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Human case of West Nile neuroinvasive disease in Portugal, summer 2015

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A case of West Nile virus (WNV) infection was reported in the Algarve region, Portugal, in the first week of September 2015. WNV is known to circulate in Portugal, with occasional reports in horses and birds (2004 to 2011) and very sporadically human cases (in 2004 and in 2010). Here we present the clinical and laboratory aspects related to the first human case of West Nile neuroinvasive disease reported in Portugal.

In September 2015, a case of West Nile neuroinvasive disease was reported in a patient with neurological symptoms by molecular and serological methods at the National Health Institute (INSA), the reference laboratory for the diagnosis of flaviviruses in Portugal. Although all samples were negative for the presence of West Nile virus (WNV) RNA, positive immunofluorescence results were confirmed by virus neutralisation tests meeting the European Union case definition for WNV infections.

Case description

On 21 July 2015 a man in his 70s was assessed in the emergency room of Faro Hospital with a history of six days of high fever (≥ 38.5 °C), headache and altered state of consciousness with progressive prostration, drowsiness and lethargy, confusion, gait difficulty and urinary retention. A macular rash was seen on arms and thighs.

The patient, who lives in a rural area in Almancil, Algarve had frequent contact with animals (chickens, pigs, sheep and horses), had not travelled abroad in the previous year and reported that he had never been vaccinated against flaviviruses (yellow fever, tick-borne encephalitis or Japanese encephalitis virus).

The laboratory findings in the hospital showed haemoglobin 12.4 mg/dL (norm: 13–17 g/dL), leukocytes 14.8 × 10^9/L (norm: 4–10 × 10^9/L) and C-reactive protein 40 mg/L (norm: < 5 mg/L). Cerebrospinal fluid (CSF) showed 103 cells/mm^3 (norm: < 6 cells/mm^3) with a predominance of lymphocytes, 143 mg/dL protein (norm: 15–60 mg/dL) and 58 mg/dL glucose (norm: 40–60 mg/dL).

At the emergency room, viral meningoencephalitis, rickettsial meningoencephalitis or bacterial meningitis diagnosis were considered and the patient was empirically treated with ceftriaxone (2 g, 12/12 h for 14 days) and doxycycline (100 mg, 12/12 h for 7 days). CSF sample molecular analyses were requested for neurotropic viruses (herpes simplex viruses (1 and 2), Varicella zoster virus, Epstein-Barr virus, cytomegalovirus, human herpes virus (6, 7 and 8) and enterovirus) and arboviruses (Toscana virus and WNV). Serological analysis for *Borrelia burgdorferi*, *Coxiella burnetii*, *Brucella* sp., Epstein-Barr virus, Hepatitis B and C viruses, human-immunodeficiency virus (1 and 2), *Mycoplasma pneumonia*, *Rickettsia conorii* and *Treponema pallidum* were also requested. All results became negative except for *C. burnetii* IgG 200 (cut-off: 200), and serological analysis were requested for Toscana virus and WNV.

After 14 days, the patient had a good outcome and was discharged with minimal residual neurological disease, i.e. some slowing in speech and action. Thirty-four days after the onset of symptoms he returned for further evaluation and blood tests, and neurological examination showed full recovery.

Molecular and serological diagnostics

In the National Institute of Health (INSA), a CSF sample taken on day 6 after symptoms onset was negative

for West Nile virus (WNV) in a RT-PCR specific for WNV lineages 1 and 2 [1]. A urine sample collected on day 34 after symptom onset was also negative by real-time RT-PCR [1,2]. Both samples were also negative when tested by generic pan-flavivirus conventional RT-PCR [3].

A serum sample taken on day 16 after symptom onset was tested by an in–house immunofluorescence assay was positive for WNV-specific IgM with a titre of 1,024 (cut-off: 16) and IgG with a titre of 2,048 (cut-off: 32). A second serum sample taken on day 34 after symptom onset showed a WNV-specific IgM titre of 64 and IgG titre of 4,096. Both serum samples were also subjected to immunofluorescence assays for immunoglobulins specific to other flaviviruses, such as yellow fever, tick-borne encephalitis, Dengue, Zika and Japanese encephalitis virus. All were negative for IgM and presented IgG cross-reaction. A WNV-specific IgM antibody response in CSF, a criterion for case confirmation, was not tested due to the lack of available samples after PCR diagnosis.

The serum samples from day 16 and 34 were tested by microneutralisation assay. Replicates of twofold dilutions of the inactivated test sera were incubated with 100 TCID50 of WNV (strain Egypt 101) at 37 °C for 1 hour in 96-well microtitre plates. Vero E6 cells were added (2×10^4/well) and plates were incubated at 37 °C under 5% CO_2. Plates were examined microscopically for cytopathic effects at 4 days post addition of cells. Standard positive (West Nile (strain Eg 101) immune mouse ascytis fluid, CDC, Atlanta) and negative sera were included in the assays. Virus used in each run of the test was back-titrated to confirm the validity of the test. Titres were assigned arithmetically as the dilution of serum giving a 50% neutralisation endpoint. Serum neutralising antibody titres of 10 or higher were considered significant. Microneutralisation assays of both serum samples were positive to a titre of 2,560.

Background

WNV is the most widely distributed mosquito-transmitted flavivirus in the world, and the aetiological agent of West Nile fever and West Nile neuroinvasive disease (WNND) [4]. The virus is maintained in nature in enzootic cycles involving ornithophilic mosquitoes, mainly *Culex* species, as primary vectors and some species of birds as primary reservoirs.

