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

Vol. 16 | Weekly issue 43 | 27 October 2011

Rapid communications

Research articles

Surveillance and outbreak reports

ecdc EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

www.eurosurveillance.org

Measles genotypes D4 and G3 reintroduced by multiple foci after 15 years without measles virus circulation, Gipuzkoa, the Basque Country, Spain, March to June 2011

G Cilla (gcilla@telefonica.net)[1,2], M Montes[1,2], J Artieda[3], L Piñeiro[1], L Arriola[3], E Pérez-Trallero[1,2,4]
1. Microbiology Department, Hospital Universitario Donostia-Instituto Biodonostia, San Sebastián, Spain
2. Biomedical Research Centre Network for Respiratory Diseases (CIBERES), San Sebastián, Spain
3. Public Health Division of Gipuzkoa, Basque Government, and CIBERESP, San Sebastián, Spain
4. Department of Preventive Medicine and Public Health, Faculty of Medicine, University of the Basque Country, San Sebastián, Spain

Citation style for this article:
Cilla G, Montes M, Artieda J, Piñeiro L, Arriola L, Pérez-Trallero E. Measles genotypes D4 and G3 reintroduced by multiple foci after 15 years without measles virus circulation, Gipuzkoa, the Basque Country, Spain, March to June 2011.
Euro Surveill. 2011;16(43):pii=19997. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19997

Article published on 27 October 2011

During a three-month period in spring 2011, 23 cases of measles occurred in seven independent outbreaks in a region in Spain with around 700,000 inhabitants, where the disease had been eliminated since 1997. High vaccination coverage and rapid diagnosis allowed implementation of containment measures and this prevented spread of the disease. Except for the first outbreak which affected 10 cases, each of the other six outbreaks caused a maximum of three secondary cases.

In spring 2011, 23 measles cases were detected in Gipuzkoa, a region in Spain bordering the south of France, where no measles cases had been reported since the second half of the 1990s [1]. The cases were not grouped into a single outbreak but belonged to several outbreaks, with distinct origins. This report describes measles circulation in Gipuzkoa from March to June 2011 and the control measures adopted and implemented.

Gipuzkoa is a territory of the Basque Country in northern Spain and it has a population of around 700,000 inhabitants. Coverage of the measles-mumps-rubella (MMR) vaccine has been over 90% since 1987 for the first dose in children aged 12 months and since 2002 for the second dose in children aged four years, respectively. Since 2007, coverage for the two MMR vaccine doses has been over 95%. Measles is included in the mandatory disease notification system in Spain.

Description of the outbreaks

Between 23 March and 29 June 2011, 23 cases of measles were detected through the Microbiological and Epidemiological Surveillance System of the Basque Country and they were distributed in seven independent outbreaks (Table).

In the outbreaks described here, specific IgM detection was performed through indirect ELISA (Enzygnost Anti-Measles Virus/IgM, Siemens, Germany). Viral RNA detection was carried out in pharyngeal swabs, saliva

Table

Measles outbreaks in Gipuzkoa, the Basque Country, Spain, March–June 2011

Outbreak start (2011)	Index case[a]	Number of secondary cases	Type of outbreak	Genotype (number of genotyped cases)
March	1 adolescent	3 children + 6 adults	Institutional	D4 (9)
April	1 adult	0	Isolated case	D4 (1)
May	1 child	0	Isolated case	D4 (1)
May	2 children	2 children + 1 adult	Familial-school	D4 (4)
May	1 adult	0	Isolated case	NA
May	1 adult	1 adolescent + 1 adult	Familial	G3 (3)
June	1 adult	1 adult	Familial	D4 (2)

NA: not available.
[a] Child: aged 0–14 years; adolescent: aged 15–20 years; adult: aged ≥21 years.

and/or urine through amplification of a nucleoprotein (N) gene fragment [2]. The viral RNA samples were processed in order to obtain the genotype [3]. The genetic sequences obtained were deposited in the GenBank (access number JN695499 to JN695503).

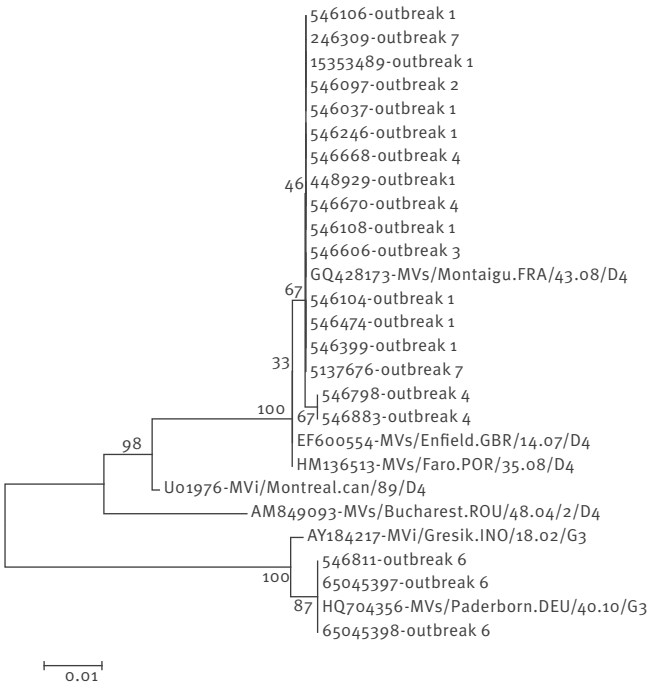
All but two cases were confirmed by viral RNA detection; one of these two cases was diagnosed by the presence of specific IgM. The second case occurred in a preschool aged boy, whose parents refused collection of biological samples from their child. However, the boy showed symptoms typical of the disease and was from a family with two confirmed measles cases. Virological tests excluded measles in 21 patients with rash in whom measles had initially been considered as diagnosis but later rash proved to be caused by other viral infections.

The first outbreak was the largest, with 10 cases, and occurred in a centre where people live collectively. Every day, the index case went to study in a neighbouring town in the Atlantic Pyrenees, a district in France where there were numerous measles cases registered during that period (incidence rate ranged from 15 per 100,000 population to 30 per 100,000 population) [4]. This outbreak affected four children, four workers in the centre and two more persons (one of them was working in a hospital and got infected after contact with a child of this outbreak in the paediatric emergency room of the hospital).

The remaining six outbreaks were smaller, mainly affecting families that were against vaccination. In all outbreaks, except one whose origin was unknown, the index case got infected outside the Basque Country. Two index cases had visited France during the 7 to 21 days before contracting the infection, two further co-index cases had visited northern France (EuroDisney) and three had visited other regions of Spain (Andalusia, Madrid and Catalonia). Seventeen of the 23 affected individuals, including the eight index cases had not been vaccinated, four had received one dose and one had received two doses, while the vaccination status of one affected individual was unknown. Four persons were hospitalised due to respiratory complications following measles (two persons in their thirties, one in their twenties and an infant under one year of age). The virus was genotyped in 20 patients: 17 belonged to genotype D4, including all those where the index case had been infected in France, and three belonged to G3 but the place of infection was unknown.

Figure

Phylogenetic tree of 20 measles viruses detected in Gipuzkoa compared with six reference strains, the Basque Country, Spain, March–June 2011



The tree was constructed through the neighbor-joining method with 1,000 bootstrap replications and shows bootstrap values in the branches.

Control measures

Persons with suspected measles were recommended to stay away from school or work and remain at home for seven days after the onset of the rash or until the diagnosis was excluded, if established before the end of the seven-day period. Children and adults from household or school, aged less than 40 years, who had had contact with a measles case and who had not previously received two vaccine doses, were offered MMR vaccination. All contacts, or in the case of children, their legal guardians, were informed about the symptoms of measles and were advised to seek medical attention if they experienced one or more of the following symptoms: fever, rash, red eyes, malaise and sore throat. In addition, the Department of Public Health alerted the network of primary care physicians with regards to the epidemiological situation of measles through electronic reports sent by email or through telephone calls.

Discussion and conclusion

After more than a decade with no measles cases detected in the region [1], seven separate outbreaks were detected within a few months in this area in the Basque Country. This striking viral activity coincided with a substantial increase in measles circulation in other European regions, in particular in neighbouring France [5]. This report shows that imported cases of measles pose a risk even to regions with high vaccination coverage in which endemic measles has been eliminated. In Gipuzkoa, the spread of the disease was probably contained by the high vaccination coverage in previous years and the rapid response of the different partners involved in the primary healthcare system and surveillance services. As in other recent outbreaks in Europe [6] most affected individuals had not been vaccinated, infection in persons who had received two vaccine doses being exceptional. Four of the seven

outbreaks were related to groups who were against vaccination.

Unvaccinated people pose a substantial risk to the general public, and if they refuse vaccination, they should restrict their contacts with the general population (school, day-care) in epidemic situations. In one of the outbreaks in Gipuzkoa, there was resistance to comply with the containment measures recommended. Notwithstanding the absence of any legislation, the community has a responsibility to protect those who cannot be vaccinated – this can be done by ensuring herd immunity. More than half of the cases occurred in young persons, without prior contact with the virus, who were born around the time when vaccination campaigns started (1975-1990).

The D4 genotype, the main genotype detected, was predominant in recent outbreaks in France, Spain and other European countries [5,7]. The G3 genotype was introduced in Europe in 2010 and one imported case has been reported in Spain [8]. Despite an exhaustive epidemiological investigation, we were unable to determine the origin of the outbreak caused by the G3 genotype.

Reaching and consolidating high vaccination coverage (with two doses) is essential to eradicate measles, a World Health Organization goal for Europe by 2015 [9]. However, the risk of measles resurgence will remain for as long as the virus continues to circulate in other regions of the world. Therefore, rapid diagnosis and notification, which allow implementation of containment measures, are crucial in the fight against this disease.

References

1. Cilla G, Basterretxea M, Artieda J, Vicente D, Pérez-Trallero E. Interruption of measles transmission in Gipuzkoa (Basque Country), Spain. Euro Surveill. 2004;9(5):pii=468. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=468
2. Mosquera Mdel M, de Ory F, Moreno M, Echevarría JE. Simultaneous detection of measles virus, rubella virus, and parvovirus B19 by using multiplex PCR. J Clin Microbiol. 2002; 40(1):111-6.
3. El Mubarak HS, van de Bildt MW, Mustafa OA, Vos HW, Mukhtar MM, Ibrahim SA, et al. Genetic characterization of wild-type measles viruses circulating in suburban Khartoum, 1997-2000. J Gen Virol. 2002;83(Pt6):1437-43.
4. Institut de Veille Sanitaire (InVS). Epidémie de rougeole en France. Actualisation des données de surveillance au 5 octobre 2011. [Measles outbreak in France. Update on surveillance data 5 October 2011]. Paris: InVS. 6 Oct 2011. Available from: http://www.invs.sante.fr/Dossiers-thematiques/Maladies-infectieuses/Maladies-a-prevention-vaccinale/Rougeole/Points-d-actualites/Archives/Epidemie-de-rougeole-en-France.-Actualisation-des-donnees-de-surveillance-au-5-octobre-2011
5. European Centre for Disease Prevention and Control (ECDC). European monthly measles monitoring (EMMO), June 2011. Stockholm: ECDC; 2011. [Accessed 26 Oct 2011]. Available from: http://ecdc.europa.eu/en/publications/Publications/2011_June_measles_montly.pdf
6. Muscat M. Who gets measles in Europe? J Infect Dis. 2011; 204 Suppl 1: S353-65.
7. Freymuth F, Vabret A. Measles, a re-emerging disease in France? Clin Microbiol Infect. 2011;17(6):793
8. Brown KE, Mulders MN, Freymuth F, Santibanez S, Mosquera MM, Cordey S, et al. Appearance of a novel measles G3 strain in multiple European countries within a two month period, 2010. Euro Surveill. 2011;16(17):pii=19852. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19852
9. World Health Organization (WHO). Regional Committee for Europe, Sixtieth session. Resolution. Renewed commitment to elimination of measles and rubella and prevention of congenital rubella syndrome by 2015 and sustained support for polio-free status in the WHO European Region. Moscow, 13–16 September 2010. [Accessed 26 Oct 2011]. Available from: http://www.who.int/immunization/sage/3_Resolution_EURO_RC60_eRes12.pdf

Human case of autochthonous West Nile virus lineage 2 infection in Italy, September 2011

P Bagnarelli (p.bagnarelli@univpm.it)[1], K Marinelli[1], D Trotta[1], A Monachetti[1], M Tavio[2], R Del Gobbo[2], M R Capobianchi[3], S Menzo[3], L Nicoletti[4], F Magurano[4], P E Varaldo[1]

1. Università Politecnica Marche, Virology Unit, Department of Biomedical Sciences and Public Health, Ancona, Italy
2. Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona, Infectious Disease Unit, Department of Gastroenterology and Transplantation, Ancona, Italy
3. National Institute of Infectious Diseases L.Spallanzani, Rome, Italy
4. Istituto Superiore di Sanitá, Rome, Italy

Citation style for this article:
Bagnarelli P, Marinelli K, Trotta D, Monachetti A, Tavio M, Del Gobbo R, Capobianchi MR, Menzo S, Nicoletti L, Magurano F, Varaldo PE. Human case of autochthonous West Nile virus lineage 2 infection in Italy, September 2011..
Euro Surveill. 2011;16(43):pii=20002. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20002

Article published on 27 October 2011

On 10 September 2011, a patient in his 50s was admitted to hospital in Ancona, Italy, after six days of high fever and no response to antibiotics. West Nile virus (WNV) infection was suspected after tests to determine the aetiology of the fever were inconclusive. On 20 September, WNV-specific IgM and IgG antibodies were detected in the patient's serum. Genomic sequencing of the viral isolate showed that the virus belonged to WNV lineage 2.

