



Impact
factor **5.7**

Eurosurveillance

Europe's journal on infectious disease epidemiology, prevention and control

Vol. 20 | Weekly issue 45 | 12 November 2015

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Refugee crisis demands European Union-wide surveillance!

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Citation style for this article:

Catchpole M, Coulombier D. Refugee crisis demands European Union-wide surveillance!. *Euro Surveill.* 2015;20(45):pii=30063. DOI: <http://dx.doi.org/10.2807/1560-7917.ES.2015.20.45.30063>

Article submitted on 12 November 2015 / accepted on 12 November 2015 / published on 12 November 2015

The conflicts in the Middle-East and instability in Libya and some parts of Asia and Africa have resulted in a dramatic influx of refugees to the European Union (EU) in recent years. In the first nine months of 2015, more than 600,000 applications for asylum were filed in the EU [1]. With no prospect of change of the international context in the near future, it is likely that the influx of refugees into the EU will continue and may even increase in coming months.

We have witnessed numerous large displacements of populations in recent years and 'Refugee health' has become an area of concern for national and international, governmental and non-governmental organisations. Much has been learned from responding to these humanitarian crises.

Although refugees are facing a similar spectrum of non-communicable diseases to those experienced by the indigenous population of their countries of origin, trauma and injuries, sexual and reproductive health issues, violence and psychosocial disorders are among the most frequent health problems refugees encounter. Disruption of healthcare delivery systems in their countries of origin and limited access to healthcare during their journey result in the interruption of treatments often required for the control of chronic diseases [2].

Refugee populations entering the EU/European Economic Area (EEA), and particularly children, are at risk of exposure to infectious diseases in the same way as other EU residents, and in some cases may be more vulnerable because of the interruption of public health programmes, notably for immunisation, in their country of origin, as well as through various barriers to access healthcare such as language, culture etc. It is therefore important that they benefit from protection from infectious diseases, including those prevented through routine vaccinations. In addition, these refugees may be at specific risk for certain infectious diseases in relation to their country of origin, countries traversed during their migration, and the conditions they experienced during their mostly difficult journeys.

It is important to note that refugees should not be seen as representing a threat to Europeans regarding infectious diseases, but rather as being themselves vulnerable for such diseases. For example, poor living conditions and close contact in crowded shelters and refugee camps may increase the risk for the spread of lice and/or fleas, which in rare cases can carry diseases such as louse-borne diseases (relapsing fever due to *Borrelia recurrentis*, trench fever due to *Bartonella quintana*, epidemic typhus due to *Rickettsia prowazekii*), murine typhus and mites (scabies). In recent months, sporadic cases of louse-borne relapsing fever (LBRF) have been reported in Belgium, Finland, Germany and the Netherlands among migrants from Eritrea, Somalia and Sudan [3-5]. LBRF is a disease transmitted by body lice that caused major epidemics in the first half of the 20th century in Europe [6,7] and is known to have occurred occasionally among homeless people in recent years, without spreading to the general population [8]. Recent reports from Italy indicate that transmission of LBRF is likely to have occurred in shelters for refugees in the EU, resulting in the risk of cross-border spread as refugees are frequently moving to other countries [9,10]. Media are reporting outbreaks of scabies and diarrhoea, notably in Calais, France, in relation to poor housing and hygiene conditions [11].

Meningococcal disease outbreaks have been associated with overcrowding overall and in refugee settings. Contributing factors include sharing dormitories, poor hygiene, and limited access to medical care [12] and that meningococcal carriage rates have been shown to be higher in individuals in overcrowded settings. Most cases are acquired through exposure to asymptomatic carriers [13]. Meningococcal disease has usually been reported in children, but is still a leading cause of both meningitis and sepsis in adolescents, young adults and adults. In addition, overcrowding has been associated with increased transmission of measles, varicella and influenza.

As we are approaching winter, the travelling and living conditions for refugees in transit to Europe or in

reception centres after their arrival is likely to deteriorate, with even more overcrowding in shelters with insufficient hygiene and therefore increased risk of transmission of communicable diseases. With the start of the influenza season, there is obviously a risk of increased influenza transmission.

Given the numbers and mobility of the refugee populations, the infectious disease risk can only be contained through a coordinated response at the EU level. That includes (i) raising awareness of the risks and types of infection that refugees may have been exposed to and may continue to be exposed to in reception centres, (ii) providing appropriate hygienic and medical countermeasures and (iii) ensuring ready access to medical diagnosis and treatment services. However, such a response will require that Europe has good information on the health situation of the refugees on the move in the EU.

Currently, the basic information that would allow a competent assessment of the situation is not available. The exact number of refugees is not known, and its assessment is hampered because refugees may avoid registration in fear of being sent back [14] and because they continue to move through different European countries. No comprehensive surveillance data is currently being gathered and only sporadic reports by organisations and institutions providing care for these populations are available.

Refugees are not currently a threat for Europe with respect to communicable diseases, but they are a priority group for communicable disease prevention and control efforts because they are more vulnerable.

The scale of the current influx of refugees is inevitably putting pressure on public health systems in frontline receiving countries. Protecting the health of this vulnerable group is complicated further by the potential occurrence of communicable diseases that have not been commonly or widely seen within Europe, creating challenges in terms of recognition and case management. It is vital to ensure that public health authorities have the right information to target resources and provide appropriate measures.

Given these challenges, the European Centre for Disease Prevention and Control (ECDC) will continue to work with its partners in Europe, including public health authorities in the Member States and the European Commission, to strengthen the evidence base guiding prevention and control measures and adding to the current evidence which pinpoint adequate hygiene conditions and vaccination services as the most immediate needs. Strengthening and coordinating surveillance will require continuing efforts to improve the quantity and quality of surveillance data collected through a EU-wide surveillance scheme. It will allow to ensure that interventions aimed at improving health of

the refugees are relevant, proportionate, appropriately targeted and coordinated.

Conflict of interest

None declared.

Authors' contributions

Mike Catchpole and Denis Coulombier jointly drafted the editorial.

References

1. European Asylum Support Office (EASO). Latest asylum trends. Number of applications for international protection in the EU+1. Valetta: EASO; September 2015. Available from: <https://easo.europa.eu/wp-content/uploads/Latest-Asylum-Trends-snapshot-Sept-2015.pdf>
2. World Health Organization (WHO). Frequently asked questions on migration and health. Geneva: WHO. [Accessed 11 Nov 2015]. Available from: <http://www.who.int/features/qa/88/en/>
3. Goldenberger D, Claas GJ, Bloch-Infanger C, Breidhardt T, Suter B, Martínez M, et al. Louse-borne relapsing fever (*Borrelia recurrentis*) in an Eritrean refugee arriving in Switzerland, August 2015. *Euro Surveill.* 2015;20(32):21204. DOI: 10.2807/1560-7917.ES2015.20.32.21204 PMID: 26290486
4. European Centre for Disease Prevention and Control (ECDC). Louse-borne relapsing fever in the Netherlands. Rapid risk assessment. Stockholm: ECDC; July 2015. Available from: <http://ecdc.europa.eu/en/publications/Publications/loose-borne-relapsing-fever-netherlands-rapid-risk-assessment.pdf>
5. Cutler S. Refugee crisis and re-emergence of forgotten infections in Europe. *Clin Microbiol Infect.* 2015 Oct 20. pii: S1198-743X(15)00918-0.
6. European Centre for Disease Prevention and Control (ECDC). Factsheet: Louse-borne relapsing fever. Stockholm: ECDC. [Accessed 11 Nov 2015]. Available from: http://ecdc.europa.eu/en/healthtopics/emerging_and_vector-borne_diseases/loose-borne-diseases/Pages/loose-borne-relapsing-fever.aspx
7. Raoult D, Roux V. The body louse as a vector of reemerging human diseases. *Clin Infect Dis.* 1999;29(4):888-911. DOI: 10.1086/520454 PMID: 10589908
8. Brouqui P, Stein A, Dupont HT, Gallian P, Badiaga S, Rolain JM, et al. Ectoparasitism and vector-borne diseases in 930 homeless people from Marseilles. *Medicine (Baltimore).* 2005;84(1):61-8. DOI: 10.1097/01.md.0000152373.07500.6e PMID: 15643300
9. Ciervo A, Mancini F, di Berenardo F, Giammanco A, Vitale G, Dones P, et al. Louse-borne relapsing fever in young migrants, Sicily, Italy, July–September 2015. *Emerg Infect Dis.* 2015;22(1). Ahead of print.
10. Lucchini A, Lipani F, Costa C, Scarvaglieri M, Balbiano R, Carosella S, et al. Louseborne Relapsing Fever among East African Refugees, Italy, 2015. *Emerg Infect Dis.* 2015;22(2). Ahead of print. DOI: 10.3201/eid2202.151768
11. Capon F. Migrants Suffering From Gangrene, Scabies at Calais Camps. *Newsweek*; July 2015. Available from: <http://europe.newsweek.com/migrants-suffering-gangrene-scabies-calais-camps-331338>
12. Lam E, McCarthy A, Brennan M. Vaccine-preventable diseases in humanitarian emergencies among refugee and internally-displaced populations. *Hum Vaccin Immunother.* 2015;11(11):2627-36. DOI: 10.1080/21645515.2015.1096457 PMID: 26406333
13. Deutch S, Labouriau R, Schønheyder HC, Ostergaard L, Nørgård B, Sørensen T. Crowding as a risk factor of meningococcal disease in Danish preschool children: a nationwide population-based case-control study. *Scand J Infect Dis.* 2004;36(1):20-3.
14. The Balkans. Asylum seekers, migrants and refugees in transit. Geneva: ACAPS. [Accessed 11 Nov 2015]. Available from: <http://acaps.org/img/documents/b-acaps-bn-balkans-asylum-seekers-migrants-refugees-in-transit-6-nov-2015.pdf>

Investigation of a staphylococcal food poisoning outbreak combining case–control, traditional typing and whole genome sequencing methods, Luxembourg, June 2014

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Citation style for this article:

Mossong J, Decruyenaere F, Moris G, Ragimbeau C, Olinger C, Johler S, Perrin M, Hau P, Weicherding P. Investigation of a staphylococcal food poisoning outbreak combining case–control, traditional typing and whole genome sequencing methods, Luxembourg, June 2014. *Euro Surveill.* 2015;20(45):pii=30059. DOI: <http://dx.doi.org/10.2807/1560-7917.ES.2015.20.45.30059>

Article submitted on 24 November 2014 / accepted on 02 July 2015 / published on 12 November 2015

In June 2014, a staphylococcal food poisoning outbreak occurred at an international equine sports event in Luxembourg requiring the hospitalisation of 31 persons. We conducted a microbiological investigation of patients and buffet items, a case–control study and a carriage study of catering staff. Isolates of *Staphylococcus aureus* from patients, food and catering staff were characterised and compared using traditional typing methods and whole genome sequencing. Genotypically identical strains (sequence type ST8, *spa*-type to24, MLVA-type 4698, enterotoxin A FRI100) were isolated in 10 patients, shiitake mushrooms, cured ham, and in three members of staff. The case–control study strongly suggested pasta salad with pesto as the vehicle of infection ($p < 0.001$), but this food item could not be tested, because there were no leftovers. Additional enterotoxigenic strains genetically unrelated to the outbreak strain were found in four members of staff. Non-enterotoxigenic strains with livestock-associated sequence type ST398 were isolated from three food items and two members of staff. The main cause of the outbreak is likely to have been not maintaining the cold chain after food preparation. Whole genome sequencing resulted in phylogenetic clustering which concurred with traditional typing while simultaneously characterising virulence and resistance traits.

Introduction

Food poisoning caused by enterotoxigenic *Staphylococcus aureus* is one of the most common foodborne diseases [1]. In France, which has a long-established foodborne disease surveillance system able to detect fairly rare events [2], staphylococcal food poisoning (SFP) has ranked in recent years as the first cause of foodborne outbreaks: of 1,288 reported foodborne outbreaks in

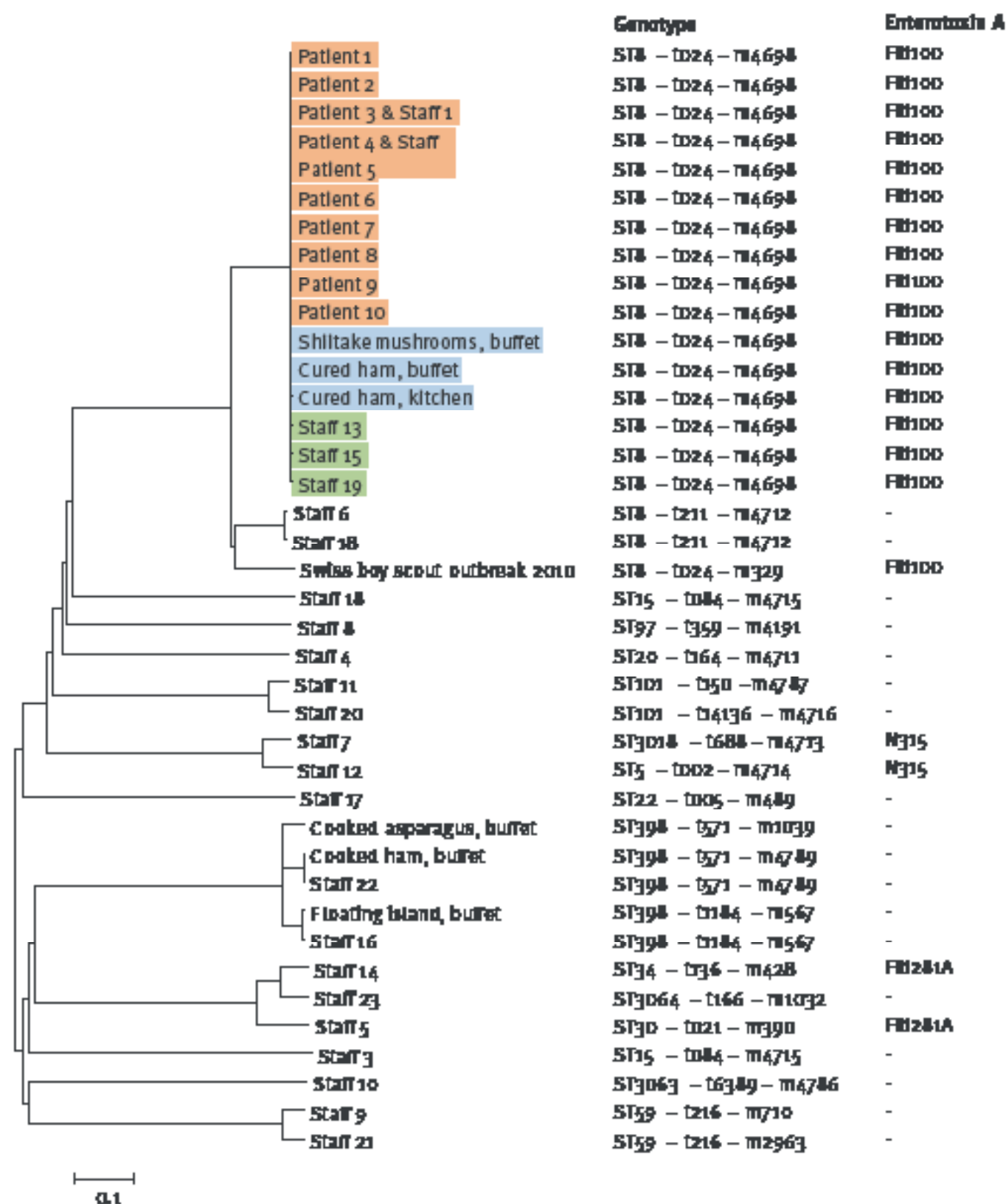
2012, 300 (23%) were due to SFP [3]. SFPs are thought to be under-reported for several reasons. First, because of the short duration of symptoms, only an estimated 10% of SFP patients visit a hospital [4]. Even if patients seek medical care, the physician often does not deem a stool analysis necessary. If a stool analysis is performed, the microbiological routine procedures often do not include testing for the presence of enterotoxigenic *S. aureus* unless specifically requested by the physician [5]. In addition, staphylococcal enterotoxin (SE) is highly stable and heat-resistant. Although the bacteria may have been inactivated by heating the food prior to consumption and can therefore be isolated neither from food nor the stool of the patient, the highly stable enterotoxins performed by *S. aureus* in the food may still be emetically active [6].

In contrast to most other gastrointestinal infections, the onset of SFP symptoms is very rapid, usually within a few hours after ingestion of the contaminated food. The median incubation period of aetiologically confirmed SFP outbreaks occurring in the United States between 1998 and 2008 was estimated to be four hours (5–95 percentile: two to seven hours) [7]. Symptoms in cases in these outbreaks typically included abdominal cramps (72%), vomiting (87%), and diarrhoea (89%). Fever (9%) was infrequently reported. The median duration of illness was 15 hours (5–95 percentile: 4–60 hours) [7].

Here, we report a SFP outbreak that occurred in a buffet restaurant at an international show-jumping event in Luxembourg in June 2014. A total of 31 persons had to be transferred by ambulance from the event site to emergency departments of three local hospitals. We describe findings of the ensuing epidemiological case–control study, the microbiological contamination

FIGURE

Clonal relationship between patient, food, and catering staff isolates, staphylococcal food poisoning outbreak, Luxembourg, 12–13 June 2014



A phylogenetic dendrogram (neighbour joining tree) was generated for 39 *Staphylococcus aureus* isolates based on the allelic profiles of 1,625 available of 1,878 queried MLST+ target genes. The scale bars indicate the number of differing alleles comprising the calculated distance. The colours represent the origin of outbreak-related strains (orange: stool samples from hospitalised patients; blue: food samples; green: throat or nose samples from colonised staff members). The genotype column shows the combined data of multilocus sequence typing (prefix ST), *spa*-typing (prefix t), and MLVA typing (prefix m).

TABLE

Results from analytical case-control study of food exposures, staphylococcal food poisoning outbreak, Luxembourg, June 2014

Food item at VIP buffet	Cases N (%)	Controls N (%)	Odds ratio	p value
Cooked ham	10 (45%)	6 (27%)	2.08 (0.50–9.04)	0.25
Cured ham	13 (59%)	10 (45%)	1.59 (0.41–6.28)	0.45
Grilled shrimps	12 (55%)	8 (36%)	1.95 (0.49–7.84)	0.28
Lamb	13 (59%)	11 (50%)	1.31 (0.33–5.18)	0.66
Melon	13 (59%)	12 (55%)	1.08 (0.27–4.30)	0.90
Panna cotta	13 (59%)	9 (41%)	1.93 (0.49–7.68)	0.29
Pesto pasta salad	18 (82%)	3 (14%)	27 (4.35–195.43)	<0.0001
Potato gratin	15 (68%)	13 (59%)	1.32 (0.31–5.59)	0.67
Potato salad	10 (45%)	11 (50%)	0.76 (0.19–2.94)	0.65
Prawns	3 (14%)	1 (5%)	3.16 (0.22–173.90)	0.32
Raw vegetables	13 (59%)	11 (50%)	1.31 (0.33–5.18)	0.66
Risotto	12 (55%)	9 (41%)	1.6 (0.41–6.31)	0.44
Ruccola salad	10 (45%)	10 (45%)	0.92 (0.24–3.57)	0.89
Salmon tartare	3 (14%)	2 (9%)	1.5 (0.15–19.7)	0.67

of food samples, and colonisation by *S. aureus* of catering employees at the event. In particular, we characterised the *S. aureus* isolates from patients, food items obtained from the buffet, and food handlers using traditional typing methods (PCR, *spa*-typing, and multilocus variable-number tandem repeat analysis (MLVA)), as well as whole genome sequencing.

The event

From 12 to 15 June 2014, an equestrian show-jumping event with approximately 140 participating international athletes and 300 horses took place in Luxembourg. Approximately one to three hours after eating a buffet lunch in the tented VIP restaurant on 12 June, 11 persons with symptoms of vomiting, diarrhoea, and prostration were taken by ambulance to the emergency departments of two hospitals where they received parenteral fluids. The official health inspection service was informed immediately of the incident and microbiological analysis of stool samples from hospitalised patients was ordered. Official food safety inspectors proceeded immediately to take samples from remaining buffet items for microbiological analysis. An inspection of the professional caterer's onsite restaurant and offsite kitchen did not reveal any major food safety deficiencies as specified in regulation (EC) 853/2004. The next morning, on 13 June, local newspapers announced salmon tartare as a potential culprit. A few hours after having the buffet lunch in the VIP restaurant on 13 June, a further 20 persons fell ill with the same symptoms and were transferred by ambulance to hospital emergency departments. The event organiser stopped serving any prepared meals for the remainder of the event. On 14 and 15 June, there were no further reports of gastrointestinal illness related to the event. While approximately 150–200 persons were estimated to have consumed the buffet lunch in the VIP

restaurant on both days and a total of 31 persons were admitted to hospital emergency departments over the two days, the exact number of affected persons is unknown. There were no reports of illness among those people who ate at the other food-serving premises at the event: a non-VIP lunch buffet operated by the same caterer but with different menus, and a barbecue stall hosted by non-professional club members.

Methods

Microbiological examination of stool samples

Culture of stool samples for bacterial pathogens (including *Salmonella*, *Campylobacter* and verotoxinogenic *Escherichia coli*) conducted in three hospital laboratories revealed the presence of *S. aureus* in ten patients and *Enterococcus* in one patient. Isolates of *S. aureus* were immediately referred to the National Health Laboratory for further molecular characterisation.

Case-control study

Following their recovery from illness and after the food samples had been analysed, eight cases who had been admitted to emergency care were contacted by telephone to get initial information on potential food exposures. All food items and symptoms reported by cases were included in a final questionnaire administered by telephone to 22 cases and 21 controls. Cases were defined as persons with sudden gastrointestinal illness (at least one symptom: vomiting, diarrhoea, abdominal cramps or nausea) who had eaten buffet lunch at the VIP restaurant on 12 or 13 June. Controls were defined as persons who had eaten buffet lunch at the VIP restaurant on 12 or 13 June, without any gastrointestinal symptoms. Non-hospitalised cases and controls were contacted using information provided by the event organiser.

Testing of food samples

Food samples were tested by accredited methods for aerobic plate count, *E. coli*, coagulase-positive staphylococci (ISO 6888-2:1999), *Salmonella*, and *Bacillus cereus*. The salmon tartare and floating island dessert samples were additionally tested for *Listeria monocytogenes*.

Staphylococcal carriage study among caterer's employees

Following the detection of *S. aureus* in patients, a staphylococcal carriage study was conducted on 19 and 20 June among the caterer's employees who worked in the onsite restaurant or in the offsite kitchen where buffet items were prepared, including the slicing of ham. Catering employees screened included waiters, cooks, and other kitchen staff. Throat and nose swabs were taken by doctors and sent the same day to the laboratory where they were streaked onto selective Chapman media (reference 51053, BioMérieux, Marcy l'Etoile, France).

Characterisation of *S. aureus* isolates and whole genome sequencing

Isolates of *S. aureus* obtained from patients, food, and catering employees were confirmed by MALDI-TOF mass spectrometry (Bruker, Brussels, Belgium). Confirmed isolates of *S. aureus* were further characterised for the presence of *nuc*, *mecA*, toxic shock syndrome toxin 1 (TSST-1), and Pantone-Valentine leukocidine (PVL) [8] as well as genes coding for staphylococcal enterotoxins A (*sea*), B, C, D E, H, I, and J [9]. Isolates exhibiting *sea* were further characterised by sequencing the PCR products and compared to strains containing allelic *sea* variants FRI100, FRI287A, and N315. In addition, isolates were subjected to *spa*-typing [8] and MLVA typing [10]. Whole genome sequencing of isolates was performed on a Miseq Desktop Sequencer using the Nextera DNA sample preparation kit (Illumina, Eindhoven, The Netherlands) with an average coverage of 59 fold (range 27–140 fold). Antimicrobial resistance genes, virulence factors and multi-locus sequence types (MLST) were determined by submitting the raw read files to public webserver tools hosted by the Center for Genomic Epidemiology in Denmark [11–13]. After sequencing, whole genome MLST+ was conducted using the Seqsphere+ v2.3 pipeline (Ridom, Münster, Germany). Briefly, after trimming reads until the average quality was 30 in a window of 30 bases, the trimmed reads were mapped to the reference genome NC_002951.2 and the allelic profiles of 1,878 target genes were determined based on the MLST+ scheme developed previously [14]. Final phylogenetic analysis was based on 10 patient isolates, 6 food isolates, 22 carrier isolates of members of staff, and an isolate with *spa*-type to24 from an unrelated Swiss outbreak in 2010. The whole genome reads were deposited at the European Nucleotide Archive (study accession no. PRJEB7847). For the purpose of this study, we use the terminology 'genotype' to denote the combined typing results of MLST, *spa*- and MLVA typing.

Results

Fifteen (48%) of the 31 patients admitted by ambulance to emergency care were women. The median age was 35 years (range 14–58 years). Three patients were catering staff at the event. Six patients were non-residents of Luxembourg. Information on clinical symptoms was available for 22 of the patients and included diarrhoea (20 patients), nausea (19 patients), cramps (19 patients) and vomiting (17 patients). All admitted patients were discharged within 48 hours. All 10 isolates obtained from patients' stool samples represented the same genotype (MLST sequence type (ST)-8, *spa*-type to24, MLVA-type 4698), possessed genes encoding *sea* allele FRI100 and conferring penicillin resistance mediated by *blaZ*.

Results from the analytical epidemiological case-control study (Table) implicated consumption of pasta salad with pesto as the most likely vehicle of SFP. Eighteen of 22 cases reported eating this food item compared to 3 of 21 controls ($p < 0.0001$). All 14 interviewed cases who had been hospitalised reported eating the pasta salad with pesto. Unfortunately, there were no leftovers of the pasta salad with pesto when sampling was taking place and so this dish was not available for microbiological testing. Eating cured ham or salmon tartare were not statistically significant risk factors ($p = 0.45$). One interviewed patient reported not having eaten ham at the buffet for religious reasons.

Food samples

Isolates of *S. aureus* with a genotype identical to patient isolates (MLST ST-8, *spa*-type to24, MLVA-type 4698) were detected in cured ham samples (range <40–5,200 colony-forming units (CFU)/g and shiitake mushrooms (<40 CFU/g) sampled at the event site and in cured ham samples (enumeration range <40–120 CFU/g) obtained at the offsite catering kitchen where the ham was sliced and stored (Figure). Non-enterotoxigenic isolates of *S. aureus* with a different genotype to patient isolates were found in cooked asparagus (<40 CFU/g, MLST ST-398, *spa*-type t571, MLVA type 1039), the floating island dessert (<40 CFU/g, MLST ST-398, *spa*-type t1184, MLVA-type 567) and several samples of cooked ham (range 50–320 CFU/g, MLST ST-398, *spa*-type t571, MLVA-type 4789). Unsliced complete legs of cured and cooked hams obtained from the supplying butcher were negative for *S. aureus*. All 18 food items sampled from the event buffet were negative for *Salmonella* and *E. coli*. One food item (cooked asparagus) was positive for presumptive *Bacillus cereus* (840 CFU/g).

