to eight days) and the first patient had developed aspecific symptoms of gastroenteritis during the afternoon of 18 June, food poisoning at the beginning of the sailing trip in Turkey was considered most likely. Therefore, the food questionnaires focused on food consumed on 17 June (lunch prepared on the ship and food served in a restaurant) and 18 June (all meals prepared on the ship). By 4 July, all seven tourists and the cook had returned to the Netherlands and the patients were interviewed by their local municipal health services. The ship owner, who stayed in Turkey, sent his questionnaire by mail.

Since the canned food items came from a small stock on board that had also been used in previous sailing trips, the 15 participating tourists of these previous trips were contacted by email to inquire whether they had developed symptoms. They did not report any symptoms and therefore were not included in the study.

The food items served and consumed by the nine group members are listed in Table 1.

Since the group was small, no statistical analysis could be done. In the food histories, special attention was given to differences in food items eaten by the patients compared to the only group member without symptoms, number 9. On board, this person did not eat the locally purchased black olives in the salad served during lunch on the first day. Among the food items prepared on board, the black olives were therefore suspected to be the most likely source of the outbreak. The preserving conditions for these olives before packing had not been controlled, and therefore may have been more prone to contamination. Unfortunately, no leftovers of these olives were available for investigation.

Regarding food eaten on the mainland, the person without symptoms only ate salad and fried calamari in the restaurant during the dinner of 17 June. The patient with the fewest symptoms, case number 3, also ate little at that restaurant: only one cheese roll and a bit of fish. From food items served at the restaurant, the meze (a Turkish starter) and the fish were most suspected because these specific items were eaten by the confirmed cases. The Turkish NFP was informed on these results.

Microbiological investigation

Serum and faecal samples of all seven hospitalised patients were collected in the Netherlands for laboratory confirmation of botulism. The ship owner, who stayed in Turkey, and the group member without symptoms did not participate in the laboratory investigation. At the Central Veterinary Institute of Wageningen UR (CVI), detection of botulism toxins in serum and faeces was performed by the mouse bio-assay according to the protocol of the United States Centers for Disease Control and Prevention [5] with some minor changes. Faeces were cultured for isolation and detection of *C. botulinum*. Typing of botulism toxins was done by the mouse neutralisation bio-assay with toxin-specific antibodies.

In four patients, the diagnosis of botulism was confirmed by faecal culture of *C. botulinum* type B. In none of their sera or faeces, botulism toxin could be detected. This resulted in four confirmed cases, four probable cases (three patients who tested negative and the ship owner who was not tested) and one 'non-case' (the person without symptoms).

Analysis of food items

The cook had bought all canned food items at several local supermarkets. After each sailing trip she discarded all open cans and thoroughly cleaned the kitchen of the ship. Because the ship owner preferred investigation in the Netherlands, he sent unopened locally purchased food items, from the stock on board that was used for the meals, to the CVI in the Netherlands

Table 2

Laboratory investigation of food items consumed by cruise participants in Turkey on 17 and 18 June 2008

Food	Packaging	pH	Detection of botulinum toxin	Detection of *C. botulinum*	Molecular typing
Black olives	Home-packed	5,00	No	No	
Pickles	Can	3,97	No	No	
Pesto	Jar	4,23	No	No	
Garlic	Jar	4,14	No	No	
Green peas	Can	4,73	No	Yes	*C. botulinum* type D
Pineapple	Can	3,89	No	No	
Coconut milk	Can	5,50	No	No	
Corn	Can	5,60	No	No	
Corn	Can	5,56	No	No	

for further investigation. Food specimens were tested for *C. botulinum* toxin and organisms according to the above protocol, and the pH was measured.

The results of the laboratory investigation of the food samples done in the Netherlands are summarised in Table 2. The pH of five of nine items was above 4.6, which is considered as the minimum pH at which *C. botulinum* can grow. Together with other factors such as temperature, water activity and redox potential, pH plays a role in preventing the growth and toxin production of *C. botulinum* during the conservation process and storage [5]. *C. botulinum* type D was isolated from a can of green peas. Although this toxin type differs from the one diagnosed in the patients, its presence in canned food shows at least an inadequate conservation process. This result was communicated to the Turkish authorities.