WNV transmission via blood transfusion or organ transplantation is a public health threat because WNV disease symptoms are estimated to occur in only about 20% of infected people (of those, less than 1% may develop WNND). Most infections are asymptomatic (80%) and asymptomatic viraemic donors can transmit the virus to immunocompromised or vulnerable recipients [5]. The acknowledgement of the risk of infected blood donations in the affected areas and the emergence of WNV in Europe in the past 10 years prompted the European Commission to release a preparedness plan for WNV and blood safety in 2012 [6].

WNV is known to circulate in Portugal with frequent detections in horses and birds [7]. INSA performs reference laboratory diagnosis of flaviviruses in Portugal and previously identified a probable human case in 2010 [8], triggering a WNV survey in horses living in the same area. The survey identified two WNV-positive horses [7]. The first confirmed human cases were diagnosed in two tourists in Ireland after a trip to Algarve in 2004 [9]; they had acquired the infection in the proximity of the human case identified this year.

Public health measures

The patient reported here represents the first serum-positive case to date in Portugal. After the communication of the clinical suspicion of a probable case of WNND, clinical, epidemiological and serological surveillance was implemented by the local health authorities in order to assess the possible presence of WNV in susceptible species in the area [10].

Although the detection of viral RNA is an unambiguous prove of WNV infection, it is known to be challenging in patients with symptomatic infections because viraemia can be low or absent at the time of symptom onset [2]. The negative PCR results in CSF and urine were not unexpected seeing as the samples were collected after the beginning of symptoms. The positive immunofluorescence results were confirmed by virus neutralisation test to ascertain the case confirmation according to the European Union case definition for WNV infections [11].

On 3 September, the General Directorate of Veterinary (DGAV; Direcção Geral de Alimentação e Veterinária, Ministério da Agricultura e do Mar) reported three new outbreaks in horses in Loulé, Algarve municipality, and so far, four of 82 horses analysed were positive for WNV infection [12].

The Algarve region possesses a large a coastal area characterised by marshlands, salt marshes, small islands, dunes and beaches. Several wetlands and bird sanctuaries are present. Fishery, aquaculture and salt extraction are important human activities, as is tourism particularly in summer. A nationwide vector surveillance programme (REVIVE) has covered the Algarve region since 2008 [13].

Conclusion

Veterinary, human and vector surveillance was initiated in the Algarve municipality after the laboratory report of the WNND human case and is still ongoing. This case highlights the essential role of laboratory diagnostics for early detection and implementation of control measures in vector-borne diseases outbreaks.

Conflict of interest

None declared.

Authors' contributions

LZZ: manuscript preparation and molecular diagnosis at INSA; PP: clinical data and laboratory findings in Algarve; LZZ, MJA, SG, TL, PP: serological diagnosis at INSA; MJA, MF: virus neutralisation diagnosis; MJA: laboratory coordination at INSA. All authors collaborated in the work and participated in the final revision of the manuscript.

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Knowledge, attitudes and practices concerning Middle East respiratory syndrome among Umrah and Hajj pilgrims in Samsun, Turkey, 2015

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We performed a questionnaire study to determine knowledge, attitudes and practices concerning Middle East respiratory syndrome (MERS) among people intending to participate in the Hajj or Umrah Muslim pilgrimages. Of the 381 respondents aged between 17 and 85 years, 55% had never heard of MERS, while only one in three knew that it is a respiratory disease. Approximately half were insufficiently informed about protective measures. Prospective pilgrims do not seem prepared to take such precautions.

We performed a survey among people intending to visit the Arabian Peninsula for the Hajj or Umrah pilgrimages and aimed to determine their awareness about Middle East respiratory syndrome (MERS).

Hajj in 2015 will take place from 21 to 25 September. Umrah is a similar pilgrimage that can be undertaken at any time of the year but is likely to be more crowded during the month of Ramadan (18 June to 17 July 2015). In 2015, 55,540 people from Turkey secured the right to perform the Hajj [1]. Because of the ability of infectious diseases to spread rapidly at mass gatherings, the Saudi Ministry of Health (MoH) advises people 65 years and older, pregnant women and children under 12 years and individuals with weak immune systems or chronic diseases to postpone travelling as long as there is the risk of MERS in the area [2]. People wishing to perform the Hajj or Umrah are advised to follow general hygiene measures such as regular hand washing, using disposable materials and using masks [2-4]. Knowledge and application of basic hygiene principles and measures in such an environment is therefore vitally important.

Enrolment in the study

Residents of Samsun, Turkey, intending to perform the Hajj or the Umrah and applying for immunisation to community health centres before the busiest months for pilgrimages were enrolled during the period from 4 May to 24 July 2015. Samsun is a city with a population of 1.25 million on the north coast of Turkey. It has seven community health centres providing immunisation services for prospective pilgrims (four in the central area and three in outlying districts). Two of these (one in the centre and one outlying) were chosen for this study. In face-to-face interviews, study participants were administered a questionnaire with 16 questions on demographic data and knowledge, attitudes and practices concerning MERS. The participants were informed about the study before the interview. The study was approved by the ethics committee of the Ondokuz Mayıs University Clinical Research Ethics Commission.