The pasta salad with pesto could not be sampled during food inspection, as there were no leftovers from this dish. The primary ingredients used to make the pesto sauce for the pasta salad (fresh basil, hard cheese, and pine nuts) were all negative for *S. aureus*.

Staphylococcal carriage study

Thirty-eight of the 49 catering employees at the event were screened for nasal/throat carriage of *S. aureus*.

Median age of the screened employees was 32.5 years (range 17–50 years), and 11 were women. Twenty-two employees were found to be colonised by *S. aureus*: three staff members were colonised by strains identical to those found in patients (Figure). Another four employees were colonised by *S. aureus* isolates exhibiting *sea*, but a different genotype than the outbreak strain. None of the seven employees colonised by isolates exhibiting *sea* reported wounds or gastrointestinal disease prior to the event. Overall, 17 different genotypes were observed among the 22 colonised employees. None of the isolates in food, patients, or catering employees were meticillin-resistant or exhibited *pvl*.

Whole genome sequencing

The whole genome phylogeny (Figure), as determined by 1,625 of 1,878 MLST and MLST+ target genes that were present in all 39 isolates, clearly delineated the outbreak isolates. *S. aureus* isolates found in 10 patients were identical to those isolated from cured ham, shiitake mushrooms and from three catering employees. Interestingly, the Luxembourg outbreak strain had 347 allele differences with a strain that led to the intoxication of 27 boy scouts in Switzerland in 2010, although both strains share a common *spa*-type to24 [15]. Two of the three food isolates which differed from the outbreak strain were also observed among catering employees. These belonged to livestock-associated sequence type ST398 with *spa*-types t571 or t1184.

Discussion

Studies of foodborne outbreaks, in which enterotoxigenic isolates were detected in patients, food, and food handlers, are rare [16–18]. Our report shows that, even in the era of whole genome sequencing, public health investigations of foodborne outbreaks remain very dependent on classical case–control investigations for interpretation of events. Whereas initial microbiological typing results suggested cured ham as the main vehicle for the intoxication, the case–control study clearly identified the pasta salad with pesto as the most likely source, which was no longer available for microbiological testing.

In our outbreak, there was good evidence that the pathogen responsible for the outbreak was *S. aureus*, because identical enterotoxigenic strains of *S. aureus* with a common *spa*-type but rare MLVA type were recovered from the stools of 10 hospitalised cases. Because three catering employees were colonised by a strain with the same genotype, it is likely that at least one of them may represent the source of food contamination, either via manual contact or through respiratory secretions [19]. However, because catering employees were screened a week after the outbreak, it cannot also be ruled out that some staff members became colonised only during or after the event [20].

One of the probable factors contributing to the outbreak may have been the unusually hot weather for the season, with maximum temperatures ranging between 25 °C and 32 °C during the week preceding the event, compared with a historical average of 21 °C. The food safety inspection at the catering facility revealed that a fridge had stopped working properly a few days prior to the event, although the catering staff denied using this fridge to store any of the dishes. The pasta salad with pesto was reported to have been pre-cooked and sealed into plastic bags in 2 kg portions, and then cooled down in a fast refrigeration unit. Nevertheless, the fact that *S. aureus* was detected in several dishes including cured and cooked ham, at concentrations up to 5,200 CFU/g, suggests that the cold chain before or during the event was interrupted to allow sufficient microbial growth during or following food manipulation.

A major limitation of our study is that the food item identified by the case–control study, pasta salad with pesto, was no longer available for testing and thus there is no microbiological evidence that the pasta salad with pesto was contaminated with the outbreak strain. However, matrices with similar biochemical properties like potato salad have been confirmed before as vehicles of SFP in France [21] and Switzerland [15]. In the latter case, a strain with identical *spa* type to24 and enterotoxin A FRI100 allele led to the intoxication of 27 boy scouts. The *sea* gene found in our outbreak strain is the dominant *sea* allele described in *S. aureus* isolates that are associated with food poisoning outbreaks worldwide [19,21–23] and in enterotoxigenic isolates recovered from food handlers [24].

The epidemiological results from our carriage study are consistent with previous findings in similar studies. Our finding of 58% carriers among food handlers concurs with longitudinal studies showing that approximately 20% of persons are persistent nasal carriers and an additional 30% are intermittent carriers of *S. aureus* [25]. The high genetic diversity among asymptomatic carriers was also observed in similar studies in Germany [26], Switzerland [27], and Bosnia [28]. Interestingly, we found meticillin-susceptible livestock-associated strains with ST398 *spa* type t571 and variants thereof in both catering employees and in food. Similar clones have recently emerged causing severe infections in neighbouring France and Belgium [29,30], while remaining rare in Germany [31].

Although WGS has been applied to meticillin-resistant *S. aureus* in hospital and long-term care settings [32–34] and to other foodborne pathogens [35,36], to our knowledge our study is the first to report WGS as a tool in a staphylococcal food poisoning outbreak. While WGS showed virtually identical groupings to MLVA, one major advantage of WGS is that it is a universal method applicable to any bacterial species and that it provides further data on the presence of genes encoding virulence and resistance factors.

Acknowledgments

We thank the hospital biologists Drs Cynthia Oxacelay, Nawfal Faik, and Alain Hakim, who provided us with the patient isolates and the occupational health service who took throat and nasal swab samples from the catering employees.

Conflict of interest

None declared.

Authors' contributions

JM coordinated the various investigations, collated strains from different sources, constructed phylogenies, conducted the statistical analysis for the case-control study, and wrote the manuscript; FD conducted the classical genotyping including MLVA, spa typing and virulence factor detection by PCR; GM was responsible for the laboratory analysis of food items; CR and CO conducted the whole genome sequencing; CO assisted with bioinformatics and with preparing the figure; SJ provided reference material and assisted with interpretation; MP was responsible for the microbiological analysis of human strains; PH led the food inspection; PW was responsible for the public health response and the case-control data collection.

References

1. Hennekinne JA, De Buyser ML, Dragacci S. *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiol Rev*. 2012;36(4):815-36. .DOI: 10.1111/j.1574-6976.2011.00311.x PMID: 22091892
2. Ostyn A, De Buyser ML, Guillier F, Groult J, Felix B, Salah S, et al. First evidence of a food poisoning outbreak due to staphylococcal enterotoxin type E, France, 2009. *Euro Surveill*. 2010;15(13).PMID: 20394711
3. Institut de Veille Sanitaire (InVS). Surveillance des toxi-infections alimentaires collectives. Données de la déclaration obligatoire. [Surveillance of foodborne illness. Data from mandatory reporting]. Saint-Maurice, France: INVS; 2012. French. Available from: www.invs.sante.fr/content/download/85413/313604/version1/file/tiac_donnees_2012.pdf
4. Holmberg SD, Blake PA. Staphylococcal food poisoning in the United States. New facts and old misconceptions. *JAMA*. 1984;251(4):487-9. .DOI: 10.1001/jama.1984.03340280037024 PMID: 6690814
5. Cornaglia G, Courcol R, Herrmann J-L, Kahlmeter G, Peigue-Lafeuille H, Vila J, editors. *European Manual of Clinical Microbiology*. 1st ed. Basel: European Society for Clinical Microbiology and Infectious Diseases and Société Française de Microbiologie; 2012.
6. Le Loir Y, Baron F, Gautier M. *Staphylococcus aureus* and food poisoning. *Genet Mol Res*. 2003;2(1):63-76.PMID: 12917803
7. Bennett SD, Walsh KA, Gould LH. Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*--United States, 1998-2008. *Clin Infect Dis*. 2013;57(3):425-33. .DOI: 10.1093/cid/cit244 PMID: 23592829
8. Mossong J, Gelhausen E, Decruyenaere F, Devaux A, Perrin M, Even J, et al. Prevalence, risk factors and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) colonization in residents of long-term care facilities in Luxembourg, 2010. *Epidemiol Infect*. 2013;141(6):1199-206. .DOI: 10.1017/S0950268812001999 PMID: 22953727
9. Kwon NH, Kim SH, Park KT, Bae WK, Kim JY, Lim JY, et al. Application of extended single-reaction multiplex polymerase chain reaction for toxin typing of *Staphylococcus aureus* isolates in South Korea. *Int J Food Microbiol*. 2004;97(2):137-45. .DOI: 10.1016/j.ijfoodmicro.2004.04.014 PMID: 15541800
10. Schouls LM, Spalburg EC, van Luit M, Huijsdens XW, Pluister GN, van Santen-Verheul MG, et al. Multiple-locus variable number tandem repeat analysis of *Staphylococcus aureus*: comparison with pulsed-field gel electrophoresis and spa-typing. *PLoS ONE*. 2009;4(4):e5082. .DOI: 10.1371/journal.pone.0005082 PMID: 19343175
11. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother*. 2012;67(11):2640-4. .DOI: 10.1093/jac/dks261 PMID: 22782487
12. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. *J Clin Microbiol*. 2012;50(4):1355-61. .DOI: 10.1128/JCM.06094-11 PMID: 22238442
13. Joensen KG, Scheut F, Lund O, Hasman H, Kaas RS, Nielsen EM, et al. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J Clin Microbiol*. 2014;52(5):1501-10. .DOI: 10.1128/JCM.03617-13 PMID: 24574290
14. Leopold SR, Goering RV, Witten A, Harmsen D, Mellmann A. Bacterial whole-genome sequencing revisited: portable, scalable, and standardized analysis for typing and detection of virulence and antibiotic resistance genes. *J Clin Microbiol*. 2014;52(7):2365-70. .DOI: 10.1128/JCM.00262-14 PMID: 24759713
15. Johler S, Leyer F, Stephan R. Comparison of virulence and antibiotic resistance genes of food poisoning outbreak isolates of *Staphylococcus aureus* with isolates obtained from bovine mastitis milk and pig carcasses. *J Food Prot*. 2011;74(11):1852-9. .DOI: 10.4315/0362-028X.JFP-11-192 PMID: 22054185
16. Wei HL, Chiou CS. Molecular subtyping of *Staphylococcus aureus* from an outbreak associated with a food handler. *Epidemiol Infect*. 2002;128(1):15-20. .DOI: 10.1017/S0950268801006355 PMID: 11895086
17. Schmid D, Gschel E, Mann M, Huhulescu S, Ruppitsch W, Bohm G, et al. Outbreak of acute gastroenteritis in an Austrian boarding school, September 2006. *Euro Surveill*. 2007;12(3):224.PMID: 17439809
18. Johler S, Tichaczek-Dischinger PS, Rau J, Sihto HM, Lehner A, Adam M, et al. Outbreak of *Staphylococcal* food poisoning due to SEA-producing *Staphylococcus aureus*. *Foodborne Pathog Dis*. 2013;10(9):777-81. .DOI: 10.1089/fpd.2013.1503 PMID: 23767854
19. Argudín MA, Mendoza MC, Rodicio MR. Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins (Basel)*. 2010;2(7):1751-73. .DOI: 10.3390/toxins2071751 PMID: 22069659
20. Kadariya J, Smith TC, Thapaliya D. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *Biomed Res Int*. 2014;2014:827965.
21. Kérouanton A, Hennekinne JA, Letertre C, Petit L, Chesneau O, Brisabois A, et al. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *Int J Food Microbiol*. 2007;115(3):369-75. .DOI: 10.1016/j.ijfoodmicro.2006.10.050 PMID: 17306397
22. Yan X, Wang B, Tao X, Hu Q, Cui Z, Zhang J, et al. Characterization of *Staphylococcus aureus* strains associated with food poisoning in Shenzhen, China. *Appl Environ Microbiol*. 2012;78(18):6637-42. .DOI: 10.1128/AEM.01165-12 PMID: 22798367
23. Veras JF, do Carmo LS, Tong LC, Shupp JW, Cummings C, Dos Santos DA, et al. A study of the enterotoxigenicity of coagulase-negative and coagulase-positive staphylococcal isolates from food poisoning outbreaks in Minas Gerais, Brazil. *Int J Infect Dis*. 2008;12(4):410-5. .DOI: 10.1016/j.ijid.2007.09.018 PMID: 18206412
24. Argudín MA, Mendoza MC, González-Hevia MA, Bances M, Guerra B, Rodicio MR. Genotypes, exotoxin gene content, and antimicrobial resistance of *Staphylococcus aureus* strains recovered from foods and food handlers. *Appl Environ Microbiol*. 2012;78(8):2930-5. .DOI: 10.1128/AEM.07487-11 PMID: 22327598
25. Wertheim HFL, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis*. 2005;5(12):751-62. .DOI: 10.1016/S1473-3099(05)70295-4 PMID: 16310147
26. Monecke S, Luedicke C, Slickers P, Ehrlich R. Molecular epidemiology of *Staphylococcus aureus* in asymptomatic carriers. *Eur J Clin Microbiol Infect Dis*. 2009;28(9):1159-65. .DOI: 10.1007/s10096-009-0752-2 PMID: 19434432
27. Wattinger L, Stephan R, Leyer F, Johler S. Comparison of *Staphylococcus aureus* isolates associated with food intoxication with isolates from human nasal carriers and human infections. *Eur J Clin Microbiol Infect Dis*. 2012;31(4):455-64. .DOI: 10.1007/s10096-011-1330-y PMID: 21761125
28. Uzunović S, Ibrahimagic A, Kamberovic F, Rijnders MIA, Stobberingh EE. Molecular Characterization of Methicillin-Susceptible And Methicillin-Resistant *Staphylococcus aureus* in Food Handlers in Bosnia and Herzegovina. *Open Infect Dis J*. 2013;7(1):15-20. .DOI: 10.2174/1874279301307010015
29. Brunel AS, Bañuls AL, Marchandin H, Bouzinbi N, Morquin D, Jumas-Bilak E, et al. Methicillin-sensitive *Staphylococcus*

- aureus CC398 in intensive care unit, France. *Emerg Infect Dis.* 2014;20(9):1511-5. .DOI: 10.3201/eid2009.130225 PMID: 25148514
30. Vandendriessche S, Kadlec K, Schwarz S, Denis O. Methicillin-susceptible *Staphylococcus aureus* ST398-t571 harbouring the macrolide-lincosamide-streptogramin B resistance gene *erm*(T) in Belgian hospitals. *J Antimicrob Chemother.* 2011;66(11):2455-9. .DOI: 10.1093/jac/dkr348 PMID: 21868413
 31. Cuny C, Layer F, Köck R, Werner G, Witte W. Methicillin susceptible *Staphylococcus aureus* (MSSA) of clonal complex CC398, t571 from infections in humans are still rare in Germany. *PLoS ONE.* 2013;8(12):e83165. .DOI: 10.1371/journal.pone.0083165 PMID: 24367584
 32. Köser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, et al. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. *N Engl J Med.* 2012;366(24):2267-75. .DOI: 10.1056/NEJMoa1109910 PMID: 22693998
 33. Harris SR, Cartwright EJP, Török ME, Holden MTG, Brown NM, Ogilvy-Stuart AL, et al. Whole-genome sequencing for analysis of an outbreak of methicillin-resistant *Staphylococcus aureus*: a descriptive study. *Lancet Infect Dis.* 2013;13(2):130-6. .DOI: 10.1016/S1473-3099(12)70268-2 PMID: 23158674
 34. Price JR, Golubchik T, Cole K, Wilson DJ, Crook DW, Thwaites GE, et al. Whole-genome sequencing shows that patient-to-patient transmission rarely accounts for acquisition of *Staphylococcus aureus* in an intensive care unit. *Clin Infect Dis.* 2014;58(5):609-18. .DOI: 10.1093/cid/cit807 PMID: 24336829
 35. Schmid D, Allerberger F, Huhulescu S, Pietzka A, Amar C, Kleta S, et al. Whole genome sequencing as a tool to investigate a cluster of seven cases of listeriosis in Austria and Germany, 2011-2013. *Clin Microbiol Infect.* 2014;20(5):431-6. .DOI: 10.1111/1469-0691.12638 PMID: 24698214
 36. Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, et al. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS ONE.* 2011;6(7):e22751. .DOI: 10.1371/journal.pone.0022751 PMID: 21799941

Persistent occurrence of serogroup Y/sequence type (ST)-23 complex invasive meningococcal disease among patients aged five to 14 years, Italy, 2007 to 2013

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Citation style for this article:

Fazio C, Neri A, Renna G, Vacca P, Antonetti R, Barbui A, Daprai L, Lanzafame P, Rossi L, Santino I, Tascini C, Vocale C, Stefanelli P. Persistent occurrence of serogroup Y/sequence type (ST)-23 complex invasive meningococcal disease among patients aged five to 14 years, Italy, 2007 to 2013. Euro Surveill. 2015;20(45):pii=30061. DOI: <http://dx.doi.org/10.2807/1560-7917.ES.2015.20.45.30061>

Article submitted on 27 January 2015 / accepted on 20 September 2015 / published on 12 November 2015

In Italy, the incidence of invasive meningococcal disease (IMD) has remained stable since 2007 (around 0.3 cases/100,000 inhabitants). However, as reported for other European countries, an increase of serogroup Y *Neisseria meningitidis* has been observed. In this study we report IMD cases from 2007 to 2013 in Italy and investigate the clinical and epidemiological features of cases affected by serogroup Y. Molecular characteristics of serogroup Y strains are also described. During the study period, the proportion of IMD cases due to serogroup Y increased, ranging from 2% in 2007 to 17% in 2013 (odds ratio (OR): 8.8), whereby the five to 14 years age group was mostly affected ($p < 0.001$). Overall 81 serogroup Y IMD cases were identified, with a median age of 18 years, ranging from three months to 84 years. Of the 81 respective patient samples, 56 were further subject to molecular typing. The sequence type (ST)-23 complex (clonal complex (cc)23) was predominant among serogroup Y meningococci (54/56 samples), and included nine different STs. Presumably, ST-23 was the founding genotype, with all the other STs presenting as single-locus variants. All cc23 isolates analysed harboured mutations in the *lpxL1* gene; however, no associations among *lpxL1* mutations, ST and age group were identified. Overall, these findings generate scientific evidence for the use of the quadrivalent meningococcal conjugate vaccine in the five to 14 years age group.

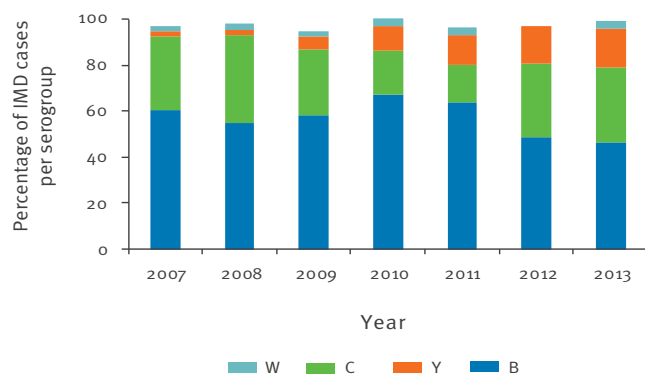
Introduction

Since the 1990s, some significant changes in serogroup Y *Neisseria meningitidis* (MenY) epidemiology have been reported worldwide. During the beginning of this period, an increase of MenY cases was observed in the United States (US) [1], as well as in Latin American countries [2]. In Colombia the proportion of MenY cases peaked at 50% in 2006 [3]. MenY incidence increased also in Europe [4,5]. In France, MenY accounted for only 5.5% of all cases of invasive meningococcal disease (IMD) in 2010 but for 10% in 2013 [6]. In Norway and in Finland, MenY represented respectively 31% and 38% of all cases reported in 2010 [7,8]. MenY emergence was observed also in Sweden, with an increase of the incidence from <0.05 cases per 100,000 inhabitants in 2000 to 0.23 in 2010 [9].

In Italy, although the incidence of IMD remained stable since 2007 (around 0.3 cases/100,000 inhabitants; data from National Surveillance System <http://www.iss.it/mabi/>), some changes were noted in the frequency distribution of specific meningococcal serogroups. In our country, similarly to other European countries, serogroup B and C are responsible for the majority of IMD cases, however, an increase in the proportion of MenY has been observed, from 4% before 2005 to 7% in 2006 [10]. Some changes in the distribution of serogroups may be due to the introduction of the meningococcal serogroup C conjugate (MCC) vaccination (between 2005 and 2007), which has been included in the 2012 to 2014 national immunisation plan (NIP), in accordance with regional policies; the

FIGURE 1

Annual proportion of invasive meningococcal disease (IMD) cases due to serogroups B, C, Y, W, Italy, 2007–2013



The serogroups B, C, W and Y are represented by different colours as specified in the legend.

vaccine is recommended to all children between 13 and 15 months of age, and to 11 to 18 year-old individuals, if not previously vaccinated, and to those belonging to risk categories [11].

The aims of present study were: (i) to describe the trend of MenY IMD cases from 2007 to 2013 in Italy and (ii) to investigate the clinical and epidemiological features and the molecular characteristics of MenY cases.

Methods

Bacterial isolates

In Italy, notification of all cases of IMD is mandatory. Clinical and epidemiological information and meningococcal isolates are collected in the frame of the National Surveillance System coordinated by the National Reference Laboratory (NRL) of the Istituto Superiore di Sanità.

Every year, the NRL receives an average of 75% of the meningococci isolated by local hospital laboratories throughout the country. Epidemiological and microbiological data for each IMD case are managed using a dedicated database. Local laboratories send the isolates to the NRL, where they are stored at -80°C before complete microbiological characterisation.

Microbiological analyses

Serogroup is confirmed by slide agglutination with commercial antisera (Remel Europe, Ltd, United Kingdom) or by multiplex polymerase chain reaction (PCR) [12]. Susceptibility to ceftriaxone, ciprofloxacin, penicillin G and rifampicin is determined by E-test method (bioMérieux SA - France) on Mueller-Hinton agar (Oxoid) supplemented with 5% of sheep blood. The breakpoints are those recommended by the European Committee

on Antimicrobial Susceptibility Testing – EUCAST version 5.0, 1 January 2015 (<http://www.eucast.org/>).

Molecular typing

Chromosomal DNA is extracted by using the QIAamp DNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Multilocus sequence typing (MLST), porin A (PorA) and ferric enterobactin transport protein A (FetA) typing are defined as described in <http://neisseria.org/>. The finetype is identified as follows: capsular group: *porA* (P1). Variable region (VR)1, VR2: *fetA* VR: sequence type (ST) (clonal complex (cc)). The *lpxL1* gene amplification and sequencing were performed as indicated by Ladhani et al. [13].

eBurst

MLST data were analysed by eBURST, version 3, (<http://eburst.mlst.net>) [14]. eBurst analysis was set up referring to the most stringent setting of identity of alleles in six of the seven housekeeping genes.

Statistical analysis

The data were analysed using EpiInfo (version 3.4.5, July 30, 2013). Odds ratios (OR), 95% confidence intervals (CI) and p values, were obtained to measure the strength of the association between serogroup Y and other variables. Statistical differences were tested using standard tests (i.e. chi-squared and chi-squared for trend); the level of statistical significance is set at $p < 0.05$.

Results

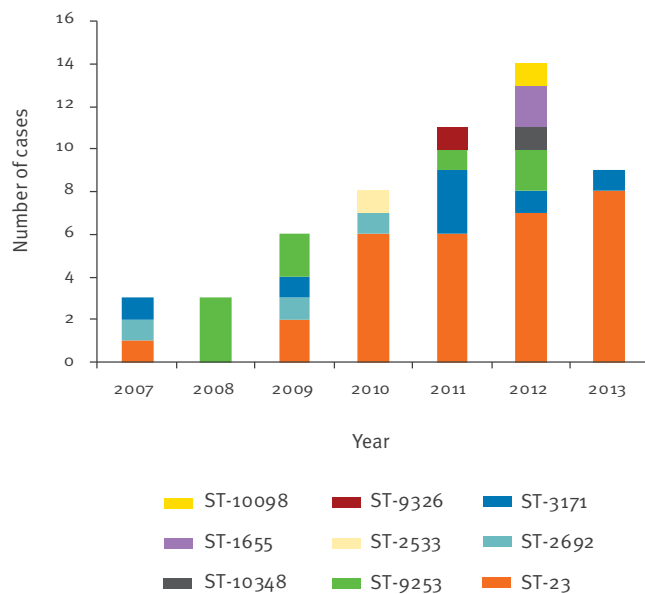
From 2007 to 2013, a total of 1,157 IMD cases were detected in Italy, with an average annual incidence of 0.27 cases per 100,000 inhabitants. The annual proportions of IMD cases attributable to the principal serogroups (B, C, Y, W) by year are shown in Figure 1. The serogroup was obtained for 902 cases (78%), including 514 (57%) for serogroup B, 253 (28%) for serogroup C, 81 (9%) for serogroup Y, 23 (3%) for serogroup W, 10 (1%) for serogroup A, and 21 (2%) for other serogroups. The proportion of MenY IMD cases increased over the years, ranging from 2% in 2007 (3/134 cases) to 17% (20/119 cases) in 2013 (the OR for 2013 compared with 2007 was 8.8, $p < 0.001$).

The median age of the patients infected by MenY was 18 years, ranging from three months to 84 years; patients infected with serogroup Y appeared to be older than patients infected by other serogroups (18 years vs 16). Overall, 35% (28/81) of MenY cases occurred in the age group comprising five to 14 year-olds, and this age group was the most affected since 2008.

The distribution of cases attributable to MenY and to other serogroups by single variable of interest is shown in the Table. In the study period, among all patients in the age group five to 14 years, almost 20% (28/141) were infected with MenY; patients in this age group were more likely to be infected with MenY compared

FIGURE 2

Distribution of sequence types (STs) belonging to clonal complex (cc) 23 (n=54)^a among a subset of samples from serogroup Y *Neisseria meningitidis* cases, which were typed, Italy, 2007–2013



^a During the study period, a total of 56 samples from serogroup Y invasive meningococcal disease cases were analysed. Of these, samples from 54 cases belonged to cc23 and are depicted in the figure according to ST.

with all the other age groups (OR:3.3; 95% CI:1.94–5.59), (data not shown).

Differences with regard to the risk of being infected with MenY according to sex were not statistically significant.

As expected, meningitis and septicaemia represented the main clinical pictures among IMD cases. There was no significant difference in MenY infection among cases with different clinical presentation. However, among patients with MenY, an increase of septicaemia, from 19% (3/16 cases) in 2011 to 42% (8/19 cases) in 2013 was observed. The respective proportion of serogroup Y in the south and the islands was higher than in northern and central Italy, with an OR of 2.18 (Table). The outcome, available for 52 of 81 cases, was fatal for three patients: two women (67 and 45 years-old) and a six year-old child with sepsis, corresponding to a case fatality ratio of six per cent.