Case report

On 4 September 2011, a man in his late 50s in Ancona, Italy, first became unwell, with general malaise and fever (body temperature higher than 39.0 °C). For these reasons, his general practitioner (GP) prescribed antibiotics, but as the patient's fever persisted after six days of treatment, he was admitted to hospital (Infectious Disease Unit of the Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona). On 10 September, the ward physician reported that the origin of the fever was unknown and that there were no pulmonary or other organ-specific symptoms, except the persistence of a general malaise. A chest examination was normal, as were the laboratory tests (haemocultures, urine cultures, haemocytometer analysis, liver and renal biochemical tests, erythrosedimentation rate, C-reactive protein and blood electrolytes). He did not have neuroinvasive disease. He was discharged on 27 September and has completely recovered.

The patient was a local fisherman who lived in Ancona, close to the harbour on the Adriatic coast. In the month before his symptoms began, he had neither travelled outside the Marche region (where Ancona is the main port) in central Italy, nor had he been at sea.

Virological analysis

Tests on a blood sample taken from the patient on 13 September (tested at the Ancona Virology Laboratory) excluded cytomegalovirus, Epstein–Barr virus, human immunodeficiency virus and Toscana virus as the cause of the fever.

On 20 September, the same sample was also tested for West Nile virus (WNV), based on a recent protocol adopted by the laboratory for differential diagnosis of meningoencephalitis and for patients with fever of unknown origin in the summer months: WNV serological tests are performed in all patients negative for Toscana virus infection. The sample was found to be positive for WNV-specific IgM and IgG (with index values (signal/cut-off ratio) of ›5.4 and 2.0, respectively) using the IgM capture DxSelect ELISA and IgG DxSelect ELISA kits (Focus Diagnostics, United States) (Table).

Following the positive ELISA results for WNV, RNA extracted from serum (collected on 13 September) and urine (collected on 21 September) was reverse transcribed and amplified by PCR using an in-house assay that uses degenerate primers designed to recognise a region in the NS5 gene that is conserved (on the basis of an alignment of database sequences) in most animal and human *flaviviruses*. In the case of WNV, the resulting PCR product is 273 bp long using the forward primer (5'-TGCATITWCAACATGITGGG-3') and reverse primer (5'-GTRTCCCAICCIGCIGTGTCATC-3').

The patient's serum sample tested negative, while the urine sample was positive. Sequencing the amplified product – in both forward and reverse directions with the same primers used for amplification – showed that the WNV NS5 gene sequence had been amplified.

BLAST analysis of the 273 bp sequence showed highest homology (100%) to the Nea Santa/Greece/2010 WNV strain detected in *Culex pipiens* in 2010 [1] as well as to other strains belonging to WNV lineage 2.

The full genomic sequence of the virus was subsequently obtained from RNA extracted from the patient's

urine sample of 21 September: this confirmed that the virus was lineage 2. It also showed 99% identity to the complete genome of isolate goshawk-Hungary/04 (10, 380 of 10, 423 nucleotides identical and no gaps) and to the more recent Nea Santa-Greece-2010 (10, 374 of 10,423 nucleotides identical and no gaps) [1-2]. The sequences obtained were submitted to GenBank: the accession numbers are JN797253 (NS5 fragment) and JN858070 (complete genome). The case was notified to the regional health authorities on 23 September and to the Istituto Superiore di Sanità on 26 September.

Another serum sample collected from the patient on 26 September showed an increased WNV-specific IgG antibody titre and a high level of WNV-specific IgM. Plasma collected on the same day was negative for WNV by RT-PCR.

The serum samples collected on 13 and 26 September and the urine sample collected on 21 September were tested at the National Reference Laboratory for WNV surveillance at the Istituto Superiore di Sanità, which confirmed the diagnosis by amplification of a different region of the NS5 gene [3].

Urine and plasma samples collected on 28 September were also tested at the National Institute of Infectious Diseases L.Spallanzani, which further confirmed the diagnosis by amplification of WNV sequences in urine but not in plasma using the cobas TaqScreen West Nile Virus Test (Roche Molecular Diagnostics, United States). The plasma sample was also tested at the National Institute of Infectious Diseases L.Spallanzani for the presence of WNV-specific IgG and IgM by an indirect immunofluorescence assay (Euroimmun, Italy), which indicated high antibody titres. In addition, a microneutralisation assay against both lineage 1 and 2 strains [4] revealed cross-neutralising activity. This does not demonstrate co-circulation of the two lineages; antibodies elicited by one WNV lineage are not expected to be highly lineage-specific, because of extensive antigenic similarity between the lineages.

WNV RNA was still detectable by the in-house RT-PCR analysis at the Ancona Virology Laboratory in the urine sample collected on 29 September, 25 days after symptom onset.

The results of all serological and molecular investigations performed on the patient's samples are shown in the Table.

WNV infection in Italy

WNV infections have been reported in both humans and horses since the summer of 2008 in north-eastern Italy [5-8] but until now, as far as we are aware, only WNV lineage 1 infections have been described in the country.

To the best of our knowledge, WNV infection has never been reported before in horses or other sentinel animals in the Marche region. A possible arthropod reservoir has never been investigated, but given the absence of infection in sentinel animals and the absence of diagnosed cases of WNV meningoencephalitis, the region was considered to be at lower risk than the WNV-affected areas of north-east of the country [9].

In contrast, Toscana virus is endemic in the Marche, as well as in the rest of central Italy, and is routinely investigated in all cases of meningoencephalitis reported in the summer and in patients with fever of unknown origin. Due to the circulation of WNV in the north-east of the country, our laboratory testing algorithm was revised, introducing molecular (in summer 2010) and serological (in summer 2011) assays for the diagnosis of WNV infection. These assays are performed for

Table

Serological and molecular test results on samples from the patient with West Nile virus lineage 2 infection, Italy, September 2011

Date of sample collection (2011)	Sample type	ELISA IgG (index)[a]	ELISA IgM (index)[b]	IFA IgG (dilution)	IFA IgM (dilution)	MNTA titre lineage 1	MNTA titre lineage 2	RT-PCR[c]
13 Sep	Serum	2.00	>5.40	ND	ND	ND	ND	Negative
21 Sep	Urine	NA	NA	NA	NA	NA	NA	Positive
26 Sep	Serum (ELISA) Plasma (RT-PCR)	3.01	>5.40	ND	ND	ND	ND	Negative
28 Sep	Plasma (IFA and RT-PCR) Urine (RT-PCR)	ND	ND	>1:320	>1:320	1:20	1:40	Plasma negative Urine positive
29 Sep	Urine	NA	NA	NA	NA	NA	NA	Positive

ELISA: enzyme-linked immonosorbent assay; IFA: immunofluorescence assay; MNTA: microneutralisation assay; NA: not applicable; ND: not done; RT-PCR: reverse-transcription polymerase chain reaction; WNV: West Nile virus.

[a] An index (signal/cut-off ratio) value of >1.50 indicates the presence of IgG antibodies to WNV.

[b] An index (signal/cut-off ratio) value of >1.10 indicates the presence of IgM antibodies to WNV.

[c] Carried out using an in-house protocol that uses degenerate primers designed to recognise a region in the NS5 gene that is conserved (on the basis of an alignment of database sequences) in most animal and human *flaviviruses* or, for the samples collected on 28 September 2011, using the cobas TaqScreen West Nile Virus Test, a real-time RT-PCR (Roche Molecular Diagnostics).

hospitalised patients in the Marche region during the months when mosquitoes and other insect vectors are active, generally from early June to late October. Although tests, carried out at the Ancona Virology Laboratory, are usually performed on blood and cerebrospinal fluid (CSF), we recently detected WNV from urine from a kidney transplant patient with encephalitis in the context of an investigation into WNV transmission through organ transplants (unpublished data): in this transplant patient, the virus was detectable in urine by molecular tests for a longer period than in serum, plasma or CSF, consistent with the fact that the kidney is a well-established site of active WNV replication in animals such as birds, dogs and rodents [10-12]. Persistent replication of the virus in kidneys in humans is supported by studies reporting WNV shedding in urine, not only early post-infection [13], but even years after the initial infection [14], although the issue is still debated [15]. In our modified algorithm, a urine sample – the preferred sample for virus detection – is currently requested from patients whose serological tests for WNV are positive in order to confirm the serological results by detecting WNV RNA.

Discussion

A number of cases of human WNV infection have been reported over the past few years in Italy [16], but never in or close to the Marche region, with the exception of one infection acquired through a kidney transplant from a donor from an affected region [17]. In the Marche, since the summer of 2010, tests for WNV infection have been performed exclusively for the differential diagnosis of meningoencephalitis cases in the summer months . Since the summer of 2011, WNV serological tests are carried also out for patients with fever of unknown origin who are negative for Toscana virus. Had this diagnostic algorithm not been adopted, the cause of the patient's febrile illness would not have been determined and the WNV lineage 2 strain would not have been identified.

This case report suggests that screening for human cases of WNV infection should be further strengthened in the summer, for cases with neuroinvasive disease and for patients with fever of unknown origin, in regions of the country not previously affected by WNV. It is well known that there is an extensive cross-reactive antibody response to members of the *Flavivirus* genus, thus molecular tests should be performed to confirm the clinical diagnosis and identify the causative virus.

Our data show that tests to detect WNV RNA in serum or plasma may give false-negative results due to the short duration of viraemia. Urine samples may be more appropriate when looking for the presence of WNV, because of longer shedding and higher viral load. Whole WNV genome reconstruction was also easily achieved from the urine sample.

The clinical presentation of the case here described was relatively mild. However, since this is the first case of WNV lineage 2 infection detected so far in the country, it is not possible to draw any conclusions on the virulence and neurotropism of the viral strain. Investigation of any future cases, as well as molecular analysis of the complete genome, could give further information about the presence of genetic determinants of virulence.
It should be noted, however, that the incidence of meningoencephalitis or fever of unknown origin did not increase this summer in the Ancona province.

Autochthonous WNV human infection has been reported in several European countries this summer, including those of the Mediterranean. As of 20 October 2011, 89 confirmed human cases of West Nile fever have been reported in the European Union (66 in Greece, 13 in Italy and 10 in Romania) and 149 in neighbouring countries (121 in the Russian Federation, 21 in Israel, 3 in Turkey, 2 in Albania and 2 in the Former Yugoslav Republic of Macedonia) [18,19]. Notably, cases of WNV infection in Greece in 2011 occurred in areas that had not been affected in 2010 [19].

The WNV lineage 1 sequences from human infections in 2008 to 2009 in Italy were grouped into a distinct cluster within the western Mediterranean cluster [12], suggesting autochthonous spread of a single virus strain, without de novo introduction. The finding of the case in Ancona described in this report might suggest that viral strains circulating in other European countries during this summer might be spreading to Italy. It is possible that the lineage 2 virus reached Ancona via infected mosquitoes carried by ships or via birds from the eastern part of Europe. An epidemiological investigation is under way in the Ancona area to identify risk factors for infection and the possible local spread of lineage 2 WNV among insect vectors and birds.

Whatever the origin of the virus, the finding of a case of WNV lineage 2 infection in the country deserves further attention, as it suggests that viral circulation routes may be expanding, and, possibly, that there is an increased opportunity for this lineage 2 virus to adapt to new environments and ecological niches. It will be important to determine whether the present molecular diagnostic assays, designed mainly to detect lineage 1 WNV, perform equally well for lineage 2 WNV. This may have important implications for effective screening of blood and organ donors.