Survey participants

Of the 381 participants, 49% (185 people) were male and 51% (196 people) female. Mean ages were 59 ± 12 years (range: 17-85). Education above primary school level was recorded for 26% of the participants (99 people). Sixty-four per cent (244 people) met at least one of the conditions for which the Saudi MoH advises postponement of travel. Chronic diseases, hypertension, diabetes and obesity were significantly more common in women than men ($p<0.05$). The influenza vaccination coverage (2014/15 season) was only 7.1% (27 people). Table 1 shows demographic data and comorbidities for Umrah and Hajj pilgrims who participated in the study.

A quarter (n = 97) participants had gone on the Hajj or Umrah at least once previously. The mean number of previous pilgrimages they had made was 1.85 ± 1.71.

Knowledge about Middle East respiratory syndrome

The proportion of participants who had not heard of MERS was 55.6% (212 people). Awareness of MERS was significantly higher among those who had gone on

Table 1

Demographic data and comorbidities in prospective Umrah and Hajj pilgrims, Samsun, Turkey, May–July 2015 (n = 381)

	Women n = 196	Men n = 185	Total n = 381	p
People older than 65 years	69 (35%)	67 (36%)	136 (36%)	0.460
Children younger than 12 years	0 (0%)	0 (0%)	0 (0%)	NA
Pregnant women	1 (1%)	NA	1 (0%)	NA
People with chronic diseases	117 (60%)	81 (44%)	198 (52%)	0.001
• Hypertension	88 (45%)	44 (24%)	132 (35%)	0.000
• Diabetes	51 (26%)	28 (15%)	79 (21%)	0.006
• Respiratory diseases	14 (7%)	9 (5%)	23 (6%)	0.237
• Heart diseases	28 (14%)	21 (11%)	49 (13%)	0.242
• Kidney diseases	7 (4%)	7 (4%)	14 (4%)	0.563
People with cancer	3 (2%)	0 (0%)	3 (1%)	0.135
People with weakened immune system or taking immunosuppressive drugs	1 (1%)	0 (0%)	1 (0%)	0.514
At least one condition for which the Saudi MoH recommends postponement of the Hajj and Umrah	133 (68%)	111 (60%)	244 (64%)	0.068
Obesity	36 (18%)	17 (9%)	53 (14%)	0.007
People vaccinated against influenza (2014/15 season)	14 (7%)	13 (7%)	27 (7%)	0.562

MoH: Ministry of Health; NA: not applicable.
A p value of $p<0.05$ was considered significant.

a pilgrimage before (chi-square test: 6.748; p = 0.007) and those with university education level (chi-square test: 46,718; p<0.001). Only 34% of participants (129 people) knew that MERS is a respiratory disease. Of those 169 who were aware of MERS, 60% (101 people) had heard of it through newspapers or television, 25% (43 people) from healthcare workers and only 4% (seven people) from religious officials. Once informed about MERS, almost half of the participants realised the importance of protective measures against MERS-Cov infections such as hand washing, mask use and avoiding contact with sick people (Table 2). However, only 22.83% (87 people) knew that antibiotics are ineffective against MERS.

While 76% of participants (288 people) said they did not intend to take protective measures against MERS-CoV infections during the Hajj or Umrah, 21% (78 people) said they would wear a mask, 14% (54 people) that they would take care in regard to hand washing and 0.5% (two people) that they would use hand disinfectants. People with university degrees were significantly more likely to take protective measures than others (chi-square test: 8.093; p=0.005).

Discussion

Although this study cannot be generalised to the entire country, it was the first of its kind in Turkey. According to data from the Ministry of Hajj on 16 July 2015, 5,715,051 Muslims have visited Saudi Arabia to perform Umrah this year [5]. Since its emergence in 2012, the majority of MERS cases have been reported from Saudi Arabia [6]. Cases have also been reported from 25 other countries [7]. According to data from the World Health Organization on 7 November 2015, there have been 1,495 confirmed cases with 528 deaths to date [8]. The first fatal case from Turkey occurred in 2014 [9].

On the basis of Saudi MoH recommendations, 64% of our survey participants should have postponed travelling, but none of them decided to postpone the pilgrimage. That proportion was 18% in an Australian in 2014 and 50% in a French study in 2013 [11,12].

More than half of the prospective pilgrims had never heard of MERS. In previous studies, 65% of French pilgrims and 35% of Australian pilgrims knew about MERS circulation on the Arabian Peninsula [11,12]. Most of those who were aware had learned of it through newspapers or television. Healthcare personnel and religious officials were much rarer sources of information. However, studies have shown that community leaders (e.g. Imams) and healthcare professionals play an important role in health promotion measures [13,14].

The awareness of healthcare personnel and religious officials concerning basic hygiene principles and measures should be increased through effective education and communication strategies. Health education programmes at the entry points to Saudi Arabia have been shown to improve pilgrims' knowledge [15]. Compared with one French (90%) and one Australian study (64%), the level of information concerning protective measures was generally insufficient [11,16]. This rate was 42% in our study, where most pilgrims did not understand the importance of MERS coronavirus infection. Only one in four of our participants intended to

Table 2

Knowledge of prospective Umrah and Hajj pilgrims about protective measures against Middle East respiratory syndrome and other respiratory diseases, Samsun, Turkey, May–July 2015 (n = 381)

Protective measures	Number	Percentage
Washing hands with soap and water	218	57
Using disposable/single-use materials	107	28
Not touching the mouth or nose before hand washing	121	32
Avoiding direct contact with sick individuals	184	48
Using masks, particularly in crowded areas	188	49
Using hand disinfectants	145	38

take precautions during their pilgrimage. Higher education level appeared to be linked to the intention to take precautions, but attitudes did not always change with knowledge. For example, Iranian pilgrims of old age and with low education level had little knowledge about health subjects, but they had a good health attitude and practice [17].

