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Outbreak of trichinellosis related to eating imported wild boar meat, Belgium, 2014

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Trichinellosis is a rare parasitic zoonosis caused by *Trichinella* following ingestion of raw or undercooked meat containing *Trichinella* larvae. In the past five years, there has been a sharp decrease in human trichinellosis incidence rates in the European Union due to better practices in rearing domestic animals and control measures in slaughterhouses. In November 2014, a large outbreak of trichinellosis occurred in Belgium, related to the consumption of imported wild boar meat. After a swift local public health response, 16 cases were identified and diagnosed with trichinellosis. Of the 16 cases, six were female. The diagnosis was confirmed by serology or the presence of larvae in the patients' muscle biopsies by histology and/or PCR. The ensuing investigation traced the wild boar meat back to Spain. Several batches of imported wild boar meat were recalled but tested negative. The public health investigation allowed us to identify clustered undiagnosed cases. Early warning alerts and a coordinated response remain indispensable at a European level.

Introduction

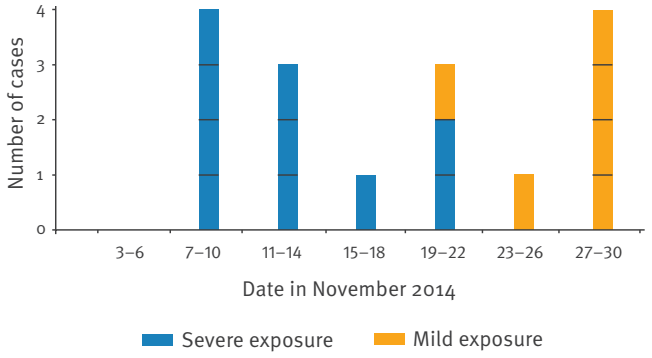
Trichinellosis is a parasitic zoonosis caused by nematodes of the genus *Trichinella*. The parasite infects domestic and wild animals and has a worldwide distribution [1]. The life cycle of the parasite consists of a domestic cycle in mainly pigs and a sylvatic cycle in a wider range of animals such as bears and wild boar [2-5]. Humans become infected after eating raw or undercooked meat from domestic pigs, horses or game containing *Trichinella* larvae [6-10]. The most important prevention measure is to freeze the meat or when preparing it, to ensure the core of the meat is cooked at a minimum of 67 °C, measured with a food thermometer, in order to kill the larvae. Different minimum temperatures and necessary duration of cooking are recommended according to the meat source [11].

Trichinella has unusual features, in comparison with other helminths. After ingestion, infective larvae are released from the muscular fibre and invade the epithelium of the host's small intestine. Sexually mature adult worms produce larvae in the small intestine, which subsequently disseminate in the host and invade muscle tissue [12,13]. Once the parasite completes development in the muscle, it remains infective for months or years. The pathological mechanisms of disease are complex and are partly related to direct lesions caused by invasion of the parasite into the host's muscle. A large inflammatory reaction mediated by eosinophils triggers numerous clinical manifestations during the acute stage of the disease [14].

The clinical picture is usually described by two stages: an intestinal stage within the first or second week after infection resulting in nausea or diarrhoea and a later muscular stage with periorbital oedema, myalgia or muscle weakness as the major symptoms [3]. The disease is mostly self-limiting: the adult worms live a mean of two to three weeks and the muscular phase is the end-stage of the infection [3]. However, major complications may arise during invasion of the muscle, including myocarditis, encephalitis and pulmonary superinfection. Fatalities have been described in infections with a high inoculum [15]. Cardiac involvement is the most frequent cause of death in human trichinellosis [16-18]. Although muscular symptoms usually

Figure 1

Epidemic curve of a trichinellosis outbreak, Belgium, November–December 2014 (n = 16)



The number of new cases is displayed according to the date of symptom onset, per time frame of four days, according to the type of exposure. Severe exposure was defined as having eaten a full dish of slowly roasted wild boar fillet; mild exposure was defined as having eaten small portions of slowly roasted wild boar fillet or wild boar stew

subside within two to four weeks, even in mild infections, muscular fatigue may last up to six months. Treatment consists of administration of antiparasitic agents with or without systemic glucocorticoid treatment [3,9].