Conclusion

This is the first notified outbreak of botulism among Dutch nationals. Eight tourists were affected during a sailing trip in Turkey by *C. botulinum* type B. In the literature, clusters of food-borne botulism are often described as resulting from home-preserved products (mainly tofu, green olives and fish [6-8]) or canned products (fish, asparagus, roasted mushrooms [1]). In Turkey, clusters of botulism have resulted from canned roasted mushrooms [9] and çakşir (*Ferula orientalis*, a regionally grown vegetable) [10]. More recently, a cluster of 10 patients with botulinum poisoning from eating süzme yoghurt has been described [11]. The authors of that article also noted that botulism in Turkey is mostly associated with the consumption of home-prepared foods, especially vegetables such as green beans, tomato or red pepper preserves.

For this cluster, the restaurant visited on 17 June was initially considered as the most likely source, because the one group member that had no symptoms had only eaten salad at this restaurant and because home-made products may have been served. The most likely sources indicated by the food questionnaires were the meze (in particular the cheese rolls) and fish served at the restaurant. However, because fish is more usually associated with *C. botulinum* type E, whereas *C. botulinum* type B was isolated from four of the patients described here, the fish is not likely to have been the source of the outbreak. Although a contamination in the meze cannot be ruled out, no other cases associated with this restaurant, nor feedback from the local investigation of the restaurant were reported by the Turkish national authorities. Therefore the location is now considered less likely as the source of the outbreak.

The meals on board were prepared from locally purchased food such as commercial canned items, bread and home-packed black olives, as well as Dutch cheese that the cruise members had brought from the Netherlands. The laboratory investigation in the Netherlands of food items purchased in Turkey revealed *C. botulinum* type D in a can of green peas, which had the same trademark as the peas used for the dinner of 18 June. However, *C. botulinum* type D is not associated with human illness. In addition, canned peas were ruled out by the food questionnaires because the first patient had already reported gastrointestinal symptoms earlier that day before the dinner with the canned peas was served. While these symptoms may have been coincidental and had another cause, the person without symptoms did report eating the green peas which made this food item also a less likely source.

The pH of the home-packed olives, the coconut milk and the corn was not low enough to prevent growth of *C. botulinum*. Since growth of *C. botulinum* is influenced by many other factors as well, it is difficult to draw conclusions from this finding. However, according to the food questionnaire, the black olives remain the most likely source for the cluster, although this cannot be confirmed as no leftovers were available for investigation.

Discussion

The lack of locally available antitoxin for treatment of the patients in Turkey was a major issue in this outbreak and the reason for the repatriation of the Dutch patients. Essential in botulism treatment is timely administration of antitoxin, preferably within 24 hours [4] after onset of the disease, as the antitoxin prevents free toxin from binding to the presynaptic membrane resulting in paralysis.

Botulism is a rare disease, and antitoxin is expensive and has a short shelf-life. Also the stock of antitoxin in the Netherlands is therefore small. In this particular incident, it was possible to purchase emergency supplies of antitoxin from the German manufacturer. In preparation for future potential food-borne outbreak of botulism it would be useful to share information among European countries on national stocks of antitoxin that could be available for exchange in outbreak situations involving several patients. This approach could also be valuable for other antitoxins such as diphtheria antitoxin as well. The European Centre for Disease Prevention and Control has been approached with the request for such an inventory.