Attitudes and behaviours of prospective pilgrims can be improved by emphasising the importance of basic hygiene principles and measures through well-structured education programmes, both on MERS and other infectious diseases.

When pilgrims visit health centres for immunisations or mosques for religious rituals, these are occasions for health professionals and imams to inform about MERS.

Conflict of interest

None declared.

Authors' contributions

Mustafa Kürşat Şahin: study design, results interpretation, writing the manuscript; Servet Aker: statistics, analysing results, reviewing the manuscript; Ebru Kaynar Tunçel: data collection.

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Hospital-based *Clostridium difficile* infection surveillance reveals high proportions of PCR ribotypes 027 and 176 in different areas of Poland, 2011 to 2013

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As part of the European *Clostridium difficile* infections (CDI) surveillance Network (ECDIS-Net), which aims to build capacity for CDI surveillance in Europe, we constructed a new network of hospital-based laboratories in Poland. We performed a survey in 13 randomly selected hospital-laboratories in different sites of the country to determine their annual CDI incidence rates from 2011 to 2013. Information on C. *difficile* laboratory diagnostic testing and indications for testing was also collected. Moreover, for 2012 and 2013 respectively, participating hospital-laboratories sent all consecutive isolates from CDI patients between February and March to the Anaerobe Laboratory in Warsaw for further molecular characterisation, including the detection of toxin-encoding genes and polymerase chain reaction (PCR)-ribotyping. Within the network, the mean annual hospital CDI incidence rates were 6.1, 8.6 and 9.6 CDI per 10,000 patient-days in 2011, 2012, and 2013 respectively. Six of the 13 laboratories tested specimens only on the request of a physician, five tested samples of antibiotic-associated diarrhoea or samples from patients who developed diarrhoea more than two days after admission (nosocomial diarrhoea), while two tested all submitted diarrhoeal faecal samples. Most laboratories (9/13) used tests to detect glutamate dehydrogenase and toxin A/B either separately or in combination. In the two periods of molecular surveillance, a total of 166 strains were characterised. Of these, 159 were toxigenic and the majority belonged to two PCR-ribotypes: 027 (n = 99; 62%) and the closely related ribotype 176 (n = 22; 14%). The annual frequency of PCR-ribotype 027 was not significantly different during the surveillance periods (62.9% in 2012; 61.8% in 2013). Our results indicate that CDIs caused by PCR-ribotype 027 predominate in Polish hospitals participating in the surveillance, with the closely related 176 ribotype being the second most common agent of infection.

Introduction

Clostridium difficile infection (CDI) is a common nosocomial problem, which can affect patients following antibiotic treatment [1]. Since 2003, reports of outbreaks of severe CDI have increased in Canada and the United States [2-4]. This increase coincides with the emergence and rapid spread of a more virulent strain of *C. difficile* belonging to the North American Pulsotype 1/BI, which is referred to in Europe as polymerase chain reaction (PCR)-ribotype 027 [5]. Some of the characteristics of this strain are higher *in vitro* production of toxins A (TcdA) and B (TcdB) and the presence of a third toxin called the binary toxin. The increase in toxin production is related to two mutations in the toxin regulatory gene *tcdC*: an 18 base-pair (bp) deletion, and deletion at position 117 [6]. In Europe, the epidemic strain was first observed in Belgium, France, the Netherlands, and the United Kingdom [7-10] and most recently caused outbreaks in Austria, Portugal and Romania [11-14]. Outbreaks of CDI caused by PCR-ribotype 027 have been associated with fluoroquinolone use in particular, and circulating PCR-ribotype 027 clones exhibit high levels of resistance against newer-generation fluoroquinolones [15]. The first Polish isolate of *C. difficile* PCR-ribotype 027 was detected in 2005 and a closely related PCR-ribotype 176 was discovered in 2008 [16,17]. CDI outbreaks associated with ribotypes 027 and 176 have been documented in three hospitals in Poland between 2008 and 2010 [18].

Based on participation in the European *Clostridium difficile* Infection surveillance Network (ECDIS-Net), which is a European Centre for Disease Prevention and Control (ECDC)-supported programme to build capacity

TABLE 1

List of collaborating hospital-laboratories included in the Polish surveillance programme for *Clostridium difficile* infection and annual incidence rates, Poland, 2011–2013

Hospital			2011		2012		2013	
Hospital-laboratory ID	Number of Beds	Type	CDI cases N	Annual CDI incidence per 10,000 patient-days	CDI cases N	Annual CDI incidence per 10,000 patient-days	CDI cases N	Annual CDI incidence per 10,000 patient-days
H1	250	S	11	1.7	18	2.9	26	4.4
H2	267	P	102	16.7	120	19.0	41	6.6
H3	269	P	11	1.7	73	11.4	106	15.8
H4	455	U	57	4.7	73	5.2	63	5.1
H5	620	U	4	0.3	56	4.5	73	5.3
H6	636	P	2	0.2	52	3.8	41	3.1
H7	718	P	19	0.9	65	2.9	106	4.7
H8	780	S	242	18.3	272	17.7	354	21.4
H9	895	U	16	0.7	48	2.3	128	6.0
H10	897	P	122	5.3	183	7.7	214	8.6
H11	1,016	U	69	2.5	70	10.8	158	26.6
H12	1,064	U	566	19.3	492	16.7	349	12.4
H13	1,310	U	247	7.1	274	7.1	182	5.2