In Europe, four species of *Trichinella* (*T. spiralis*, *T. nativa*, *T. britovi* and *T. pseudospiralis*) are endemic in domestic and wild animals [19]. Since 1992, the European Union (EU) Council Directive 92/45 has required the examination of meat of wild boars (*Sus scrofa*), domestic pigs and horses for the presence of *Trichinella* species before processing and marketing [20,21]. Before implementation of the EU directives, high incidence rates of human trichinellosis were observed in eastern European countries (2.46–5.45 cases/100,000 persons/year), but they have decreased sharply in the past five years [22]. According to the European Centre for Disease Prevention and Control (ECDC), 320 confirmed human cases were reported in the EU during 2014 [23]. In Belgium, the last reported cases in humans after eating indigenous wild boar meat occurred in 1979 [24]. In Belgium, *Trichinella* infection has not been detected in domestic pigs or horses since 1992, although serological evidence has pointed towards the presence of *Trichinella* species in wild boar and foxes [25,26].

The event

At the end of November 2014, 10 patients were admitted to three different hospitals in Belgium with fever, periorbital swelling, muscular pain and remarkable eosinophilia after eating wild boar meat in three different restaurants. A diagnosis of trichinellosis was confirmed by serology and PCR on the patients' muscle biopsies, in which *T. spiralis* was identified. In order to determine the extent of the outbreak, to identify its source and to implement control measures, an epidemiological study was conducted.

In Flanders, the northern region of Belgium, foodborne illnesses are notified to the Flemish Agency for Care and Health, which is responsible for investigating the source of disease and limiting its further spread. This is done in collaboration with the Federal Agency for the Safety of the Food Chain (FASFC), which operates nationwide to monitor and protect food safety. On 3 December, after the first diagnoses of human trichinellosis that day, both agencies were notified by email and other informal channels. Alerts were sent out regionally and all relevant public health authorities were informed. Through a ProMED Mail posting [27], this warning was communicated to the broader infectious disease community. The European Early warning and Response System (EWRS) and the Rapid Alert Safety for Food and Feed (RASFF) were alerted, to inform public health authorities in all EU Member States. Radio and television broadcasts and newspapers reported on the disease outbreak. Primary care physicians in the affected regions were asked to stay alert for patients with symptoms possibly related to trichinellosis and to ask symptomatic patients for details of potential exposure. Suspected cases had to be reported to the Flemish Agency for Care and Health.

Methods

Outbreak case definition

A probable case of trichinellosis was defined as a person who had consumed wild boar meat between 1 November and 6 December 2014 (two days after the first diagnosis and the start date of the outbreak investigation), with eosinophilia of > 500 cells/µL (norm: 0–450 cells/µL) with symptoms of myositis with or without fever (body temperature > 38 °C). Myositis was defined as muscle pain or muscle tenderness on physical examination and/or creatinine kinase levels > 200 international units (IU)/L (norm: 0–171 IU/L).