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The Russian gonococcal antimicrobial susceptibility programme (RU-GASP) – national resistance prevalence in 2007 and 2008, and trends during 2005-2008

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Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* is a major problem worldwide. In the former Soviet countries including Russia, the knowledge regarding AMR has been highly limited. However, in 2004 the Russian gonococcal antimicrobial susceptibility programme (RU-GASP) was initiated. The aims of this study were to examine and describe the prevalence of *N. gonorrhoeae* AMR in 2007 and 2008 in Russia, and reveal trends in the period from 2005 to 2008. Gonococcal isolates (660 in 2007 and 900 in 2008) from 36 surveillance sites were examined using agar dilution method. From 2005 to 2008, the proportion of isolates resistant to spectinomycin increased from 0% to 7.2%, and remained high for those resistant to ciprofloxacin (approximately 49%). The resistance to azithromycin was 2.3% and 0.4% in 2007 and 2008, respectively. All isolates between 2005 and 2008 were susceptible to ceftriaxone. In conclusion, the AMR of *N. gonorrhoeae* in Russia is high, as in most countries in the European Union, and ceftriaxone should be the first line for treatment. If there is no access to ceftriaxone or in the presence of severe beta-lactam antimicrobial allergy, spectinomycin should be used; however, the resistance to spectinomycin has increased. Regular, quality-assured national and international surveillance of AMR in *N. gonorrhoeae* is crucial globally for public health.

Introduction

Gonorrhoea remains one of the most common sexually transmitted infections (STIs) in most countries [1]. In Russia, the estimated gonorrhoea incidences were 60.8 and 56.4 cases per 100,000 inhabitants in 2007 and 2008, respectively. However, the incidence varied substantially in the seven federal districts (FDs) of Russia. The incidence in Russia remains high. Nevertheless, the incidence from 1993 to 2008, with exception of the years 1999 and 2000, decreased from 230.9 to 56.4 cases per 100,000 inhabitants [2].

The impact of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* on effective treatment and, accordingly, control of gonorrhoea is of long-standing concern. Emergence and transmission of resistance in *N. gonorrhoeae* to most antimicrobials used for treatment are major problems globally [3-14], and the World Health Organization (WHO) has recently published initiatives to enhance global *N. gonorrhoeae* AMR surveillance that focus on multidrug- and extensively drug-resistant *N. gonorrhoeae* [3]. Expanded, but quality-assured and quality-controlled, AMR surveillance worldwide is crucial to inform STI management and treatment guidelines and, accordingly, for public health [3,4,8,13,14].

In the European Union (EU) countries, a *N. gonorrhoeae* AMR surveillance programme, the European gonococcal antimicrobial susceptibility programme (EURO-GASP), has been running since 2004 [11]. However, in eastern Europe and the former Soviet countries, including Russia and its highly diverse federal districts (FDs), the knowledge regarding AMR in *N. gonorrhoeae*, which is crucial for the empirical treatment, has been highly limited [15-18]. However, in 2004 the national Russian GASP (RU-GASP), coordinated at the State Research Center of Dermatology and Venereology of the Russian Ministry of Health (SRCDV), Moscow, was initiated with the main objective to inform the STI management and treatment guidelines in Russia. In this programme, SRCDV initially implemented optimised and quality-assured systems for collection, storage and transportation of clinical specimens and *N. gonorrhoeae* cultures from the different FDs of Russia to SRCDV. In 2008, RU-GASP published the first ever international report that described the *N. gonorrhoeae* AMR in 2005 and 2006 in Russia [10]. The aims of the present study were to examine and describe the prevalence of *N. gonorrhoeae* antimicrobial resistance in 2007 and

2008 in Russia, including all seven FDs, and to reveal trends in the resistance from 2005 to 2008.

Materials and methods

Study population

As previously described [10], dermato-venereological clinics situated all over Russia are surveyed in RU-GASP. In the present study, *N. gonorrhoeae* isolates from 36 surveillance sites, which were selected to represent all the FDs of Russia, were examined and the results were compared to the previously published results from 2005 and 2006 [10].

Representative, i.e. mainly consecutive, culture-positive patients attending the clinics from January 2007 to December 2008 were included. The inclusion criterion was: male or female patient (12 to 60 years of age), with diagnosed (clinically and using culture) symptomatic uncomplicated gonorrhoea. Exclusion criteria were: i) refusing participation and ii) presence of serious somatic pathology or disease of the central nervous system.