A total of 59 samples from the 81 serogroup Y IMD cases, were received by the NRL, allowing further typing. Moreover bacterial isolates derived from 50 patients respectively, were also obtained, and could be used for antibiotic susceptibility testing. All MenY isolates retrieved from cases were susceptible to ceftriaxone, ciprofloxacin and rifampicin. Moreover, 21 of 50 isolates showed a decreased susceptibility to penicillin G (minimum inhibitory concentration (MIC)₅₀ and MIC₉₀ were 0.047 and 0.125mg/L, respectively).

Molecular analyses

Molecular analyses were performed on 56 of 59 MenY samples received by the NRL. MLST identified the ST-23/clonal complex (cc) 23 as the major cc (54/56 samples). The remaining two belonged to ST-167 complex (cc167), one was ST-767 and one was ST-884. Nine different STs were found in the cc23: ST-23 (30 samples), ST-9253 (8 samples), ST-3171 (7 samples), ST-2692 (3 samples), ST-1655 (2 samples), ST-2533 (1 sample), ST-9326 (1 sample), ST-10348 (1 sample) and ST-10098 (1 sample) corresponding to a new MLST profile defined for the first time in this study (Figure 2). ST-23 was detected during the whole period, with 10 samples in the age group comprising five to 14 year-olds. ST-1655, ST-10098 and ST-10348 appeared in Italy for the first time in 2012.

eBURST analysis clustered all STs belonging to the cc23 in a same group. The ST-23 was determined to be the founding genotype, with all others STs as single-locus variants (SLV).

PorA typing

Among the 56 samples which were typed, *porA* VR1 identified two different types: P1.5–2 in 45 samples and P1.5–1 in 11 samples (20%). *porA* VR2 identified eight different types, including P1.10–2 (35 samples), P1.10–1 (8 samples), P1.2–2 (8 samples), and P1.10–4, P1.10–8, P1.10–28, P1.10–92, P1.13–2 as singletons. The 5–2, 10–2 was the *porA* VR1, VR2 combination more frequently detected (35 samples).

FetA

F2–13 was present in 28 samples, F4–1 in 13 and the F5–8 in eight. Moreover, seven *fetA* types (F1–3, F1–12, F1–15, F1–23, F1–80, F2–9, F5–8) were identified as single samples.

Finetypes

A total of 25 different finetypes was identified. The two main were Y: P1.5–2,10–2: F2–13: ST-23 (cc23) (16 samples) and Y: P1.5–2,10–2: F2–13: ST-9253 (cc23) (7 samples). The former one appeared in 2009 and increased through 2013; differently, the latter finetype decreased from 2008 through 2012.

lpxL1

All 56 serogroup Y samples were analysed for the *lpxL1* gene however the gene amplification using the existing primers failed for eight samples. These belonged either to the finetypes Y: P1.5–1,2–2: F5–8: ST-23 (cc23) or Y: P1.5–1,2–2: F5–8: ST-3171 (cc23).

All cc23 samples analysed (n=48) harbour a mutation in the *lpxL1* gene. In particular, 38 were *lpxL1* type XVII, seven type VI, two type V and one type XVI. However, no associations between a specific *lpxL1* type, ST and age group were identified. Moreover, no differential association with the clinical picture of meningitis and septicaemia was found. Isolates belonging to cc167

TABLE

Epidemiological and clinical data of patients with invasive meningococcal disease due to serogroup Y compared with other serogroups, Italy, 2007–2013 (n=902)

Variable	Serogroup Y patients (N=81) n (%)	Other serogroups patients (N=821) n (%)	OR (95% CI)	Total patients (N=902) n (%)
Age group in years ^a				
<5	8 (10)	274 (33)	1	282 (31)
5–14	28 (35)	113 (14)	8.49 (3.56–20.91) ^b	141 (16)
15–24	12 (15)	166 (20)	2.48 (0.92–6.78)	178 (20)
≥24	33 (41)	266 (32)	4.25 (1.84–10.17) ^b	299 (33)
Sex ^a				
Male	45 (56)	420 (51)	1.19 (0.73–1.93)	465 (52)
Female	36 (44)	399 (49)	1	435 (48)
Clinical picture				
Meningitis	44 (54)	371 (45)	1.21 (0.69–2.15)	415 (46)
Septicaemia	22 (27)	225 (27)	1	247 (27)
Meningitis + septicaemia	14 (17)	223 (27)	0.64 (0.30–1.35)	237 (26)
Other	1 (1)	2 (1)	NA	3 (1)
Geographical area				
North	44 (54)	541 (66)	1	585 (65)
Centre	18 (22)	173 (21)	1.28 (0.69–2.35)	191 (21)
South and islands	19 (23)	107 (13)	2.18 (1.18–4.029) ^b	126 (14)

CI: confidence interval; NA: not applicable; OR: odds ratio.

^a Age and sex were unknown in two cases.

^b $p \leq 0.01$.

showed a *lpxL1* sequence identical to the reference (GenBank accession number: AE002098.2).

The *lpxL1* genotype was analysed in a subsample of non-serogroup Y meningococci. Among 20 serogroup C strains only one showed the mutation type III in *lpxL1* gene.

Discussion

As already reported, a stable increase of MenY cases has been observed in Italy since 2004 [10]. Noteworthy, the proportion of MenY among IMD cases increased almost eight times between 2007 (2%) and 2013 (17%).

Previous studies reported that, relative to other *N. meningitidis* serogroups, MenY is usually found in older patients [15–17]. Nevertheless, recent data analyses from several countries yield conflicting results as to the principal age groups affected by MenY: 20 to 29 years in Sweden (2000–2010) [9], ≥45 years in England and Wales (2007–2009) [13] and <5 years in South Africa (2003–2007) [18]. In Italy, the most affected age group comprised 45 to 64 year-olds until 2007, shifting to the five to 14 years age group from 2008 onwards; in this regard, from 2007 to 2013, ca 20% of patients in the latter age group were affected by MenY.

Of note, since 2011, an increase of septicaemia cases attributed to serogroup Y was observed. Overall, the case fatality ratio among IMD cases caused by serogroup Y was six per cent. As already reported [10], a

high proportion (42%) of MenY isolates with decreased sensitivity to penicillin was found.

Several reports indicated the cc23 as one of the most frequently detected in invasive MenY cases: in particular, it was responsible for an increase of IMD incidence in the 1990s in the US [1] and was associated with 94% of serogroup Y meningococci isolated between 2000 and 2005 [19]. From 1999 to 2003, in Canada 65.7% of invasive MenY strains were cc23 [20], whereas in Taiwan this cc characterised 11 of 13 MenY causing disease between 2001 and 2002 [21]. In South Africa, during the years 2003 to 2007, 11% of invasive MenY belonged to cc23 [18]. In Europe, during the 1990s, the cc23 was isolated more frequently from healthy carriers than from invasive meningococcal cases [22,23]. Nevertheless, in Sweden, from 2000 to 2010, the cc23 was identified in the three major clones responsible for the increased number of IMD cases [9] and in England it was found in the 56% of MenY causing IMD during the years from 2007 to 2009 [13]. In Italy, cc23 was the main cc among invasive MenY: it was detected in 89% of MenY samples from 1998 to 2006 [10], and in 54 of 56 (96%) of samples from 2007 to 2013. However, the identification in this study of 25 different finetypes suggests that more than a single strain is responsible for the MenY increase in Italy.

All the cc23 isolates analysed in this work harboured a mutation in the *lpxL1* gene, and in particular, the mutation XVII was the most frequently found (79%). Several

studies have demonstrated the presence of *lpxL1* mutations in *N. meningitidis* carrier strains cc23 and in meningococci isolated from cases of chronic meningococcaemia and meningitis [13,24-27]. In contrast, as shown from the results reported here, MenY cc23 with a mutated *lpxL1* was associated indifferently with meningitis or septicaemia.

In conclusion, in Italy, IMD due to serogroup Y is steadily increasing, especially among five to 14 year-old patients, with predominance of isolates belonging to cc23 and harbouring *lpxL1* mutation. Overall, these results have significant public health implications. They support the potential utility of vaccination with the quadrivalent-meningococcal vaccine (ACWY) and/or the opportunity of a booster dose with this vaccine among children and young adolescents previously immunised with the MCC vaccine. More than 10 years since the beginning of vaccination with the MCC vaccine, there is evidence of a different epidemiology of IMD in Italy. The results are consistent with those of other studies that reported an increase of MenY and, more recently, of MenW infections [6,28] and provide further information which can be used to decide if and when the quadrivalent vaccination should be introduced. The quadrivalent meningococcal vaccine (ACWY) is safe and immunogenic; however, the cost-effectiveness of a booster with MCC vs the latter vaccine is still debated. In Italy, the use of quadrivalent vaccine is currently recommended for people at risk and for people who live in or travel to countries where meningococcal disease is hyperendemic or epidemic; this policy is likely to change. In fact, the dynamic nature of IMD epidemiology is well known [29]. In this respect, monitoring changes in the trend of the different serogroups and the microbiological features of meningococci is key to generate scientific evidence which is essential for producing appropriate vaccine recommendations.

Acknowledgements

The authors thank Stefano Boros for statistical support; Luigina Ambrosio, for technical assistance; Maria Chironna, Anna Di Taranto, Maria Laura Garlaschi, Maria Paola Landini, Daniela Lombardi, Laura Moschella, Maria Grazia Pascucci, Alessandra Piatti and Damiano Picicco for samples and clinical data. This publication made use of the *Neisseria* Multi Locus Sequence Typing website developed by Keith Jolley and sited at the University of Oxford [29]. The development of this site has been funded by the Wellcome Trust and European Union. This work was funded by the Ministry of Health-CCM Project "Sorveglianza delle malattie invasive da *Neisseria meningitidis*, *Streptococcus pneumoniae* ed *Haemophilus influenzae*", 2012-2013.

Conflict of interest

None declared.

Authors' contributions

Cecilia Fazio provided insight on microbiological investigation and drafted the manuscript. Arianna Neri contributed in the molecular analyses and provided insight into interpretation of results. Giovanna Renna and Paola Vacca carried out the laboratory analyses. Raffaele Antonetti, Anna Maria Barbui, Laura Daprai, Paolo Lanzafame, Lucia Rossi, Iolanda Santino, Carlo Tascini and Caterina Vocale were involved in the invasive meningococcal diseases at the local level. They were in charge of the data collection and management. Paola Stefanelli designed the purpose of this article and drafted the manuscript. All authors participated in the drafting and revision of this manuscript and gave their final approval of this version.

References

- Krauland MG, Dunning Hotopp JC, Riley DR, Daugherty SC, Marsh JW, Messonnier NE, et al. Whole genome sequencing to investigate the emergence of clonal complex 23 *Neisseria meningitidis* serogroup Y disease in the United States. *PLoS ONE*. 2012;7(4):e35699. DOI: 10.1371/journal.pone.0035699 PMID: 22558202
- Abad R, Agudelo CI, Brandileone MC, Chanto G, Gabastou JM, Hormazabal JC, et al. Molecular characterization of invasive serogroup Y *Neisseria meningitidis* strains isolated in the Latin America region. *J Infect*. 2009;59(2):104-14. DOI: 10.1016/j.jinf.2009.06.001 PMID: 19576638
- Agudelo CI, Sanabria OM, Ovalle MV. Serogroup Y meningococcal disease, Colombia. *Emerg Infect Dis*. 2008;14(6):990-1. DOI: 10.3201/eid1406.071357 PMID: 18507927
- Bröker M, Bukovski S, Culic D, Jacobsson S, Koliou M, Kuusi M, et al. Meningococcal serogroup Y emergence in Europe: high importance in some European regions in 2012. *Hum Vaccin Immunother*. 2014;10(6):1725-8. DOI: 10.4161/hv.28206 PMID: 24608912
- Bröker M, Jacobsson S, Kuusi M, Pace D, Simões MJ, Skoczynska A, et al. Meningococcal serogroup Y emergence in Europe: update 2011. *Hum Vaccin Immunother*. 2012;8(12):1907-11. DOI: 10.4161/hv.21794 PMID: 23032167
- Bröker M, Emonet S, Fazio C, Jacobsson S, Koliou M, Kuusi M, et al. Meningococcal serogroup Y disease in Europe: Continuation of high importance in some European regions in 2013. *Hum Vaccin Immunother*. 2015;11(9):2281-6. DOI: 10.1080/21645515.2015.1051276 PMID: 26036710
- Vainio A, Toropainen M, Kuusi M, Virolainen A. Characteristics of invasive meningococcal serogroup Y isolates in Finland, 1995-2010. Poster session presented at: 11th European Meningococcal Disease Society (EMGM) meeting; 2011 May 18-20; Ljubljana Slovenia. P037.
- Caugant DA, Lovoll Ø, Blystad H. Meningococcal disease in Norway, 2009-2010: emergence of serogroup Y. Poster session presented at: 11th European Meningococcal Disease Society (EMGM) meeting; 2011 May 18-20; Ljubljana Slovenia. P040.
- Hedberg ST, Törös B, Fredlund H, Olcén P, Mölling P. Genetic characterisation of the emerging invasive *Neisseria meningitidis* serogroup Y in Sweden, 2000 to 2010. *Euro Surveill*. 2011;16(23):19885. PMID: 21679677
- Fazio C, Neri A, Starnino S, Sofia T, Mastrantonio P, Stefanelli P. Characterization of invasive serogroup Y meningococci in Italy: prevalence of ST-23 Complex/Cluster A3. *New Microbiol*. 2008;31(4):467-72. PMID: 19123301
- Ministero della Salute. Piano Nazionale Prevenzione Vaccinale 2012-2014. Italian. Available from: www.salute.gov.it/imgs/c_17_pubblicazioni_1721_allegato.pdf.
- Zhu H, Wang Q, Wen L, Xu J, Shao Z, Chen M, et al. Development of a multiplex PCR assay for detection and genotyping of *Neisseria meningitidis*. *J Clin Microbiol*. 2012;50(1):46-51. DOI: 10.1128/JCM.00918-11 PMID: 22090406
- Ladhani SN, Lucidarme J, Newbold LS, Gray SJ, Carr AD, Findlow J, et al. Invasive meningococcal capsular group Y disease, England and Wales, 2007-2009. *Emerg Infect Dis*. 2012;18(1):63-70. DOI: 10.3201/eid1801.110901 PMID: 22261040
- Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol*. 2004;186(5):1518-30. DOI: 10.1128/JB.186.5.1518-1530.2004 PMID: 14973027
- Munford RS. Meningococcal infections. In Braunwald E, Hauser SL, Fauci AS, Longo DL, Kasper DL and Jameson JL (ed.).

- Harrison's principles of internal medicine. 15th ed., volume 1. Washington DC: McGraw-Hill; 2001;927-30.
16. Centers for Disease Control and Prevention (CDC). Serogroup Y meningococcal disease--Illinois, Connecticut, and selected areas, United States, 1989-1996. *MMWR Morb Mortal Wkly Rep*. 1996;45(46):1010-3. PMID: 8965799
 17. Racoosin JA, Whitney CG, Conover CS, Diaz PS. Serogroup Y meningococcal disease in Chicago, 1991-1997. *JAMA*. 1998;280(24):2094-8. DOI: 10.1001/jama.280.24.2094 PMID: 9875877
 18. du Plessis M, Wolter N, Crowther-Gibson P, Hamstra HJ, Schipper K, Moodley C, et al. Meningococcal serogroup Y lpxL1 variants from South Africa are associated with clonal complex 23 among young adults. *J Infect*. 2014;68(5):455-61. DOI: 10.1016/j.jinf.2013.12.011 PMID: 24393652
 19. Harrison LH, Shutt KA, Schmink SE, Marsh JW, Harcourt BH, Wang X, et al. Population structure and capsular switching of invasive *Neisseria meningitidis* isolates in the pre-meningococcal conjugate vaccine era--United States, 2000-2005. *J Infect Dis*. 2010;201(8):1208-24. DOI: 10.1086/651505 PMID: 20199241
 20. Tsang RS, Henderson AM, Cameron ML, Tyler SD, Tyson S, Law DK, et al. Genetic and antigenic analysis of invasive serogroup Y *Neisseria meningitidis* isolates collected from 1999 to 2003 in Canada. *J Clin Microbiol*. 2007;45(6):1753-8. DOI: 10.1128/JCM.02134-06 PMID: 17442798
 21. Chiou CS, Liao JC, Liao TL, Li CC, Chou CY, Chang HL, et al. Molecular epidemiology and emergence of worldwide epidemic clones of *Neisseria meningitidis* in Taiwan. *BMC Infect Dis*. 2006;6(1):25-32. DOI: 10.1186/1471-2334-6-25 PMID: 16478548
 22. Yazdankhah SP, Kriz P, Tzanakaki G, Kremastinou J, Kalmusova J, Musilek M, et al. Distribution of serogroups and genotypes among disease-associated and carried isolates of *Neisseria meningitidis* from the Czech Republic, Greece, and Norway. *J Clin Microbiol*. 2004;42(11):5146-53. DOI: 10.1128/JCM.42.11.5146-5153.2004 PMID: 15528708
 23. Claus H, Maiden MCJ, Wilson DJ, McCarthy ND, Jolley KA, Urwin R, et al. Genetic analysis of meningococci carried by children and young adults. *J Infect Dis*. 2005;191(8):1263-71. DOI: 10.1086/428590 PMID: 15776372
 24. Van der Ley P, Rodenburg G, Fransen F, Bogaert D, Schipper K, Claus H, et al. Naturally occurring lipid A variants among meningococcal carriage and disease isolates. In: Abstracts of the Seventeenth International Pathogenic *Neisseria* Conference (IPNC); 2010 Sep 11-16; Banff, Canada; Abstract OM12. Available from: <http://neisseria.org/ipnc/history.shtml>.
 25. Fransen F, Heckenberg SGB, Hamstra HJ, Feller M, Boog CJP, van Putten JPM, et al. Naturally occurring lipid A mutants in *neisseria meningitidis* from patients with invasive meningococcal disease are associated with reduced coagulopathy. *PLoS Pathog*. 2009;5(4):e1000396. DOI: 10.1371/journal.ppat.1000396 PMID: 19390612
 26. Brouwer MC, Spanjaard L, Prins JM, van der Ley P, van de Beek D, van der Ende A. Association of chronic meningococemia with infection by meningococci with underacylated lipopolysaccharide. *J Infect*. 2011;62(6):479-83. DOI: 10.1016/j.jinf.2011.03.010 PMID: 21459106
 27. Persa OD, Jazmati N, Robinson N, Wolke M, Kremer K, Schweer K, et al. A pregnant woman with chronic meningococcaemia from *Neisseria meningitidis* with lpxL1-mutations. *Lancet*. 2014;384(9957):1900. DOI: 10.1016/S0140-6736(14)61645-7 PMID: 25457917
 28. Ladhani SN, Beebejaun K, Lucidarme J, Campbell H, Gray S, Kaczarski E, et al. Increase in endemic *Neisseria meningitidis* capsular group W sequence type 11 complex associated with severe invasive disease in England and Wales. *Clin Infect Dis*. 2015;60(4):578-85. DOI: 10.1093/cid/ciu881 PMID: 25389259
 29. Halperin SA, Bettinger JA, Greenwood B, Harrison LH, Jelfs J, Ladhani SN, et al. The changing and dynamic epidemiology of meningococcal disease. *Vaccine*. 2012;30(Suppl 2):B26-36. DOI: 10.1016/j.vaccine.2011.12.032 PMID: 22178525
 30. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*. 2010;11(1):595. DOI: 10.1186/1471-2105-11-595 PMID: 21143983

Carbapenemase-producing *Enterobacteriaceae* in Europe: assessment by national experts from 38 countries, May 2015

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Citation style for this article:

Albiger B, Glasner C, Struelens M, Grundmann H, Monnet D, the European Survey of Carbapenemase-Producing *Enterobacteriaceae* (EuSCAPE) working group. Carbapenemase-producing *Enterobacteriaceae* in Europe: assessment by national experts from 38 countries, May 2015. *Euro Surveill.* 2015;20(45):pii=30062. DOI: <http://dx.doi.org/10.2807/1560-7917.ES.2015.20.45.30062>

Article submitted on 01 October 2015 / accepted on 06 November 2015 / published on 02 November 2015

In 2012, the European Centre for Disease Prevention and Control (ECDC) launched the 'European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE)' project to gain insights into the occurrence and epidemiology of carbapenemase-producing *Enterobacteriaceae* (CPE), to increase the awareness of the spread of CPE, and to build and enhance the laboratory capacity for diagnosis and surveillance of CPE in Europe. Data collected through a post-EuSCAPE feedback questionnaire in May 2015 documented improvement compared with 2013 in capacity and ability to detect CPE and identify the different carbapenemase genes in the 38 participating countries, thus contributing to their awareness of and knowledge about the spread of CPE. Over the last two years, the epidemiological situation of CPE worsened, in particular with the rapid spread of carbapenem-hydrolysing oxacillinase-48 (OXA-48)- and New Delhi metallo-beta-lactamase (NDM)-producing *Enterobacteriaceae*. In 2015, 13/38 countries reported inter-regional spread of or an endemic situation for CPE, compared with 6/38 in 2013. Only three countries replied that they had not identified one single case of CPE. The ongoing spread of CPE represents an increasing threat to patient safety in European hospitals, and a majority of countries reacted by establishing national CPE surveillance systems and issuing guidance on control measures for health professionals. However, 14 countries still lacked specific national guidelines for prevention and control of CPE in mid-2015.

Introduction

The global rise of carbapenemase-producing *Enterobacteriaceae* (CPE) is alarming and represents

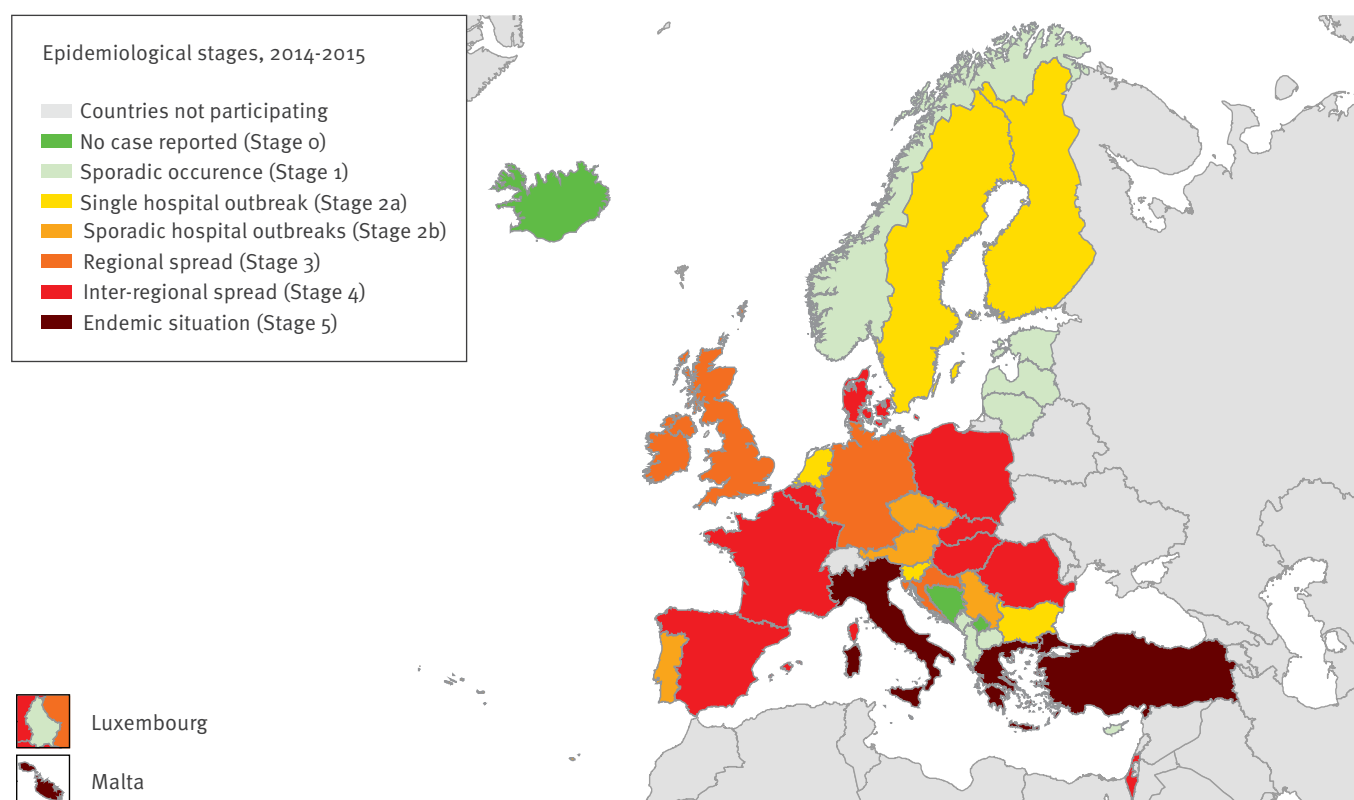
an increasing threat to healthcare delivery and patient safety in Europe and beyond.

In 2012, the European Centre for Disease Prevention and Control (ECDC) launched the 'European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE)' project to improve the understanding of the occurrence and epidemiology of CPE, to increase awareness of the spread of CPE and to build laboratory capacity for diagnosis and surveillance in Europe.

In February 2013, a self-assessment questionnaire was sent to one national expert (NE) from each of the EuSCAPE participating countries (i.e. 28 European Union (EU) Member States, Iceland, Norway, the seven EU enlargement countries (Albania, Bosnia and Herzegovina, Kosovo*, Montenegro, the former Yugoslav Republic of Macedonia, Serbia and Turkey) and Israel, to gather information on the current awareness of and knowledge about the spread of CPE, the public health responses and the available national guidelines on detection, surveillance, prevention and control, as well as on the capacity for laboratory diagnosis and surveillance. NEs were chosen based on their national and international laboratory and/or epidemiological experience in CPE among experts from national reference or expert laboratories, from the European Antimicrobial Resistance Surveillance Network (EARS-Net), and from the ECDC Coordinating Competent Bodies (National Focal Points for antimicrobial resistance and National Focal Points for microbiology) and ECDC National Correspondents for EU enlargement countries. The answers collected from the NEs showed that the epidemiological situation for CPE

FIGURE 1

Occurrence of carbapenemase-producing *Enterobacteriaceae* based on self-assessment by national experts, 38 European countries, May 2015



had worsened since 2010 and CPE continued to spread in European hospitals [1-3]. Answers also indicated that the knowledge and awareness of the spread of CPE and the laboratory capacity for diagnosis and surveillance were heterogeneous among countries [1,2]. These findings highlighted the urgent need for a coordinated European effort towards early diagnosis, active surveillance and guidance on infection control measures [1,2].

In September and October 2013, the EuSCAPE project supported laboratory capacity building for diagnosis and surveillance by hosting a ‘train-the-trainer’ workshop at the European level for national laboratory experts on the identification and confirmation of CPE, and by carrying out an external quality assessment (EQA) of national reference/expert laboratories. The workshop and the EQA aimed at ensuring performance quality, consistency and comparability of data between participating countries and laboratories. Between November 2013 and April 2014, 36 European countries participated in the first European-wide structured survey of CPE (data not shown). The participating reference/expert laboratories were asked to collect CPE isolates of *Klebsiella pneumoniae* and *Escherichia coli* together with clinical data on these CPE-related infections to gain an understanding on the prevalence and epidemiology of CPE, as well as the risk factors associated with CPE infections in Europe.