As a result of this case of WNV infection, the same precautionary measures in force in the WNV-affected regions in north-east Italy were immediately adopted in the Marche, for the sake of safety of organ donation in the region. In particular, these measures concern the need to use a nucleic acid amplification test (NAAT) to check for the presence of WNV RNA in blood taken from organ donors living in the Marche region or who stayed at least one night in the region in the 28 days before notification of the case described in this report.

In conclusion, stronger vector control programmes, integrated human and animal WNV surveillance and implementation of diagnostic procedures that include testing of urine samples for WNV detection could provide a useful contribution to controlling WNV spread and human disease.

References

1. Papa A, Bakonyi T, Xanthopoulou K, Vázquez A, Tenorio A, Nowotny N. Genetic characterization of West Nile virus lineage 2, Greece, 2010.Emerg Infect Dis. 2011;17(5):920-2.
2. Bakonyi T, Ivanics E, Erdélyi K, Ursu K, Ferenczi E, Weissenböck H, et al.. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. Emerg Infect Dis. 2006;12(4):618-23.
3. Tanaka M. Rapid identification of flavivirus using the polymerase chain reaction. J Virol Methods. 1993;41(3):311-22.
4. Capobianchi MR, Sambri V, Castilletti C, Pierro AM, Rossini G, Gaibani P, et al. Retrospective screening of solid organ donors in Italy, 2009, reveals unpredicted circulation of West Nile virus. Euro Surveill. 2010;15(34):pii=19648 Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19648
5. Rossini G, Cavrini F, Pierro A, Macini P, Finarelli AC, Po C, et al. First human case of West Nile virus neuroinvasive infection in Italy, September 2008 – case report. Euro Surveill. 2008;13,(41):pii=19002. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19002
6. Barzon L, Squarzon L, Cattai M, Franchin E, Pagni S, Cusinato R, et al. West Nile virus infection in Veneto region, Italy, 2008-2009. Euro Surveill. 2009;14(31):pii=19289. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19289
7. Macini P, Squintani G, Finarelli AC, Angelini P, Martini E, Tamba M, et al. Detection of West Nile virus infection in horses, Italy, September 2008. Euro Surveill. 2008;13(39):pii=18990. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18990
8. Rizzo C, Vescio F, Declich S, Finarelli AC, Macini P, Mattivi A, et al. West Nile virus transmission with human cases in Italy, August - September 2009. Euro Surveill. 2009;14(40):pii=19353. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19353
9. Ministero della Salute Italiana. Sorveglianza dei casi umani delle malattie trasmesse da vettori con particolare riferimento alla Chikungunya, Dengue e West Nile Disease - 2011 [Surveillance of human cases of vector-borne diseases with special reference to Chikungunya, dengue fever and West Nile disease - 2011]. Ministero della Salute Italiana: Rome; June 2011. Italian. Available from: http://www.normativasanitaria.it/normsan-pdf/0000/39170_1.pdf
10. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis. 2003;9:311–22.
11. Buckweitz S, Kleiboeker S, Marioni K, Ramos-Vara J, Rottinghaus A, Schwabenton B, et al. Serological, reverse transcriptase-polymerase chain reaction, and immunohistochemical detection of West Nile virus in a clinically infected dog. J Vet Diagn Invest. 2003;15(4):324-9.
12. Tonry JH, Xiao SY, Siirin M, Chen H, da Rosa AP, Tesh RB. Persistent shedding of West Nile virus in urine of experimentally infected hamsters. Am J Trop Med Hyg. 2005;72(3):320-4.
13. Tonry JH, Brown CB, Cropp CB, Co JK, Bennett SN, Nerurkar VR, et al. West Nile virus detection in urine. Emerg Infect Dis. 2005;11(8):1294-6.
14. Murray K, Walker C, Herrington E, Lewis JA, McCormick J, Beasley DW, et al. Persistent infection with West Nile virus years after initial infection. J Infect Dis. 2010;201(1):2-4.
15. Gibney KB, Lanciotti RS, Sejvar JJ, Nugent CT, Linnen JM, Delorey MJ, et al. West Nile Virus RNA not detected in urine of 40 people tested 6 years After acute West Nile virus disease. J Infect Dis. 2011;203(2):344-7.
16. Rossini G, Carletti F, Bordi L, Cavrini F, Gaibani P, Landini MP, et al. Phylogenetic analysis of West Nile Virus isolates, Italy, 2009-2009.. Emerg Infect Dis. 2011;17(5):903-6.
17. Nanni Costa A, Capobianchi MR, Ippolito G, Palù G, Barzon L, Piccolo G, et al. West Nile virus: the Italian national transplant network reaction to an alert in the north-eastern region, Italy 2011. Euro Surveill. 2011;16(41):pii=19991. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19991
18. European Centre for Disease Prevention and Control (ECDC). West Nile fever maps. Reported cases of West Nile fever for the EU and neighbouring countries. Stockholm: ECDC. [Accessed 20 Oct 2011]. Available from: http://ecdc.europa.eu/en/activities/diseaseprogrammes/emerging_and_vector_borne_diseases/Pages/West_Niles_fever_Risk_Maps.aspx
19. Danis K, Papa A, Papanikolaou E, Dougas G, Terzaki I, Baka A, et al. Ongoing outbreak of West Nile virus infection in humans, Greece, July to August 2011. Euro Surveill. 2011;16(34):pii=19951. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19951

First *Neisseria gonorrhoeae* strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011

M Unemo (magnus.unemo@orebroll.se)[1], D Golparian[1], A Stary[2], A Eigentler[3]
1. Swedish Reference Laboratory for Pathogenic Neisseria, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden
2. Outpatients Centre for Diagnosis of Infectious Venero-Dermatological Diseases, Vienna, Austria
3. Microbiological Laboratory Doz. Moest, Innsbruck, Austria

Citation style for this article:
Unemo M, Golparian D, Stary A, Eigentler A. First Neisseria gonorrhoeae strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011. Euro Surveill. 2011;16(43):pii=19998. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19998

Article published on 27 October 2011

We describe the first cefixime-resistant *Neisseria gonorrhoeae* strain in Austria that caused treatment failure. It follows the first five cases in Europe of cefixime treatment failure, reported in Norway in 2010 and the United Kingdom in 2011. Effective treatment of gonorrhoea is crucial for public health control and, at present, requires substantially enhanced awareness, more frequent test-of-cure, interaction with experts after therapeutic failure, tracing and therapy of contacts, and surveillance of gonococcal antimicrobial resistance and treatment failures worldwide.

We report here the first *Neisseria gonorrhoeae* strain with resistance to cefixime in Austria, which caused a treatment failure with cefixime.

Gonorrhoea is the second most prevalent bacterial sexually transmitted infection (STI) worldwide, and the aetiological agent, *N. gonorrhoeae*, has developed resistance to all antimicrobials used as first-line treatments. In most countries, the currently recommended first-line drugs are the extended-spectrum cephalosporins (ESCs) ceftriaxone (injectable) and cefixime (oral). However, the susceptibility of *N. gonorrhoeae* to both is decreasing worldwide [1,2]. Cefixime standard treatment (400 mg single oral dose) has been preferred in many countries due to its effectiveness, the ease of an oral, single-dose regimen, and because, before 2010, verified treatment failures had only been reported in Japan [3]. However, two cases of clinical failures with cefixime standard treatment were described in 2010 in Norway [4], which were strictly verified using the World Health Organization (WHO) criteria [1], and three cases in 2011 in the United Kingdom [5,6]. Furthermore, the first gonococcal strain with high-level clinical resistance to ceftriaxone (the last remaining option for empirical first-line treatment), i.e. the first extensively drug-resistant gonococcus [1], was recently found in Japan [7]. It is now possible that gonorrhoea will become untreatable in certain circumstances and especially some settings [1,7].

Case report

In early July 2011, an Austrian man-who-has-sex-with-men (MSM) had unprotected sex with one anonymous MSM in a gay sauna in Munich, Germany. Some days later, the Austrian man presented to an urologist in the region of Innsbruck, Austria (day 1), with symptoms of urethritis (urethral discharge and dysuria) that had been present for two days. The patient was administered cefixime at a 400 mg oral dose once a day for seven days. On day 4, a urine sample taken on day 1 was shown to contain *N. gonorrhoeae*- as well as *Chlamydia trachomatis*-specific DNA using the Abbott m2000rt RealTime CT/NG PCR (Abbott Molecular Diagnostics). On day 8, he presented to a general practitioner with persisting symptoms, and the same treatment was prescribed for an additional 14 days. On day 22, the patient returned with persisting symptoms to the urologist he had visited initially, and microscopy of a urethral smear displayed urethritis and intracellular Gram-negative diplococci within polymorphonuclear leukocytes. Furthermore, *N. gonorrhoeae* was cultured from an additional urethral sample taken on that day, and *N. gonorrhoeae*- as well as *C. trachomatis*-specific DNA was found in a urine sample using the Abbott PCR. The patient was on the same day given one oral dose of 2 g azithromycin. On day 43, follow-up examination showed that the symptoms and signs had resolved, and a PCR test (urine sample; Abbott PCR) was negative for *N. gonorrhoeae* as well as for *C. trachomatis*). The patient repeatedly denied (on each visit) any sexual activities after recognition of symptoms and, in particular, between first treatment and test-of-cure.

Characterisation of the cefixime-resistant *Neisseria gonorrhoeae* strain

Unfortunately, no pre-treatment *N. gonorrhoeae* isolate was available. The post-treatment strain (cultured on day 22) was however species-confirmed using culture on selective agar medium, rapid oxidase production, presence of Gram-negative diplococci, and two species-verifying assays, an in house sugar utilisation

test and Phadebact GC Monoclonal Test (Bactus AB, Sweden). The results of the characterisation of the strain are summarised in the Table.

The strain was assigned to serovar Bpyut, multilocus sequence typing (MLST) ST1901 and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) ST1407, performed as previously described [7,8]. The strain had minimum inhibitory concentrations (MICs) of five antimicrobial drugs as follows: 1.0 mg/L of cefixime (average of 0.8 mg/L in four Etest determinations), 0.5 mg/L of ceftriaxone (average of 0.3 mg/L in four Etest determinations), 0.25 mg/L of azithromycin, 8 mg/L of spectinomycin, and >32 mg/L of ciprofloxacin (see Table). Accordingly, the strain was resistant to cefixime (>0.12 mg/L), ceftriaxone (>0.12 mg/L), and ciprofloxacin (>32 mg/L), based on the breakpoints stated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [9]. The strain did not produce any beta-lactamase.

Sequencing of resistance determinants for penicillins and ESCs (*penA, mtrR, porB1b, ponA* and *pilQ* alterations) were performed as previously described [7,8,10]. The strain contained a *penA* mosaic XXXIV allele [7] with a single additional amino acid alteration (T534A) in the gene for penicillin binding protein 2, and additionally the *mtrR* and *penB* resistance determinants, which all together caused the high MICs of the ESCs [7,10,11] (Table). Transformation experiments, performed as previously described [7,12], confirmed that the only new resistance determinant, i.e. the novel *penA* mosaic allele (*penA* mosaic XXXIV allele with an additional T534A alteration), was responsible for the resistance to ESCs.

Table

Characteristics of the first *Neisseria gonorrhoeae* strain with resistance to cefixime and causing treatment failure, Austria, 2011

MIC (mg/L)		MLST	NG-MAST	*penA* allele	*mtrR*	*penB*	*ponA*
IX	TX						
1.0	0.5	ST1901	ST1407	Mosaic (novel)[a]	A-del in promoter[b]	G120K A121N[c]	L421P[d]

IX: cefixime; MIC: minimum inhibitory concentration (Etest was used and only whole MIC dilutions are presented); MLST: multilocus sequence typing; NG-MAST: *Neisseria gonorrhoeae* multiantigen sequence typing; PCR: polymerase chain reaction; ST: sequence type; TX: ceftriaxone.

[a] Mosaic allele encodes a mosaic penicillin binding protein 2 (PBP2), which causes decreased susceptibility to extended-spectrum cephalosporins.

[b] Characteristic single nucleotide (A) deletion in the inverted repeat of the promoter region of *mtrR* that causes overexpression of the MtrCDE efflux pump, which results in a further decreased susceptibility to extended-spectrum cephalosporins.

[c] Alterations of amino acids 120 and 121 in the porin PorB1b that cause a decreased intake of extended-spectrum cephalosporins and, accordingly, a further decreased susceptibility to extended-spectrum cephalosporins .

[d] Alteration of amino acid 421 in the penicillin-binding protein 1 (PBP1), which results in decreased susceptibility to penicillins.