Diagnostics, culture conditions and preservation of *N. gonorrhoeae* isolates

A clinical examination was performed, and specimens (urethral and cervical from females, and urethral from males) were collected.

All specimens were cultured on selective culture media and the *N. gonorrhoeae* isolates were species-verified, preserved in cryomedium, and transported to SRCDV as previously described [10].

Antimicrobial susceptibility testing

At SRCDV, the susceptibility to ciprofloxacin, spectinomycin, ceftriaxone, and also azithromycin (not included before 2007) was determined using agar dilution method, according to the recommendations of Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) [19]. Accordingly, the minimum inhibitory concentration (MIC) breakpoints for susceptibility or resistance were ≤0.06/≥1 for ciprofloxacin, ≤32/≥128 for spectinomycin and ≤0.25 (susceptible) for ceftriaxone [19]. For azithromycin, CLSI does not describe any breakpoints, and the MIC breakpoints (≤0.25/≥1) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.escmid.org/research_projects/eucast) were used. The susceptibility to penicillin G and tetracycline was also analysed using the CLSI method [19]. However, because penicillin G and tetracycline are not recommended for treatment of gonorrhoea, the susceptibility to these was not comprehensively analysed. For quality control, the CLSI-recommended *N. gonorrhoeae* reference strain ATCC 49226 was examined in each run [19]. However, during 2009, the 2008 WHO *N. gonorrhoeae* reference strains intended for quality assurance and quality control of gonococcal AMR surveillance [4] were also included in the quality control. Beta-lactamase production was identified using nitrocefin discs, according to the manufacturer's instructions (Cefinase discs; Becton Dickinson).

Results

Patient characteristics

N. gonorrhoeae isolates (one isolate per patient) from 660 patients in 2007 (594 males and 66 females) and 900 patients in 2008 (766 males and 134 females) were examined. The mean ages of the men were 25 years (median age: 27 years; range: 15 to 45 years) and 22 years (median age: 23 years; range: 14 to 57 years) in 2007 and 2008, respectively. The mean ages of the women were 21 years (median age: 23 years; range: 12 to 35 years) and 19 years (median age: 20 years; range: 12 to 60 years) in 2007 and 2008, respectively. In order to reveal any trends, the results of the AMR testing for these patients were compared to previously published results from 2005 and 2006 [10]. The gender distribution and age distribution were relatively similar during the four years compared.

Antimicrobial susceptibility of *N. gonorrhoeae* isolated in Russia in 2007 and 2008

The proportions of the *N. gonorrhoeae* isolates that displayed resistance and intermediate susceptibility to the four antimicrobials used in the recommended gonorrhoea treatment in 2007 and 2008 are described in Table 1.

Briefly, the proportions of isolates displaying resistance in 2007 and 2008, respectively, were: 49.6% and 49.1% for ciprofloxacin, 2.3% and 0.4% for azithromycin, 0.9% and 7.2% for spectinomycin, and 0% and 0% for ceftriaxone (Table 1). Of the *N. gonorrhoeae* isolates, 0.0% and 2.2% were beta-lactamase-producing

Table 1

Proportion of *Neisseria gonorrhoeae* isolates in Russia displaying resistance and intermediate susceptibility to the four antimicrobials used in the recommended gonorrhoea treatment, Russia, 2007 (n=660) and 2008 (n=900)

	Proportion of isolates (%)			
	Intermediate susceptible		*Resistant*	
	2007	2008	2007	2008
Ciprofloxacin (S≤0.06 mg/l; R≥1 mg/l)	5.5	7.5	49.6	49.1
Spectinomycin (S≤32; R≥128)	3.6	1.1	0.9	7.2
Ceftriaxone (S≤0.25)	0	0	0	0
Azithromycin (S≤0.25; R≥1)	7.4	4.8	2.3	0.4