CDI: *Clostridium difficile* infection; S: specialised; P: provincial; U: university.

for CDI surveillance in Europe, we previously set up a surveillance network of hospital-based laboratories in Poland. Here, a new network made up of a number of randomly selected Polish hospital-laboratories was constructed to conduct surveillance from 2011 to 2013. The aim of the study was to determine the annual CDI incidence rates in these institutions. In addition, periodical microbiological surveillance (February–March in both 2012, and 2013) was conducted to characterise *C. difficile* isolates obtained in the same hospitals.

Methods

Selection of hospitals

The aim was to include secondary and tertiary care hospitals. Funding only allowed to include a maximum of 20 hospitals in Poland, so an invitation to participate in the study was sent to 20 clinical hospital-laboratories which were selected at random among 600 healthcare facilities in different parts of the country. The number of people living in the areas of the 20 hospitals-laboratories is 10,867,100. Of the 20 hospital-laboratories contacted, seven declined to participate. Reasons for not enrolling in the study included not performing CDI surveillance (n=2 laboratories) or insufficient capacity (n=1 laboratory). In some cases (n=4), the reasons were not listed. Of the 13 hospitals (designated H1 to H13) that responded favourably, 11 provided secondary (n=5) or tertiary care (n=6), and two were specialised in pulmonology/thoracic surgery (H1) and oncology (H8). The number of beds among the hospitals varied from 250 (H1) to 1,310 (H13). Although the hospitals did not cover all Polish provinces, and three were in Warsaw, the 13 hospitals were located in 10 different cities across Poland, namely Bystra (H1), Bydgoszcz (H9), Krakow (H13), Łańcut (H3), Maków Mazowiecki (H2), Piła (H7), Płock (H10), Poznań (H4), Szczecin (H5), Warsaw (H8, H11, and H12), and Włocławek (H6).

Data collection

Before the start of the study (in January 2012), surveys were sent to participating hospital-laboratories, with requests for epidemiological data in order to calculate the annual CDI incidence rates for 2011, 2012 and 2013. Questions about *C. difficile* diagnostic testing were also asked. The surveys were completed by early 2014. In addition, between 1 February and 31 March in the two consecutive years 2012 and 2013 respectively, participating hospitals sent strains from patients identified with CDI to the Anaerobe Laboratory in Warsaw for molecular characterisation.

Determining incidence rates of *Clostridium difficile* infections

The study design was a hospital-based surveillance, using CDI case definitions based on ECDIS-Net recommendations as previously described by Kuijper et al. [19]. Hospitalised patients were included as a CDI case if onset of symptoms (abdominal pain, diarrhoea, ileus, toxic megacolon) occurred within the surveillance period. The detection of patients with CDI was based on the finding of clinical specimens testing positive for *C. difficile* in the laboratory. Annual hospital incidence rates were calculated per 10,000 patient-days. Numerator data included all reported initial CDI episodes of hospitalised patients above the age of two years, as well as recurrent episodes that occurred more than eight weeks after the onset of a preceding

episode. Age and sex of patients with CDI were registered. Denominator data comprised reported annual numbers of admissions and patient-days per hospital (in 2011, 2012, and 2013). The incidence rates of all participating hospitals were used to calculate a mean incidence rate.

Diagnostic tests used for *Clostridium difficile* infection and indications for testing

The epidemiological surveys also comprised questions on *C. difficile* laboratory diagnostic testing, and indications for testing. Participating laboratories were asked to report the type of screening test such as enzyme immunoassay for TcdA only, TcdA and/or B or glutamate dehydrogenase (GDH), molecular tests, toxigenic culture, or any other tests. Subsequently, participants were asked if they used a confirmation test. For both questions, there was a possibility to report more than one test.

Furthermore, decision criteria to perform *C. difficile* diagnostic testing were assessed, i.e. testing based on a physicians' request, testing in cases of antibiotic-associated diarrhoea, testing all diarrhoeal stools, or testing of diarrhoeal stools in a hospitalised patient from the third day of admission (nosocomial diarrhoea).

Molecular characterisation of isolates

Faecal specimens sent by the clinicians for routine *C. difficile* detection were tested in hospital-laboratories according to their standard methodology. All *C. difficile* strains (max 30) isolated from consecutive faeces samples testing positive for CDI in February and March of 2012 and 2013, respectively, were sent to the Anaerobe Laboratory, Medical University of Warsaw for detection of toxin encoding genes and PCR-ribotyping. Only one sample per patient was included in the study. Faecal samples were inoculated anaerobically on selective media for 48 h or 24 h, and *C. difficile* colonies were sub-cultured on blood agar and identified using standard methods, as described previously [18].

The toxigenicity was characterised by testing *C. difficile* isolates for *tcdB* and binary toxin encoding genes using the GeneXpert CD assay (Cepheid; Sunnyvale, California, United States), which is based on a real-time PCR method. PCR ribotyping was performed according to the method described by Stubbs et al. [20]. The Cardiff-ECDC collection of reference isolates (n = 23) of *C. difficile* was used as a reference set.