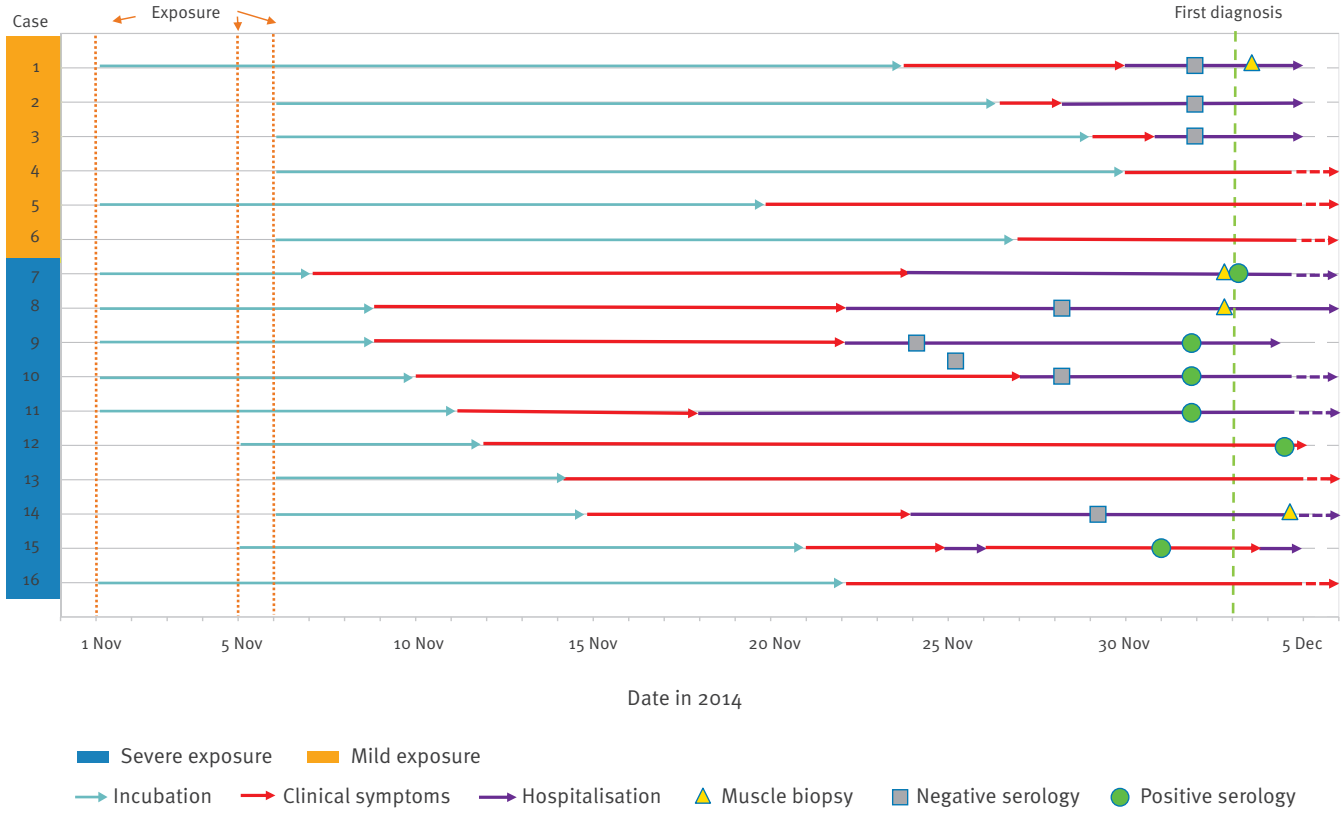
A confirmed case was defined as a probable case with positive serology or seroconversion, detected by anti-*Trichinella* IgG, or the presence of intramuscular larvae in a muscle biopsy as demonstrated by histology and PCR.

Laboratory analysis

The choice of diagnostic workup was at the discretion of the treating clinician and usually included blood counts, serum biochemical testing, electrocardiography, echocardiography, imaging studies and electromyography. Serological testing was performed at the National Reference Laboratory for Infectious and Tropical diseases at the Antwerp Institute of Tropical Medicine (ITM) using a commercially available assay based on excretory/secretory *Trichinella* antigens (Trichinella Microwell Serum ELISA, SciMedx Corporation, Denville, NJ, United States). ELISA-positive sera were confirmed by an in-house ELISA and western blot. Muscle biopsies

Figure 2

Timeline showing exposure, incubation period and diagnostic examinations, trichinellosis outbreak, Belgium, 1 November–6 December 2014 (n = 16)



Cases 1–6 had mild exposure (defined as having eaten small portions of slowly roasted wild boar fillet or wild boar stew); Cases 7–16 had severe exposure (defined as having eaten a full dish of slowly roasted wild boar fillet).