R: resistant; S: susceptible

in 2007 and 2008, respectively. The proportions of isolates displaying resistance or intermediate susceptibility to penicillin G and tetracycline in 2007 and 2008 (in parenthesis) were: 72.4% (81.3%) and 67.2% (85.5%), respectively. The susceptibility to these antimicrobials was not further evaluated, because they are not recommended for treatment. From 2005 to 2008, the proportion of isolates resistant to spectinomycin increased significantly from 0% to 7.2%, and remained high to ciprofloxacin (at approximately 49%) [10]. All isolates (100%) from these four years were susceptible to ceftriaxone (Table 1; [10]). Nevertheless, examining the MIC distribution of ceftriaxone in general, the MIC values of ceftriaxone increased in the period from 2005 to 2008, and isolates at the breakpoint (especially in 2007: MIC=0.25 mg/l, n=14) were increasingly identified (data not shown).

Furthermore, multiple resistance to several of the four antimicrobials used in the recommended treatment of gonorrhoea was common (Table 2). In the period from 2007 to 2008, the level of *N. gonorrhoeae* isolates resistant to ciprofloxacin+spectinomycin and to ciprofloxacin+spectinomycin+azithromycin increased from 0% to 6.0% and from 0% to 0.4%, respectively.

The levels of resistance or intermediate susceptibility to the antimicrobials in the seven FDs of Russia in 2007 and 2008 are summarised in Table 3.

Substantial regional differences regarding prevalence of gonococcal AMR in the different FDs of Russia were identified. The levels of resistance or intermediate susceptibility to ciprofloxacin were high in all the FDs. Most disquieting, resistance or intermediate susceptibility to spectinomycin and azithromycin was found in six and five, respectively, of the seven FDs.

Discussion

This study is the second ever international report from RU-GASP that annually, since the programme's initiation in 2004, surveys the antimicrobial resistance of *N. gonorrhoeae* in Russia. The present multicentre study comprehensively describes the antimicrobial resistance of *N. gonorrhoeae* in 2007 and 2008, including the trends during the period form 2005 to 2008, in all seven highly diverse FDs of the Russian Federation.

In Russia, the level of resistance of *N. gonorrhoeae* to all antimicrobials used in the traditional gonorrhoea treatment (penicillins, tetracycline and ciprofloxacin) is exceedingly high. However, between 2005 and 2008, all Russian isolates were susceptible to ceftriaxone and still no gonorrhoea treatment failures using ceftriaxone of appropriate quality and dosage has been described for urogenital gonorrhoea worldwide [3]. Nevertheless, in Russia and in many other countries [3] the MIC values of ceftriaxone have increased. Resistance to azithromycin was also identified, dispersed in five of the

Table 2

Proportion of *Neisseria gonorrhoeae* isolates in Russia displaying multiple resistance to several of the antimicrobials used in the recommended gonorrhoea treatment, Russia, 2007 (n=660) and 2008 (n=900)

Year	CIP+SPM	CIP+SPM+AZM
2007 (n=660)	0 (4.5)	0 (2.6)
2008 (n=900)	6.0 (6.5)	0.4 (1.5)

AZM: azithromycin; CIP: ciprofloxacin; SPM: spectinomycin.
Resistance (resistance or intermediate susceptibility) are shown.
All isolates were susceptible to ceftriaxone.

Table 3

Proportion of *Neisseria gonorrhoeae* isolates displaying resistance or intermediate susceptibility to the four antimicrobials used in the recommended gonorrhoea treatment in 2007 and 2008 in all the seven federal districts (FDs) of Russia