Results

Clostridium difficile infection incidence

During the three year-surveillance period, the annual mean incidence for the collaborating hospitals was 8.17 CDI per 10,000 patient-beds. In 2011 the annual CDI rate ranged from 0.2 to 19.3 per 10,000 patient-days (hospital mean: 6.1/10,000 patient-days), in 2012 from 2.3 to 19.0 per 10,000 patient-days (hospital mean: 8.6/10,000 patient-days), and in 2013 from 3.1 to 26.6 per 10,000 patient-days (hospital mean: 9.6/10,000 patient-days) (Table 1).

The highest incidence rates of CDI were observed in university hospitals, for example, H4 (range: 4.7–5.2 per 10,000 patients-days), H12 (range: 12.4–19.3 per 10,000 patients-days), and H13 (range: 5.2–7.1 per 10,000 patients-days), and the lowest in provincial hospitals such as H6 (range: 0.2–3.8 per 10,000 patients-days) and H7 (range: 0.9–4.7 per 10,000 patients-days).

TABLE 2

Types of diagnostic tests for *Clostridium difficile* infection used by hospital-laboratories in Poland, 2011–2013 (n=13 hospital-laboratories)

Hospital-laboratory ID	Test used to diagnose CDI
H1	EIA TOX A/B, TC[a]
H2[b]	GDH+TOX A/B
H3	EIA TOX A/B
H4[b]	EIA GDH and EIA TOX A/B; TC, qPCR[a]
H5[b]	GDH+TOX A/B
H6[b]	GDH+TOX A/B
H7	GDH+TOX A/B, TC[a]
H8	EIA TOX A/B, TC[a]
H9[b]	EIA GDH and EIA TOX A/B, TC[a]
H10	GDH+TOX A/B
H11[b]	GDH+TOX A/B, TC[a]
H12[b]	EIA TOXA/B or qPCR; TC[a]
H13[b]	GDH+TOX A/B, Illuminigene[a]

CDI: *Clostridium difficile* infection; EIA: enzyme immunoassay; GDH: glutamate dehydrogenase; TOX A/B: toxins A and B.

Laboratory tests were named as follow: EIA GDH (TechLab, USA): EIA to detect GDH; EIA GDH and EIA TOX A/B: EIA test for GDH alone and EIA confirmation test for TOX A/B; EIA TOX A/B: different EIA to detect toxins A and B (mainly TOX A/B, Wampole, USA); GDH+TOXA/B: combined test detecting both TOX A and/or B and GDH (The C. Diff Quik Chek Complete (TechLab; Blacksburg, VA, USA and Alere; Waltham, MA, USA)); TC: toxigenic culture; qPCR: The Xpert kit (Cepheid, Sunnyvale, CA, USA).

[a] Hospitals where two different tests were used for screening and confirmation.

[b] Indicates whether diagnostic changes occurred per hospital in 2012–2013 (TC, GDH+TOXA/B; qPCR).

Diagnostic tests for *Clostridium difficile* infection, and decision criteria for testing

Nine of the 13 laboratories used separate or combined assays for GDH and TcdA/B toxins in order to test for *C. difficile*. Twelve of the 13 laboratories used a two-step or three-step algorithm to diagnose CDI of which seven applied the C. Diff Quik Chek Complete (TechLab; Blacksburg, VA, USA and Alere; Waltham, MA, USA) test, and two applied a combination of two separate enzyme immunoassays (Table 2). The C. Diff Quik Chek Complete is one test but recognises two different targets and can therefore be considered as a two-step algorithm. Three laboratories used only an enzyme immunoassay for Tcd A/B detection. In addition, one laboratory used the Illumigene *C. difficile* Kit (Illumigene *C. difficile*

TABLE 3

Proportion of PCR-ribotype 027 per toxigenic strains in hospital-laboratories participating in the surveillance programme for *Clostridium difficile* infection, Poland, 2012 and 2013 (n=159 strains)

Hospital-Laboratory	Ribotype 027 per toxigenic strains	
	2012	2013
H1	0/0	1/1
H2	1/9	2/4
H3	Nd/0	8/10
H4	14/16	5/9
H5	Nd/0	2/5
H6	3/4	5/7
H7	3/3	3/6
H8	1/1	3/10
H9	2/2	5/7
H10	3/3	7/10
H11	2/4	8/10
H12	7/18	6/10
H13	8/10	Nd
Total	**44/70**	**55/89**

Nd: data from hospital not included in the table (hospital was included in the surveillance in only one year); PCR: polymerase chain reaction.

DNA Amplification Test, Meridian Bioscience, Inc., Cincinnati, OH) that detects a conserved 5' sequence of *tcdA* gene of *C. difficile*. Two laboratories used commercial qPCR, such as the GeneXpert C. *difficile* assay (Cepheid; Sunnyvale, CA, USA) that detects *tcdB* gene, the binary toxin encoding genes (*cdt*) and the deletion at nucleotide 117 on *tcdC* (Δ117) as surrogate markers for presumptive identification of 027/NAP1/BI strains. Seven laboratories used the toxigenic culture test as confirmation test. Of seven laboratories applying toxigenic culture, five introduced the toxigenic culture test on the request of the coordinator study before this survey to collect clinical isolates for characterisation.