Discussion

We report here the first *N. gonorrhoeae* strain with resistance to cefixime in Austria, which caused a treatment failure with the internationally recommended first-line treatment cefixime. This treatment failure as well as two of the three previously reported ones in the United Kingdom [5,6] occurred in MSM, and an enhanced focus on prevention and control of gonorrhoea in MSM may be necessary. The present treatment failure was identified in an Austrian patient, however, the infection was contracted in Germany, which suggests that this strain may be present in the MSM community in Germany.

Because no pre-treatment isolate was available, the same situation as for two of the three reported cases of cefixime treatment failures in the United Kingdom [5,6], it was not possible to verify the treatment failure in full accordance to the WHO criteria [1]. However, a detailed clinical history was recorded, reinfection was ruled out as much as is possible (denied on each visit by the patient), the post-treatment isolate was in vitro highly resistant to cefixime, and the strain contained genetic resistance determinants explaining the high cefixime MIC. Furthermore, the high cefixime MIC of the strain makes it most likely that this was a treatment failure. According to Monte Carlo simulations, a 400 mg dose of cefixime results in a median time of free cefixime above MIC ($fT_{>MIC}$) of only 6.8 h (3.8-9.6 h) for the detected MIC of 1.0 mg/L [13]. Furthermore, one day (ca. 24 h) after administration of 400 mg cefixime the concentration of free cefixime is very low (ca. 0.03 mg/L) [13]. Consequently, especially due to the short half life of cefixime (3.4 hours) administration of one 400 mg dose per day for several days does not substantially extend the cefixime $fT_{>MIC}$ unless the MIC of the strain is relatively low [13]. This regimen accordingly does not provide any major benefits, compared with the recommended cefixime single-dose regimen, for the treatment of gonorrhoea, is evidently not able to clear an infection with a gonococcal strain that has an MIC of cefixime of 1.0 mg/L; rather, it may select for higher ESC resistance. This emphasises the importance of adhering to appropriate treatment guidelines (i.e. using a 400 mg cefixime single-dose regimen, and, if failure is confirmed or suspected, another antimicrobial drug), and of involving STI experts when the commonly used recommended treatment fails in a patient. It is now evident that cefixime treatment failures have occurred in several European countries. In many cases they may not be recognised because azithromycin is additionally administered to many of the gonorrhoea patients (due to suspicion of chlamydial infection) [1,13], follow-up examination and test-of-cure are rarely performed [1], or treatment failures are not appropriately verified and reported. The recently updated treatment guidelines in the United States [14] and the United Kingdom [15] recommend cefixime 400 mg only as an alternative treatment (if ceftriaxone is not an option), and this change may need to be considered also in the European [16] and other treatment

guidelines. In all countries, it is crucial to maintain as much capacity as possible to culture and perform antimicrobial resistance (AMR) testing of gonococci, for AMR surveillance purposes but also for adequate verification of treatment failures and, if needed, for informing the antimicrobial treatment of individual patients.

The present treatment failure was caused by a gonococcal strain of ST1407, which is multidrug-resistant and is spreading in many countries worldwide [17,18]. ST1407 or closely related subtypes also caused the recent treatment failures in Norway [4] and the United Kingdom [5].

In conclusion, clinical failures of gonorrhoea treatment with the internationally recommended first-line treatment cefixime have occurred in three European countries. Improved prevention (e.g. condom use) and control of gonorrhoea, enhanced awareness of cefixime treatment failures, more frequent follow-up examination including test-of-cure and appropriate collection of demographic and behavioural data (e.g. sexual orientation), and surveillance of gonococcal AMR and treatment failures (appropriately verified and subsequently reported) are crucial worldwide to mitigate the spread and minimise the impact of ESC-resistant gonococcal strains and, accordingly, to ensure that gonorrhoea remains a treatable infection. Furthermore, adherence to appropriate treatment guidelines and timely evidence-based revision of these guidelines, involvement of STI experts when the commonly used recommended treatment fails in a patient, as well as tracing and therapy also of sexual contacts of the index case are imperative.

Acknowledgments

We are grateful to Maria Haller, Outpatients' Centre for Diagnosis of Infectious Venero-Dermatological Diseases, Vienna for her valuable support in this project.

References

1. Tapsall JW, Ndowa F, Lewis DA, Unemo M. Meeting the public health challenge of multidrug- and extensively drug-resistant Neisseria gonorrhoeae. Expert Rev Anti Infect Ther. 2009;7(7):821-34.
2. Lewis DA. The gonococcus fights back: is this time a knock out? Sex Transm Infect. 2010;86(6):415-21.
3. Yokoi S, Deguchi T, Ozawa T, Yasuda M, Ito S, Kubota Y, et al. Threat to cefixime treatment of gonorrhea. Emerg Infect Dis. 2007;13(8):1275-7.
4. Unemo M, Golparian D, Syversen G, Vestrheim DF, Moi H. Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. Euro Surveill. 2010;15(47):pii=19721. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19721
5. Ison CA, Hussey J, Sankar KN, Evans J, Alexander S. Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. Euro Surveill. 2011;16(14):pii:19833. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19833
6. Forsyth S, Penney P, Rooney G. Cefixime-resistant Neisseria gonorrhoeae in the UK: a time to reflect on practice and recommendations. Int J STD AIDS. 2011;22(5):296-7.
7. Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, et al. Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhea? Detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob Agents Chemother. 2011;55(7):3538-45.
8. Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J. Phenotypic and genetic characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. J Antimicrob Chemother. 2009;63(6):1142-51.
9. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 1.3. European Society of Clinical Microbiology and Infectious Diseases; 5 Jan 2011. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf
10. Lindberg R, Fredlund H, Nicholas R, Unemo M. Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime and ceftriaxone: Association with genetic polymorphisms in penA, mtrR, porB1b, and ponA. Antimicrob Agents Chemother. 2007;51(6):2117-22.
11. Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas R. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in Neisseria gonorrhoeae. Antimicrob Agents Chemother. 2009;53(9):3744-51.
12. Ohnishi M, Watanabe Y, Ono E, Takahashi C, Oya H, Kuroki T, et al. Spreading of a chromosomal cefixime-resistant penA gene among different Neisseria gonorrhoeae lineages. Antimicrob Agents Chemother. 2010;54(3):1060-7.
13. Chisholm SA, Mouton JW, Lewis DA, Nichols T, Ison CA, Livermore DM. Cephalosporin MIC creep among gonococci: time for a pharmacodynamic rethink? J Antimicrob Chemother. 2010;65(10):2141-8.
14. Workowski KA, Berman S; Centers for Disease Control and Prevention (CDC). Sexually transmitted diseases treatment guidelines, 2010. MMWR Recomm Rep. 2010;59(RR-12):1-110.
15. Bignell C, Fitzgerald M; (Guideline Development Group). UK national guideline for the management of gonorrhoea in adults, 2011. Int J STD AIDS. 2011;22(10):541-7.
16. Bignell C; IUSTI/WHO. 2009 European (IUSTI/WHO) guideline on the diagnosis and treatment of gonorrhoea in adults. Int J STD AIDS. 2009;20(7):453-7.
17. Golparian D, Hellmark B, Fredlund H, Unemo M. Emergence, spread and characteristics of Neisseria gonorrhoeae isolates with in vitro decreased susceptibility and resistance to extended-spectrum cephalosporins in Sweden. Sex Transm Infect. 2010;86(6):454-60.
18. Chisholm SA, Alexander S, Desouza-Thomas L, Maclure-Webster E, Anderson J, Nichols T, et al. Emergence of a Neisseria gonorrhoeae clone showing decreased susceptibility to cefixime in England and Wales. J Antimicrob Chemother. 2011;66(11):2509-12.

Highly heterogeneous temperature sensitivity of 2009 pandemic influenza A(H1N1) viral isolates, northern France

I Pelletier[1,2], D Rousset[3,4,5,6], V Enouf[3,4,5,6], GROG[7], F Colbère-Garapin[1,2] S van der Werf[3,4,5,6], N Naffakh (nadia.naffakh@pasteur.fr)[3,4,5]
1. Institut Pasteur, Unité de Biologie des Virus Entériques, Département de Virologie, Paris, France
2. INSERM U994 (French National Institute of Health and Medical Research) Paris, France
3. Institut Pasteur, Unité de Génétique Moléculaire des virus à ARN, Département de Virologie, Paris, France
4. CNRS URA3015 (French National Centre for Scientific Research), Paris, France
5. Université Paris Diderot, Sorbonne Paris Cité, Unité de Génétique Moléculaire des virus à ARN, Paris, France
6. Institut Pasteur, Centre National de Référence des virus influenzae (Région Nord), Paris, France
7. Groupes Régionaux d'Observation de la Grippe, Open Rome, Paris, France

Citation style for this article:
Pelletier I, Rousset D, Enouf V, GROG, van der Werf S, Naffakh N. Highly heterogeneous temperature sensitivity of 2009 pandemic influenza A(H1N1) viral isolates, northern France.
Euro Surveill. 2011;16(43):pii=19999. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19999

Article published on 27 October 2011

We assayed the temperature sensitivity of 2009 pandemic influenza A(H1N1) viral isolates (n=23) and seasonal influenza A(H1N1) viruses (n=18) isolated in northern France in 2007/08 and 2008/09. All isolates replicated with a similar efficiency at 34 °C and 37 °C, and with a lower efficiency at 40 °C. The pandemic viral isolates showed a stronger heterogeneity in their ability to grow at the highest temperature, as compared with the seasonal isolates. No statistically significant difference in temperature sensitivity was observed between the pandemic viral isolates from severe and mild cases of influenza. Our data point to the impact of temperature sensitivity on the genetic evolution and diversification of the pandemic influenza A(H1N1) virus since its introduction into the human population in April 2009, and call for close surveillance of this phenotypic marker related to host and tissue tropism.

Introduction

A novel influenza A(H1N1) virus emerged in April 2009 [1-3] and rapidly spread all over the world. In France, the first cases were identified in early May 2009. The 2009 pandemic A(H1N1) virus presented a unique combination of genomic segments that had not been reported previously [4]. The segments coding for the neuraminidase (NA) and the matrix (M) proteins of the virus were related to the Eurasian lineage of swine influenza A(H1N1) viruses, whereas the six remaining gene segments were related to triple swine–human–avian influenza A(H1N1) reassortants that have been isolated from humans in contact with pigs in North America since 1998 [5,6]. Although the properties of isolates of the 2009 pandemic influenza A(H1N1) virus have already been largely examined in vitro and in vivo (for a review, see [7]), sensitivity to elevated temperature has not been characterised precisely. Temperature sensitivity is an important viral phenotypic marker, as it may be involved in host species restriction, tissue specificity and/or virulence [8-11]. In humans and pigs, influenza A viruses initially replicate in the upper respiratory tract at temperatures close to 33 °C and 37 °C, respectively, whereas in aquatic birds, influenza A viruses with low pathogenicity preferentially replicate in the intestinal tract at a temperature close to 40 °C [12-14]. The sensitivity of avian influenza A viruses to low temperature (33 °C) has been clearly demonstrated [15,16]. In contrast, no reduction in viral multiplication at 33 °C was observed for the swine viruses, and it has been proposed that temperature sensitivity might represent a specific, host-dependent signature of influenza A viruses [17]. Depending on the optimal temperature for viral multiplication, fever in infected patients may either limit or facilitate viral multiplication and consequently the administration of anti-pyretic drugs may or not be beneficial. Treatment of ferrets infected with influenza virus with sodium salicylate (an anti-pyretic) resulted in increased viral loads in nasal washes [18].

In order to characterise and compare the temperature sensitivity of both pandemic influenza A(H1N1) viral isolates and seasonal viruses isolated in northern France in 2007/08 and 2008/09 before the emergence of the pandemic virus, we developed a test to compare viral multiplication at 34 °C, 37 °C and 40 °C.

Methods

Virus samples and reference isolates

We included 23 isolates of 2009 pandemic influenza A(H1N1) virus and 18 seasonal influenza A(H1N1) viral isolates in our study. The pandemic isolates were collected in northern France between weeks 39 and 51 (24 September to 16 December) in 2009; pandemic activity in this area started at week 42 in 2009, peaked at week 49 and ended at week 2 in 2010 [19]. The isolates for the 2007/08 season were collected in northern France between week 44 (29 October) in 2007 and week 3 (14

January) in 2008 and those for the 2008/09 season between week 45 (3 November) in 2008 and week 4 (19 January) in 2009.