Serological tests were performed after the initial diagnosis was made by muscle biopsy, in some cases retrospectively. Some cases were diagnosed or remained hospitalised after 5 December (shown as horizontal dotted lines).

were examined by the local pathologist. For this purpose, 3 µm sections were cut from formalin-fixed, paraffin-embedded muscle biopsy specimens and stained with haematoxylin and eosin. Portions of the biopsies were also sent to the National Reference Laboratory for Trichinella at ITM, for additional examination including trichinoscopy and magnetic stirrer artificial digestion [28]. After HCl-pepsin digestion, isolated larvae were characterised by multiplex PCR following DNA extraction from single larvae [29,30].

Statistical analysis

Statistical analysis of the data was performed with SPSS 19. After normality testing using the Shapiro–Wilk test and assessment of the equality of variances with the Levene test, Student's t-test was used to determine differences in continuous variables between subgroups. The differences between other epidemiological parameters were evaluated with Fisher's exact test. An α-error of $p < 0.05$ was considered statistically significant.

Trace-back investigation

The FASFC and the Flemish Agency for Care and Health conducted a trace-back investigation focusing on the supply chain of the suspected meat. A detailed questionnaire about time of consumption of wild boar meat, time and duration of symptoms and treatment modalities was sent to all confirmed cases. Clinical and laboratory data from the individual patient files were reviewed after obtaining informed consent. Serological testing for trichinellosis was performed for asymptomatic persons accompanying confirmed cases at the restaurants and reporting the same consumption.

Results

Epidemiological characteristics of the cases

During the last 2 weeks of November 2014 and the first 2 weeks of December 2014, 16 patients were identified as confirmed cases of trichinellosis. They all reported eating wild boar meat during the first week of November in three restaurants in the Belgian provinces of Limburg and Antwerp. The exact date of meat consumption was

known for all the cases. Their median age was 37 years (interquartile range (IQR): 31–48; standard deviation (SD): 11); six were female (Table). Two subgroups were distinguished according to the type of exposure: cases who had eaten a full dish of slowly roasted wild boar fillet (classed as 'severe' exposure, n = 10) and those who had eaten small portions of slowly roasted wild boar fillet or wild boar stew (classed as 'mild' exposure, n = 6).

None of the cases reported eating other game meat during the investigation period. The epidemic curve of the outbreak is shown in Figure 1 and a timeline with data on exposure, incubation period, and clinical and laboratory data are presented in Figure 2.

Clinical and laboratory data

The cases' first symptoms appeared after a mean period of 15 days (range: 6–24; SD: 7) post-exposure. Cases with severe exposure had a significantly shorter incubation period compared with those with mild exposure: mean 10 days (range: 6–21; SD: 5) vs 22 days (range: 19–24; SD: 2), $p < 0.000$ (Table). The most frequent symptoms are summarised in the Table. Periorbital oedema lasted on average six days (range: 2–13 days; SD: 3). Muscular pain or tenderness was reported, ranging from moderate to severe and involved both upper and lower extremities (8/16) or was limited to the lower limbs (6/16). Symptoms related to the intestinal stage were reported by five cases. A total of 10 cases were hospitalised for a mean period of 14 days (range: 2–75; SD: 21); three required admission to an intensive-care unit.

Six cases initially presented with neurological complaints, including photophobia, neck stiffness and headache. Brain computed tomography (CT) of all cases showed no abnormalities. Three underwent lumbar puncture: their cerebrospinal fluid showed no cellular or biochemical abnormalities and bacterial cultures remained negative. One of the three who underwent lumbar puncture developed generalised muscle weakness 10 days after exposure, which remained severely debilitating two months later despite treatment with albendazole and corticosteroids. This case, a man in his early 50s, had, however, underlying conditions, which could obscure the clinical picture. A full-body positron emission tomography/CT and electromyography analysis were consistent with diffuse myositis [31]. The persisting symptoms included tremor, impaired coordination, fine motor control and loss of strength in the main muscle groups.