Different decision criteria were applied to perform diagnostic tests for CDI on faeces specimens. Two of the total 13 laboratories tested all diarrhoeal faecal samples submitted to the laboratory. Six tested specimens only on the request of a physician and five applied additional criteria for CDI diagnostics, such as testing samples in case of antibiotic-associated diarrhoea and testing all diarrhoeal samples from patients who developed diarrhoea more than two days after admission (nosocomial diarrhoea).

Molecular characterisation of *Clostridium difficile* isolates

A total of 13 hospital-laboratories (one laboratory per one hospital; 11 in 2012 and an additional two new laboratories in 2013) participated in the two-month periods of molecular surveillance and sent a total of 166 *C. difficile* isolates to the central laboratory. Of these further data were available for 100 patients. The median age of patients was 62.8 years (range: 7–95 years) and 50 patients (50%) were female.

Among the 166 *C. difficile* isolates, 159 were toxigenic and seven non-toxigenic. Using support of the Reference Laboratory in Leiden, 27 different PCR ribotypes were identified of which one was not present in the Leiden University Medical Centre (LUMC) database. A majority of the toxigenic isolates belonged to PCR-ribotype 027 (n = 99; 62.3%) and the closely related ribotype 176 (n = 22; 13.8%). The remaining 45 (toxigenic and non-toxigenic) *C. difficile* isolates belonged to 25 different ribotypes. Of the 25 ribotypes, 19 and six contained toxigenic and non-toxigenic isolates, respectively. The 19 toxigenic PCR ribotypes included types 001, 002 (n=3 strains), 003 (n=3), 005, 012, 014 (n=8), 017, 018 (n=2), 023 (n=6), 045, 046 (n=2), 053, 056, 081, 087, 112, 152, 231 and one new ribotype (two strains with the same pattern) which was not recognised. Non-toxigenic types included types 009, 010 (n=2 strains), 031, 035, 039, and 207 (though results of type 207 need molecular confirmation).

Proportion of *Clostridium difficile* ribotypes 027 and 176 in the collaborating hospitals

The epidemic PCR-ribotype 027 strain was detected in all hospitals, with overall proportion of 62.9% (44/70) in 2012 (ranging from: 0–100%) and 61.8% (55/89) in 2013 (ranging from: 30.0–100%) among toxigenic strains (Table 3).

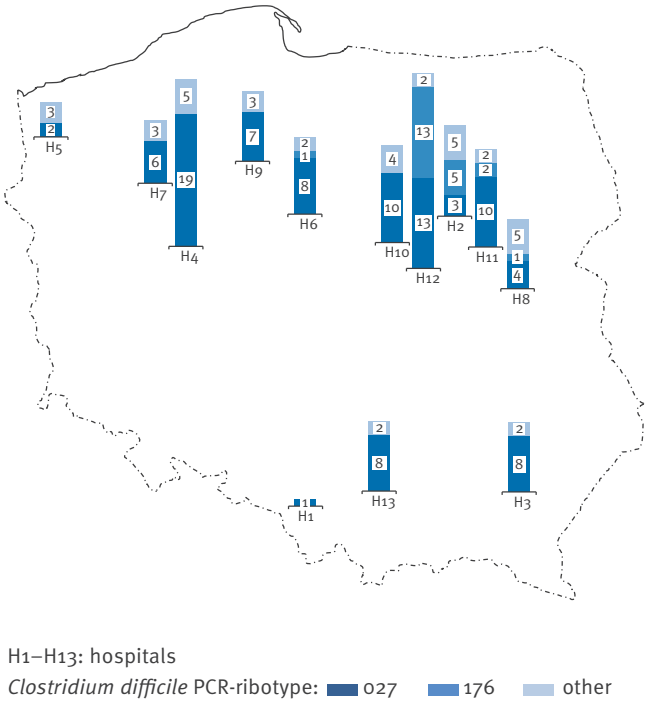
The annual proportion of PCR-ribotype 027 per toxigenic strains did not increase significantly during the surveillance periods (2012: 62.9%, 95% confidence interval (CI): 50–74; and 2013: 61.8%, 95% CI: 51–72). A high percentage (≥75%) was found in several hospitals located in different cities H3, H4, H9, H10 and H13. However, differences were observed between hospitals in the same city. The distributions of *C. difficile* PCR-ribotypes in 2012 and 2013 are shown in the Figure.

Discussion

Stimulated by the ECDC capacity-building network for CDI surveillance (ECDIS-Net) we developed a surveillance programme to estimate the incidence of CDI in hospitalised patients in Poland, comprising annual epidemiological surveys and periodical molecular surveillance. The main objective of the Polish surveillance was to encourage local laboratories to develop local diagnostic algorithms and to support surveillance studies that use internationally agreed-upon definitions. We found an annual mean incidence of 8.17 CDI per 10,000 patient-beds during the three year-surveillance period in the collaborating hospitals. The CDI incidence rate seems to have increased from 6.1/10,000 patient-days in 2011 to 9.6/10,000 patient-days in 2013. This incidence rate is in agreement with CDI incidence rate of 8.2 per 10,000 patient-days as reported for Poland in the EUropean, multi-centre, prospective bi-annual point prevalence study of *CLostridium difficile* Infection

Figure

Number of *Clostridium difficile* PCR-ribotypes 027, 176 and other per toxigenic strain, in study-participating hospitals (n=13) located in 10 cities, Poland, 2012–2013 (n=159 strains)



H1–H13: hospitals

Clostridium difficile PCR-ribotype: 027 176 other

PCR: polymerase chain reaction.

in hospitalised patients with Diarrhoea (EUCLID) study in 2012 in which Poland participated with 27 hospitals [21].