One of the 2007/08 seasonal influenza A(H1N1) viral isolates, (A/Paris/1149/2008), was included in most experiments (12/13, due to a technical problem in one) as a control to assess the reproducibility of our experimental conditions. A further 12 seasonal influenza A(H1N1) viral isolates from 2007/08, either susceptible or resistant to oseltamivir, and five seasonal influenza A(H1N1) viral isolates from 2008/09, all resistant to oseltamivir, were also included. These seasonal viral isolates were chosen at the beginning and peak of the influenza seasons in northern France, as for the pandemic isolates, and, for the 2007/08 seasonal isolates, we also took into account the co-circulation of viruses sensitive or naturally resistant to oseltamivir.

Among the 23 pandemic influenza A(H1N1) viral isolates included in our study, we defined two distinct groups of viruses according to the disease severity of the patients (Table1). Information about the existence of underlying conditions prone to increase disease severity was noted when available (Table 1). Severe influenza cases were those who were hospitalised in an intensive care unit or died as a result of their infection. Patients with mild disease were matched as much as possible by the week and geographical area of collection.

Two representative isolates from the human North American triple reassortant influenza A(H1N1) viruses (A/Illinois/09/2007 and A/Ohio/02/2007) and from the swine Eurasian influenza A(H1N1) and Hong Kong triple reassortant internal gene (TRIG) influenza A(H1N2) lineages (A/Swine/Cotes d'Armor/0231/2006 and A/

Table 1

Origin and characteristics of 2009 pandemic influenza A(H1N1) viral isolates from mild and severe influenza cases, northern France, 24 September–16 December 2009 (weeks 39–51) (n=23)

Viral isolate[a]	Sample type	Week of sampling	Type of patient[b]	Age of patient (years)	Disease severity	Additional information	Haemagglutinin residue 222[c]	Neuraminidase residue 275[c]
20097639	Nasal and pharyngeal	51	Outpatient	40	Mild	NA	E	H
20097214	Nasal and pharyngeal	49	Outpatient	47	Mild	NA	D	Y
20096074	Nasal and pharyngeal	45	Outpatient	16	Mild	NA	D	H
20095771	Nasal and pharyngeal	44	Outpatient	24	Mild	NA	D	H
20095509	Nasal and pharyngeal	42	Outpatient	45	Mild	NA	D	H
20095501	Nasal and pharyngeal	43	Outpatient	8	Mild	NA	D	H
20095383	Nasal and pharyngeal	42	Outpatient	29	Mild	NA	D	H
20095016	Nasal and pharyngeal	41	Outpatient	14	Mild	NA	D	H
20097391	Nasal and pharyngeal	49	Inpatient	44	Severe	Deceased	D	H
20097367	Nasal and pharyngeal	48	Inpatient	26	Severe	NA	D	H
20097155	Nasal and pharyngeal	48	Inpatient	2.5	Severe	NA	D	H
20097097[d]	Lung	49	Inpatient	6	Severe	Deceased	D	H
20097101[d]	Brain	49	Inpatient	6	Severe	Deceased	G[e]	H
20096934	Nasal and pharyngeal	47	Inpatient	63	Severe	Haemopathy	D	H
20095911	Nasal and pharyngeal	43	Inpatient	10	Severe	Chronic respiratory insufficiency	D	H
20096365	Nasal	45	Inpatient	55	Severe	Chronic obstructive bronchopneumopathy	E	H
20094517	Nasal and pharyngeal	39	Inpatient	20	Severe	NA	D	H
20094518	Nasal and pharyngeal	39	Inpatient	45	Severe	NA	E	H
20094785	Nasal and pharyngeal	40	Inpatient	29	Severe	NA	E	H
20096928	Nasal and pharyngeal	45	Inpatient	22	Severe	Acute respiratory distress syndrome	D	H
20097105	Lung	48	Inpatient	46	Severe	Deceased	G	H
20097208	Nasal and pharyngeal	48	Inpatient	51	Severe	Deceased	D	H
20097388	Nasal and pharyngeal	49	Outpatient	19	Severe	Deceased (at home)	D	H

NA: not available.

[a] 2009XXXX stands for A/Paris/XXXX/2009.

[b] Samples from outpatients are from the Groupes Régionaux d'Observation de la Grippe (GROG), the national network of sentinel general practitioners and paediatricians and from the Réseau National des Laboratoires (RENAL), a network of hospital laboratories.

[c] Sequence information refers to the viruses isolated after one passage in Madin-Darby canine kidney (MDCK) cells.

[d] Viruses 20097097 and 20097101 were isolated from the lung and brain, respectively, of the same patient.

[e] The sequence of the virus present in the original specimen was also determined, and a D was found at residue 222 of the haemagglutinin.

Swine/Hong Kong/1578/2003) [20], respectively, were tested in parallel.

Preparation and analysis of viral isolates

In order to produce suitable viral stocks, all isolates were amplified by two serial passages at a multiplicity of infection of 10^{-3} plaque forming units per cell at 35 °C in MDCK cells in serum-free minimal essential medium (MEM) containing 1µg/ml trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK). We assumed that the pandemic viral isolates would be able to grow efficiently at 35 °C (as the temperature of the human upper respiratory tract is about 33 °C) and we therefore chose to amplify the virus at 35 °C rather than 37°C in order to avoid the preselection of variants that grow preferentially at high temperature. Viral stocks were clarified and aliquots for single use were kept frozen at –80 °C.

Viral RNA was prepared using the QIAamp Viral RNA Mini Kit (Qiagen). Reverse transcription PCR was carried out using the SuperScript One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) and oligonucleotides specific for the haemagglutinin (HA) and NA segments. The amplicons were sequenced using a Big Dye terminator sequencing kit and an automated sequencer (Applied Biosystems). In some cases, pyrosequencing was used to determine specifically the sequence at residue 222 of the HA or at residue 275 of the NA. For the H275Y mutation, the primers GRswN1-780Fw/090206 (5'-GGGGAAGATTGTYAAATCAGTYGA-3') and GRswN1-1273Rv/090207 (5'-biotin-CWACCCAGAAR-CAAGGYCTTATG-3') were used for amplification, and GRswN1-804Fw/090208 (5'-GYTGAATGCMCCTAATT-3') for sequencing, as previously described [21]. For the D222G mutation, the primers GRswH1-672Fw (5'-CAAGAAGTTCAAGCCGGAAATAGC-3') and GRswH1-821 Rv (5'-biotin-ATTGCGAATGCATATCTCGGTAC-3') were used for amplification, and GRswH1-693Fw (5'-AGCAATAAGACCCAAAG-3') for sequencing. Primers were designed using the Pyrosequencing Assay Design Software (Biotage). Pyrosequencing reactions were performed on purified biotinylated amplicons as previously described [22].

Temperature-sensitivity assays

Confluent three-day-old cultures of MDCK cells in 96-well plates, prepared in MEM containing 5% foetal calf serum and 50 µg/ml gentamycin, were washed twice with serum-free MEM before infection. Serum-free MEM (170 µl/well) containing trypsin-TPCK (1µg/ml) and gentamycin (50 µg/ml) were added to cultures. Ten-fold dilutions of each virus sample in MEM (30 µl/well, 10 wells/dilution, 3 plates/virus sample) were added to cells. Plates were sealed with an adhesive membrane and covered with lids and incubated at 34 °C, 37 °C and 40 °C. Incubators were used for incubation at 34 °C and 37 °C, whereas a water bath was used to incubate plates at exactly 40 °C.

Cytopathic effects were observed under the microscope three days after infection and virus titres as 50% tissue culture infectious doses ($TCID_{50}$) per mL were determined as previously described by Reed and Muench [23]. The reproductive capacity at the high, potentially restrictive temperature of 40 °C (RCT_{40} value) is the difference, in log values, between the titres at 40 °C and at 37 °C for each viral isolate. Similarly, the reproductive capacity at 34 °C (RCT_{34} value) is the difference in log values between the viral titres at 34 °C and at 37 °C. Both RCT values are expressed as the mean ± standard deviation (SD) from at least three independent experiments.

Results

For all isolates tested, viral titres were similar at 34°C and 37°C; the RCT_{34} values varied between –0.63±0.53 and +0.50±0.16 (Figure). In contrast, significant differences were observed between isolates grown at 40 °C, since the RCT_{40} values varied between 0.00±0.16 and –4.23±0.42. The RCT_{40} value of the pool of 2007/08 and 2008/09 seasonal viruses varied between –2.40±0.29 and –3.97±0.12, indicating that the titres of these viruses were about 250- to 9,300-fold lower at 40 °C than at 37 °C. The pandemic viruses showed RCT_{40} values ranging from –1.30±0.29 to –4.23±0.42, indicating that their titres were about 20- to 17,000-fold lower at 40 °C than at 37 °C.

On average, pandemic viral isolates were about three-fold less sensitive at 40 °C than the pool of the 2007/08 and 2008/09 seasonal viruses (RCT_{40} values of –2.55±0.82 and –3.06±0.46, respectively; $p<0.05$, Student's *t*-test) and showed a significantly higher variability in temperature sensitivity (variance ratio: 3.18; $p<0.025$, Fisher's exact test). No statistically significant differences were seen in RCT_{40} values regardless of whether the pandemic viral isolates had been isolated from severe cases with or without underlying condition (n=15) or from mild cases (n=8) (Table 2).

Interestingly, two human isolates representative of the North American triple reassortant influenza A(H1N1) viruses (A/Illinois/09/2007 and A/Ohio/02/2007) grew similarly at 40 °C and 37 °C (Table 2). Their growth was thus clearly more resistant to high temperature than that of the pandemic viral isolates. The Hong Kong TRIG swine influenza A(H1N2) and Eurasian swine influenza A(H1N1) viruses included in our study showed an intermediate phenotype between the triple reassortant and pandemic viruses (Table 2 and Figure).

A D222G substitution in the receptor binding site of HA was seen in two of the viral isolates included in our study (isolates 20097101 and 20097105). This substitution has been detected sporadically, with some degree of correlation between the presence of the substitution and the severity of the disease [24-27]. Isolates 20097101 and 20097105 showed RCT_{40} values of –2.25±0.57 and –1.33±0.12, respectively (data not shown). The 20097101 virus was isolated from the

brain of a young patient who died after infection and showed a G residue at position 222 of the HA (Table 1). The viruses detected in the initial brain specimen showed a D at this position, but probably contained a low, undetectable fraction of viruses of the HA-222G genotype upon amplification in MDCK cells.

The 20097097 virus isolated from the lung of the same patient showed a D residue at position 222 of the HA (Table 1). No statistically significant difference in temperature sensitivity was observed between the 20097101 and 20097097 isolates.

One of the pandemic viral isolates (20097214) included in our study had the H275Y substitution in the NA (Table 1) that is associated with oseltamivir resistance [28,29] and was characterised by a marked sensitivity to high temperature, with an RCT_{40} value of –3.90±0.57. However, the two panels of oseltamivir-resistant and -sensitive seasonal isolates from 2007/08 showed no statistically significant difference in temperature sensitivity (Table 2 and Figure). Overall, our results suggest that neither the D222G substitution in the HA nor the H275Y substitution in the NA have a major impact on the viral sensitivity to high temperature.

The NA and M gene sequences of the 23 pandemic viral isolates included in our study were determined: the Global Initiative on Sharing Avian Influenza Data (GISAID) accession numbers are shown in Table 3. The NA and M1 amino acid sequences of the 23 pandemic viral isolates included in our study were were aligned with the corresponding sequences of the swine and triple reassortant viruses. The pandemic virus-derived sequences showed very few variations: their NA and M1 sequence shared about 91% and 94% identity with the respective Eurasian swine virus-derived sequences and 81% and 88% identity with the respective triple reassortant virus-derived sequences.