Laboratory analysis of all cases showed marked eosinophilia, reaching a peak on four weeks after exposure, with a mean of 34% (range: 7–65%; SD: 13) white blood cells (norm: 1–6%). Serum creatinine kinase (CK) levels were elevated in all but two cases (mean: 666 units (U)/L (range: 101–1,564; SD: 450; norm: < 171 U/L), with a peak concentration coinciding with maximum eosinophilia levels.

In four of five cases tested for troponin T, elevated levels pointed to myocarditis. Electrocardiogram analysis pointed towards myocarditis in four cases. One such case had signs of anteroseptal myocardial oedema without a dynamic gradient on transthoracic ultrasound evaluation, which had normalised at a second examination three weeks later. Serial follow-up of troponin levels in three cases showed a prolonged elevation compared with CK levels.

All cases received anthelmintic treatment, consisting of oral mebendazole 300–500 mg every eight hours (n = 14) or albendazole 400mg every 12 hours (n = 2) for 14 days. The hospitalised cases (n = 10) received methylprednisolone for a minimum of two days. There was a mean of 32 days (range: 26–39; SD: 4) between exposure and treatment start. Clinical improvement was observed after 48–72 hours after treatment. All but the above-mentioned case had an uneventful recovery.

Serology and microbiological identification

The clinical suspicion of *Trichinella* infection was confirmed by serology in all 16 cases. Biopsies of quadriceps muscle from three cases, weighing 0.24 g, 0.26 g and 0.30 g, revealed lymphohistiocytosis, eosinophilic infiltration and first-stage *Trichinella* larvae, which were identified as *T. spiralis* by multiplex PCR. One additional biopsy revealed eosinophilic myositis without detection of larvae.

Three persons who ate slowly roasted wild boar fillet or wild boar stew at the same time in the same restaurants as eight of the cases stayed healthy without developing any symptoms. Paired samples tested serologically at five and nine weeks after consumption of the dish remained negative.

Source tracing

The FASFC conducted an investigation, focusing on five suppliers of wild boar meat, in collaboration with the three restaurants involved. On inspection, there was evidence that the cold chain had been respected in all three restaurants. The investigation identified a single distributor of wild boar meat, imported from a certified supplier in north-eastern Spain. As there was a delay of several weeks between eating the suspected meat and the start of the investigation, no Spanish wild boar meat from this distributor remained in the restaurants where the cases had eaten. A week before the cases had eaten the suspected meat, all three restaurants received supplies of wild boar meat of the same batch on the same day. One supplier still had meat from the same batch. The remaining meat from this batch, as well as other batches from the same Spanish exporter, were recalled. A total of 58 samples from 21 different batches were examined by magnetic stirrer artificial digestion at the ITM, using 100 g per sample in 1g portions. None of these samples contained *Trichinella* larvae.

Table

Characteristics of trichinellosis cases according to level of exposure[a], Belgium, November–December 2014 (n = 16)

Characteristics	Number of cases[b] among all cases n = 16	Number of cases[b] among those with severe exposure[a] n = 10	Number of cases[b] among those with mild exposure[a] n = 6	P value[c]
Median age in years (IQR)	37 (31–48)	47 (34–50)	30 (20–39)	0.02
Female	6	3	3	0.61
Median time to symptom onset after eating wild boar meat, in days (IQR)	13 (8–22)	9 (8–13)	22 (21–23)	<0.00
Intestinal-stage gastrointestinal symptoms	6	4	2	1.00
Symptoms reported at presentation				
Fatigue	16	10	6	1.00
Fever	14	9	5	1.00
Night sweats	14	10	4	0.12
Periorbital oedema	14	9	5	1.00
Ophtalmological inflammation	14	9	5	1.00
Photophobia	6	4	2	1.00
Headache	12	7	5	1.00
Muscular pain	14	9	5	1.00
Abdominal pain	5	3	2	1.00
Rash	1	1	0	1.00
Lymphadenopathy	1	1	0	1.00
Outcome				
Hospitalisation	10	7	3	0.65
Myocarditis	4	4	0	0.23
Complete recovery	15	9	6	1.00

IQR: interquartile range.