In March 2015, after the completion of the EuSCAPE project, a post-EuSCAPE feedback questionnaire was sent to the participating countries to document whether (i) knowledge and awareness regarding the occurrence and spread of CPE had increased, and (ii) national capacity for containment of CPE had changed in terms of surveillance, laboratory reference services, and availability of guidance on infection prevention and control measures for these bacteria, since February 2013.

In this report, we present the analysis of the NES’ answers on behalf of their countries to the post-EuSCAPE feedback questionnaire and provide summaries of the current epidemiological situation of the spread of CPE in each country.

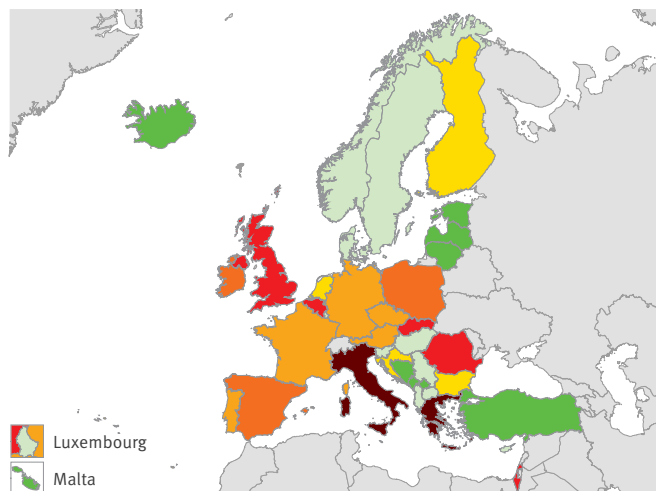
Methods

The post-EuSCAPE feedback questionnaire was derived from the self-assessment questionnaire issued in February 2013 [1,2]. The questionnaire was divided in five sections. The first two sections explored awareness and knowledge about the occurrence of CPE in each country and collected information on the current national capacity for containment of CPE. The third and fourth sections collected the participants’ feedback on the EuSCAPE activities, e.g. laboratory capacity building workshop, EQA exercise and on the impact of the EuSCAPE project on collaborations and networking capacity, respectively. The fifth section investigated

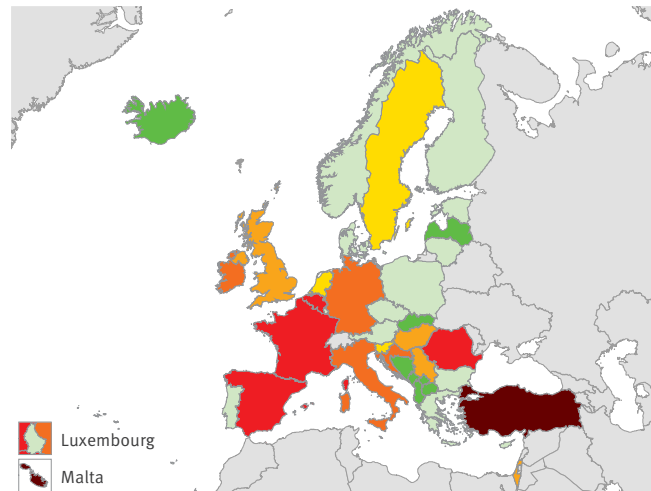
FIGURE 2

Geographic distribution of carbapenemase-producing *Enterobacteriaceae* by resistance mechanism, based on self-assessment by national experts, 38 European countries, May 2015

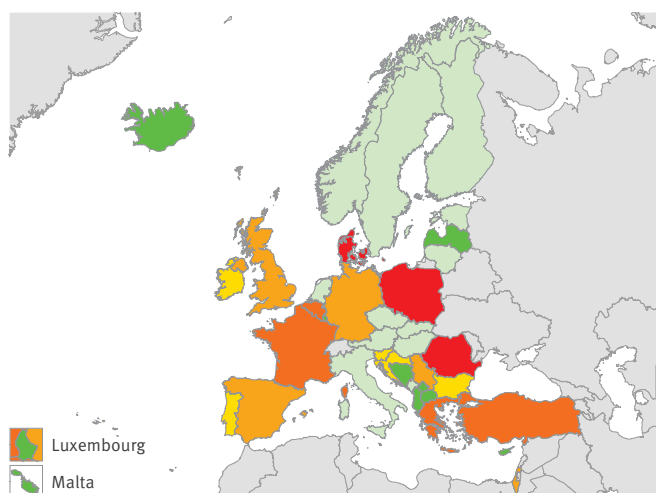
A. *Klebsiella pneumoniae* carbapenemase (KPC)



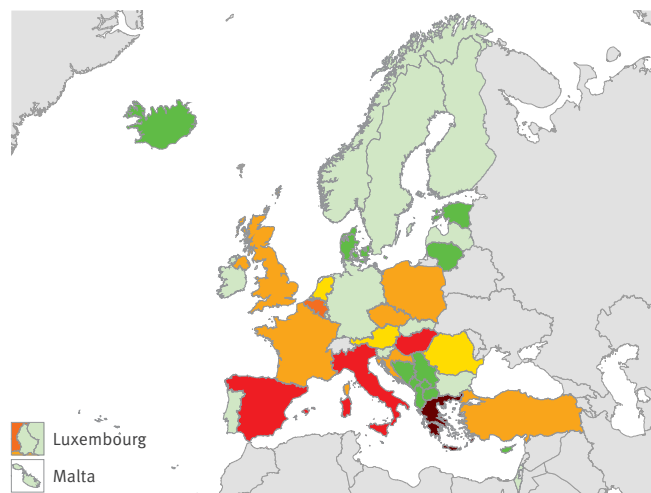
B. Oxacillinase-48 (OXA-48)



C. New Delhi metallo-beta-lactamase (NDM)



D. Verona integrin-encoded metallo-beta-lactamase (VIM)



Epidemiological stages, 2014-2015

- | | |
|---|---|
| <ul style="list-style-type: none"> Countries not participating No case reported (Stage 0) Sporadic occurrence (Stage 1) Single hospital outbreak (Stage 2a) | <ul style="list-style-type: none"> Sporadic hospital outbreaks (Stage 2b) Regional spread (Stage 3) Inter-regional spread (Stage 4) Endemic situation (Stage 5) |
|---|---|

desired areas for future ECDC activities on carbapenem-resistant Gram-negative bacteria. The questionnaire was sent to the same NEs who participated in a similar survey in February 2013, with the exception of France and the Netherlands. They were invited to coordinate their replies with colleagues in their countries i.e. the ECDC National Focal Points for antimicrobial resistance and the ECDC National Correspondents for EU enlargement countries) to reflect the national situation and to complete the questionnaire online between 3

March and 30 April 2015 (questionnaire available upon request from the corresponding author). They were also asked to provide a description of the emergence and spread of CPE in their country beyond *K. pneumoniae* and *E. coli* isolates collected during the EuSCAPE structured survey. The answers were based on their knowledge of national clinical and microbiological data and/or their personal judgement. When necessary, the respondents were contacted for clarification, and corrections were made accordingly. The latest data from

TABLE 1*Epidemiological stages of carbapenemase-producing Enterobacteriaceae spread [1,3]*

Epidemiological scale	Description	Stage
No cases reported	No cases reported.	0
Sporadic occurrence	Single cases, epidemiological unrelated.	1
Single hospital outbreak	Outbreak defined as two or more epidemiologically-associated cases with indistinguishable geno- or phenotype in a single institution.	2a
Sporadic hospital outbreaks	Unrelated hospital outbreaks with independent, i.e. epidemiologically-unrelated introduction or different strains; no autochthonous inter-institutional transmission reported.	2b
Regional spread	More than one epidemiologically-related hospital outbreak confined to hospitals that are part of the same region or health district, suggestive of regional autochthonous inter-institutional transmission.	3
Inter-regional spread	Multiple epidemiologically-related outbreaks occurring in different health districts, suggesting inter-regional autochthonous inter-institutional transmission.	4
Endemic situation	Most hospitals in a country are repeatedly seeing cases admitted from autochthonous sources.	5

EARS-Net provided an additional source of information on the percentage of carbapenem resistance in invasive isolates, in the EU/ European Economic Area (EEA) Member States.

For the presentation, countries were arbitrarily grouped in geographic entities independently of the epidemiological stages of CPE spread, geopolitical or economic considerations.

Using the same epidemiological staging system as in 2010 and 2013 (Table 1), all participating countries self-assessed their epidemiological situation of CPE, thereby documenting the progression of CPE within countries and dissemination in Europe between 2013 and 2015. All countries provided a self-assessment of the current national situation.

Results

Overall occurrence of carbapenemase-producing *Enterobacteriaceae*

Three countries reported not having identified one single case of CPE, whereas 13 reported regional and inter-regional spread, and four reported an endemic situation. Nine countries reported sporadic occurrence, five reported single hospital outbreak and four reported sporadic hospital outbreaks (Figure 1, Table 2).

Table 2 documents the epidemiological stages and dissemination for CPE within countries in the years 2010, 2013 and 2015 and indicates the changes in status between surveys in 2013 and 2015.

Occurrence of carbapenemase-producing *Enterobacteriaceae* by type of carbapenemase

All countries were able to rate the occurrence and spread of CPE by type of carbapenemase. As of May 2015, *K. pneumoniae* carbapenemase (KPC)-producing

Enterobacteriaceae still had the widest dissemination in Europe, but carbapenem-hydrolysing oxacillinase-48 (OXA-48)-producing *Enterobacteriaceae* had almost reached the same spread, a change compared with February 2013, with eight countries reporting regional or inter-regional spread and another two countries reporting an endemic situation (Figure 2). The distribution of KPC- and OXA-48-producing *Enterobacteriaceae* varies and does not necessarily overlap, for example, Greece seeing predominantly KPC-producing *Enterobacteriaceae* and rarely OXA-48-producing *Enterobacteriaceae*, and Malta seeing almost exclusively OXA-48-producing *Enterobacteriaceae*.

The European epidemiology for CPE also changed between 2013 and 2015 for New Delhi metallo-beta-lactamase (NDM)-producing *Enterobacteriaceae*; five countries reported sporadic hospital outbreaks, and seven countries regional or inter-regional spread. No country reported an endemic situation. The epidemiological situation for Verona integron-encoded metallo-beta-lactamase (VIM)-producing *Enterobacteriaceae* remained stable with some minor country-specific changes. Imipenemase (IMP)-producing *Enterobacteriaceae* remained rare in Europe (Table 3).

Description of the emergence and spread of carbapenemase-producing *Enterobacteriaceae*

The NEs participating in the EuSCAPE provided a description of the emergence and spread of CPE in their country beyond *K. pneumoniae* and *E. coli* isolates collected during the EuSCAPE structured survey.

Denmark, Iceland, Finland, Norway, Sweden and the Netherlands

In Denmark, only sporadic occurrence of CPE, mostly related to foreign travel, was observed until 2012 when the situation for CPE changed to sporadic hospital outbreaks with the spread of VIM-4 producing *E.*

TABLE 2

Comparison of epidemiological stages of carbapenemase-producing *Enterobacteriaceae*, 38 European countries, 2010–2015

Country	Epidemiological stage for the spread of CPE			Change in epidemiological situation for CPE between 2013 and 2015
	2010 ^a	2013 ^b	2014–2015 ^c	
Albania	NA	2a	1	↓
Austria	0	2b	2b	→
Belgium	2b	3	4	↑
Bosnia and Herzegovina	1	1	0	↓
Bulgaria	0	2a	2a	→
Croatia	1	3	3	→
Cyprus	2a	2a	1	↓
Czech Republic	1	2b	2b	→
Denmark	1	2a ^d	4	↑
Estonia	0	2a	1	↓
Finland	1	2a	2a	→
France	3	3	4	↑
Germany	3	3	3	→
Greece	5	5	5	→
Hungary	3	4	4	→
Iceland	0	0	0	→
Ireland	1	4	3	↓
Israel	5	4	4	→
Italy	4	5	5	→
Kosovo*	NA	2b	0	↓
Latvia	1	1	1	→
Lithuania	1	1	1	→
Luxembourg	NA	1	1	→
Malta	1	5	5	→
Montenegro	NA	0	1	↑
The Netherlands	2a	2b	2a	↓
Norway	2a	2a	1	↓
Poland	4	3	4	↑
Portugal	1	1	2b	↑
Romania	1	1	4	↑
Serbia	1	1	2b	↑
Slovakia	NA	2a	4	↑
Slovenia	0	1	2a	↑
Spain	2b	3	4	↑
Sweden	2a	2b	2a	↓
The former Yugoslav Republic of Macedonia	NA	0	1	↑
Turkey	NA	2a	5	↑
United Kingdom	2b	3	3	→

CPE: carbapenemase-producing *Enterobacteriaceae*; NA: not available.

↑: increase in the epidemiological stage between 2013 and 2015; ↓: decrease in the epidemiological stage between 2013 and 2015; →: unchanged epidemiological stage between 2013 and 2015.

Grey: countries with no data available.

Dark green: no case reported (Stage 0).

Light green: sporadic occurrence (Stage 1).

Light yellow: single hospital outbreak (Stage 2a).

Dark yellow: sporadic hospital outbreaks (Stage 2b).

Orange: regional spread (Stage 3).

Red: inter-regional spread (Stage 4).

Brown: endemic situation (Stage 5).

^a The results were based on data obtained through a Europe-wide consultation during a workshop at the Dutch National Institute for Public Health and the Environment (RIVM) on 29–30 April 2010 [3].^b The results were based on data obtained through a self-assessment questionnaire (February 2013) to the national experts who participated in the 'European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE)' project [1,2].^c This online survey (March–May 2015).^d Data provided in 2015.

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coli, the identification of NDM-4 producing *E. coli* and an outbreak of NDM-1 producing *Citrobacter freundii* [4,5]. Since 2013, the number of CPE cases in Denmark has further increased with multiple epidemiologically-related hospital outbreaks of OXA-48- and NDM-producing *Enterobacteriaceae* in different regions of the country [6]. In 2014, most of the CPE cases had no history of recent travel abroad. Denmark is now facing an inter-regional spread of CPE.

The situation in **Iceland** has remained unchanged since 2010 despite active screening. Iceland is one of the few countries in Europe that has not reported any case of CPE.

In **Norway**, the occurrence of CPE, KPC-, OXA-48- and NDM-producing *Enterobacteriaceae*, has remained sporadic with still a small number of CPE cases (ca 10 cases per year, including colonisation) since 2013. The majority of the identified cases had a link with foreign travel.

In **Finland**, 74 CPE isolates from 66 patients have been obtained since 2009, with an increase from five cases in 2009 to 18 cases in 2014. About 70% of the patients with CPE had a history of foreign travel, mostly to Asia or southern Europe. Until 2013, the predominant CPE in Finland were OXA-48-producing *Enterobacteriaceae* [7]. In 2013, Finland experienced its largest and first outbreak of CPE, involving nine patients without direct link to travel abroad. This outbreak of colonisation was due to KPC-producing *K. pneumoniae* ST 512 making them the predominant CPE in Finland, although still at a very low prevalence [8]. In 2015, eight NDM-producing *Enterobacteriaceae*, six KPC-producing *Enterobacteriaceae* and four OXA-48-producing *Enterobacteriaceae* have been isolated so far (data not shown).

In **Sweden**, most identified cases had a history of foreign travel. In 2014, there was a slight increase in the number of CPE cases due to an outbreak that was only detected through identification of a secondary colonised case. Until 2013, the predominant CPE in Sweden were NDM-producing *Enterobacteriaceae* closely followed by OXA-48-producing *Enterobacteriaceae* [9]. Since 2014, OXA-48-producing *Enterobacteriaceae* became predominant over NDM-producing *Enterobacteriaceae*, however both are still at low level.

In **the Netherlands**, KPC-, OXA-48- and NDM-producing *Enterobacteriaceae* have so far only been responsible for single hospital outbreaks, although a recent inter-institutional outbreak of KPC-producing *K. pneumoniae* occurred following the transfer of a patient from a nursing home to a hospital [10,11].

Estonia, Latvia and Lithuania

The Baltic countries only recently started to report CPE cases [12,13].

In **Estonia**, the first case of CPE, i.e. German imipenemase (GIM)-producing *Enterobacter aerogenes*, was identified in 2015 (personal communication, Paul Naaber, 26 June 2015).

In **Latvia**, only three cases of CPE have been identified so far, of which the first two VIM-producing isolates were identified during the EuSCAPE structured survey (data not shown).

In **Lithuania**, surveillance of CPE became mandatory in 2014. Between 1 January and 31 December 2014, 13 CPE cases were reported, consisting of two cases of OXA-48-producing *K. pneumoniae*, nine cases of NDM-producing *Enterobacter cloacae*, one case of NDM-producing *E. aerogenes* and one case of VIM-producing *E. cloacae*.

Ireland and United Kingdom

In **Ireland**, sporadic occurrence of CPE, i.e. KPC-, VIM- and NDM-1-producing *Enterobacteriaceae*, had been reported until 2011, with the majority of cases being related to travel abroad [14-16]. In 2011, an outbreak of epidemiologically-related KPC-producing *K. pneumoniae* in two hospitals from two different regions resulted in epidemiological stage 4 of CPE spread in the country [17]. This was concomitant with the first hospital outbreak of OXA-48-producing *K. pneumoniae* [18]. Since 2013, although the spread of CPE was limited to regional spread in some regions, the overall national situation is considered to have worsened due to an increase in the overall number of reported CPE cases. Furthermore, increasing numbers of hospitals and regions where CPE had not been encountered before 2013, have since reported sporadic cases or outbreaks of CPE. Prior to 2013, KPC-producing *Enterobacteriaceae* were the main type of CPE responsible for hospital outbreaks, but from 2013 onwards, OXA-48- and NDM-producing *Enterobacteriaceae* were also responsible for outbreaks.

The **United Kingdom (UK)**, reported the emergence and the spread of NDM-1-producing CPE soon after its first isolation in 2008 from a patient repatriated to Sweden from a hospital in India, and this led to a National Resistance Alert 3 notice by the Department of Health [19,20]. To date, the UK has reported the largest number of NDM-producing CPE cases among European countries and has seen multiple NDM variants. The number of CPE isolates received by the national reference laboratory has increased continuously since 2008. In 2014, an increasing number of NDM- or OXA-48-producing isolates was reported compared with previous years with a marked increase in carbapenemase-producing *E. coli*.

Austria, Czech Republic, Germany, Luxembourg and Slovenia

In **Austria**, the epidemiological situation worsened between 2010 and 2013, but has since remained unchanged with a low occurrence of CPE and sporadic hospital outbreaks [21–24]. Between 2010 and 2015, the most frequently confirmed carbapenemase genes by the reference laboratory were *bla*_{VIM} and *bla*_{KPC}, but also *bla*_{OXA-48} and *bla*_{NDM} were also found in low numbers. In April 2015, Austria initiated the Austrian surveillance project ‘Carba-Net Austria’ and organised four laboratory capacity building workshops on the identification of CPE and characterisation of carbapenemases based on the EuSCAPE protocols and training curriculum.

In the **Czech Republic**, the occurrence of CPE was rare until 2011 with only sporadic cases, and a total of three cases detected between 2009 and 2010. In 2011, however, the number of CPE increased due to the repatriation of patients from hospitals in Italy and Greece and an outbreak following the transfer of a patient from Italy [25]. To contain this increase, the national surveillance included CPE isolates from active screening samples as part of its surveillance scheme and the Ministry of Health issued, in 2012, official national guidelines for the control of CPE covering both infected and colonised cases. No further increase in the occurrence of CPE was observed in 2012 and 2013, and only one outbreak restricted to five patients and four sporadic cases was reported until mid-2013 [26]. During the EuSCAPE survey, the Czech Republic reported only two confirmed CPE cases, of which one involved NDM-1-producing *K. pneumoniae* from a patient transferred from Ukraine [27].

In **Germany**, there has been an increasing number of CPE referred to the German National Reference Laboratory for CPE and the German national antibiotic resistance surveillance has showed an increase of resistance to meropenem in *K. pneumoniae* from 0.1% in 2010 to 0.5% in 2014. Both observations possibly indicated an increase in the prevalence of CPE in Germany albeit on a low level. Several outbreaks with KPC-2-, KPC-3-, NDM-1- and OXA-48-producing *Enterobacteriaceae* have been documented; notably a protracted KPC-2 outbreak involving over 100 patients and a polyclonal KPC-2 outbreak involving other species besides *K. pneumoniae* [28]. The most prevalent CPE are in order of importance OXA-48-, KPC-2-, VIM-1-, NDM-1- and KPC-3-producing *Enterobacteriaceae*. Despite the dominance of OXA-48-producing *Enterobacteriaceae*, mostly KPC-producing *K. pneumoniae* outbreaks have been reported in Germany.

Luxembourg has only experienced sporadic cases of VIM-producing CPE [29].

In **Slovenia**, only sporadic cases of CPE were detected until 2013, with a large proportion of the cases being related to patient transfers from foreign hospitals [30]. The situation changed in October 2014 with the first outbreak of both OXA-48- and NDM-1-producing *E. coli* and *K. pneumoniae* affecting several wards in a single

hospital. While one of the first identified patients had been transferred from a foreign hospital, other patients had no history of travel abroad. Some CPE-positive patients belonging to this outbreak were transferred to other hospitals across the country, but no further spread occurred in these hospitals.

Hungary, Poland, Romania and Slovakia

In **Hungary**, ca 600 VIM-4-producing *Enterobacteriaceae* isolates – the predominant type of CPE in Hungary – have been collected since 2008. The first KPC-2-producing *K. pneumoniae* isolates were reported from 2008 to 2009 during a local outbreak in the north-eastern part of Hungary and the index case was a patient previously hospitalised in Greece [31]. About 20 KPC-producing isolates, from sporadic cases and mostly associated with medical treatment abroad, have since been collected, with an average of 1 to 2 isolates per year. These were KPC-producing *K. pneumoniae* until 2015 when the first KPC-producing *E. coli* was isolated. Only two small outbreaks caused by OXA-48-like-producing *K. pneumoniae* were reported, in 2012 and 2014, and both were linked to patient transfers from Romania and Ukraine, respectively [32]. In total, 20 OXA-48-producing *Enterobacteriaceae* have been identified so far in Hungary. Since 2013, only sporadic cases of NDM-producing CPE, primarily *E. cloacae*, have been identified, of which some but not all were linked to Romania.

In **Poland**, KPC-producing *K. pneumoniae* were predominant between 2008 and 2012 [33,34]. Since 2012, the epidemiology of CPE has changed with a decreasing number of KPC-producing *K. pneumoniae* and an increasing number of NDM-1-producing *K. pneumoniae*. The former primarily occurred in the regions that had experienced outbreaks of KPC-producing *K. pneumoniae* in 2008–2012. The latter was a consequence of a large inter-regional outbreak of NDM-producing *K. pneumoniae* that started at the end of 2012 [35], just a few months after the first case of NDM-1-producing *K. pneumoniae* was found in a patient with previous travel history to Africa [36].

In **Romania**, the first confirmed cases of OXA-48- and NDM-1-producing *Enterobacteriaceae*, mostly *K. pneumoniae*, were isolated in 2011 and both OXA-48- and NDM-1-producing *Enterobacteriaceae* were the predominant CPE in Romania until 2013. During the EuSCAPE structured survey, mostly OXA-48-producing *Enterobacteriaceae* were found (data not shown) [37–39].

Prior to 2013, **Slovakia** experienced only one small local epidemic in two hospitals, following an imported case of NDM-1-producing *K. pneumoniae* [40]. However, the situation changed in December 2013 after the identification of the first case of KPC-2-producing *K. pneumoniae* in a patient who had been hospitalised in Greece and the subsequent spread of CPE to more than

TABLE 3

Comparison of epidemiological stages of carbapenemase-producing *Enterobacteriaceae* by type of carbapenemase, 38 European countries, 2010–2015

Country	Epidemiological stage for the spread of CPE by type of carbapenemase									
	KPC		OXA48		VIM		NDM		IMP	
	2013 ^a	2014–2015 ^b	2013 ^a	2014–2015 ^b	2013 ^a	2014–2015 ^b	2013 ^a	2014–2015 ^b	2013 ^a	2014–2015 ^b
Albania	1	1	2a	0	1	0	0	0	2a	0
Austria	1	2b	1	1	2b	2a	2a	1	0	0
Belgium	3	4	3	4	3	3	1	3	0	0
Bosnia and Herzegovina	0	0	0	0	0	0	0	0	0	0
Bulgaria	NA	2a	NA	1	2a	1	NA	2a	NA	0
Croatia	3	2a	1	3	1	2b	1	2a	1	1
Cyprus	NA	1	NA	1	NA	0	NA	0	1	0
Czech Republic	2b	2b	0	1	2a	2b	1	1	0	0
Denmark	1	1	1	1	2a ^c	0	1	4	0	0
Estonia	NA	0	NA	1	NA	0	NA	1	NA	0
Finland	2a	2a	1	1	1	1	1	1	0	1
France	2b	2b	3	4	2b	2b	2a	3	2a	1
Germany	3	2b	2b	3	2b	1	2a	2b	1	1
Greece	5	5	1	1	5	5	0	3	0	0
Hungary	3	1	1	2b	4	4	0	1	0	0
Iceland	0	0	0	0	0	0	0	0	0	0
Ireland	4	3	3	3	2a	1	1	2a	1	1
Israel	4	4	2b	2b	0	1	2a	2b	0	0
Italy	5	5	2b	3	4	4	2b	1	0	0
Kosovo*	2b	0	2b	0	0	0	1	0	0	0
Latvia	1	0	NA	0	NA	1	NA	0	NA	0
Lithuania	0	0	1	1	0	0	0	1	0	0
Luxembourg	1	1	0	1	1	1	0	0	NA	1
Malta	0 ^c	0	5	5	0 ^c	1	0 ^c	0	1	1
Montenegro	NA	0	NA	0	NA	0	NA	1	NA	0
The Netherlands	2a	2a	2b	2a	1	2a	1	1	1	1
Norway	2a	1	1	1	1	1	1	1	0	1
Poland	3	3	1	1	1	2b	1	4	0	DNK
Portugal	1	2b	0	1	1	1	0	2a	0	0
Romania	NA	4	1	4	NA	2a	1	4	NA	NA
Serbia	NA	1	NA	2b	NA	0	NA	2b	NA	0
Slovakia	NA	4	NA	0	NA	1	2a	1	NA	0
Slovenia	1	1	1	2a	1	1	1	2a	0	0
Spain	2b	3	3	4	3	4	1	2b	2b	1
Sweden	2a	1	1	2a	1	1	2a	1	0	1
The former Yugoslav Republic of Macedonia	NA	1	NA	0	NA	0	NA	0	NA	NA
Turkey	2b	0	1	5	1	2b	1	3	1	1
United Kingdom	3	4 ^d	3	2b	3	2b	2b	2b	1	0

CPE: carbapenemase-producing *Enterobacteriaceae*; DNK: do not know; IMP: imipenemase; KPC: *Klebsiella pneumoniae* carbapenemase; NA: not available; NDM: New Delhi metallo-beta-lactamase; OXA-48: carbapenem-hydrolysing oxacillinase-48; VIM: Verona integron-encoded metallo-beta-lactamase.