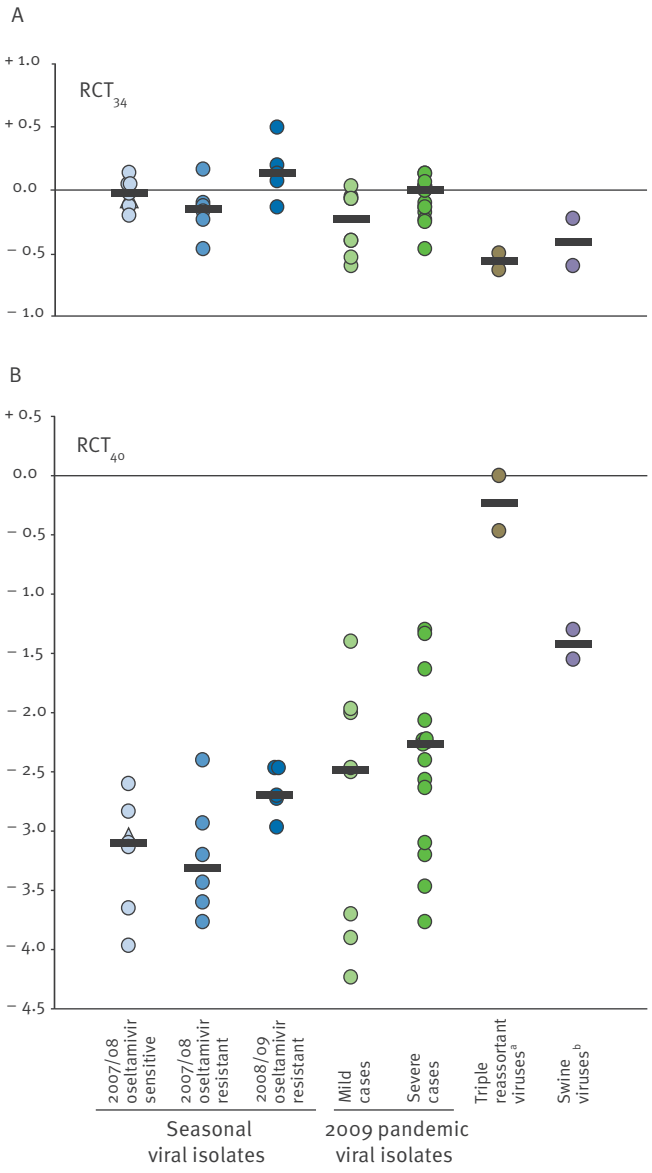
Discussion and conclusion

A panel of seasonal and pandemic influenza A(H1N1) viral isolates from northern France in 2007/08 to 2008/09 grew with similar efficiency at 34 °C and 37 °C, suggesting that these viruses are well adapted to the physiological temperatures of the upper and lower respiratory tract. In contrast, they replicated less efficiently at 40 °C than at 37 °C. As compared with seasonal isolates, the pandemic viral isolates showed a marked heterogeneity in temperature sensitivity as indicated by a significantly higher variability in the corresponding RCT_{40} values. This heterogeneity probably reflects ongoing evolution and genetic diversification of the virus since its introduction in the human population in April 2009.

The sensitivity to high temperature of isolates of the pandemic virus from severe cases of influenza was not statistically significantly different from that of isolates from mild cases, but the numbers were small. These results suggest that there was little or no correlation between temperature sensitivity of pandemic viruses and clinical severity. However, this finding should be confirmed by analysing a larger panel of viruses, given

Figure

Reproductive capacity of 2009 pandemic influenza A(H1N1) viral isolates (n=23) and 2007/08 and 2008/09 seasonal influenza A(H1N1) viral isolates (n=18) at 34 °C and 40 °C, relative to 37 °C, northern France



RCT: reproductive capacity at a given temperature.

RCT34 (panel A) and RCT40 (panel B) values are shown for 2008 and 2009 seasonal influenza A(H1N1) viral isolates (blue symbols) and 2009 pandemic influenza A(H1N1) viral isolates (green symbols). Two North American influenza A(H1N1) triple reassortant viruses (brown symbols) and two swine influenza viruses (purple symbols) were included for comparison. The 2008 seasonal influenza A(H1N1) viral isolate used as reference in most experiments is indicated with a triangle, whereas other influenza strains are indicated with circles. The line at 0.0 separates viral isolates that replicate more efficiently at 34 °C or 40 °C than at 37 °C (RCT values >0) from those that replicate less efficiently at 34 °C or 40 °C than at 37 °C (RCT values <0). Median values are indicated by horizontal black bars.

[a] Swine–human–avian triple reassortant influenza A(H1N1) viruses isolated from humans.

[b] A Eurasian swine influenza A(H1N1) virus and a Hong Kong triple reassortant internal gene (TRIG) swine influenza A(H1N2) virus.

Table 2

Reproductive capacity of 2009 pandemic influenza A(H1N1) viral isolates (n=23) and 2007/08 and 2008/09 seasonal influenza A(H1N1) viral isolates (n=18) at 34 °C and 40 °C, northern France

Type of viral isolate	Viral isolates[a]	Mean RCT_{34} ± SD	Mean RCT_{40} ± SD
2007/08 seasonal isolates, oseltamivir sensitive n=7	20081149	−0.03±0.11	−3.19±0.43
	20081207		
	20081129		
	20080730		
	20080658		
	20080552		
	20080286		
2007/08 seasonal isolates, oseltamivir resistant n=6	20081093	−0.15±0.19	−3.22±0.46
	20081019		
	20080749		
	20080577		
	20081170		
	20081166		
2008/09 seasonal isolates, oseltamivir resistant n=5	20090244	+0.16±0.21	−2.67±0.19
	20091401		
	20091349		
	20090639		
	20090445		
2009 pandemic isolates from mild cases n=8	20097639	−0.26±0.23	−2.77±0.97
	20097214		
	20096074		
	20095771		
	20095509		
	20095501		
	20095383		
	20095016		
2009 pandemic isolates from severe cases among inpatients and/or deceased patients n=15	20097391	−0.07±0.16	−2.43±0.70
	20097367		
	20097155		
	20097097		
	20097101		
	20096934		
	20095911		
	20096365		
	20094517		
	20094518		
	20094785		
	20096928		
	20097105		
	20097208		
	20097388		
Swine–human–avian triple reassortant influenza A(H1N1) viruses isolated from humans	A/Illinois/09/2007	−0.57±0.07	−0.23±0.23
	A/Ohio/02/2007		
Swine viruses: a Eurasian swine influenza A(H1N1) virus and a Hong Kong TRIG swine influenza A(H1N2) virus	A/Swine/Cotes d'Armor/0231/2006	−0.41±0.19	−1.43±0.13
	A/Swine/Hong Kong /1578/2003		

RCT: reproductive capacity at a given temperature; SD: standard deviation.

[a] 2009XXXX stands for A/Paris/XXXX/2009.

the strong heterogeneity in temperature sensitivity, the possible bias due to the fact that the severity of the disease in up to 25% of severe cases during the pandemic was due to bacterial secondary infections rather than the characteristics of the pandemic virus [30,31] and the fact that host factors, such as underlying conditions identified as risk factors, seem to have contributed substantially to the clinical course of severe cases with 2009 pandemic influenza A(H1N1) [32,33].

The Eurasian swine influenza A(H1N1) virus, a Hong Kong TRIG swine influenza A(H1N2) virus and two A(H1N1) triple reassortant viruses included in our study showed a lower sensitivity to elevated temperature (40 °C) than the pandemic and seasonal viral isolates on average, in agreement with the fact that the normal body temperature of pigs varies between 38.5 °C and 39.2 °C [34]. All the pandemic viral isolates included in our study replicated less efficiently at 40 °C than did the triple reassortant viruses although their genomic segments, except for the NA and matrix (M) segments, are phylogenetically related to the triple reassortants.

No specific sequence signature was observed for the viruses that showed the highest RCT_{40} (data not shown). Overall, our observations suggest that the sensitivity to high temperature of the pandemic viral isolates is determined by complex gene constellation and/or mutation effects.

In conclusion, our small dataset shows that the pandemic viruses that circulated in northern France in 2009 were more heterogeneous with respect to their ability to grow at high temperature (40 °C) than the seasonal viruses that circulated there in 2007/08 and 2008/09. They point to the impact of viral temperature sensitivity on the genetic evolution and diversification of the pandemic virus during the first year after its introduction into the human population and they call for a close monitoring of this phenotypic marker related to host and tissue tropism during the coming years.

Table 3

GISAID accession numbers of 2009 pandemic influenza A(H1N1) viral isolates, northern France (n=23)

Viral isolate[a]	NA sequence	M1 sequence
20094517	EPI320178	EPI320177
20094518	EPI320180	EPI320179
20094785	EPI320181	EPI320182
20095016	EPI320184	EPI320183
20095383	EPI320186	EPI320185
20095501	EPI320188	EPI320187
20095509	EPI320190	EPI320189
20095771	EPI320192	EPI320191
20095911	EPI320194	EPI320193
20096074	EPI320196	EPI320195
20096365	EPI320198	EPI320197
20096928	EPI320200	EPI320199
20096934	EPI320202	EPI320201
20097097	EPI320204	EPI320203
20097101	EPI320206	EPI320205
20097105	EPI320208	EPI320207
20097155	EPI320210	EPI320209
20097208	EPI320212	EPI320211
20097214	EPI320214	EPI320213
20097367	EPI320216	EPI320215
20097388	EPI320218	EPI320217
20097391	EPI320220	EPI320219
20097639	EPI320222	EPI320221

GISAID: Global Initiative on Sharing Avian Influenza Data; M: matrix; NA: neuraminidase.

[a] 2009XXXX stands for A/Paris/XXXX/2009.

Acknowledgments

We are indebted to the members of the GROG and Réseau National des Laboratoires (RENAL) sentinel networks who provided the specimens from which viruses were isolated. We thank G. Simon (French Agency for Food, Environmental and Occupational Health & Safety (ANSES, Ploufragan, France), M. Peiris (The University of Hong Kong, Hong Kong) and N. Cox (Centers for Disease Control and Prevention (CDC), Atlanta, United States) for providing the swine and triple reassortant strains. We gratefully acknowledge the contribution of the members of the National Influenza Center (Northern-France), Mathilde Benassaya, David Briand, Frédérique Cuvelier, Sébastien Legal, Jennifer Martinez, Vanessa Roca and Jessy Vandekerkhove for isolation and characterisation of the viruses. This work was funded by the Institut Pasteur and by the European Community's Seventh Framework Programme (FP7/2007-2013) under the project 'European Management Platform for Emerging and Re-emerging Infectious disease Entities' (EMPERIE) EC grant agreement number 223498.

References

1. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009 18;360(25):2605-15.
2. Naffakh N, van der Werf S. April 2009: an outbreak of swine-origin influenza A(H1N1) virus with evidence for human-to-human transmission. Microbes Infect. 2009;11(8-9):725-8.
3. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature. 2009;459(7249):931-9.
4. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009;325(5937):197-201.
5. Olsen CW. The emergence of novel swine influenza viruses in North America. Virus Res. 2002;85(2):199-210.
6. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, et al. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. N Engl J Med. 2009;360(25):2616-25.
7. Girard MP, Tam JS, Assossou OM, Kieny MP. The 2009 A (H1N1) influenza virus pandemic: a review. Vaccine. 2010;28(31):4895-902.
8. Richman DD, Murphy BR. The association of the temperature-sensitive phenotype with viral attenuation in animals and humans: implications for the development and use of live virus vaccines. Rev Infect Dis. 1979;1(3):413-33.
9. Mackenzie J. Virulence of temperature-sensitive mutants of influenza virus. Br Med J. 1969;3(5673):757-8.
10. Sabin A, Lwoff A. Relation between reproductive capacity of polioviruses at different temperatures in tissue culture and neurovirulence. Science. 1959;129:1287-8.