Since 2003, a rising incidence of CDI in North America and Europe has coincided with outbreaks of *C. difficile* PCR ribotype 027 and a changing epidemiology [22]. A large hospital-based survey conducted in November 2008, involving 106 laboratories in 34 European countries, showed that ribotype 027 was not among the most prevalent European types [23]. However, a recently completed study in 2013 across 14 European countries revealed a re-emergence of PCR-ribotype 027 as the predominant type in acute care hospitals in Austria, Belgium, Denmark, Germany, Hungary, Romania, and Serbia [24]. Increased incidence of *C. difficile* PCR ribotype 027 were also observed in Hesse, Germany from 2011 to 2013 [25]. In another European survey performed in 2011 and 2012 in 20 countries (EUCLID), 1,211 *C. difficile* isolates were collected of which *C. difficile* 027 was the most prevalent [21]. However, 88% of *C. difficile* type 027 were in only four countries: Germany (43.5% of all PCR-ribotype 027s), Hungary (17.5%), Poland (16.1%) and Romania (11.7%). In that survey it was found 19% of all CDI cases were not diagnosed in Poland due to lack of clinical suspicion, in comparison with an average 23.1% of all European countries. A second important conclusion from EUCLID was that only 39.9% of all participating laboratories used optimised methodology as defined by European guidelines. In our survey however, 12 of 13 participating laboratories applied a two-step algorithm, illustrating the importance of standardised diagnostics in ECDIS-Net.

In our study, *C. difficile* PCR-ribotype 027 was prevalent in all participating hospitals. A particularly high incidence was observed in university hospitals, H4, H11, H12, and H13. The CDI incidence varied considerably among the participating hospitals, not only related to the hospital sample size, but also due to the background of the hospitals, such as university or provincial hospitals with specific services (e.g. transplant medicine, haematology) or specialised hospitals (pulmonology/thoracic surgery and oncology).

The high incidence of PCR-ribotype 027 strains in these hospitals is likely a reflection of multiple exposures to the environment of healthcare facilities, antibiotic consumption and disruption of intestinal microbiota, and immunosuppression. However, we did not analyse antibiotic consumption among patients in this study. We observed that Polish university hospitals experienced higher number of CDI episodes compared with provincial hospitals.

After PCR-ribotype 027, PCR-ribotype 176 (13.8%) was the most common ribotype found among the *C. difficile* strains of Polish hospitals participating in this surveillance. Whole-genome sequencing studies (personal communication Trevor Lawley, Welcome Trust, Cambridge, UK, May 2014) have revealed that *C. difficile* isolates belonging to PCR-ribotype 176 are closely related to those of PCR-ribotype 027, a well-recognised hypervirulent strain. PCR-ribotype 176 has also been found in the Czech Republic [17,26]. Other PCR ribotypes found in Poland were PCR-ribotype 014 and PCR-ribotype 018. PCR-ribotype 014 accounted for 4.5% of the isolates in Poland, which is lower than that identified for Europe (16%) in 2008 [23]. We also detected *C. difficile* PCR-ribotype 018, which is the most frequently found ribotype in Italy [27]. We found seven non-toxigenic isolates belonging to six uncommon PCR ribotypes. It is likely that these isolates were derived from patients with mixed infections of both toxigenic and non-toxigenic isolates. Other PCR-ribotypes were detected sporadically, i.e., once or twice, during the two study periods.

Our study has a few limitations. First, of the 20 hospital-based laboratories invited, only 13 laboratories participated. This may be attributed to the voluntary nature of participation of the survey and lack of funding, but may have resulted in selection bias. Second, our study also included three smaller hospitals with 260 to 265 beds, which influenced the precision of our calculated incidence rates. Overall, the results of this surveillance programme were not yet validated. Lastly, we could only characterise a part of the *C. difficile* strains from patients with diagnosed CDI in the participating hospitals.

An important achievement of our study is the construction of a network to survey CDI in Poland. Hospitals collect a minimum amount of clinical and epidemiological data and send their isolates to a central laboratory. Our next steps are to validate the surveillance programme, to standardise the diagnostics of CDI and optimise patient selection for CDI testing. The identification of the (re)emergence of PCR-ribotypes 027 and PCR-ribotype 176 through molecular surveillance in this study is of concern and needs to be addressed through a national approach to combat CDI. Further studies evaluating the virulence factors and epidemiology of PCR-ribotypes 027 and 176 are urgently needed. Our study underscores the need for local and regional surveillance in Poland to detect and control CDI.

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Conflict of interest

None declared

Authors' contribution

Hanna Pituch designed and conducted the survey. Piotr Obuch- Woszczatyński, Dominika Lachowicz, Dorota Wultańska and Paweł Karpiński performed the laboratory investigations and data analyses Hanna Pituch and Grażyna Młynarczyk performed data analyses. Sofie van Dorp supported data analyses and interpretation of the data. Hanna Pituch and Ed Kuijper interpreted the data and wrote the manuscript. The Polish C. difficile Study Group, coordinated the study in the hospitals, enrolled patients, and collected epidemiological data.

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