Grey: countries with no data available.

Dark green: no case reported (Stage 0).

Light green: sporadic occurrence (Stage 1).

Light yellow: single hospital outbreak (Stage 2a).

Dark yellow: sporadic hospital outbreaks (Stage 2b).

Orange: regional spread (Stage 3).

Red: inter-regional spread (Stage 4).

Brown: endemic situation (Stage 5).

^a The results were based on data obtained through a self-assessment questionnaire (February 2013) to the national experts that participated in the 'European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE)' project [1,2].

^b This online survey (March–May 2015).

^c Data provided in 2015.

^d For Scotland, it was not possible to determine the epidemiological stage for each enzyme at the time of the questionnaire however, KPC-producing *Enterobacteriaceae* are sporadic (Stage 1).

*This designation is without prejudice to positions on status, and is in line with United Nations Security Council resolution 1244/99 and the International Court of Justice Opinion on the Kosovo declaration of independence.

10 hospitals. The number of patients infected or colonised with KPC-2-producing *K. pneumoniae* has now reached 150. In addition, two small local outbreaks of VIM-producing *E. cloacae* and NDM-producing *K. pneumoniae* were reported up to 2015.

Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Kosovo*, Montenegro, Serbia and the former Yugoslav Republic of Macedonia

Albania organised two laboratory capacity building workshops and urged hospitals not involved in the EuSCAPE project to initiate surveillance studies. This led to the identification of the first confirmed case of KPC-3-producing *K. pneumoniae* from a patient with no recent travel history, but with a previous admission in the intensive care unit at the University Hospital in Tirana in 2014 [41].

Bosnia and Herzegovina did not report any CPE, but NDM-1-producing *K. pneumoniae* had previously been reported in Croatia from a patient transferred from Bosnia and Herzegovina [42,43].

In **Bulgaria**, the occurrence of CPE has increased since 2012. KPC-2- and VIM-1-producing *K. pneumoniae* were isolated from a hospitalised patient in Varna and an outbreak caused by NDM-1-producing *E. coli* was reported from the Military Medical Academy Hospital of Sofia [44,45].

In **Croatia**, the first reported case of CPE was a NDM-1-producing *K. pneumoniae* isolated in 2009 in the University Hospital Centre of Zagreb from a patient repatriated from Bosnia and Herzegovina [42]. In February 2011, the first KPC-producing *K. pneumoniae* was isolated from a patient at the same hospital [46]. A multicentre study performed from 2011 to 2012 in four large hospital centres in Croatia identified a higher prevalence of VIM-1-producing *Enterobacteriaceae* than of NDM- and KPC-producing *Enterobacteriaceae* [47]. Since 2014, the epidemiology of CPE in Croatia has changed with the rapid spread of OXA-48-producing *Enterobacteriaceae* whereas incidence of KPC isolates declined.

Kosovo* is one of the few countries that have not reported any cases of CPE isolated from normally sterile body fluids such as blood cultures and cerebrospinal fluid (CSF), although NDM-1-producing *K. pneumoniae* were previously reported in Austria, Belgium and in Germany from patients being transferred from hospitals in Kosovo* [43,48,49].

In **Montenegro**, a laboratory capacity building workshop was organised and phenotypic methods of detection of CPE were implemented in the participating laboratories, leading to the identification of NDM-1-producing *K. pneumoniae* during the EuSCAPE structured survey (data not shown).

In **Serbia**, NDM- and OXA-48-producing *Enterobacteriaceae*, as well as NDM- and OXA-48- co-producing *Enterobacteriaceae* have been isolated during the EuSCAPE structured survey (data not shown). The latter type of CPE was also identified in a patient transferred from Serbia to Switzerland in December 2013 [50].

In the former Yugoslav Republic of Macedonia, only KPC-producing *K. pneumoniae* have been isolated so far through the EuSCAPE structured survey (data not shown).

Belgium, France, Portugal and Spain

In **Belgium**, the situation of CPE has seriously worsened with a rapid spread of CPE since 2012, i.e. a doubling in prevalence and incidence in acute care hospitals between 2012 and 2015 and more than 80% of the reported cases being confirmed as autochthonous acquisition, i.e. not travel-related. In addition, there has been an increase in the number of documented regional and inter-regional transmissions of epidemiologically related clusters and/or outbreaks, especially for OXA-48-producing *Enterobacteriaceae* and to a lesser extent for KPC-producing *Enterobacteriaceae*. There has also been an increase in the number of outbreaks with one third of the country's hospitals reporting outbreaks of CPE. Another major change in Belgium in 2015 was the marked increase, compared with 2013, in the number of non-travel-related NDM cases with inter-institution regional spread and multiple large difficult-to-control outbreaks occurring in several hospitals.

In **France**, the number of cases and outbreaks of CPE has steadily increased since 2009 with the sharpest increase during the last quarter of 2014. KPC-producing *Enterobacteriaceae* however, have been declining since 2012. Most cases were acquired abroad, i.e. through hospitalisation or travel. However, there has been an increase in the number of autochthonous cases, usually OXA-48-producing *Enterobacteriaceae*. In 2014, the most frequent CPE are OXA-48-producing *K. pneumoniae* and *E. coli*, followed by NDM-, VIM- and KPC-producing *Enterobacteriaceae*.

In **Spain**, the situation of CPE has worsened in the last few years with an increasing trend in the number of CPE cases and a wide geographic spread [51-54]. The spread of CPE has currently affected 34/50 Spanish provinces, resulting in a potential inter-regional spread of CPE [47,49, unpublished data]. The most predominant CPE are OXA-48- and VIM-producing *K. pneumoniae* [51-53]. In general, the prevalence of KPC- and NDM-producing *Enterobacteriaceae* in Spain is low but increasing [51,54]. Recently, an inter-hospital spread of NDM-7-producing *K. pneumoniae* that belonged to MLST type 437 was described in Madrid [51]. Although not frequent, detection of the polyclonal dissemination of OXA-48-producing *E. coli* is worrying.

In **Portugal**, only sporadic isolates or single hospital cases have been described. The most predominant CPE

TABLE 4A

National capacity for surveillance and containment of carbapenemase-producing *Enterobacteriaceae*, 38 European countries, May 2015***

Country	National system for surveillance	Officially nominated national reference laboratory, or expert laboratory	National recommendation or obligation for reporting (notification) to health authorities	National plan for containment of (or preparedness to contain) CPE	National recommendation or guideline on infection control measures	Reference or URL for recommendation or guideline on infection control measures
Albania	..a, b	..a		..a		
Austria	• ^c	•	• ^d		•	http://www.analyse.eu/content/inhalte/nationales_referenzzentrum/station%C3%A4re_patienten_mit_auslandsanamnese/index.html
Belgium	• ^e	•	• ^f	•	•	http://www.health.belgium.be/internet2Prd/groups/public/@public/@shc/documents/ie2divers/19074512.pdf http://www.sante.belgique.be/internet2Prd/groups/public/@public/@shc/documents/ie2divers/19074512_nl.pdf
Bosnia and Herzegovina	..a, b		..a	..a		
Bulgaria	•	•	•	..a	•	
Croatia	• ^e	•	• ^f	•	•	
Cyprus	..e, g	• ^h				
Czech Republic	• ^e	•	• ^f	•	•	http://www.mzcr.cz/Legislativa/dokumenty/vestnik-c8/2012_6865_2510_11.html http://www.szu.cz/narodni-referencni-laborator-pro-antibiotika
Denmark	• ^c	•				
Estonia	..g		• ^d			
Finland	• ^e	•	• ^f		•	http://urn.fi/URN:ISBN:978-952-302-260-7
France	• ^e	•	• ^f	•	•	http://www.sante.gouv.fr/fichiers/bo/2014/14-02/ste_20140002_0000_0064.pdf
Germany	• ^c	•	..a		•	http://edoc.rki.de/documents/rki_ab/resuFmoGFF7o/PDF/210bND4dxM.pdf
Greece	• ^e	• ^h	• ^f	•	•	http://www.keelpno.gr/el-gr/νοσήματαθέματαυγείας/πολυανθεκτικάπαθογόναστανοσοκομεία.aspx
Hungary	• ^e	• ^h	• ^f	..a	•	http://www.oek.hu/oek.web?nid=1067&pid=1&to=&lang=hun
Iceland	• ^e	•	• ^f	•	•	
Ireland	• ^e	•	• ^d		•	http://www.hpsc.ie/hpsc/A-Z/MicrobiologyAntimicrobialResistance/InfectionControlandHAI/Guidelines/File,12922,en.pdf
Israel	• ^e	•	• ^f	•	•	
Italy	• ^c	• ^h	• ^d	..a	•	http://www.trovanorme.salute.gov.it/rendeNormsanPdf?anno=0&codLeg=45499&parte=1%20&serie=
Kosovo*	..a, b	• ^h			..a	
Latvia	• ^e	• ^h	• ^f	..a		
Lithuania	• ^e	•				
Luxembourg	• ^c	• ^h	• ^d		•	

CPE: carbapenemase-resistant *Enterobacteriaceae*.

In the table cells, a dot signifies 'in place' and the absence of dot signifies 'absent'. Black dots indicate that the system or document was already in place in 2013. Blue cells indicate a change reported in 2015, as compared with 2013.

^a In preparation.

^b No national system for surveillance, but country reports carbapenem-resistant invasive isolates (*Klebsiella pneumoniae* and *Escherichia coli*) to the Central Asian and Eastern European Surveillance on Antimicrobial Resistance (CAESAR).

^c Voluntary participation of the laboratories.

^d Voluntary notification to health authorities.

^e Mandatory participation of the laboratories (for the United Kingdom, only mandatory for Scotland).

^f Mandatory notification to health authorities (for the United Kingdom, only mandatory for Scotland).

^g No national system for surveillance, but country reports carbapenem-resistant invasive isolates (*K. pneumoniae* and *E. coli*) to the European Antimicrobial Resistance Surveillance Network (EARS-Net).

^h An expert laboratory fulfils a similar role to that of a national reference laboratory.

*This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the Internal Court of Justice Opinion on the Kosovo declaration of independence.

TABLE 4B

National capacity for surveillance and containment of carbapenemase-producing *Enterobacteriaceae*, 38 European countries, May 2015***

Country	National system for surveillance	Officially nominated national reference laboratory, or expert laboratory	National recommendation or obligation for reporting (notification) to health authorities	National plan for containment of (or preparedness to contain) CPE	National recommendation or guideline on infection control measures	Reference or URL for recommendation or guideline on infection control measures
Malta	• ^e	• ^h		- ^a	•	
Montenegro	- ^{a, b}	- ^a	• ^f			
The Netherlands	• ^c	• ^h		- ^a	•	http://www.rivm.nl/dsresource?objectid=rivmp:46410&type=org&disposition=inline&ns_nc=1
Norway	• ^e	•	• ^f	- ^a	•	http://www.fhi.no/dokumenter/96331178b9.pdf http://www.fhi.no/eway/default.aspx?pid=239&trg=List_6212&Main_6157=6263:0:25.6493&MainContent_6263=6464:0:25.6513&List_6212=6453:0:25.6499:1:0:0::0:0
Poland	• ^e	•	• ^f		•	http://www.antybiotyki.edu.pl/pdf/kpc-20120713.pdf
Portugal	• ^e	•	• ^f	•	- ^a	
Romania	• ^c	• ^h			- ^a	http://www.srm.ro
Serbia	• ^c	•				
Slovakia	• ^e	• ^h	• ^f	•	•	http://www.ruvztn.sk/OU%20MZ%20SR.pdf
Slovenia	- ^{a, g}	• ^h	- ^a		•	http://www.mz.gov.si/fileadmin/mz.gov.si/pageuploads/mz_dokumenti/delovna_podrocja/zdravstveno_varstvo/zdravstveno_varstvo_v_posebnih/NAKOB0_oktober_2010/PRIPOROCILA_ESBL_26.10.10.pdf
Spain	• ^c	• ^h	• ^d	- ^a	•	http://www.aemps.gob.es/publicaciones/publica/docs/plan-estrategico-antibioticos.pdf http://www.madrid.org/cs/Satellite?blobcol=urldata&blobheader=application%2Fpdf&blobheadername1=Content-disposition&blobheadername2=cadena&blobheadervalue1=filename%3DPLAN+PREVENCION%2F93N+Y+CONTROL+EPC+CM_v1_sept+2013.pdf&blobheadervalue2=language%3Des%26site%3DPortalSalud&blobkey=id&blobtable=MungoBlobs&blobwhere=1352838664739&ssbinary=true http://safh.org/wp-content/uploads/2014/10/Programa-para-el-control-de-las-EPC_SSPA.pdf
Sweden	• ^e	•	• ^f	•	•	http://www.folkhalsomyndigheten.se/pagefiles/17838/ESBL-producerande%20tarmbakterier.pdf
The former Yugoslav Republic of Macedonia	• ^e	• ^h	• ^f			
Turkey	•	• ^h	• ^d			
United Kingdom	• ^{c, e}	•	• ^{d, f}	•	•	https://www.gov.uk/government/publications/carbapenemase-producing-enterobacteriaceae-early-detection-management-and-control-toolkit-for-acute-trusts http://www.documents.hps.scot.nhs.uk/hai/amr/cpe-guidance.pdf

CPE: carbapenemase-resistant *Enterobacteriaceae*.

In the table cells, a dot signifies 'in place' and the absence of dot signifies 'absent'. Black dots indicate that the system or document was already in place in 2013. Blue cells indicate a change reported in 2015, as compared with 2013.

^a In preparation.

^b No national system for surveillance, but country reports carbapenem-resistant invasive isolates (*Klebsiella pneumoniae* and *Escherichia coli*) to the Central Asian and Eastern European Surveillance on Antimicrobial Resistance (CAESAR).

^c Voluntary participation of the laboratories.

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^e Mandatory participation of the laboratories (for the United Kingdom, only mandatory for Scotland).

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^g No national system for surveillance, but country reports carbapenem-resistant invasive isolates (*K. pneumoniae* and *E. coli*) to the European Antimicrobial Resistance Surveillance Network (EARS-Net).

^h An expert laboratory fulfils a similar role to that of a national reference laboratory.

*This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the Internal Court of Justice Opinion on the Kosovo declaration of independence.

were KPC-producing *Enterobacteriaceae*, but OXA-48-producing *Enterobacteriaceae* have also been recently reported [55,56].

Cyprus, Greece, Israel, Italy, Malta and Turkey

During the EuSCAPE structured survey, **Cyprus** collected only three CPE isolates (data not shown). In line with this, the latest data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) showed a decreasing trend in the percentage of carbapenem-resistant isolates among invasive, i.e. blood and cerebrospinal fluid (CSF), *K. pneumoniae* isolates from 15.7% to 5% during 2011 to 2014 [12,57].

Since the early 2000s, **Greece** has been first facing a nationwide epidemic of polyclonal VIM-producing *K. pneumoniae* followed by a nation-wide occurrence of mainly monoclonal KPC-2-producing *K. pneumoniae*. The first NDM-1-producing *K. pneumoniae* isolate was reported in 2012 in a patient repatriated from Albania [58,59]. Since 2012, NDM-producing *Enterobacteriaceae* have been isolated from patients in 15 Greek hospitals and several clonal outbreaks of NDM-1-producing *Enterobacteriaceae* have been reported with only a few cases in each outbreak, therefore of much smaller magnitude than earlier and concurrent outbreaks with KPC-producing *Enterobacteriaceae*. OXA-48-producing *Enterobacteriaceae* are still rarely isolated. According to EARS-Net data, Greece had the highest percentage of carbapenem-resistant isolates among invasive *K. pneumoniae* in Europe in 2014, with more than 62% of *K. pneumoniae* invasive isolates being carbapenem-resistant, but with a decreasing trend from 68.2% in 2011 to 62.3% in 2014.

In **Israel**, CPE were rarely detected until 2006 when the situation changed dramatically, with the nationwide spread of KPC-producing *K. pneumoniae*. This led the Ministry of Health to initiate a nationwide intervention plan aiming to contain the spread [60]. The situation of CPE is now stable and the spread of CPE has been contained for several years, but CPE are not eradicated. Recently, several reports have indicated that NDM- and OXA-48-producing *Enterobacteriaceae*, are now present in Israel [61-63].

In **Italy**, it was not until 2010 that CPE became a major issue when KPC-producing *K. pneumoniae* became endemic, due to a rapid countrywide diffusion mostly caused by strains of clonal complex 258 [64]. This increase in percentages of carbapenem resistance in invasive *K. pneumoniae* isolates has been documented by EARS-Net since 2010 and the latest data from EARS-Net reported that 32.9% of *K. pneumoniae* invasive isolates were carbapenem-resistant [12]. NDM-1- and OXA-48-producing *Enterobacteriaceae* have been reported but their dissemination was still limited and cases were mostly acquired abroad [65-67]. In an effort to control and prevent the further spread of CPE, the Ministry of Health issued a circular letter in 2013 asking

the public health offices across the country to report all cases of bacteraemia caused by CPE to the regional and national authorities. Although there is still under-reporting of CPE, more than 2,000 CPE bacteraemia cases have been reported since publication of the circular letter. One worrisome recent development is the rapid and country-wide dissemination of resistance to colistin in KPC-producing *K. pneumoniae* [68] and the presence of pandrug-resistant (PDR) strains (data not shown).

In **Malta**, dissemination of OXA-48-producing *Enterobacteriaceae* had changed the country's epidemiological level from rare sporadic occurrence before 2010 to an endemic situation by 2013 [1,2]. It is thought that the influx of injured Libyan war victims to the intensive treatment unit of the country's only tertiary care hospital in 2011 contributed to the first outbreak and spread of OXA-48-producing *Enterobacteriaceae* in the country [69]. Despite initial control of the outbreak, the situation rapidly became endemic in this hospital and OXA-48-producing *Enterobacteriaceae* spread to other health and residential care entities on the Maltese islands. Until 2014, no KPC- or NDM-producing *Enterobacteriaceae* were reported while during the same period more than 400 new cases of OXA-48-producing *Enterobacteriaceae* were identified. Since then, the number of new cases of OXA-48-producing *Enterobacteriaceae* has continued to increase. In addition, sporadic cases of VIM- and NDM- producing *Enterobacteriaceae* were recently identified, mainly acquired outside the country. EARS-Net data for Malta showed an increase in the percentage of invasive carbapenem-resistant *K. pneumoniae*, OXA-48 –producing *K. pneumoniae*, from 3.8% to 9.9% during the period 2011 to 2014 [12].

In **Turkey**, OXA-48-producing *Enterobacteriaceae* are endemic, and since 2013, an increasing number of reports have demonstrated the emergence of other types of CPE (e.g. NDM-1- and KPC-producing *Enterobacteriaceae*) [70]. This was confirmed by the results of the EuSCAPE structured survey (data not shown). Reports of NDM-1-producing *Enterobacteriaceae* cases have been increasing, especially in hospitals from cities close to the Syrian border. The latter development is in accordance with recent reports on both autochthonous and imported NDM-1-producing *Enterobacteriaceae* cases in Turkish hospitals [71,72]. In 2015, the first *K. pneumoniae* co-producing OXA-48 and NDM-1 was isolated from a patient treated in the hospital of Sanliurfa, a city close to the border with Syria [73].

National capacity for surveillance and containment of carbapenamase-resistant *Enterobacteriaceae*

Table 4 summarises the existing surveillance and reference laboratory systems in place as well as the available national guidance documents for the containment of CPE in the participating countries at the time of the survey.

Surveillance of carbapenamase-resistant *Enterobacteriaceae*

Twenty-five EU Member States, Norway and Iceland had a dedicated national system for surveillance of CPE. Three EU Member States did not have a dedicated national surveillance system but reported carbapenem-resistant *K. pneumoniae* and *E. coli* from blood and CSF to EARS-Net. Slovenia, one of these, reported that at the time it was developing a dedicated national system for surveillance of CPE for implementation by the end of 2015. The Netherlands, which has a system in place, reported that enhanced surveillance of CPE will take place from 2016 onwards. In order to increase laboratory participation and coverage as well as to improve data quality, the enhanced surveillance should further optimise diagnostic testing and integrate clinical, molecular and epidemiological data for all CPE cases to determine relevant risk factors to target interventions and control potential spread.

All EU enlargement countries and Israel reported participating in the Central Asian and Eastern European Surveillance on Antimicrobial Resistance (CAESAR) network, a joint initiative of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the Dutch National Institute for Public Health and the Environment (RIVM) and the World Health Organization Regional Office for Europe (WHO/Europe). However, only Serbia, the former Yugoslav Republic of Macedonia and Turkey have so far reported data to CAESAR using the EARS-Net methodology [74]. Israel, Serbia, the former Yugoslav Republic of Macedonia and Turkey had dedicated national systems for the surveillance of CPE, while Albania, Bosnia and Herzegovina, Kosovo* and Montenegro were developing their surveillance system to be able to report data to CAESAR by 2015 or 2016.

Of 31 countries with a dedicated national surveillance system for CPE, 20 countries reported that surveillance of CPE was mandatory for all laboratories, nine countries reported that surveillance of CPE was voluntary and two countries did not specify. In Romania and Serbia, surveillance was voluntary and in form of a sentinel system of individual laboratories. In Ireland, laboratory participation was only mandatory for invasive disease caused by CPE, i.e. isolation from blood and CSF, but remained voluntary for CPE isolated from other body sites (Table 4).

Laboratory capacity for carbapenamase-resistant *Enterobacteriaceae*

Thirty-four countries reported having an officially nominated national reference laboratory for CPE or a national expert laboratory that fulfilled a similar role. Both Albania and Montenegro reported that the national reference laboratory was in development for implementation by 2015–2016 (Table 4).

Notification to health authorities for carbapenamase-resistant *Enterobacteriaceae*

Twenty-six countries reported having a national recommendation for reporting to health authorities CPE-positive patients identified by diagnostic laboratories. In most countries there is mandatory notification for all private and hospital laboratories and for all infections; only seven countries notified CPE cases on a voluntary basis. In two of the latter, notification of CPE cases was voluntary but notification of CPE outbreaks was mandatory. Slovenia and Germany reported that national recommendations or obligations for reporting were going to be implemented by the end of 2015, and for Bosnia and Herzegovina this is planned by 2016–2017 (Table 4).

National plan for containment of and infection control measures for carbapenamase-resistant *Enterobacteriaceae*

Eleven countries had implemented a national plan for the containment or for preparedness to contain CPE, and another nine countries were developing national containment plans. Spain had no national but regional specific plans.

Twenty-four countries reported having national recommendations or guidelines for infection prevention and control measures to be applied for patients confirmed as being infected or colonised with CPE: for six countries this applied to single CPE cases, for 15 to single CPE cases and outbreaks, for Greece this only applied to outbreaks and two countries did not specify the scope of their recommendations. Twelve of the national recommendations or guidelines were specific guidance documents for prevention and control of CPE, while nine were included as part of a general guidance document for multidrug-resistant organisms (MDROs) that specifically referred to CPE and three included a general guidance document for MDROs not specifically referring to prevention and control of CPE. Kosovo* and Portugal indicated that such recommendations or guidelines are in preparation for implementation by the end of 2015.

The most cited measures in such national recommendations or guidelines were isolation e.g. in single rooms, of suspected/colonised/infected patients and increased hand hygiene compliance (21 countries each), followed by active screening for early detection at admission of patients having been hospitalised abroad, implementation of contact precautions, for visitors and medical staff, and implementation of environmental hygiene procedures e.g. decontamination of equipment and disposal of waste (20 countries each), active screening for early detection of transferred patients from other wards/hospitals at admission (19 countries), cohorting of suspected/colonised/infected patients (18 countries), active screening for early detection of colonised patients at admission (16 countries),

dedicated infection control teams (14 countries), separate cohort nursing care e.g. nurses, doctors (13 countries), specialised training for nursing staff in infection control (12 countries), implementation of an antibiotic stewardship programme (11 countries), and audit and feedback to local, regional or national health authorities (10 countries).

Discussion

In 2013, at the beginning of the EuSCAPE project, knowledge about the spread and occurrence of CPE was heterogeneous among European countries [1]. Moreover, some NEs expressed concerns that under-detection affected the epidemiological self-assessment of their country. Following EuSCAPE activities including a capacity building workshop and an EQA exercise to improve the detection of CPE and the identification of the different carbapenemases circulating in Europe, the results of this follow-up survey provide evidence that the activities contributed to the desired improvement and increased awareness and knowledge of the epidemiology of CPE in many participating countries. After participation in the EuSCAPE project, all countries were able to self-assess their current situation, whereas only 26 countries could do so in 2013. In addition, all participating countries were able to rate the occurrence and spread of CPE according to the type of carbapenemase, while such data were only partially or not available in several European countries in 2013 [1,2].

In 2015, 13/38 countries reported inter-regional spread of or an endemic situation for CPE, compared with 6/38 countries in 2013. In addition, the survey documented the more frequent reporting of OXA-48- and NDM-producing *Enterobacteriaceae* compared with 2013. For OXA-48-producing *Enterobacteriaceae*, four countries had reported regional spread and only one country had reported an endemic situation in 2013, while in 2015, three countries reported regional spread, four reported inter-regional spread and two reported an endemic situation. Similarly for NDM-producing *Enterobacteriaceae*, only Italy and the UK had reported sporadic hospital outbreaks in 2013, while in 2015 six countries reported sporadic hospital outbreaks and seven countries reported regional and inter-regional spread. For the countries that were uncertain about their epidemiological stage in the 2013 survey, the results of this survey reflect an improved ability to detect CPE and identify the different carbapenemases. For the other countries, the changes in epidemiological stages observed between 2013 and 2015 likely reflect an increasing spread of CPE, as confirmed by the NEs. At the same time, increased awareness of CPE spread and surveillance might also contribute to increased detection and reporting of more advanced epidemiological stages. Indeed, countries with strict screening policies are more likely to report such advanced epidemiological stages.

The establishment of a surveillance system for CPE, based on the notification of CPE cases to health authorities, supported by reference laboratory confirmation and identification as well as, molecular typing services are the cornerstones of efficient monitoring and controlling of the spread of CPE. Many countries have developed dedicated surveillance systems and designated reference laboratories over the last two years, as well as implemented mandatory laboratory participation in CPE surveillance, or mandatory reporting of all cases of infections. However, despite the increased awareness and the worsening of the epidemiological situation in 2015, only 25 of the 38 countries that participated in the EuSCAPE project had enacted mandatory notification of CPE cases to health authorities. Active reporting of CPE cases should be encouraged by making all clinical cases notifiable to public health authorities.