11. Christodoulou C, Colbere-Garapin F, Macadam A, Taffs LF, Marsden S, Minor P, et al. Mapping of mutations associated with neurovirulence in monkeys infected with Sabin 1 poliovirus revertants selected at high temperature. J Virol. 1990;64(10):4922-9.
12. Alford RH, Kasel JA, Gerone PJ, Knight V. Human influenza resulting from aerosol inhalation. Proc Soc Exp Biol Med.1966;122(3):800-4.
13. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56(1):152-79.
14. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84(2):268-78.
15. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, et al. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog. 2007;3(10):1374-9.
16. Massin P, van der Werf S, Naffakh N. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol. 2001;75(11):5398-404.
17. Massin P, Kuntz-Simon G, Barbezange C, Deblanc C, Oger A, Marquet-Blouin E, et al. Temperature sensitivity on growth and/or replication of H1N1, H1N2 and H3N2 influenza A viruses isolated from pigs and birds in mammalian cells. Vet Microbiol. 2009 19;142(3-4):232-41.
18. Husseini RH, Sweet C, Collie MH, Smith H. Elevation of nasal viral levels by suppression of fever in ferrets infected with influenza viruses of differing virulence. J Infect Dis. 1982;145(4):520-4.
19. Vaux S, Brouard C, Fuhrman C, Turbelin C, Cohen J, Valette M, et al. Dynamique et impact de l'épidémie A(H1N1)2009 en France métropolitaine, 2009-2010 [Dynamics and impact of the A(H1N1)2009 epidemic in metropolitan France, 2009-2010]. Bulletin Epidémiologique Hebdomadaire. 2010;24-25-26:259-66. French. Available from: http://www.invs.sante.fr/beh/2010/24_25_26/beh_24_25_26_2010.pdf
20. Vijaykrishna D, Smith GJ, Pybus OG, Zhu H, Bhatt S, Poon LL, et al. Long-term evolution and transmission dynamics of swine influenza A virus. Nature. 201126;473(7348):519-22.
21. Deyde VM, Sheu TG, Trujillo AA, Okomo-Adhiambo M, Garten R, Klimov AI, et al. Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. Antimicrob Agents Chemother. 2010;54(3):1102-10.
22. Bright RA, Medina MJ, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, et al. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet. 2005;366(9492):1175-81.
23. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg. 1938;27:493-7.
24. Drews SJ, Pabbaraju K, Wong S, Tokaryk KL, May-Hadford J, Lee B, et al. Surveillance of autopsy cases for D222G substitutions in haemagglutinin of the pandemic (H1N1) 2009 virus in Alberta, Canada. Clin Microbiol Infect. 2011;17(4):582-4.
25. Ikonen N, Haanpää M, Rönkkö E, Lyytikäinen O, Kuusi M, Ruutu P, et al. Genetic diversity of the 2009 pandemic influenza A(H1N1) viruses in Finland. PLoS One. 2010;5(10):e13329.
26. Kilander A, Rykkvin R, Dudman SG, Hungnes O. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010. Euro Surveill. 2010;15(9):pii=19498. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19498
27. Mak GC, Au KW, Tai LS, Chuang KC, Cheng KC, Shiu TC, et al. Association of D222G substitution in haemagglutinin of 2009 pandemic influenza A (H1N1) with severe disease. Euro Surveill. 2010;15(14);15(14):pii=19534. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19534
28. Gubareva LV. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res. 2004;103(1-2):199-203.
29. Wang MZ, Tai CY, Mendel DB. Mechanism by which mutations at his274 alter sensitivity of influenza A virus N1 neuraminidase to oseltamivir carboxylate and zanamivir. Antimicrob Agents Chemother. 2002;46(12):3809-16.
30. Shieh WJ, Blau DM, Denison AM, Deleon-Carnes M, Adem P, Bhatnagar J. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. Am J Pathol. 2010;177(1):166-75.
31. Viasus D, Paño-Pardo JR, Pachón J, Campins A, López-Medrano F, Villoslada A, et al. Factors associated with severe disease in hospitalized adults with pandemic (H1N1) 2009 in Spain. Clin Microbiol Infect. 2011;17(5):738-46.
32. Van Kerkhove MD, Vandemaele KA, Shinde V, Jaramillo-Gutierrez G, Koukounari A, Donnelly CA, et al. Risk factors for severe outcomes following 2009 Influenza A (H1N1) Infection: a global pooled analysis. PLoS Med. 2011;8(7):e1001053.
33. Yu H, Feng Z, Uyeki TM, Liao Q, Zhou L, Feng L, et al. Risk factors for severe illness with 2009 pandemic influenza A (H1N1) virus infection in China. Clin Infect Dis. 2011;52(4):457-65.
34. Ingram DL, Mount LE. The metabolic rates of young pigs living at high ambient temperatures. Res Vet Sci. 1965;6:300-6.

Nosocomial and non-nosocomial *Clostridium difficile* infections hospitalised patients in Belgium - compulsory surveillance data from 2008 to 2010

N Viseur (natacha.viseur@wiv-isp.be)[1], M L Lambert[1], M Delmée[2], J Van Broeck[2], B Catry[1]
1. Public Health and Surveillance Department, Scientific Institute for Public Health, Brussels, Belgium
2. Microbiology Unit, Catholic University of Louvain, Brussels, Belgium

Citation style for this article:
Viseur N, Lambert ML, Delmée M, Van Broeck J, Catry B. Nosocomial and non-nosocomial Clostridium difficile infections hospitalised patients in Belgium - compulsory surveillance data from 2008 to 2010.
Euro Surveill. 2011;16(43):pii=20000. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20000

Article published on 27 October 2011

Surveillance of *Clostridium difficile* infection (CDI) is compulsory in Belgian hospitals. Our objectives were to compare incidence and case characteristics of nosocomial infections (Nc-CDI) with onset of diarrhoea more than two days after hospital admission, with non-nosocomial cases (Nnc-CDI). The database included inpatients from 2008 to 2010. Of 8,351 cases reported by 150 hospitals, 3,102 (37%) were classified as Nnc-CDI and 5,249 (63%) as Nc-CDI. In 2010, the mean incidence per 1,000 admissions was 0.95 for Nc-CDI and 0.56 for Nnc-CDI. Both incidences were relatively stable over the three years, with a slight decrease in 2010 ($p<0.01$). Onset of symptoms in Nnc-CDI cases took place in the community (57.1%), nursing homes (14.2%) or hospitals (17.5%); data for 11.2% were missing. Nnc-CDI cases were younger than Nc-CDI (median age 75 vs. 79 years, $p<0.001$), and more likely to be women (62% vs. 57%, $p<0.001$) and to have pseudomembranous colitis (5.3% vs. 1.6%, $p<0.001$). In 2009, *C. difficile* ribotype 027 was found in 32 of 70 reporting hospitals compared with 19 of 69 in 2010 ($p<0.03$). Although our study population only included hospitalised patients, the results do not support the hypothesis of an increase in the incidence of severe community-associated CDI.

Introduction

Clostridium difficile infection (CDI) is the leading cause of diarrhoea in healthcare settings. In recent years, an increase in the incidence and the severity of nosocomial (Nc) or healthcare-associated CDI has been reported in Canada [1], the United States [2], and several European countries [3]. This rise has in part been explained by the emergence of a new virulent strain, PCR ribotype 027 [4], although in some countries, for instance Germany, an increase in CDI incidence had been described several years before the virulent strain 027 occurred [5].

In Belgium, CDI incidence and mortality more than doubled between 1998 and 2007 [6]. PCR ribotype 027 was identified for the first time in 2005 from a hospital outbreak [7]. In that context, a nation-wide prospective surveillance of CDI in hospitals was introduced by the Institute of Public Health (WIV-ISP) in 2006 which became compulsory in July 2007.

Although strains attributed to severe pathology are generally found in hospital inpatients, recent reports suggest that the occurrence and severity of CDI in the community is also increasing [8,9]. Moreover, CDI is increasingly recognised as a cause of diarrhoea in populations previously considered to be at low risk [10-12]. Recent reports have shown that CDI, including severe cases, can also occur in infants and children [13,14], healthy young people living in the community, and peripartum women [8,15].

In this article, using data from the compulsory surveillance of CDI in hospitals in Belgium, we compared incidence of Nc and non-nosocomial (Nnc) cases over the last three years (2008–2010) and compared the cases in terms of age, sex, and severity of the infection.

Methods

Prospective surveillance of CDI in hospitals in Belgium was introduced in July 2006 and became compulsory in July 2007 for all hospitals. Psychiatric and chronic care hospitals of less than 150 beds are statutory excluded, but their voluntary participation is encouraged. The full surveillance protocol [16] and the annual report of the surveillance [17] are available in Dutch and French at our dedicated website.

Study design

We analysed all CDI cases recorded in the hospital-based surveillance for hospitals participating in the period from 1 January 2008 to 31 December 2010.

Definitions

Recent recommendations for the definition of CDI cases [18] were followed in the protocol. A CDI case

was defined as a patient with symptoms of diarrhoea or toxic megacolon combined with a positive result of a laboratory assay and/or endoscopic or histopathologic evidence of pseudomembranous colitis. Participating hospitals were asked to apply this definition to all hospitalised patients recorded during the surveillance. Fulfilment of the definition was checked for each individual case by the WIV-ISP.

Cases were classified as Nc-CDI cases if onset of diarrhoea took place more than two days after admission to the reporting hospital. All other cases were classified as Nnc-CDI cases, and the place of onset of symptoms was recorded.

Data analysis

Mean annual incidence was calculated as the sum of CDI cases across all reporting hospitals in a given year divided by the sum of denominators (i.e. number of hospital-days or admissions). Incidences for Nc-cases are expressed by hospital-days and by admissions. For Nnc and total cases, the denominator of hospital-days is irrelevant, and incidences were calculated only by admissions.

The STATA 10.1SE (StataCorp. College Station, Texas, United States) statistical package was used for analysis.

Ribotyping

In the surveillance protocol, each hospital is asked to send five consecutive isolates from CDI patients to the national *C. difficile* reference laboratory every year. All strains are ribotyped by PCR, based on the comparison of patterns of PCR products of the 16S–23S rRNA intergenic spacer regions using primers described by Barbut et al. [19]. The size of each peak was determined using GeneScan software or GeneMapper V.4 software (AB(Applied Biosystems).

Results

From 1 January 2008 to 31 December 2010, a total of 8,882 episodes of CDI were registered by 150 hospitals. We excluded 531 episodes (6%), which did not meet the case-definition for CDI (e.g. symptoms of diarrhoea without a positive result of toxin detection). Descriptive data were analysed from a total of 8,351 CDI episodes from 7,646 patients. For our incidences analysis, only hospitals that contributed for an entire calendar year were selected, which left a total of 6,733 CDI episodes registered by 136 hospitals. Of these, 4,214 were defined as nosocomial.

Incidences

The mean annual incidence of CDI across hospitals that contributed surveillance data for an entire year in the study period is shown in Table 1. The slightly decreasing incidence of Nc-CDI over the three consecutive years and the large variability of incidences between hospitals are shown in Figure 1. Overall, the mean incidence of CDI was 1.87, 1.82 and 1.52 per 1,000 admissions in 2008, 2009 and 2010, respectively and 37% of CDI were considered to be Nnc-cases. Trends over time of incidences of Nc and Nnc-CDI evolved in parallel and seemed to be stable (Table 1).

Ribotyping data from the reference laboratory

In 2010, *C. difficile* ribotype 027 was identified in 19 of 69 hospitals sending CDI stool samples for typing

Table 1

Incidence of *Clostridium difficile* infection in hospitals in Belgium, 2008–2010 (n=6,733)

Year	2008	2009	2010
Number of hospitals	107	107	91
Reported CDI			
Number of total cases	2,354	2,321	2,058
Proportion of nososomial cases[a]	63.3%	61.4%	63.3%
Mean incidence of CDI[b]			
All cases			
per 1,000 admissions	1.87	1.82	1.52[c]
Nosocomial cases[a]			
per 1,000 admissions	1.18	1.12	0.95
per 10,000 hospital-days	1.48	1.46	1.26
Non-nosocomial cases			
per 1,000 admissions	0.69	0.70	0.56

CDI: *Clostridium difficile* infections.

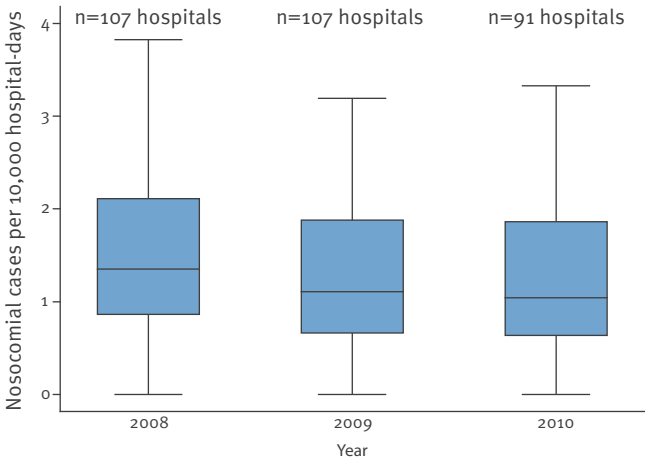
[a] Symptom onset (diarrhoea) more than two days after admission to reporting hospital.

[b] Total cases across reporting hospitals/total denominators.

[c] p<0.01, Pearson's chi-squared test: difference to 2008 incidence.

Figure 1

Distribution of incidences of nosocomial *Clostridium difficile* infection per 10,000 hospital-days in Belgian hospitals, 2008–2010 (n=4,214)



Boxes contain the middle 50% of the data; line in the box: median value; upper edge (hinge): 75th percentile; lower edge (hinge): 25th percentile; the range of the middle two quartiles is known as the inter-quartile range; the ends of the vertical lines (whiskers) indicate the minimum and maximum data values, unless outliers are present in which cases the whiskers extend to a maximum of 1.5 times the inter-quartile range.

Statistical outliers were excluded.

(Table 2). This proportion was lower than previous years (28% and 46% in 2010 and 2009, respectively; p<0.03).

Characteristics of patients with *Clostridium difficile* infection

The median age of the 7,646 CDI patients that met the case definition was 78 years (P25-P75: 64-85 years), and 59% were female. Of the 8,351 episodes of CDI associated with these patients, 5,249 episodes (63%) occurred more than two days after admission to the reporting hospital (Nc-CDI). In comparison with Nc-CDI patients, Nnc-CDI patients were younger (75 years for Nnc vs. 79 years for Nc cases, p<0.001 (Mann-Whitney test)) (Figure 2) and with a higher proportion of women (62% for Nnc vs. 57% for Nc cases, p<0.001 (Pearson's chi-squared test)).