Federal district	Year (no. of isolates)	Ciprofloxacin (S≤0.06 mg/l; R≥1 mg/l)	Spectinomycin (S≤32; R≥128)	Ceftriaxone (S≤0.25)	Azithromycin (S≤0.25; R≥1)
Central	2007 (n=99)	52.5	5.1	0	9.0
	2008 (n=210)	58.8	6.0	0	10.1
North-western	2007 (n=185))	51.5	2.9	0	10.0
	2008 (n=112)	53.8	12.1	0	9.6
Southern	2007 (n=75)	24.0	4.0	0	8.0
	2008 (n=115)	34.0	8.7	0	5.4
Volga	2007 (n=198)	60.3	2.7	0	10.0
	2008 (n=256)	50.7	9.6	0	3.4
Urals	2007 (n=10)	40.0	10.0	0	0
	2008 (n=86)	83.3	6.5	0	0
Siberian	2007 (n=47)	73.7	13.2	0	21.0
	2008 (n=121)	71.4	6.0	0	0
Far-eastern	2007 (n=46)	71.1	0	0	0
	2008[a]	nd[a]	nd[a]	nd[a]	nd [b]

nd: not done; R: resistant; S: susceptible.
[a] Unfortunately, it was not possible to receive any viable *N. gonorrhoeae* isolates from the Far-eastern federal district in 2008.

seven FDs (range: 3.4% to 21% during 2007 and 2008), which may reflect the fact that azithromycin is commonly used in these FDs. The resistance to spectinomycin, which had not been identified in Russia in 2005 [10] and is rare internationally, also increased during 2007 and 2008 (0.9%-7.2%), which may reflect a frequent use [20]. A representative selection of these isolates was also confirmed as resistant using Etest and/or genetic methods; however, they only displayed a low level of resistance. The 2008 WHO *N. gonorrhoeae* reference strains intended for quality assurance and quality control of gonococcal AMR surveillance [4], which were implemented for quality control in Russia in 2009, will from now on confirm the validity of all the AMR results in RU-GASP.

Major longitudinal trends of the *N. gonorrhoeae* AMR in the different FDs of Russia remain difficult to interpret due to the limited and divergent sample sizes from each FD and the short time period for studying dynamics. In RU-GASP, efforts are continuously made to increase the representativeness and number of examined isolates, and the number has increased by 77% from 2005 (n=509 [10]) to 2008 (n=900).

A main objective of the RU-GASP, as for all AMR surveillance programmes, is to form the basis for continuous revision and updating of the Russian STI management and treatment guidelines. As previously stated [10], the RU-GASP has clearly highlighted that penicillins and tetracycline, as well as the fluoroquinolones used frequently since the 1990s should not be used for empirical gonorrhoea treatment. Furthermore, oral azithromycin is not recommended in empirical treatment because it needs to be administered in doses of 2 g to avoid treatment failures, i.e. doses that commonly give adverse gastro-intestinal effects, and resistant strains are spreading in Russia and increasingly in many other countries, including high-level resistance in England, Wales, Scotland and Italy [3,7,11,12,21-23]. Fluoroquinolones and azithromycin are not recommended for use in the gonorrhoea treatment unless MIC results are available for the specific isolates. The recommended first-line antimicrobial should be ceftriaxone (250 mg, 1×intramuscularly) and, if there is no access to ceftriaxone or in the presence of severe allergy to beta-lactam antimicrobials, spectinomycin (2 g, 1× intramuscularly) should be used. However, increasing levels of resistance to spectinomycin have been observed in Russia, and adequate monitoring of its use and of the treated patients is crucial.

RU-GASP has implemented optimised, harmonised and quality-assured culture diagnostics, as well as quality-assured and quality-controlled AMR testing in Russia, in accordance with national and international recommendations [17,19, 24-33]. In addition, the 2008 WHO *N. gonorrhoeae* reference strains intended for quality assurance and quality control of gonococcal AMR surveillance [3,4], a prerequisite for any global WHO AMR surveillance programme for *N. gonorrhoeae*, were implemented during 2009 in quality assurance and control. The further rationale and applications for, and uses of, these reference strains are provided in WHO documents elsewhere [13]. These are used for the provision of internationally valid and comparable phenotypic and genetic AMR data worldwide. Genetic typing of resistance mechanisms, an additional aim for RU-GASP, is becoming increasingly relevant for surveillance of resistance to antimicrobials, especially for the expanded-spectrum, third generation cephalosporins [3,4,34,35], even though current testing remains based on MIC determinations.