Twenty countries had either implemented a national or regional plan for the containment of, or preparedness to contain, CPE or were developing national containment plans. However, national guidance documents on infection prevention and control of CPE were not available in 14 countries. In an effort to support health-care professionals, hospital administrators and public health professionals, ECDC published an online directory of guidance documents on prevention and control of carbapenem-resistant *Enterobacteriaceae* by EU/EEA Member States, ECDC, international and national agencies and professional societies [75,76].

A major impending threat to public health as a consequence of the expanding CPE epidemic in Europe is the emergence of PDR strains causing untreatable infections. Polymyxins, and particularly colistin, represent a last-line option for the treatment of patients infected with CPE. The latest data available from the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) show that consumption of polymyxins, mainly colistin, in Europe, almost doubled between 2009 and 2013 [77]. In parallel to this increasing colistin consumption, colistin resistance is increasing in carbapenem-resistant *Enterobacteriaceae* [68,77,78]. In Italy, 43% of KPC-producing *K. pneumoniae* isolates collected during the EuSCAPE structured survey in 2013 to 2014 [68] and 13% of carbapenem-resistant *K. pneumoniae* isolates from blood cultures reported to EARS-Net in 2014 were resistant to colistin [12]. Approximately 20% of carbapenem-resistant *K. pneumoniae* isolates from blood cultures reported to EARS-Net in 2014 were resistant to colistin in Romania and Greece [12]. In February 2015, a Greek hospital reported an outbreak of PDR *Enterobacteriaceae*, via the acquisition of *bla*_{VIM} by the naturally colistin-resistant *Providencia stuartii*, in an intensive care unit occurring in September to November 2011 [79].

The accumulation of other resistance markers in CPE strains in addition to colistin resistance makes it likely that Europe will soon witness an increasing number of

outbreaks of extensively drug-resistant (XDR) or PDR *Enterobacteriaceae* [57], for which few or even no treatment options are available. The United States Food and Drug Administration (FDA) recently approved the use of a combination of a well-established β -lactam antibiotic, ceftazidime, with a novel β -lactamase inhibitor, avibactam, for treatment of serious infections caused by resistant Gram-negative pathogens. Ceftazidime-avibactam is active against OXA-48- and KPC-producing *Enterobacteriaceae* but not NDM- or VIM-producing *Enterobacteriaceae* and would offer a partial solution to treat infections due to XDR or PDR Gram-negative bacteria.

In conclusion, the EuSCAPE project and this follow-up survey confirm the urgent need for a coordinated European effort for surveillance, control and prevention of CPE in Europe. The project contributed to the improvement of the capacity and ability to detect CPE in Europe by creating a European network of national reference/expert laboratories able to provide information for monitoring incidence and spread of carbapenemases in the 38 participating countries. Furthermore, results presented here show the need to develop an EU-wide system for public health surveillance of high-risk CPE clones and mobile genetic vectors of epidemic carbapenemases across the healthcare systems in Europe for informing risk assessment and control programmes.

**This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo declaration of independence.*

****Authors' correction**

On 10 December 2015, upon request of the authors, the following text was added to the Acknowledgements section: 'The authors would like to thank Liselotte Diaz Högberg for providing data and the analysis of the data on the percentage of carbapenem resistance in invasive *Klebsiella pneumoniae* isolates in the European Union / European Economic Area Member States from the European Antimicrobial Resistance Surveillance Network (EARS-Net).'

*****Erratum**

Table 4 was corrected and replaced on 22 September 2016.

The European Survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE) working group comprises the EuSCAPE national experts, ECDC National Focal Points for antimicrobial resistance, ECDC National Correspondents for EU enlargement countries, the EuSCAPE scientific advisory board and External consulted experts

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Acknowledgements

The authors would like to thank Liselotte Diaz Högberg for providing data and the analysis of the data on the percentage of carbapenem resistance in invasive *Klebsiella pneumoniae* isolates in the European Union / European Economic Area Member States from the European Antimicrobial Resistance Surveillance Network (EARS-Net).** The European Survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE) was funded by ECDC following an open call for tender (OJ/25/04/2012-PROC/2012/036).

Conflict of interest

None declared.

Authors' contributions

Barbara Albiger: modified, adapted and further developed the self-assessment questionnaire issued in February 2013 for the purpose of this study, supervised and coordinated the post-EuSCAPE survey collecting the data, performed the data analysis and wrote the manuscript.

Dominique L. Monnet: reviewed and provided feedback on the questionnaire and reviewed and approved the data, the analysis and the manuscript.

Marc J. Struelens: contributed to drafting and the review of the manuscript

Corinna Glasner and Hajo Grundmann: reviewed and provided feedback on the questionnaire and the manuscript.

The national experts, the ECDC National Focal Points for antimicrobial resistance and the ECDC National Correspondents for EU enlargement countries: answered the survey providing country specific data, provided country specific profile, approved the final data and the analysis, and reviewed and provided feedback the manuscript.

The EuSCAPE scientific advisory board and the external consulted experts: reviewed and provided feedback on the manuscript.

References

1. European Survey on Carbapenemase-Producing Enterobacteriaceae (EuSCAPE) Working Group, Glasner C, Albiger B, Buist G, Tambić Andrasević A, Canton R, Carmeli Y, et al. . Carbapenemase-producing Enterobacteriaceae in Europe: a survey among national experts from 39 countries, February 2013. *Euro Surveill.* 2013;18(28):20525. DOI: 10.2807/1560-7917.ES2013.18.28.20525 PMID: 23870096
2. European Centre for Disease Prevention and Control (ECDC). Carbapenemase-producing bacteria in Europe: interim results from the European Survey on carbapenemase-producing Enterobacteriaceae (EuSCAPE) project 2013. Stockholm: ECDC. 2014. Available from: <http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-carbapenemase-producing-bacteria-europe.pdf>
3. CNSE Working Group, Grundmann H, Livermore DM, Giske CG, Canton R, Rossolini GM, Campos J, et al. . Carbapenem-non-susceptible Enterobacteriaceae in Europe: conclusions from a meeting of national experts. *Euro Surveill.* 2010;15(46). PMID: 21144429
4. Jakobsen L, Hansen F, Stegger M, Andersen PS, Hammerum AM, Fuglsang-Damgaard D. Use of whole-genome sequencing for detection of the spread of VIM-4-producing *Escherichia coli* between two patients in Denmark. *Int J Antimicrob Agents.* 2015;45(3):327-9. DOI: 10.1016/j.ijantimicag.2014.12.003 PMID: 25595952
5. Jakobsen L, Hammerum AM, Hansen F, Fuglsang-Damgaard D. An ST405 NDM-4-producing *Escherichia coli* isolated from a Danish patient previously hospitalized in Vietnam. *J Antimicrob Chemother.* 2014;69(2):559-60. DOI: 10.1093/jac/dkt356 PMID: 24013194
6. Hammerum AMHF, Olesen B, Struve C, Holzknecht BJ, Andersen PS, Thyse A-M, et al. Investigation of a possible outbreak of NDM-5-producing ST16 *Klebsiella pneumoniae* among patients in Denmark with no history of recent travel using whole-genome sequencing. *J Glob Antimicrob Resist.* 2015;3(3):219-21. DOI: 10.1016/j.jgar.2015.05.003
7. Österblad M, Kirveskari J, Hakanen AJ, Tissari P, Vaara M, Jalava J. Carbapenemase-producing Enterobacteriaceae in Finland: the first years (2008-11). *J Antimicrob Chemother.* 2012;67(12):2860-4. DOI: 10.1093/jac/dks299 PMID: 22855858
8. Kanerva M, Skogberg K, Rynänen K, Pakkamäki A, Jalava J, Öllgren J, et al. Coincidental detection of the first outbreak of carbapenemase-producing *Klebsiella pneumoniae* colonisation in a primary care hospital, Finland, 2013. *Euro Surveill.* 2015;20(26):21172. DOI: 10.2807/1560-7917.ES2015.20.26.21172 PMID: 26159309
9. Löfmark S, Sjöström K, Mäkitalo B, Edquist P, Tegmark Wisell K, Giske CG. Carbapenemase-producing Enterobacteriaceae in Sweden 2007-2013: Experiences from seven years of systematic surveillance and mandatory reporting. *Drug Resist Uptat.* 2015;20:29-38. DOI: 10.1016/j.drug.2015.05.001 PMID: 26004211
10. Dautzenberg MJ, Ossewaarde JM, de Kraker ME, van der Zee A, van Burgh S, de Greeff SC, et al. Successful control of a hospital-wide outbreak of OXA-48 producing Enterobacteriaceae in the Netherlands, 2009 to 2011. *Euro Surveill.* 2014;19(9):20723. DOI: 10.2807/1560-7917.ES2014.19.9.20723 PMID: 24626209
11. Bathoorn E, Friedrich AW, Zhou K, Arends JP, Borst DM, Grundmann H, et al. Latent introduction to the Netherlands of multiple antibiotic resistance including NDM-1 after hospitalisation in Egypt, August 2013. *Euro Surveill.* 2013;18(42):20610. DOI: 10.2807/1560-7917.ES2013.18.42.20610 PMID: 24176580
12. European Centre for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC. 2015; (Forthcoming).
13. Pavelkovich A, Balode A, Edquist P, Egorova S, Ivanova M, Kaftyreva L, et al. Detection of carbapenemase-producing enterobacteriaceae in the baltic countries and st. Petersburg area. *Biomed Res Int.* 2014;2014:548960.
14. Prior AR, Roche C, Lynch M, Kelly S, O'Rourke K, Crowley B. First identified case of VIM-producing carbapenem-resistant *Klebsiella pneumoniae* in the Republic of Ireland associated with fatal outcome. *Euro Surveill.* 2010;15(50). PMID: 21172170
15. Roche C, Cotter M, O Connell N, Crowley B. First identification of class A carbapenemase-producing *Klebsiella pneumoniae* in the Republic of Ireland. *Euro Surveill.* 2009;14(13). PMID: 19341609
16. McDermott H, Morris D, McArdle E, O'Mahony G, Kelly S, Cormican M, et al. Isolation of NDM-1-producing *Klebsiella pneumoniae* in Ireland, July 2011. *Euro Surveill.* 2012;17(7). PMID: 22370013
17. Morris D, Boyle F, Morris C, Condon I, Delannoy-Vieillard AS, Power L, et al. Inter-hospital outbreak of *Klebsiella pneumoniae* producing KPC-2 carbapenemase in Ireland. *J Antimicrob Chemother.* 2012;67(10):2367-72. DOI: 10.1093/jac/dks239 PMID: 22744760
18. Wrenn C, O'Brien D, Keating D, Roche C, Rose L, Ronayne A, et al. Investigation of the first outbreak of OXA-48-producing *Klebsiella pneumoniae* in Ireland. *J Hosp Infect.* 2014;87(1):41-6. DOI: 10.1016/j.jhin.2014.03.001 PMID: 24746608
19. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother.* 2009;53(12):5046-54. DOI: 10.1128/AAC.00774-09 PMID: 19770275
20. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis.* 2010;10(9):597-602. DOI: 10.1016/S1473-3099(10)70143-2 PMID: 20705517
21. Zarfel G, Hoenigl M, Würstl B, Leitner E, Salzer HJ, Valentin T, et al. Emergence of carbapenem-resistant Enterobacteriaceae in Austria, 2001-2010. *Clin Microbiol Infect.* 2011;17(11):E5-8. DOI: 10.1111/j.1469-0691.2011.03659.x PMID: 21939472
22. Grisold AJ, Hoenigl M, Ovcina I, Valentin T, Fruhwald S. Ventilator-associated pneumonia caused by OXA-48-producing *Escherichia coli* complicated by ciprofloxacin-associated rhabdomyolysis. *Journal of infection and chemotherapy: official journal of the Japan Society of Chemotherapy.* 2013;19(6):1214-7.
23. Leitner E, Zarfel G, Luxner J, Herzog K, Pekard-Amenitsch S, Hoenigl M, et al. Contaminated handwashing sinks as the source of a clonal outbreak of KPC-2-producing *Klebsiella oxytoca* on a hematology ward. *Antimicrob Agents Chemother.* 2015;59(1):714-6. DOI: 10.1128/AAC.04306-14 PMID: 25348541
24. Heller I, Grif K, Orth D. Emergence of VIM-1-carbapenemase-producing Enterobacter cloacae in Tyrol, Austria. *J Med Microbiol.* 2012;61(Pt 4):567-71. DOI: 10.1099/jmm.0.038646-0 PMID: 22194339
25. Czech Participants of European Antimicrobial Resistance Surveillance Network, Hrabák J, Papagiannitsis CC, Studentová V, Jakub V, Fridrichová M, Zemlicková H, et al. . Carbapenemase-producing *Klebsiella pneumoniae* in the Czech Republic in 2011. *Euro Surveill.* 2013;18(45):20626. DOI: 10.2807/1560-7917.ES2013.18.45.20626 PMID: 24229789
26. Studentova V, Dobiasova H, Hedlova D, Dolejska M, Papagiannitsis CC, Hrabak J. Complete nucleotide sequences of two NDM-1-encoding plasmids from the same sequence type 11 *Klebsiella pneumoniae* strain. *Antimicrob Agents Chemother.* 2015;59(2):1325-8. DOI: 10.1128/AAC.04095-14 PMID: 25421477

27. Hrabák J, Študentová V, Jakubů V, Adámková V, Dvořáková L, Balejová M, et al. Prevalence study on carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates in Czech hospitals--results from Czech Part of European Survey on Carbapenemase-Producing Enterobacteriaceae (EuSCAPE). *Epidemiol Mikrobiol Immunol*. 2015;64(2):87-91. PMID: 26099612
28. Ducomble T, Fauchoux S, Helbig U, Kaisers UX, König B, Knaust A, et al. Large hospital outbreak of KPC-2-producing *Klebsiella pneumoniae*: investigating mortality and the impact of screening for KPC-2 with polymerase chain reaction. *J Hosp Infect*. 2015;89(3):179-85. DOI: 10.1016/j.jhin.2014.11.012 PMID: 25623204
29. Bogaerts P, de Castro RR, Deplano A, Bouchahrouf W, Tsoho C, Denis O, et al. Detection of a VIM-27-producing *Klebsiella pneumoniae* isolate in a patient following surgical tourism in Greece. *Antimicrob Agents Chemother*. 2011;55(9):4488-9. DOI: 10.1128/AAC.00688-11 PMID: 21746946
30. Pirš M, Andlovic A, Cerar T, Žohar-Čretnik T, Kobola L, Kolman J, et al. A case of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* in a patient transferred to Slovenia from Libya, November 2011. *Euro Surveill*. 2011;16(50):20042. PMID: 22221496
31. Tóth A, Damjanova I, Puskás E, Jánvári L, Farkas M, Dobák A, et al. Emergence of a colistin-resistant KPC-2-producing *Klebsiella pneumoniae* ST258 clone in Hungary. *Eur J Clin Microbiol Infect Dis*. 2010;29(7):765-9. Error! Hyperlink reference not valid. DOI: 10.1007/s10096-010-0921-3 PMID: 20401676
32. Jánvári L, Damjanova I, Lázár A, Rácz K, Kocsis B, Urbán E, et al. Emergence of OXA-162-producing *Klebsiella pneumoniae* in Hungary. *Scand J Infect Dis*. 2014;46(4):320-4. DOI: 10.3109/00365548.2013.879993 PMID: 24552581
33. KPC-PL Study Group, Baraniak A, Grabowska A, Izdebski R, Fiett J, Herda M, Bojarska K, et al. . Molecular characteristics of KPC-producing Enterobacteriaceae at the early stage of their dissemination in Poland, 2008-2009. *Antimicrob Agents Chemother*. 2011;55(12):5493-9. DOI: 10.1128/AAC.05118-11 PMID: 21930889
34. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, et al. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis*. 2013;13(9):785-96. DOI: 10.1016/S1473-3099(13)70190-7 PMID: 23969216
35. Baraniak A, Izdebski R, Fiett J, Gawryszewska I, Bojarska K, Herda M, et al. NDM-producing Enterobacteriaceae in Poland, 2012-14: inter-regional outbreak of *Klebsiella pneumoniae* ST11 and sporadic cases. *J Antimicrob Chemother*. 2015;dkv282. [Epub ahead of print].
36. Fiett J, Baraniak A, Izdebski R, Sitkiewicz I, Żabicka D, Meler A, et al. The first NDM metallo- β -lactamase-producing Enterobacteriaceae isolate in Poland: evolution of IncFII-type plasmids carrying the bla(NDM-1) gene. *Antimicrob Agents Chemother*. 2014;58(2):1203-7. DOI: 10.1128/AAC.01197-13 PMID: 24247128
37. Székely E, Damjanova I, Jánvári L, Vas KE, Molnár S, Bilca DV, et al. First description of bla(NDM-1), bla(OXA-48), bla(OXA-181) producing Enterobacteriaceae strains in Romania. *Int J Med Microbiol*. 2013;303(8):697-700. DOI: 10.1016/j.ijmm.2013.10.001 PMID: 24183483
38. Dortet L, Flonta M, Boudehen YM, Creton E, Bernabeu S, Vogel A, et al. Dissemination of Carbapenemase-Producing Enterobacteriaceae and *Pseudomonas aeruginosa* in Romania. *Antimicrob Agents Chemother*. 2015;59(11):7100-3. DOI: 10.1128/AAC.01512-15 PMID: 26303798
39. Gheorghe I, Czobor I, Chifiriuc MC, Borcan E, Ghiță C, Banu O, et al. Molecular screening of carbapenemase-producing Gram-negative strains in Romanian intensive care units during a one year survey. *J Med Microbiol*. 2014;63(Pt 10):1303-10. DOI: 10.1099/jmm.0.074039-0 PMID: 25060972
40. Schréterová E, Takáčová V, Nikš M, Pastvová L, Tomčo L, Siegfried L. [Methods of phenotypic determination of New Delhi metallo-beta-lactamase (NDM-1) in *Klebsiella pneumoniae* isolated in Slovakia]. *Klin Mikrobiol Infekc Lek*. 2014;20(3):79-84. PMID: 25702288
41. Kostyanov T, Tafaj S, Skenduli I, Bardhi D, Kapisiyi P, Bino S, et al. First detection of KPC-3-producing *Klebsiella pneumoniae* in Albania. *New Microbes New Infect*. 2015;4:11-2. DOI: 10.1016/j.nmni.2015.01.001 PMID: 25830029
42. Mazzariol A, Bošnjak Z, Ballarini P, Budimir A, Bedenić B, Kalenč S, et al. NDM-1-producing *Klebsiella pneumoniae*, Croatia. *Emerg Infect Dis*. 2012;18(3):532-4. DOI: 10.3201/eid1803.1103890 PMID: 22377049
43. Meletis G, Oustas E, Bagkeri M. Carbapenemase reports from the Balkans: a systematic review. *Infez Med*. 2014;22(2):85-106. PMID: 24955795
44. Markovska R, Schneider I, Stoeva T, Bojkova K, Boyanova L, Bauernfeind A, et al. First identification of KPC-2 and VIM-1 producing *Klebsiella pneumoniae* in Bulgaria. *Diagn Microbiol Infect Dis*. 2013;77(3):252-3. DOI: 10.1016/j.diagmicrobio.2013.07.019 PMID: 24041551
45. Poirel L, Savov E, Nazli A, Trifonova A, Todorova I, Gergova I, et al. Outbreak caused by NDM-1- and RmtB-producing *Escherichia coli* in Bulgaria. *Antimicrob Agents Chemother*. 2014;58(4):2472-4. DOI: 10.1128/AAC.02571-13 PMID: 24514099
46. Bedenić B, Mazzariol A, Plečko V, Bošnjak Z, Barl P, Vraneš J, et al. First report of KPC-producing *Klebsiella pneumoniae* in Croatia. *J Chemother*. 2012;24(4):237-9. DOI: 10.1179/1973947812Y.0000000017 PMID: 23040691
47. Zujic Atalić V, Bedenić B, Kocsis E, Mazzariol A, Sardelić S, Barišić M, et al. Diversity of carbapenemases in clinical isolates of Enterobacteriaceae in Croatia--the results of a multicentre study. *Clin Microbiol Infect*. 2014;20(11):O894-903. DOI: 10.1111/1469-0691.12635 PMID: 24674100
48. Bogaerts P, Bouchahrouf W, de Castro RR, Deplano A, Berhin C, Piérard D, et al. Emergence of NDM-1-producing Enterobacteriaceae in Belgium. *Antimicrob Agents Chemother*. 2011;55(6):3036-8. DOI: 10.1128/AAC.00049-11 PMID: 21444697
49. Zarfel G, Hoenigl M, Leitner E, Salzer HJ, Feiler G, Masoud L, et al. Emergence of New Delhi metallo- β -lactamase, Austria. *Emerg Infect Dis*. 2011;17(1):129-30. DOI: 10.3201/eid1701.101331 PMID: 21192874
50. Seiffert SN, Marschall J, Perreten V, Carattoli A, Furrer H, Endimiani A. Emergence of *Klebsiella pneumoniae* co-producing NDM-1, OXA-48, CTX-M-15, CMY-16, QnrA and ArmA in Switzerland. *Int J Antimicrob Agents*. 2014;44(3):260-2. DOI: 10.1016/j.ijantimicag.2014.05.008 PMID: 25123809
51. Oteo J, Miró E, Pérez-Vázquez M, Navarro F. Evolution of carbapenemase-producing Enterobacteriaceae at the global and national level: what should be expected in the future? *Enferm Infecc Microbiol Clin*. 2014;32(Suppl 4):17-23. DOI: 10.1016/S0213-005X(14)70170-3 PMID: 25542048
52. GEIH-GEMARA (SEIMC) and REIPI, Oteo J, Ortega A, Bartolomé R, Bou G, Conejo C, Fernández-Martínez M, et al. . Prospective multicenter study of carbapenemase-producing Enterobacteriaceae from 83 hospitals in Spain reveals high in vitro susceptibility to colistin and meropenem. *Antimicrob Agents Chemother*. 2015;59(6):3406-12. DOI: 10.1128/AAC.00086-15 PMID: 25824224
53. Spanish Collaborating Group for the Antibiotic Resistance Surveillance Program, Oteo J, Saez D, Bautista V, Fernández-Romero S, Hernández-Molina JM, Pérez-Vázquez M, et al. . Carbapenemase-producing enterobacteriaceae in Spain in 2012. *Antimicrob Agents Chemother*. 2013;57(12):6344-7. DOI: 10.1128/AAC.01513-13 PMID: 24041898
54. Seara N, Oteo J, Carrillo R, Pérez-Blanco V, Mingorance J, Gómez-Gil R, et al. Interhospital spread of NDM-7-producing *Klebsiella pneumoniae* belonging to ST437 in Spain. *Int J Antimicrob Agents*. 2015;46(2):169-73. DOI: 10.1016/j.ijantimicag.2015.04.001 PMID: 25982912
55. Antibiotic Resistance Surveillance Program in Portugal (ARSIP), Manageiro V, Ferreira E, Almeida J, Barbosa S, Simões C, Bonomo RA, et al. . Predominance of KPC-3 in a survey for carbapenemase-producing Enterobacteriaceae in Portugal. *Antimicrob Agents Chemother*. 2015;59(6):3588-92. DOI: 10.1128/AAC.05065-14 PMID: 25779587
56. Manageiro V, Ferreira E, Pinto M, Caniça M. First description of OXA-48 carbapenemase harbored by *Escherichia coli* and *Enterobacter cloacae* from a single patient in Portugal. *Antimicrob Agents Chemother*. 2014;58(12):7613-4. DOI: 10.1128/AAC.02961-14 PMID: 25246399
57. EARS-Net Coordination Group and EARS-Net participants, Magiorakos AP, Suetens C, Monnet DL, Gagliotti C, Heuer OE. The rise of carbapenem resistance in Europe: just the tip of the iceberg? *Antimicrob Resist Infect Control*. 2013;2(1):6. DOI: 10.1186/2047-2994-2-6 PMID: 23410479
58. Voulgari E, Zarkotou O, Ranellou K, Karageorgopoulos DE, Vriani G, Mamali V, et al. Outbreak of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* in Greece involving an ST11 clone. *J Antimicrob Chemother*. 2013;68(1):84-8. DOI: 10.1093/jac/dks356 PMID: 22945916
59. Giakkoupi P, Tryfinopoulou K, Kontopidou F, Tsonou P, Golegou T, Souki H, et al. Emergence of NDM-producing *Klebsiella pneumoniae* in Greece. *Diagn Microbiol Infect Dis*. 2013;77(4):382-4. DOI: 10.1016/j.diagmicrobio.2013.09.001 PMID: 24135413
60. Schwaber MJ, Carmeli Y. An ongoing national intervention to contain the spread of carbapenem-resistant enterobacteriaceae. *Clin Infect Dis*. 2014;58(5):697-703. DOI: 10.1093/cid/cit795 PMID: 24304707