With regard to the severity of the infection, the proportion of pseudomembranous colitis among Nnc-CDI cases was higher (5.3% for Nnc vs. 1.6% for Nc cases, p<0.001 (Pearson's chi-squared test)). In 2010, this proportion in Nnc-CDI cases was lower than in the previous years, although the difference was not statistically significant (4.9% and 6.1% in 2010 and 2008, respectively; p=0.27 (Pearson's chi-squared test)). For the 3,102 Nnc-CDI cases included in the study, onset of symptoms took place in the community (n=1,761, 57.1%), in a nursing home (n=447, 14.2%) or in the reporting or another hospital (n=543, 17.5%), while 11.2% (n=351) remained unclassified due to missing data.

Discussion

Data from the last three years showed a relatively stable incidence of CDI in Belgian hospitals, with a slight decrease in 2010. These findings do not support the hypothesis [8,9] of an increase in the incidence of severe (e.g. leading to hospitalisation) community-associated CDI. In Belgium, 75% of deaths associated with a CDI diagnosis take place in a hospital [6]. Therefore we consider our assessment of the severity of community-associated cases using only hospitalised cases a fair approximation of the reality. The large variability of incidences between hospitals seems unlikely to be attributed only to case mix variation. Health services research has led to the conclusion that unexplained variation in a number of important clinical areas is a very significant issue, and that there could be huge improvements if the poorest performers could match the best [20].

If the variation in *C. difficile* rates is not explained by case mix, then it might be due to differences in prevention practices, which would indicate that there is a potential for improved prevention. But more research is needed to test this hypothesis in the field of healthcare-associated infections. The proportion of hospitals where ribotype 027 was found decreased in 2010, but a limitation of these data is that only 70 hospitals sent samples for typing, against 110 who contributed to the epidemiological data.

The incidence of all CDI in Belgium was comparable with the incidences of 1.1 per 1,000 admissions reported in France in 2009 [21] and 1.8 per 1,000 admissions reported in the Netherlands in 2008 [22], although the data in these countries are collected voluntarily and thus not as representative compared with the compulsory Belgian surveillance. It was lower than in Germany (1.4 vs 4.6/1,000 admissions in 2007) [23]. Unlike in Belgium, participation in the national surveillance in

Table 2

Riboptypes distribution among *Clostridium difficile* infections in Belgian hospitals, 2008–2010 (n=1,663 samples)

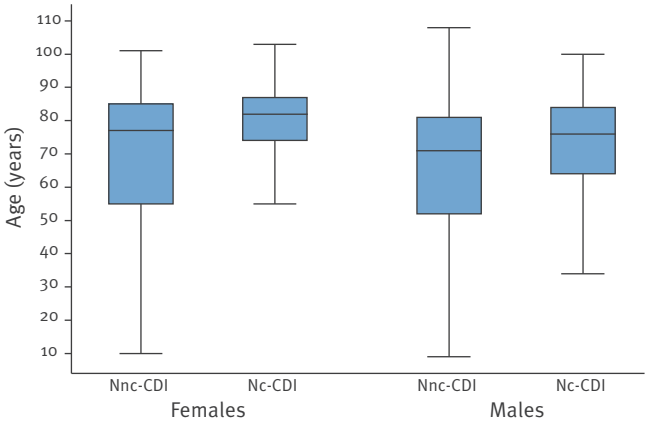
Year	2008 Jan-Jun	2009 Jan-Jun	2010 Jan-Jun
Number of hospitals who sent CDI stool samples for typing	51	70	69
Hospitals with ribotype 027			
Number	28	32	19
Proportion	55%	46%	28% [a]
Hospitals with ribotype 014			
Number	10	25	23
Proportion	20%	36%	33%
Hospitals with ribotype 078			
Number	19	8	16
Proportion	37%	11%	23%

CDI: *Clostridium difficile* infections.

[a] p<0.03, Pearson's chi-squared test: difference to 2009 proportion.

Figure 2

Age distribution of patients with *Clostridium difficile* infections in Belgian hospitals, 2008–2010 (n=7,646 patients)



CDI: *Clostridium difficile* infections; Nc: nosocomial; Nnc: non-nosocomial.

Statistical outliers were excluded.

Germany is voluntary and a bias towards a participation of hospitals with higher incidence might have been involved.

The incidence of Nc-CDI in Belgium was lower than that reported in a European-wide hospital-based survey specific for CDI, performed in 2008 (97 hospitals in 34 European countries) [24]. Importantly, the incidence of Nc-CDI in the three Belgian hospitals that participated in that survey (2.8/10,000 hospital-days) was higher than the incidence here reported in our nation-wide surveillance of 98 hospitals (1.5/10,000 hospital-days in 2008), which suggests that, at least for Belgium, the survey overestimated the incidence numbers due to the limit number of hospitals included. In England [25] and in Québec [26], Nc-CDI surveillance is also compulsory. The mean incidence of Nc-CDI in these countries in the period from April 2009 to March 2010 was higher (3.6 and 6.3/10.000 hospital-days, respectively) than in Belgium (1.3/10.000 hospital-days in 2010).

The strengths of the Belgian surveillance are the wide coverage of hospital data and the possibility for hospitals to follow their incidence in a secured web-based follow-up system in real time, thus they receive rapid feedback after recording, which can be used to make local improvements. Indeed, comparable data, i.e. data from a representative sample of hospitals and recent data, are scarce in the literature. A limitation of our system is that the data do not allow us to provide incidence per inhabitants and to estimate a cross-transmission at national level.. By definition, our surveillance only includes the more severe (hospitalised) community-associated CDI.

Conclusion

The incidence of all CDI in hospitalised patients from Belgium was stable over the period 2008 to 2010, although a slight decrease was observed in 2010. The incidence in Belgium is comparable with the incidence reported in surrounding countries like France and the Netherlands. Although our study population only included hospitalised patients, the results do not support the hypothesis [8,9] of an increase in the incidence of severe community-associated CDI. The large variability of incidence between the hospitals in our study suggests that a proportion of these infections, although hard to quantify, could be prevented by improving classical infection control methods. Current CDI surveillance in Belgium is a useful and valid tool to monitor trends in incidence and severity of cases in Belgian hospitals.

References

1. Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, et al. Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ. 2004;171(5):466-72.
2. Zilberberg MD, Shorr AF, Kollef MH. Increase in adult Clostridium difficile-related hospitalizations and case-fatality rate, United States, 2000-2005. Emerg Infect Dis. 2008;14(6):929-31.
3. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML,et al. Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill. 2008;13(31):pii=18942. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18942
4. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366(9491):1079-84.
5. Zaiß NH, Witte W, Nübel U. Fluoroquinolone resistance and Clostridium difficile, Germany. Emerg Infect Dis. 2010;16(4):675-7.
6. Gutierrez I, Lambert ML. Trends in mortality and morbidity related to Clostridium difficile infections, Belgium 1998-2007. Brussels: Scientific Institute for Public Health; April 2010. ISSN : D/2010/2505/18. Available from: http://www.nsih.be/download/CDIF/CDI_trends_mortality_morbidity_final_report_June_2010.pdf
7. Joseph R, Demeyer D, Vanrenterghem D, van den Berg R, Kuijper E, Delmee M. First isolation of Clostridium difficile PCR ribotype 027, toxinotype III in Belgium. Euro Surveill. 2005;10 (42):pii=2815. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2815
8. Centers for Disease Control and Prevention (CDC). Severe Clostridium difficile-asssociated disease in populations previously at low risk -- four states, 2005. MMWR Morb Mortal Wkly Rep. 2005;54(47):1201-5.
9. Centers for Disease Control and Prevention (CDC). Surveillance for community-associated Clostridium difficile--Connecticut, 2006. MMWR Morb Mortal Wkly Rep. 2008;57(13):340-3.
10. Kutty PK, Woods CW, Sena AC, Benoit SR, Naggie S, Frederick J, et al. Risk factors for and estimated incidence of community-associated Clostridium difficile infection, North Carolina, USA. Emerg Infect Dis. 2010;16(2):197-204.
11. McFarland LV, Clarridge JE, Beneda HW, Raugi GJ. Fluoroquinolone use and risk factors for Clostridium difficile-associated disease within a veterans administration health care system. Clin Infect Dis. 2007;45(9):1141-51.
12. Wilcox MH, Mooney L, Bendall R, Settle CD, Fawley WN. A case-control study of community-associated Clostridium difficile infection. J Antimicrob Chemother. 2008;62(2):388-96.
13. Zilberberg MD, Shorr AF, Kollef MH. Increase in Clostridium difficile-related hospitalizations among infants in the United States, 2000-2005. Pediatr Infect Dis J. 2008;27(12):1111-13.
14. Kim J, Smathers SA, Prasad P, Leckerman KH, Coffin S, Zaoutis T. Epidemiological Features of Clostridium difficile-Associated Disease Among Inpatients at Children's Hospitals in the United States, 2001-2006. Pediatrics. 2008;122(6):1266-70.
15. Rouphael NG, O'Donnell JA, Bhatnagar J, Lewis F, Polgreen PM, Beekmann S, et al. Clostridium difficile-associated diarrhea: an emerging threat to pregnant women. Am J Obstet Gynecol. 2008;198(6):635-6.
16. Scientific Institute for Public Health (ISP). Surveillance des infections à Clostridium difficile. Protocole. [Surveillance of Clostridium difficile infections. Protocol]. Version 4.0. Brussels: ISP; 2010. French. Available from: http://www.nsih.be/surv_cdif/download_fr.asp
17. Viseur N, Lambert ML. Epidémiologie des infections à Clostridium difficile en Belgique; rapport 2011. [Epidemiology of Clostidium difficile infections in Belgium ; report 2011]. Brussels: Scienific Institute for Public Health; 2011. ISSN – version online: 2034-4562. French. Available from:http://www.nsih.be/download/CDIF/CDIF-AR-2011-FR.pdf
18. Bauer MP, Kuijper EJ, van Dissel JT; European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for Clostridium difficile infection (CDI). Clin Microbiol Infect. 2009;15(12):1067-79.
19. Barbut F, Mario N, Delmée M, Gozlan J, Petit JC. Genomic fingerprinting of Clostridium difficile isolates by using a random amplified polymorphic DNA (RAPD) assay. FEMS Microbiol Lett. 1993;114(2):161-6.
20. The NHS Confederation. Variation in healthcare: does it matter and can anything be done? London: NHS Confederation; 2004. Available from: http://www.nhsconfed.org/Publications/Documents/Variation%20in%20healthcare.pdf
21. Coignard B, Eckert C, Rahib D, Hébert M, Boussat S, Jarno P, et al. Caractéristiques épidémiologiques et microbiologiques des infections à Clostridium difficile en France: résultats de l'étude ICD-RAISIN 2009. [Epidemiological and microbiological characteristics of Clostridium difficile infections in France: reults of the ICD-RAISIN 2009 study]. Powerpoint presentation. XXIth congress of the de la SFHH. 3 June 2010; Bordeaux, France. French. Available from: http://www.cleanis.fr/docs/news/fr/icd-en-france-en-2009_12784041873816680O.pdf

22. Hensgens MP, Goorhuis A, Notermans DW, van Benthem BH, Kuijper EJ. Decrease of hypervirulent Clostridium difficile PCR ribotype 027 in the Netherlands. Euro Surveill. 2009;14(45):pii=19402. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19402

23. Gastmeier P, Weitzel-Kage D, Behnke M, Eckmanns T. Surveillance of Clostridium difficile-associated diarrhoea with the German nosocomial infection surveillance system KISS (CDAD-KISS). Int J Antimicrob Agents. 2009;33 Suppl 1:S19-S23.

24. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, et al. Clostridium difficile infection in Europe: a hospital-based survey. Lancet. 2011;377(9759):63-73.

25. Health Protection Agency (HPA). Results of the mandatory Clostridium difficile reporting scheme by acute Trust in UK (2007-2010). London: HPA. [Accessed June 2011]. Available from: http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1195733750761

26. Surveillance data on Clostridium difficile Infection in Québec hospitals. ISBN no: 2-550-45949-0. Québec: Ministère de la Santé et des Services sociaux (MSSS). [Accessed June 2011]. French. Available from: http://msssa4.msss.gouv.qc.ca/en/document/publication.nsf/fb143c75e0c27b69852566aa0064b01c/9ee11991af5fce358525753c00650c88?OpenDocument