Conclusion

In conclusion, the present national RU-GASP survey emphasises that the antimicrobial resistance of *N. gonorrhoeae* across Russia is exceedingly high and ceftriaxone should be the first-line antimicrobial for gonorrhoea treatment. If there is no access to ceftriaxone or in cases of severe beta-lactam antimicrobial allergy, spectinomycin should be used. Continuous and quality-assured local, national and international surveillance of *N. gonorrhoeae* antimicrobial susceptibility/resistance is crucial for public health purposes. It is fundamental to establish, quality-assure and quality-control regional and national GASP networks in many of the other eastern European countries, something that is presently in progress under WHO protocols [3,4,13].

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Association of D222G substitution in haemagglutinin of 2009 pandemic influenza A (H1N1) with severe disease

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To the editor: The preferential binding of influenza virus to sialic acid-α2,3-galactose (α2,3 receptor) or sialic acid-α2,6-galactose (α2,6 receptors) may determine its tropism as α2,3 and α2,6 receptors are dominant on lower and upper respiratory cells respectively [1]. The recent glycan microarray analysis suggested that the haemagglutinin (HA) D222G substitution could cause a shift from α2,6 receptors to the mixed α2,3/α2,6 receptors specificity which might increase binding to α2,3 receptors [2] and contribute to severity of disease. This substitution in the HA gene has been reported in samples of viruses obtained from cases with mild to severe illness from around 20 countries, areas and territories [3]. A recent study from Norway has evaluated the clinical relevance of this substitution with severe and mild cases [4].

In an attempt to understand the relevance of HA D222G substitution among pandemic influenza A (H1N1) causing infections in Hong Kong, HA gene sequences from respiratory specimens and virus isolates of severe and non-severe cases were examined. Cases were individuals who had laboratory confirmed pandemic H1N1 influenza virus by either viral culture or reverse transcription PCR (RT-PCR) of respiratory specimens [5]. The severe cases were individuals classified by the attending physician as being in a serious or critical condition.

From 1 May 2009 to 31 January 2010, 458 respiratory samples were examined. Of 219 severe cases, nine (4.1%) showed D222G substitution while none of the 239 non-severe cases showed D222G substitution. Four of the nine cases died. The association of D222G with

Table

Comparison between severe and non-severe cases of pandemic H1N1 infection with D222G mutation[a] in the haemagglutinin gene, Hong Kong, May 2009-January 2010 (n=458)

Month[b]	All cases			Severe cases			Non-severe cases			p
	Number tested	Number with D222G	% with D222G	Number tested	Number with D222G	% with D222G	Number tested	Number with D222G	% with D222G	
2009										
May	14	0	0	0[c]	0	0	14	0	0	NA
June	17	0	0	0[c]	0	0	17	0	0	NA
July	57	3	5.3	14	3	21.4	43[d]	0	0	0.025
August	89	0	0	37	0	0	52	0	0	NA
September	107	2	1.9	57	2	3.5	50	0	0	0.563
October	55	0	0	39	0	0	16	0	0	NA
November	37	2	5.4	19	2	10.5	18	0	0	0.514
December	45	2	4.4	29	2	6.9	16	0	0	0.820
2010										
January	37	0	0	24	0	0	13	0	0	NA
Total	**458**	**9**	**2.0**	**219**	**9**	**4.1**	**239**	**0**	**0**	**0.002**

NA: not applicable; p: p-value of difference of severe and non-severe cases with D222G mutation, calculated by Fisher's exact test, doubled one-sided.

[a] Amino acid position is D239G when counted from the start codon of the strain of human swine influenza virus type A (subtype H1) A/California/4/2009, GenBank Accession: FJ966082.

[b] The number of cases in each month was based on the date of specimen collection.

[c] The first severe case was found in July 2009.