61. Gefen-Halevi S, Hindiyyeh MY, Ben-David D, Smollan G, Gal-Mor O, Azar R, et al. Isolation of genetically unrelated bla(NDM-1)-positive *Providencia rettgeri* strains in Israel. *J Clin Microbiol*. 2013;51(5):1642-3. DOI: 10.1128/JCM.00381-13 PMID: 23486709
62. Adler A, Shklyar M, Schwaber MJ, Navon-Venezia S, Dhaheer Y, Edgar R, et al. Introduction of OXA-48-producing Enterobacteriaceae to Israeli hospitals by medical tourism. *J Antimicrob Chemother*. 2011;66(12):2763-6. DOI: 10.1093/jac/dkr382 PMID: 22191089
63. Adler A, Solter E, Masarwa S, Miller-Roll T, Abu-Libdeh B, Khammash H, et al. Epidemiological and microbiological characteristics of an outbreak caused by OXA-48-producing Enterobacteriaceae in a neonatal intensive care unit in Jerusalem, Israel. *J Clin Microbiol*. 2013;51(9):2926-30. DOI: 10.1128/JCM.01049-13 PMID: 23804390
64. AMCLI-CRE Survey Participants, Giani T, Pini B, Arena F, Conte V, Bracco S, Migliaiavacca R, et al. Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. *Euro Surveill*. 2013;18(22). PMID: 23787077
65. Gaibani P, Ambretti S, Berlingeri A, Cordovana M, Farruggia P, Panico M, et al. Outbreak of NDM-1-producing Enterobacteriaceae in northern Italy, July to August 2011. *Euro Surveill*. 2011;16(47):20027. PMID: 22152705
66. Giani T, Conte V, Di Pilato V, Aschbacher R, Weber C, Larcher C, et al. *Escherichia coli* from Italy producing OXA-48 carbapenemase encoded by a novel Tn1999 transposon derivative. *Antimicrob Agents Chemother*. 2012;56(4):2211-3. DOI: 10.1128/AAC.00035-12 PMID: 22290939
67. D'Andrea MM, Venturelli C, Giani T, Arena F, Conte V, Bresciani P, et al. Persistent carriage and infection by multidrug-resistant *Escherichia coli* ST405 producing NDM-1 carbapenemase: report on the first Italian cases. *J Clin Microbiol*. 2011;49(7):2755-8. DOI: 10.1128/JCM.00016-11 PMID: 21525229
68. Network EuSCAPE-Italy, Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, et al. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Euro Surveill*. 2014;19(42):20939. DOI: 10.2807/1560-7917.ES2014.19.42.20939 PMID: 25358041
69. European Centre for Disease Prevention and Control (ECDC). Annual Epidemiological Report 2012. Reporting on 2010 surveillance data and 2011 epidemic intelligence data. Stockholm: ECDC. 2013. Available from: <http://ecdc.europa.eu/en/publications/Publications/Annual-Epidemiological-Report-2012.pdf>
70. Labarca J, Poirel L, Ozdamar M, Turkoglu S, Hakko E, Nordmann P. KPC-producing *Klebsiella pneumoniae*, finally targeting Turkey. *New Microbes New Infect*. 2014;2(2):50-1. DOI: 10.1002/nmi2.42 PMID: 25356342
71. Poirel L, Ozdamar M, Ocampo-Sosa AA, Turkoglu S, Ozer UG, Nordmann P. NDM-1-producing *Klebsiella pneumoniae* now in Turkey. *Antimicrob Agents Chemother*. 2012;56(5):2784-5. DOI: 10.1128/AAC.00150-12 PMID: 22391536
72. Poirel L, Yilmaz M, Istanbulu A, Arslan F, Mert A, Bernabeu S, et al. Spread of NDM-1-producing Enterobacteriaceae in a neonatal intensive care unit in Istanbul, Turkey. *Antimicrob Agents Chemother*. 2014;58(5):2929-33. DOI: 10.1128/AAC.02047-13 PMID: 24550328
73. Kilic A, Baysallar M. The First *Klebsiella pneumoniae* Isolate Co-Producing OXA-48 and NDM-1 in Turkey. *Ann Lab Med*. 2015;35(3):382-3. DOI: 10.3343/alm.2015.35.3.382 PMID: 25932453
74. World Health Organization, Regional Office for Europe (WHO/Europe). Central Asian and Eastern European Surveillance of Antimicrobial Resistance. Annual report 2014. Copenhagen: WHO/Europe. 2015. Available from: http://www.euro.who.int/__data/assets/pdf_file/0006/285405/CAESAR-Surveillance-Antimicrobial-Resistance2014.pdf?ua=1
75. European Centre for Disease Prevention and Control (ECDC). Directory of available online guidance documents on prevention and control of carbapenem-resistant Enterobacteriaceae. Stockholm: ECDC. 2014. Available from: http://ecdc.europa.eu/en/healthtopics/Healthcare-associated_infections/guidance-infection-prevention-control/Pages/guidance-prevention-control-infections-CRE.aspx
76. European Centre for Disease Prevention and Control (ECDC). Systematic review of the effectiveness of infection control measures to prevent the transmission of carbapenemase-producing Enterobacteriaceae through cross-border transfer of patients. Stockholm: ECDC. 2014. Available from: <http://ecdc.europa.eu/en/publications/Publications/CPE-systematic-review-effectiveness-infection-control-measures-to-prevent-transmission-2014.pdf>
77. European Centre for Disease Prevention and Control (ECDC). Summary of the latest data on antibiotic consumption in the European Union. November 2014. Stockholm: ECDC. 2014. Available from: <http://ecdc.europa.eu/en/eaad/antibiotics-get-informed/antibiotics-resistance-consumption/Pages/data-reports.aspx>
78. Weterings V, Zhou K, Rossen JW, van Stenis D, Thewissen E, Kluytmans J, et al. An outbreak of colistin-resistant *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* in the Netherlands (July to December 2013), with inter-institutional spread. *Eur J Clin Microbiol Infect Dis*. 2015;34(8):1647-55. DOI: 10.1007/s10096-015-2401-2 PMID: 26067658
79. Douka E, Perivolioti E, Kraniotaki E, Fountoulis K, Economidou F, Tsakris A, et al. Emergence of a pandrug-resistant VIM-1-producing *Providencia stuartii* clonal strain causing an outbreak in a Greek intensive care unit. *Int J Antimicrob Agents*. 2015;45(5):533-6. DOI: 10.1016/j.ijantimicag.2014.12.030 PMID: 25749199

Zanamivir-resistant influenza viruses with Q136K or Q136R neuraminidase residue mutations can arise during MDCK cell culture creating challenges for antiviral susceptibility monitoring

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Citation style for this article:

Little K, Leang S, Butler J, Baas C, Harrower B, Mosse J, Barr I, Hurt A. Zanamivir-resistant influenza viruses with Q136K or Q136R neuraminidase residue mutations can arise during MDCK cell culture creating challenges for antiviral susceptibility monitoring. *Euro Surveill.* 2015;20(45):pii=30060. DOI: <http://dx.doi.org/10.2807/1560-7917.ES.2015.20.45.30060>

Article submitted on 22 February 2015 / accepted on 24 September 2015 / published on 12 November 2015

Surveillance of circulating influenza strains for antiviral susceptibility is important to ensure patient treatment guidelines remain appropriate. Influenza A(H3N2) and A(H1N1)pdm09 virus isolates containing mutations at the Q136 residue of the neuraminidase (NA) that conferred reduced susceptibility to the NA inhibitor (NAI) zanamivir were detected during antiviral susceptibility monitoring. Interestingly, the mutations were not detectable in the viruses from respective clinical specimens, only in the cultured isolates. We showed that variant viruses containing the Q136K and Q136R NA mutations were preferentially selected in Madin-Darby canine kidney epithelial (MDCK) cells, but were less well supported in MDCK-SIAT1 cells and embryonated eggs. The effect of Q136K, Q136R, Q136H and Q136L substitutions in NA subtypes N1 and N2 on NAI susceptibility and in vitro viral fitness was assessed. This study highlights the challenges that cell culture derived mutations can pose to the NAI susceptibility analysis and interpretation and reaffirms the need to sequence viruses from respective clinical specimens to avoid misdiagnosis. However, we also demonstrate that NA mutations at residue Q136 can confer reduced zanamivir, peramivir or laninamivir susceptibility, and therefore close monitoring of viruses for mutations at this site from patients being treated with these antivirals is important.

Introduction

Neuraminidase inhibitors (NAIs) are a class of influenza antivirals that target the highly conserved enzymatic site of the neuraminidase (NA) glycoprotein on the surface of influenza A and B viruses [1]. The NAIs have become the most widely used antivirals for the treatment or prophylaxis of influenza, particularly

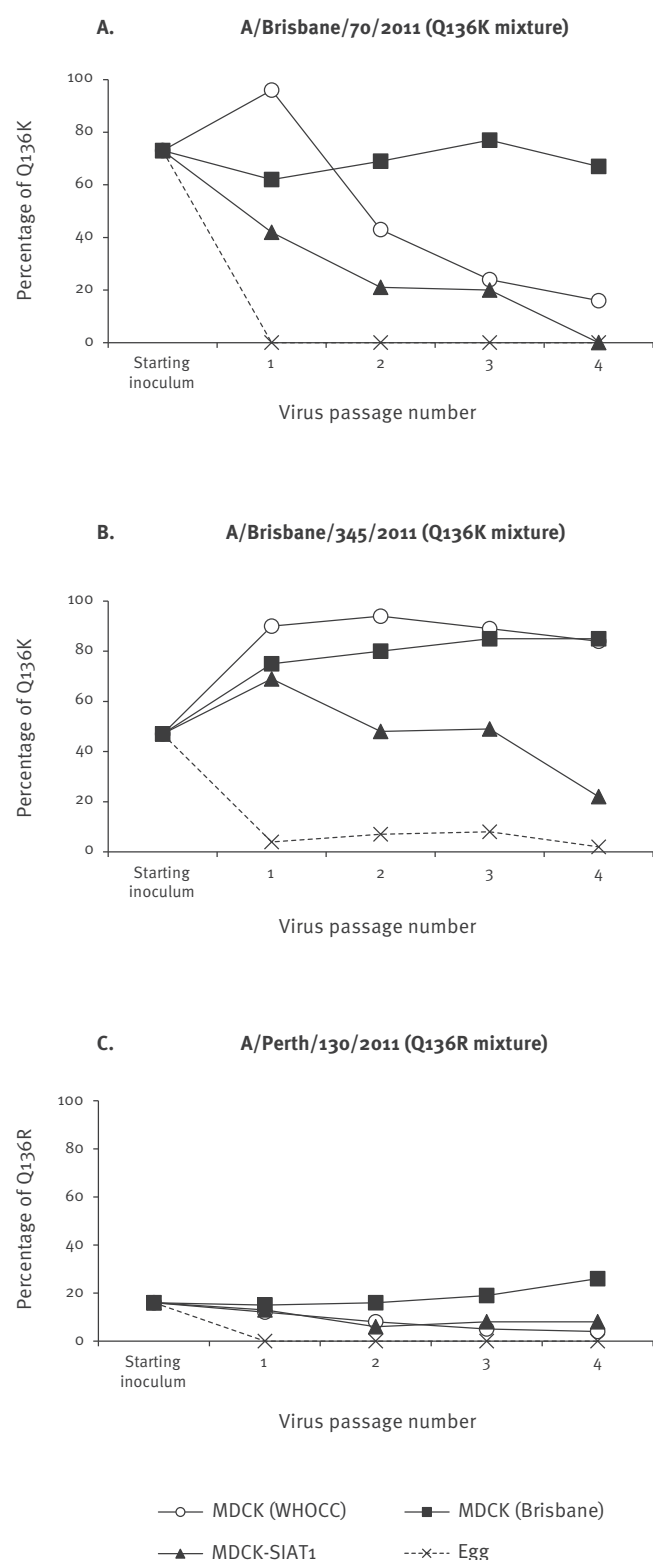
since the development of widespread resistance to the adamantanes, the older class of antivirals that block the M2 ion channel protein [2]. Two NAIs, oseltamivir and zanamivir, have been available in many countries since 1999, and two new NAIs, peramivir and laninamivir, have recently been approved for human use in Japan and a small number of other countries. Each of the NAIs is structurally different and therefore binds slightly differently within the NA active site [1]. This difference in binding is advantageous for treatment, as a virus that develops resistance against one NAI, may retain sensitivity to others. For example, the H275Y NA mutation in NA subtype N1-containing viruses confers resistance to oseltamivir but not to zanamivir [3].

Resistance to the NAIs commonly occurs as a result of amino acid mutations within the NA active site, either in the catalytic residues (those that interact directly with the NAIs), or in the framework residues (those that provide structural support for the catalytic residues) [4]. However, not all viruses with resistance to NAIs will pose a public health risk, as mutations that reduce binding can also impact the ability of the NA to interact with the natural substrate during replication [5]. However, in some cases, the mutation can affect NAI sensitivity but not compromise viral 'fitness'. The H275Y mutation that was present in seasonal influenza A(H1N1) viruses between 2007 and 2009 is such an example, as it conferred oseltamivir resistance but did not appear to affect the ability of the virus to replicate and transmit [6,7].

Oseltamivir resistant influenza viruses have been detected at a considerably higher frequency than zanamivir resistant viruses. During human clinical trials,

FIGURE 1

Serial passage of A(H1N1)pdm09 influenza virus isolates (n=3) in different Madin-Darby canine kidney (MDCK) cells and embryonated hens' eggs



WHO CC: World Health Organization Collaborating Centre for Reference and Research on Influenza.

Three isolates were used, two containing a mixture of wildtype (WT) and variant viruses with a Q136K neuraminidase (NA) mutation (panels A and B) and one containing a mixture of WT and variant viruses with a Q136R NA mutation (panel C). Proportion of Q136K or Q136R was determined by pyrosequencing analysis.

oseltamivir resistance was detected in <1–4% of adults [8,9] and 5–6% of treated children [10] undergoing oseltamivir treatment, although in observational studies the frequency of resistance in oseltamivir treated children has been as high as 27% [11]. Most significantly, oseltamivir-resistant seasonal A(H1N1) viruses with an H275Y mutation became widespread during 2008, spreading globally even in regions of low drug usage [12,13]. In comparison, there have only been a few reports of zanamivir resistance. The first was in an immunocompromised child undergoing zanamivir treatment where an influenza B virus with a R152K NA mutation was detected that caused a 40–150-fold reduction in zanamivir sensitivity [14,15]. More recently a small number of A(H1N1)pdm09 viruses with an I223R NA mutation have been detected in immunocompromised patients exposed to oseltamivir and/or zanamivir [16,17] and in a patient without previous exposure to NAIs [18], but the change in zanamivir sensitivity as a result of this mutation is relatively minor (10-fold), compared with the larger 45-fold shift in oseltamivir sensitivity [16].

Previously, our group and others reported the detection of former seasonal A(H1N1) virus isolates with a Q136K mutation that conferred a 250-fold reduction in zanamivir sensitivity [19,20]. The Q136K isolates were particularly unusual because the mutation could not be detected in the clinical specimens from which they were derived. This suggested that either the variant virus was being generated in cell culture or it was present in very low levels in the clinical specimen and then selectively amplified during cell culture. The former seasonal A(H1N1) virus stopped circulating soon after the emergence of the A(H1N1)pdm09 virus in 2009, and for the first two years after the A(H1N1)pdm09 viruses started circulating, no Q136K variants were reported. However, here we report the detection of both Q136K and Q136R substitutions in A(H1N1)pdm09 and A(H3N2) viruses between 2011 and 2014, investigate their selection in different cell lines and determine the effect that these and other amino substitutions of the Q136 residue have on NAI susceptibility and NA enzymatic function.

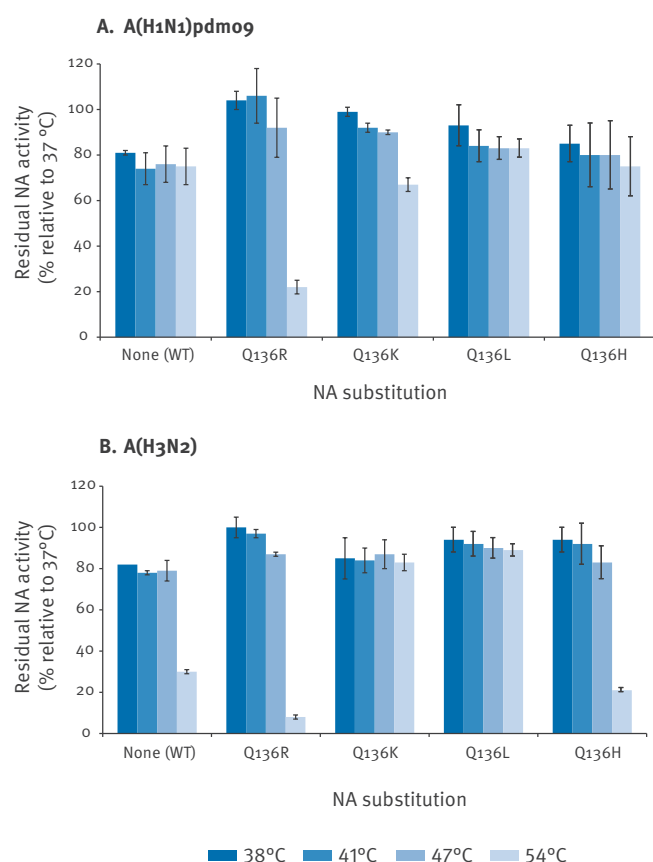
Methods

Virus strains, Madin-Darby canine kidney epithelial cells and virus culture

The influenza viruses used in this study were received at the World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza (WHOCC), Melbourne, Australia through the WHO Global Influenza Surveillance and Response System (GISRS) from countries in the Asia Pacific region. The Q136K or Q136R isolates had initially been isolated and then passaged in MDCK cells in external laboratories before being received and repassaged one to two further times at the WHOCC. WHOCC MDCK cells were originally received from ATCC (CCL-34) and used at passage level 63 to 83 and grown in Dulbecco's modified

FIGURE 2

Thermostability of influenza viruses expressing wildtype, Q136R, Q136K, Q136L or Q136H substituted neuraminidases



NA: neuraminidase; SD: standard deviation; WT: wildtype.

Residual activity is expressed as a percentage of activity relative to that at 37°C. Mean values are presented and error bars indicate \pm SD from three separate experiments.

Eagle's medium (DMEM)/Ham's F12/Coon's medium with L-glutamine (SAFC Biosciences) supplemented with 2 nM L-glutamine (SAFC Biosciences), 1X Eagle's minimum essential medium (MEM) non-essential amino acids (Sigma), 0.06% (v/v) sodium bicarbonate (Sigma), 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (Sigma), 2% penicillin-streptomycin solution (Sigma) and 10% fetal bovine serum (SAFC Biosciences). Viruses were cultured in MDCK cells in DMEM/Ham's F12/Coon's maintenance medium with L-glutamine (SAFC Biosciences) supplemented with 2 nM L-glutamine (SAFC Biosciences), 1X MEM non-essential amino acids (Sigma), 0.06% (v/v) sodium bicarbonate (Sigma), 0.02 M HEPES buffer solution (Sigma), 2% penicillin-streptomycin solution (Sigma), 2 μ g/mL fungizone (amphotericin B) (SAFC Biosciences) and 4 μ g/mL trypsin (Sigma).

Neuraminidase activity and neuraminidase inhibition assays

To determine NA activity, viruses were standardised to an equivalent haemagglutinin (HA) titre using turkey red blood cells, serially diluted (twofold) in assay buffer (32.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.5, 4 mM CaCl_2 with 0.1% nonyl phenoxy-polyethoxyethanol (NP-40)), and then mixed with an equal volume (50 μ L) of the fluorescence substrate 2-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) (0.3 mM) (Sigma) before incubation at 37°C for 60 min. The reaction was terminated by the addition of 100 μ L of stop solution (0.14 M NaOH in 83% ethanol). Quantification of the fluorescent product 4-methylumbelliferone was determined using a Fluoroscan Ascent FL (Thermo) with an excitation wavelength of 360 nm and an emission wavelength of 448 nm. Viruses were tested in duplicate in three separate assays. The NA activity of the recombinant variant viruses was calculated as a relative percentage of the NA activity of the recombinant wildtype (WT) virus at the same virus dilution.

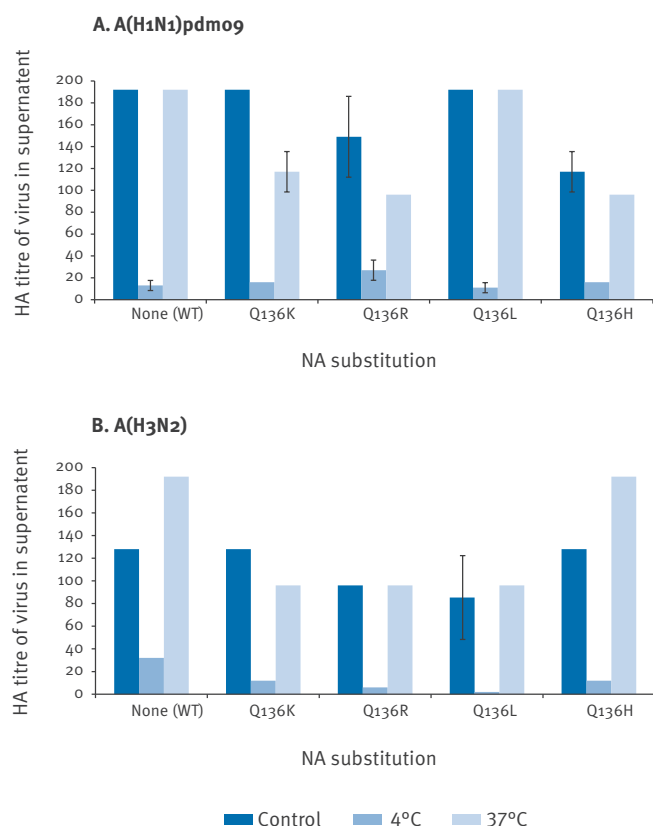
Viruses were tested for susceptibility to the NAIs laninamivir, oseltamivir carboxylate, peramivir, and zanamivir, which were kindly provided by Daiichi-Sankyo, Japan, Hoffman-La Roche Ltd, Switzerland, BioCryst Pharmaceuticals Inc., United States (US), and GlaxoSmithKline, United Kingdom (UK), respectively. To determine the drug concentration required to inhibit 50% of the NA activity (IC_{50}), 50 μ L of virus, diluted according to the NA activity assay, was mixed with varying concentrations of inhibitor in microtitre plates (FluoroNunc plates, Nunc). Final reaction mixture concentrations of the NAIs ranged from 0.01 nM to 10,000 nM. The virus/NAI mix was incubated at room temperature for 45 min before the addition of 50 μ L of MUNANA substrate (0.3 mM) and then incubated at 37°C for 60 min. The reaction was terminated by addition of 100 μ L of the stop solution. The data were plotted as the percentage of fluorescence activity inhibited against the log NAI concentration. IC_{50} values were calculated using the logistic curve fit programme 'Robosage' kindly provided by GlaxoSmithKline, UK.

Reverse transcription-polymerase chain reaction, sequencing and pyrosequencing

Reverse transcription-polymerase chain reaction (RT-PCR) was conducted using gene specific primers (sequences available on request) using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase Kit (Life Technologies) according to the manufacturer's instructions. Sequencing was conducted using the ABI Prism Dye Terminator III Cycle Sequencing Kit (Life Technologies) and analysed on the ABI 3500XL automated DNA sequencer (Life Technologies) and data aligned using the DNASTar Lasergene 8 software package. Pyrosequencing to determine the relative proportion of a Q136 variant in a viral population used the PyroMark ID System (QIAGEN). Briefly, 40 μ L biotinylated PCR product was

FIGURE 3

Haemagglutinin/neuraminidase balance of influenza viruses expressing wildtype, Q136R, Q136K, Q136L or Q136H neuraminidases following incubation at 4°C, 37°C and in the absence of epithelial cells (control)



HA: haemagglutinin; NA: neuraminidase; SD: standard deviation; WT: wildtype.

Mean values are presented and error bars indicate \pm SD from three separate experiments. Where error bars are not visible, the same HA titre was obtained across the three experiments.

immobilised on streptavidin-coated sepharose beads (QIAGEN), transferred using the PyroMark Vacuum Prep Workstation (QIAGEN) into 40 μ L of PyroMark Annealing Buffer containing pyrosequencing primers and then subjected to pyrosequencing reactions using PyroGold Reagents (QIAGEN) following the manufacturer's protocol. The relative proportion of a Q136 variant in a sample was determined using the Allele Quantitation application of the PyroMark ID software.

Serial passage experiments in Madin-Darby canine kidney epithelial cells and eggs

Three isolates containing WT (i.e. Q136) and Q136K or alternatively WT (Q136) and Q136R were passaged four times in either MDCK cells from Queensland Health Scientific Services (henceforth referred to as Brisbane MDCK cells), MDCK cells from the WHO CC, MDCK-SIAT1 cells or 11 day-old embryonated hens' eggs. The Brisbane MDCK cells were originally received from ATCC (CCL-34) and used at passage level 77 to 90 and, before use in the serial viral passage experiments, were grown

TABLE 1

Frequency of Q136K or Q136R variants detected in A(H1N1)pdm09 or A(H3N2) surveillance samples in the Asia-Pacific region, 2009–2014

Year	A(H1N1)pdm09			A(H3N2)		
	Isolates N	Q136K n (%)	Q136R n (%)	Isolates N	Q136K n (%)	Q136R n (%)
2009	1,068	0 (0.0)	0 (0.0)	117	0 (0.0)	0 (0.0)
2010	1,796	0 (0.0)	0 (0.0)	280	0 (0.0)	0 (0.0)
2011	1,172	9 (0.8)	12 (1.0)	798	1 (0.1)	0 (0.0)
2012	237	1 (0.4)	1 (0.4)	1,620	0 (0.0)	0 (0.0)
2013	960	0 (0.0)	0 (0.0)	668	1 (0.1)	0 (0.0)
2014	1,236	0 (0.0)	0 (0.0)	526	0 (0.0)	0 (0.0)
Total	6,469	10 (0.2%)	13 (0.2)	4,009	2 (<0.1)	0 (0.0)

The Global Initiative on Sharing Avian Influenza Data (GISAID) isolate identity numbers for sequences of selected Q136K and Q136R viruses detected in this study are: EPI_ISL_103643, 172961, 172962, 106853, 106854, 106855, 106856, 106849, 122566, 106851 and 128664 (www.gisaid.org).

in Opti-MEM with L-glutamine (Thermo Fisher), supplemented with 100x Antibiotic / Antimycotic (Thermo Fisher) and 10% fetal bovine serum (Thermo Fisher). The MDCK-SIAT1 cells were kindly provided by Professor Hans-Dieter Klenk, University of Marburg, Germany, and before the serial viral passage study, these cells were passaged in the same medium as described previously for the WHO CC MDCK cells except that it was further supplemented with 1 mg/mL G418 sulphate (Geneticin, GIBCO, US). Viruses were diluted 1:100 after each passage and used for inoculation of the subsequent passage. Viruses were cultured in eggs for two days at 35°C, and in cells for up to five days at 35°C in the medium used for the WHO CC MDCK cells described previously.

Plasmid construction, site directed mutagenesis and reverse genetics

The NA segments from the A(H3N2) virus A/Wyoming/3/2003 and the A(H1N1)pdm09 virus A/Auckland/0.5009 were amplified by RT-PCR, digested with the restriction enzyme *BsaI* and ligated into the *BsmBI* digested pHW2000 vector (kindly provided by St Jude Children's Research Hospital, Memphis) using the Quick Ligation Kit T4 ligase (New England Biolabs) according to the manufacturer's protocol. Plasmids were transformed into One Shot TOP10 Competent *E. coli* Cells (Life Technologies) and positive clones were inoculated into 5 mL Fast-Media Ampicillin TB (InvivoGen, US) and incubated at 37°C with shaking at 225 rpm for 16 hours. Plasmid DNA was then isolated using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's protocol and eluted into 50 μ L autoclaved water.