[d] This case was found with 222G in culture but 222D in original specimen, it was classified as 222D.

severe disease was statistically significant (p=0.002, Fisher's exact test, doubled one-sided). Other substitutions, of D222N (severe cases, n=3; non-severe cases, n=1) and D222E (only in non-severe cases, n=4) were also found. The first severe case appeared on 6 July 2009 and D222G substitution was detected in July, September, November and December of the same year (Table).

No distinct phylogenetic clusterings of the severe cases with D222G substitution have been observed (data not shown). To put this in perspective, from July 2009 to January 2010, the accumulated severe cases were 244 while the number of isolates in our laboratory was 25,625. Priority of analysis has been given to severe cases over non-severe cases, with 90% and 1 % of cases analysed respectively.

Influenza is an RNA virus which evolves rapidly, frequently changing surface structures. A recent study at the United States (US) Centers for Disease Control and Prevention reported 14 cases with D222G substitution found only in virus isolates but not in the original clinical specimens [3]. We observed similar finding with one non-severe case showing D222G substitution in a virus isolate but not in the original clinical specimen, however, for the other nine severe cases, we detected D222G substitution in both the virus isolate and original specimen. Similar to the Norwegian study, we also found mixed 222G and 222D in some severe cases [4]. Although experiments with ferrets did not support a causal link of D222G substitution with virulence [3], further study is warranted to elucidate the intriguing relationship between D222G substitution and severe disease.

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Author's reply: Association of D222G substitution in haemagglutinin of 2009 pandemic influenza A (H1N1) with severe disease

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To the editor: We appreciate the response to our paper made by Dr. Mak and colleagues, whose data from Hong Kong SAR appear to be in good agreement with what we have seen in Norway.

In our original study, we observed a significantly higher frequency of D222G in patients with severe outcomes (including fatal) compared to patients with mild disease. In fact, in both our data set and the Hong Kong data, mutant viruses were not found among several hundred mild cases. Furthermore, as can be seen from our published data, the frequency may be higher also in fatal outcomes (eight of 27 cases) versus severe non-fatal outcomes (three of 34 cases). Comparing these frequencies results in p=0.078 with Fisher's exact test (two-sided) and p=0.046 with the Mid-P Exact test (two-sided). It would be interesting to know if the new data from Hong Kong SAR can corroborate this observation. Mak *et al*. report four fatal D222G cases and five non-fatal severe D222G cases, but one would also need to know the total number of fatal cases versus non-fatal severe cases analysed to make the comparison. Hopefully, this information can be obtained.

D222G substitution in virus isolates only and not in the original clinical specimens was found in one case in Hong Kong and 14 cases reported by the United States (US) Centers for Disease Control and Prevention [1]. We have also seen this virus culture artefact in one case with mild disease. This case was counted as wild type in our data set. This further underscores the importance to perform the sequence analysis of the primary specimen.

The frequency of D222G mutant viruses in the severe cases is somewhat lower in the Hong Kong data, compared to ours (4.1 per cent in Hong Kong data versus 18 per cent in our data set). Whilst this difference may represent a real variation in frequency, it may also arise from a different composition of cases, e.g. if the proportion of fatal cases were higher in the Norwegian sample of severe plus fatal cases. Mak and colleagues also observed, as we did, that the 222G mutant sometimes occurs in a mixture with non-mutated 222D genomes. Sensitivity of detection of mutant viral genomes when occurring as the minority variant in such mixtures may thus also influence the observed frequency. We have been using a pyrosequencing assay to identify the 222 genotype. Under ideal conditions this methodology can reliably detect and quantitate a mutant when present in the total virus population at levels as low as 10% [2]. However, in our data set the initial finding of D222G mutants by pyrosequencing could uniformly be verified by conventional sequencing. Therefore, since the Hong Kong data come from a study focusing on this particular position we assume that the methodology difference is not likely to have caused the different frequencies in the two data sets. In the overall global data, however, it is possible that some cases with mutant/wild type mixtures have been overlooked and only the majority sequence recorded.

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