Single nucleotide (nt) mutations were introduced into the NA plasmids to alter the Q136 codon to residues H, K, L, or R using the QuikChange Multi Site Directed

TABLE 2

Neuraminidase (NA) activity and NA inhibitor susceptibility of reverse genetics derived Q136 variants

Subtype	NA mutation	NA activity (% of WT)	IC ₅₀ (nM) ± SD (Fold difference of IC ₅₀ compared with WT)			
			Zanamivir	Oseltamivir	Peramivir	Laninamivir
A(H1N1)pdm09	None (WT)	–	0.2 ± 0.0 (–)	0.3 ± 0.1 (–)	0.1 ± 0.0 (–)	0.2 ± 0.0 (–)
	Q136L	54.2 ± 3.6	6.3 ± 2.0 (32)	3.6 ± 0.6 (12)	0.5 ± 0.3 (5)	0.8 ± 0.1 (4)
	Q136H	98.6 ± 0.8	0.2 ± 0.0 (1)	0.2 ± 0.1 (1)	0.1 ± 0.0 (1)	0.2 ± 0.0 (1)
	Q136R	34.2 ± 0.8	161.9 ± 35.0 (810)	0.2 ± 0.0 (1)	68.2 ± 16.8 (682)	131.8 ± 30.7 (659)
	Q136K	32.9 ± 1.6	117.7 ± 11.7 (589)	0.3 ± 0.0 (1)	34.3 ± 3.7 (343)	25.5 ± 6.0 (126)
A(H3N2)	None (WT)	–	1.3 ± 0.3 (–)	0.5 ± 0.1 (–)	0.3 ± 0.0 (–)	1.2 ± 0.1 (–)
	Q136L	37.3 ± 1.3	12.8 ± 2.5 (10)	6.5 ± 1.2 (13)	0.9 ± 0.3 (3)	1.3 ± 0.4 (1)
	Q136H	109.3 ± 1.0	0.8 ± 0.2 (1)	0.2 ± 0.1 (1)	0.2 ± 0.1 (1)	1.0 ± 0.2 (1)
	Q136R	82.5 ± 3.6	3.0 ± 0.7 (2)	1.4 ± 0.2 (3)	0.8 ± 0.0 (3)	1.1 ± 0.0 (1)
	Q136K	43.2 ± 3.1	12.3 ± 2.4 (10)	6.6 ± 1.4 (13)	0.9 ± 0.3 (3)	1.6 ± 0.2 (1)

IC₅₀: drug concentration required to inhibit 50% of the NA activity; SD: standard deviation; WT: wildtype.

Figures in bold indicate a 10-fold or greater increase in IC₅₀ compared with the respective WT virus. Mean ± SD are derived from three separate experiments.

Mutagenesis Kit (Agilent Technologies) and using mutagenesis primers designed according to manufacturer's guidelines and synthesised by GeneWorks (Adelaide, Australia). The NA segment was sequenced directly from the plasmid as described previously to confirm that the desired mutation had been introduced and that no additional mutations were present.

Recombinant viruses composed of the NA gene from one of the viruses described above, and the remaining seven segments from A/Puerto Rico/8/34 (A/PR/8/34) plasmids kindly provided by Dr Robert Webster, St. Jude Children's Research Hospital, Memphis) were generated by reverse genetics. All eight plasmids were transfected into a co-culture of 293T and MDCK cells as previously described [21]. Rescued viruses were subsequently cultured in MDCK cells in maintenance media described above.

Thermostability and neuraminidase cleavage from A549 cells

The thermostability of the NA was determined by subjecting viruses to a range of temperatures and then determining the amount of residual NA activity as a percentage of the NA activity of the virus at 37°C. The lower temperatures (38 and 41°C) were physiologically relevant, while the higher temperatures (47 and 54°C) provided information about protein stability. Virus was incubated at 37, 38, 41, 47 and 54°C for 15 min using the gradient function on a thermocycler (Bio-Rad Laboratories) and the NA activity of each virus calculated based on the mean of triplicate assays.

NA cleavage efficiency was characterised by the binding and elution of WT and mutant virus to A549 adenocarcinomic human alveolar epithelial cells (ATCC) following a previously published protocol [22]. A549 cells were grown in the growth medium described previously. Virus with a known HA titre was allowed to bind

to human epithelial cells at 4°C for 30 min and the HA titre of the supernatant determined. To assess the NA efficiency, the virus/cells (previously incubated at 4°C) were then incubated at 37°C to provide an opportunity for NA to cleave the HA and release the virus from cells. The comparative HA titre of the cell supernatant, without cells (control) compared with 'after binding' (4°C) and 'after elution' (37°C) incubations, was used to indicate the efficiency of NA cleavage.

Results

Q136K and Q136R variants detected during surveillance testing

A total of 6,469 A(H1N1)pdm09 and 4,009 A(H3N2) virus isolates from the Asia-Pacific region were tested at the WHOCC for their susceptibility to the NAIs from 2009 to 2014. Prior to 2011, all A(H1N1)pdm09 and A(H3N2) virus isolates demonstrated normal inhibition to zanamivir. However, in 2011, 21 of 1,172 (1.1%) A(H1N1)pdm09 isolates tested showed either reduced- or highly reduced-inhibition to zanamivir due to a substitution of the Q136 residue in the NA to either K (n=9) or R (n=12) (Table 1). In 2012, two of 237 (0.8%) A(H1N1)pdm09 virus isolates were also considerably less inhibited by zanamivir and similarly contained either a Q136K or Q136R NA substitution. After 2012, no more A(H1N1)pdm09 virus isolates with Q136K or Q136R were detected. With regard to A(H3N2), during the period from 2011 to 2014, zanamivir inhibition was found to be reduced in two virus isolates (one in 2011 and one in 2013) due to a Q136K NA substitution (Table 1).

Only one of the Q136K A(H1N1)pdm09 isolates and three Q136R isolates appeared to be pure viral populations following pyrosequencing analysis, while all of the others were detected as mixed viral populations, with proportions of variant virus ranging from 47 to

80% for the Q136K isolates, and three to 94% in the Q136R isolates. As a result of the mixed viral populations, the zanamivir IC_{50} values of the different isolates differed markedly from each other, with the Q136K isolates ranging from 1.9 to 250 nM, and the Q136R isolates ranging from 2.1 to 92 nM. Neither the Q136K nor Q136R isolates had an increased oseltamivir IC_{50} , although they did have an increased peramivir and laninamivir IC_{50} (data not shown).

Following detection of the Q136K or Q136R variants in MDCK isolates, it was necessary to determine whether viruses with these substitutions were present in the respective original clinical specimens. Of the 25 virus isolates where a Q136K or Q136R variant was detected, 16 clinical specimens were available for analysis. Sanger sequencing or pyrosequencing failed to detect the presence of the variants in any of the clinical specimens tested, even as a minor proportion of the viral population. Clonal analysis of one of the virus samples was conducted but again failed to detect the presence of the variant in 92 clones analysed. These data demonstrated that the Q136K or Q136R variants viruses were either below the level of detection of the assays or were not present in the specimen but spontaneously generated during culture. Given these findings, it was clear that these viruses were being positively selected during MDCK cell culture. All of the Q136K or Q136R isolates had been passaged between two and four times in MDCK cells before analysis. Two Q136R A(H1N1)pdm09 isolates which contained >80% Q136R had only been passaged twice in MDCK cells. Seventeen (68%) of the 25 Q136K or Q136R isolates detected at the WHO CC, had initially been passaged and submitted to the WHO CC by a single laboratory in Brisbane, Australia. This was a disproportionately high number of viruses with a mutation at Q136 given that during the 2009 to 2014 period this laboratory submitted 24.3% (2,552/10,478) of all of the viruses tested at the WHOCC.

Serial passage of Q136K and Q136R A(H1N1)pdm09 viruses in cell and egg culture

To better understand whether the MDCK cells used by the Brisbane laboratory were more selective for the Q136K or Q136R A(H1N1)pdm09 variants than other cell lines, two isolates containing NA 136 Q/K mixtures (named A/Brisbane/345/2011 and A/Brisbane/70/2011) and one isolate with a NA 136 Q/R mixture (A/Perth/130/2011) were passaged four times in either Brisbane MDCK cells, WHOCC MDCK cells, MDCK-SIAT1 cells or hens eggs. Passage in the Brisbane MDCK cells either maintained or increased the proportion of Q136K or Q136R viruses in the viral population (Figure 1). This positive selection for the Q136K variant was also observed following passage of the A/Brisbane/345/2011 isolate in the WHOCC MDCK cells, but was not seen with the other two isolates, where passage in WHOCC MDCK cells resulted in the gradual loss of the Q136K or Q136R variant. The MDCK-SIAT1 cells consistently selected against the Q136K or Q136R variants, with the proportion of each variant gradually

decreasing after serial passage (Figure 1). The largest change in mixture proportion was seen following egg passage, which showed that growth of the variants were not well supported in embryonated eggs and were rapidly selected against, such that after a single passage in eggs the Q136K and Q136R viruses were undetectable in two of the isolates, and accounted for <10% of the viral population in the third isolate (Figure 1).

Neuraminidase activity and neuraminidase inhibitor susceptibility of reverse genetics derived Q136K, R, L and H variants

Examination (in 2014) of human and avian N1 and N2 sequences from the public sequence databases Global Initiative on Sharing Avian Influenza Data (GISAID) and GenBank revealed not only the Q136R and Q136K substitutions, but also Q136L and Q136H, present in a small number (less than 1%) of sequences from A(H3N2), A(H1N1) and A(H5N1) virus isolates. Site directed mutagenesis and reverse genetics were used to better investigate the phenotypic effect of the Q136K, Q136R, Q136L and Q136H substitutions in the NAs from A(H1N1)pdm09 and A(H3N2) viruses. 7:1 reassortants containing either the N1 or N2 WT NA (no mutations) or variant NA (Q136K, R, L or H) on a PR/8 backbone were successfully generated by reverse genetics.

For N1 reassortants, the Q136H mutant retained full NA activity, while the Q136R, Q136K and Q136L mutants had between 33 and 54% of the WT NA activity (Table 2). For the same mutations in the N2 NA, the Q136H substitution also had no effect on NA activity, whereas the Q136R substitution caused a minor reduction in NA activity (82% activity of WT), and the Q136K and Q136L substitutions caused large reductions in NA activity (40% activity of WT) (Table 2).

Analysis of the N1 reassortants for NAI susceptibility showed that the Q136H substitution had no effect, whereas the Q136L mutant demonstrated a moderate 32-fold increase in zanamivir IC_{50} and a minor 4- to 12-fold increase in oseltamivir, peramivir and laninamivir IC_{50} compared with the respective WT. In comparison, the Q136R substitution caused a 659- to 810-fold increase in zanamivir, peramivir and laninamivir IC_{50} compared with WT, while the Q136K substitution caused a 126- to 589-fold increase against the same NAIs (Table 2). Both the Q136K and Q136R substitutions had no effect on oseltamivir susceptibility.

The large effect of the Q136R and Q136K substitutions observed in the N1 NA was not observed in the N2 NA. Q136R caused only a minor (two- to threefold) change in oseltamivir, peramivir and zanamivir IC_{50} . A Q136L or a Q136K substitution caused a moderate 10- to 13-fold increase in zanamivir and oseltamivir IC_{50} and a threefold increase in peramivir IC_{50} but no change in laninamivir sensitivity compared with the WT. Q136H in N2 had no effect on NAI sensitivity, similar to that observed in the N1 NAs (Table 2).

Viral function of reverse genetics derived Q136K, R, L and H variants

The thermostability and HA/NA balance of the reassortant variant viruses were determined. The Q136L and Q136H N1 variants retained high NA activity across the 38°C to 54°C temperature range, equivalent to that of the WT virus. However the N1 reassortants with Q136K and Q136R mutations showed a substantial loss of activity at 54°C (67% and 22% remaining activity compared to 37°C respectively) (Figure 2A). The N2 reassortant WT had some loss of activity at 54°C (30% remaining activity), as did the NAs with Q136R and Q136H mutations (8–21% remaining activity), while the NAs with Q136K and Q136L mutations maintained high activity (>80%) across the entire temperature range (Figure 2B).

All N1 and N2 reassortant viruses contained the PR/8 HA and all showed good cell binding at 4°C, as indicated by low viral titres in the supernatant. After incubation at 37°C, the N1 WT reassortant and the Q136L variant showed full restoration of HA titre, demonstrating an active NA enzyme, whereas the Q136K and Q136R variants had only partial restoration of HA titre, suggesting that the NA activity was insufficient to cleave all bound virus from the cells (Figure 3A). All of the N2 reassortants, with the exception of Q136K, showed full restoration of HA titre following incubation at 37°C (Figure 3B).

Discussion

In this study we describe the detection of A(H1N1)pdm09 influenza virus isolates, and to a lesser extent A(H3N2) viruses, with amino acid substitutions at the Q136 NA residue that reduce zanamivir, peramivir and laninamivir susceptibility. Surveillance data show that the Q136K and Q136R substitutions occurred sporadically in A(H1N1)pdm09 cultured isolates, with periods such as 2011 where a relatively high detection rate was observed, compared with other years where they were absent. Importantly, in all cases, the residue substitution that was present in the isolate, was not detected in the virus from the clinical specimen demonstrating that the mutation was either arising, or being selected for, during MDCK cell culture passage. Because the 'gold-standard' for laboratory assessment of NAI susceptibility is the phenotypic NA inhibition assay, which requires a cell culture isolate for testing, there is concern that viruses such as these can be reported as being 'resistant' when in fact the virus that came from the patient was sensitive. In addition, the process of cell culture may also select against a resistant virus, meaning that a variant virus is not detected when it was present in the clinical specimen. Misdiagnosis can have an impact on the therapies being used in patient management and may unnecessarily result in therapy being stopped, modified or inappropriately continued. In addition to the Q136K/R variants described here, there are many other NA mutations that alter NAI susceptibility and also appear to be selected during MDCK cell culture [19,23]. Interestingly these seem to be increasingly reported for influenza B viruses [23–25].

Therefore sequence analysis of the influenza viruses in the original specimen remains important when laboratories detect mutations in cultured isolates.

While conventional MDCK cells selected for the Q136K and Q136R A(H1N1)pdm09 variants, their growth was not supported in eggs, with a single passage resulting in the near complete loss of the variant virus. MDCK-SIAT1 cells also did not appear to give selective growth advantage to the Q136 variants. MDCK-SIAT1 cells have enhanced binding due to an increased concentration of α 2,6-linked sialic acids on the MDCK cell surface [26], which may mean that viruses with reduced NA activity, such as the Q136K/R variants, have reduced replication in this cell line, possibly explaining the difference with the conventional MDCK cell lines. If clinical samples are available in the future that have been shown to result in MDCK isolates with Q136K/R mutations, it would be useful to test whether primary isolation into MDCK-SIAT1 or human bronchial epithelial cells prevents this initial selection of the variant virus.

Although the Q136K and Q136R variants detected here were all cell culture derived variants, other studies have reported mutations at Q136 that were detected in viruses from clinical specimens. The Q136K mutation was detected in an A(H3N2) virus, together with an E119V NA mutation, in a patient who had previously undergone a bone marrow transplant, following a treatment course of both inhaled and intravenous zanamivir and oseltamivir [27]. Mutations at Q136 have also been detected in ferrets infected with influenza A(H5N1). The Q136L variant was detected in the nasal wash of a zanamivir treated ferret infected with an A(H5N1) virus [28], while a Q136H mutation was detected in an A(H5N1) virus from a ferret not being treated with an antiviral [29]. These reports demonstrate that viruses with these mutations have the potential to infect or replicate *in vivo* both in the presence or absence of zanamivir pressure.

The zanamivir concentration in sputum 12 hours post-inhalation has been reported to be between 159 and 4,315 nM [30]. Therefore, while the correlation between the drug concentration in sputum and the drug concentration at the site of viral replication is not clear, it is anticipated that only the Q136K and Q136R mutations in the A(H1N1)pdm09 virus may potentially impact the clinical effectiveness of zanamivir. The Q136L mutation in both N1 and N2 NAs and Q136K in N2 NA caused mild increases in zanamivir and oseltamivir IC_{50} , which are expected to be below the concentrations present at the sites of replication in treated individuals [30,31].

An evaluation of the ability of the Q136K and Q136R A(H1N1)pdm09 variants to replicate and transmit in animal models will provide useful insights into the potential risk that these viruses may pose to public health. Where possible these future studies would benefit from using Q136K and Q136R variants that were naturally occurring, rather than cell culture derived or

generated by reverse genetics. One limitation of this study is that the N1 and N2 NAs with Q136 substitutions were assessed in 1:7 reassortant viruses generated by reverse genetics on a PR/8 backbone, therefore there is potential that the HA/NA balance between the variant NA molecules and the HA from PR/8 may be different from that seen in the 'natural' isolate. The in vitro assays showed that as a result of the Q136K mutation the N1 reassortant had a moderate loss in NA activity and thermostability. A reduction in NA enzyme activity and surface expression due to the Q136K NA mutation has been previously reported [32,33]. Pizzorno et al. [33] also demonstrated that an A(H1N1)pdm09 Q136K variant had compromised replication compared with a WT virus in mice, while in a ferret model the variant was able to transmit between contact ferrets, but at slower rate than for the WT virus. However, a study in guinea pigs found that the Q136K variant did not transmit between animals [32]. Taken together, these studies indicate that the replication and transmissibility of the Q136K variant in the A(H1N1)pdm09 virus appears to be compromised and therefore is unlikely to circulate through the human population. However, compensatory mutations in the NA or other genes that may occur in the A(H1N1)pdm09 virus in the future may buffer the compromising effect of the Q136K mutation and improve overall replication and transmissibility of the variant, in a manner similar to that seen for the H275Y mutation [7,34]. To date there has been no evaluation of the in vivo fitness of the Q136R A(H1N1)pdm09 variant.

In this study we have highlighted the challenges that cell culture derived mutations, such as Q136K and Q136R, can pose to the analysis and interpretation of viruses for NAi susceptibility. This further reaffirms the need to sequence viruses from the clinical specimens of any isolate that shows reduced susceptibility in a phenotypic NA inhibition assay to avoid misdiagnosis and any unnecessary change in patient management with respect to the use of antivirals. Our findings highlight the effect of mutations at the Q136 residue of N1 viruses on laninamivir, peramivir or zanamivir susceptibility, and therefore close monitoring of viruses for these mutations in patients being treated with these antivirals is important.

Acknowledgements

The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

Conflict of interest

None declared.

Authors' contributions

SKL and ACH generated antiviral susceptibility data for circulating viruses submitted to the WHO CC; KL, JB, CB, BH and

ACH conducted and analysed the mutagenesis, reverse genetics and functional assays of the variant viruses; KL and ACH wrote the draft of the manuscript and all authors further edited the manuscript and approved the final version of the paper. JM, IGB and ACH provided supervisory oversight.

References

- Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors. *Lancet*. 2000;355(9206):827-35. DOI: 10.1016/S0140-6736(99)11433-8 PMID: 10711940
- Hurt AC, Ho HT, Barr I. Resistance to anti-influenza drugs: adamantanes and neuraminidase inhibitors. *Expert Rev Anti Infect Ther*. 2006;4(5):795-805. DOI: 10.1586/14787210.4.5.795 PMID: 17140356
- Hurt AC, Holien JK, Parker MW, Barr IG. Oseltamivir resistance and the H274Y neuraminidase mutation in seasonal, pandemic and highly pathogenic influenza viruses. *Drugs*. 2009;69(18):2523-31. DOI: 10.2165/11531450-000000000-00000 PMID: 19943705
- Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature*. 1983;303(5912):41-4. DOI: 10.1038/303041a0 PMID: 6188957
- Yen H-L, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, et al. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. *Antimicrob Agents Chemother*. 2005;49(10):4075-84. DOI: 10.1128/AAC.49.10.4075-4084.2005 PMID: 16189083
- Hurt AC, Nor'e SS, McCaw JM, Fryer HR, Mosse J, McLean AR, et al. Assessing the viral fitness of oseltamivir-resistant influenza viruses in ferrets, using a competitive-mixtures model. *J Virol*. 2010;84(18):9427-38. DOI: 10.1128/JVI.00373-10 PMID: 20631138
- Bloom JD, Gong LI, Baltimore D. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science*. 2010;328(5983):1272-5. DOI: 10.1126/science.1187816 PMID: 20522774
- Neuraminidase Inhibitor Flu Treatment Investigator Group, Nicholson KG, Aoki FY, Osterhaus AD, Trottier S, Carewicz O, Mercier CH, et al. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. *Lancet*. 2000;355(9218):1845-50. DOI: 10.1016/S0140-6736(00)02288-1 PMID: 10866439
- US Oral Neuraminidase Study Group, Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, et al. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. *JAMA*. 2000;283(8):1016-24. DOI: 10.1001/jama.283.8.1016 PMID: 10697061
- Whitley RJ, Hayden FG, Reisinger KS, Young N, Dutkowski R, Ipe D, et al. Oral oseltamivir treatment of influenza in children. *Pediatr Infect Dis J*. 2001;20(2):127-33. DOI: 10.1097/00006454-200102000-00002 PMID: 11224828
- Tamura D, Sugaya N, Ozawa M, Takano R, Ichikawa M, Yamazaki M, et al. Frequency of drug-resistant viruses and virus shedding in pediatric influenza patients treated with neuraminidase inhibitors. *Clin Infect Dis*. 2011;52(4):432-7. DOI: 10.1093/cid/ciq183 PMID: 21248368
- Hurt AC, Ernest J, Deng YM, Iannello P, Besselaar TG, Birch C, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res*. 2009;83(1):90-3. DOI: 10.1016/j.antiviral.2009.03.003 PMID: 19501261
- European Influenza Surveillance Scheme, Meijer A, Lackenby A, Hungnes O, Lina B, van-der-Werf S, Schweiger B, et al. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. *Emerg Infect Dis*. 2009;15(4):552-60. DOI: 10.3201/eid1504.181280 PMID: 19331731
- Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J Infect Dis*. 1998;178(5):1257-62. DOI: 10.1086/314440 PMID: 9780244
- Wetherall NT, Trivedi T, Zeller J, Hodges-Savola C, McKimm-Breschkin JL, Zambon M, et al. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. *J Clin Microbiol*. 2003;41(2):742-50. DOI: 10.1128/JCM.41.2.742-750.2003 PMID: 12574276
- van der Vries E, Stelma FF, Boucher CA. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. *N Engl J*

- Med. 2010;363(14):1381-2. DOI: 10.1056/NEJMc1003749 PMID: 20879894
17. Nguyen HT, Fry AM, Loveless PA, Klimov AI, Gubareva LV. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir. *Clin Infect Dis.* 2010;51(8):983-4. DOI: 10.1086/656439 PMID: 20858074
 18. Eshaghi A, Patel SN, Sarabia A, Higgins RR, Savchenko A, Stojios PJ, et al. Multidrug-resistant pandemic (H1N1) 2009 infection in immunocompetent child. *Emerg Infect Dis.* 2011;17(8):1472-4. PMID: 21801626
 19. Okomo-Adhiambo M, Nguyen HT, Sleeman K, Sheu TG, Deyde VM, Garten RJ, et al. Host cell selection of influenza neuraminidase variants: implications for drug resistance monitoring in A(H1N1) viruses. *Antiviral Res.* 2010;85(2):381-8. DOI: 10.1016/j.antiviral.2009.11.005 PMID: 19917319
 20. Hurt AC, Holien JK, Parker M, Kelso A, Barr IG. Zanamivir-resistant influenza viruses with a novel neuraminidase mutation. *J Virol.* 2009;83(20):10366-73. DOI: 10.1128/JVI.01200-09 PMID: 19641000
 21. Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine.* 2002;20(25-26):3165-70. DOI: 10.1016/S0264-410X(02)00268-2 PMID: 12163268
 22. Reading PC, Pickett DL, Tate MD, Whitney PG, Job ER, Brooks AG. Loss of a single N-linked glycan from the hemagglutinin of influenza virus is associated with resistance to collectins and increased virulence in mice. *Respir Res.* 2009;10(117):117. DOI: 10.1186/1465-9921-10-117 PMID: 19930664
 23. Leang S-K, Deng YM, Shaw R, Caldwell N, Iannello P, Komadina N, et al. Influenza antiviral resistance in the Asia-Pacific region during 2011. *Antiviral Res.* 2013;97(2):206-10. DOI: 10.1016/j.antiviral.2012.12.016 PMID: 23274624
 24. Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, et al. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrob Agents Chemother.* 2008;52(9):3284-92. DOI: 10.1128/AAC.00555-08 PMID: 18625765
 25. Escuret V, Collins PJ, Casalegno JS, Vachieri SG, Cattle N, Ferraris O, et al. A novel I221L substitution in neuraminidase confers high-level resistance to oseltamivir in influenza B viruses. *J Infect Dis.* 2014;210(8):1260-9. DOI: 10.1093/infdis/jiu244 PMID: 24795482
 26. Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD. Overexpression of the α -2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J Virol.* 2003;77(15):8418-25. DOI: 10.1128/JVI.77.15.8418-8425.2003 PMID: 12857911
 27. Eshaghi A, Shalhoub S, Rosenfeld P, Li A, Higgins RR, Stojios PJ, et al. Multiple influenza A (H3N2) mutations conferring resistance to neuraminidase inhibitors in a bone marrow transplant recipient. *Antimicrob Agents Chemother.* 2014;58(12):7188-97. DOI: 10.1128/AAC.03667-14 PMID: 25246391
 28. Hurt AC, Lowther S, Middleton D, Barr IG. Assessing the development of oseltamivir and zanamivir resistance in A(H5N1) influenza viruses using a ferret model. *Antiviral Res.* 2010;87(3):361-6. DOI: 10.1016/j.antiviral.2010.06.009 PMID: 20603155
 29. Govorkova EA, Ilyushina NA, Marathe BM, McClaren JL, Webster RG. Competitive fitness of oseltamivir-sensitive and -resistant highly pathogenic H5N1 influenza viruses in a ferret model. *J Virol.* 2010;84(16):8042-50. DOI: 10.1128/JVI.00689-10 PMID: 20519385
 30. Peng AW, Milleri S, Stein DS. Direct measurement of the anti-influenza agent zanamivir in the respiratory tract following inhalation. *Antimicrob Agents Chemother.* 2000;44(7):1974-6. DOI: 10.1128/AAC.44.7.1974-1976.2000 PMID: 10858364
 31. Calfee DP, Peng AW, Cass LM, Lobo M, Hayden FG. Safety and efficacy of intravenous zanamivir in preventing experimental human influenza A virus infection. *Antimicrob Agents Chemother.* 1999;43(7):1616-20. PMID: 10390212
 32. Kaminski MM, Ohnemus A, Staeheli P, Rubbenstroth D. Pandemic 2009 H1N1 influenza A virus carrying a Q136K mutation in the neuraminidase gene is resistant to zanamivir but exhibits reduced fitness in the guinea pig transmission model. *J Virol.* 2013;87(3):1912-5. DOI: 10.1128/JVI.02507-12 PMID: 23192869
 33. Pizzorno A, Abed Y, Rhéaume C, Bouhy X, Boivin G. Evaluation of recombinant 2009 pandemic influenza A (H1N1) viruses harboring zanamivir resistance mutations in mice and ferrets. *Antimicrob Agents Chemother.* 2013;57(4):1784-9. DOI: 10.1128/AAC.02269-12 PMID: 23357777
 34. Butler J, Hooper KA, Petrie S, Lee R, Maurer-Stroh S, Reh L, et al. Estimating the fitness advantage conferred by permissive neuraminidase mutations in recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. *PLoS Pathog.* 2014;10(4):e1004065. DOI: 10.1371/journal.ppat.1004065 PMID: 24699865