[a] Severe exposure was defined as having eaten a full dish of slowly roasted wild boar fillet. Mild exposure was defined as having eaten small portions of slowly roasted wild boar fillet or wild boar stew.

[b] Unless otherwise specified.

[c] Severe exposure vs mild exposure.

As a precaution, the FASFC decided that all sampled batches of Spanish wild boar meat had to undergo heat treatment at 84 °C for 640 min, using a protocol adapted to the final product and application of the meat. Remaining meat from the suspected batch was destroyed.

Discussion

Despite the EU directive, which requires wild boars hunted for commercial purpose to be examined for *Trichinella larvae*, an infection risk for humans remains due to important reservoirs (wild carnivore mammals) [1,20,22]. The source of infection of this outbreak pointed towards wild boar meat imported from Spain, where *Trichinella* sp. infection is still endemic in the wild boar population [32,33]. According to ECDC epidemiological data, trichinellosis was most prevalent in eastern Europe (Romania, Bulgaria, Lithuania and Latvia) mainly due to eating domestic pork. In mediterranean Europe, Italy and Spain reported respectively 33 and 10 cases in 2012 [22]. Several outbreaks in these two southern European countries have been reported in the past 10 years [34-36].

No *Trichinella*-positive samples were found in the remaining imported wild boar meat. No remaining supplies of the suspected batch could be retrieved from the restaurants involved, due to the timeframe of the recall operation. The clinical pattern of the disease in humans often leads to a diagnosis several weeks after infection, which hampers source-tracing efforts. The authorities and controlling agencies in Spain reported no irregularities in the suspected slaughterhouse. No other *Trichinella* outbreaks were reported in the EU during the same time period. However, this cluster of infections highlights the importance of European-wide monitoring and an early warning system. Swift action by local networks and agencies is critical when a food-borne outbreak is detected. In this outbreak, the EWRS and RASFF made it possible to communicate efficiently and inform other potentially affected regions. Currently, the large EU-funded research Platform for European Preparedness Against (Re-) emerging Epidemics (PREPARE) is aiming to harmonise the response to severe infectious disease outbreaks and assemble real-time evidence for clinical management of patients. It should be noted, however, that due to the long incubation period and the delay of seroconversion

in parasitic food-borne infections [3], mounting a rapid response to an outbreak is often difficult.

Although *Trichinella* larvae can be destroyed by heating [3], the preparation of boar meat remains problematic given the culinary habit of serving and eating meat that is not always fully cooked. In this outbreak, the cases who consumed a full dish of slowly roasted wild boar fillet seemed to have been exposed to a higher degree than those who ate wild boar stew, prepared at higher temperatures and for a longer duration. The recommendations of the United States Centers for Disease Control and Prevention (CDC) regarding prevention of trichinellosis stress the use of a food thermometer to evaluate the internal meat temperature [11].

It has been suggested that the severity of the clinical features of trichinellosis is proportional to the number of larvae ingested [14]. In this outbreak, cases with severe exposure developed symptoms significantly earlier and showed a trend towards more severe clinical presentation, with more gastrointestinal complaints and cardiological complications compared with cases with mild exposure.

The overall clinical picture was consistent with the typical pattern as reported in literature: signs and symptoms appeared one to four weeks after exposure and included almost always the classical triad of fever, periorbital oedema and muscular pain or tenderness [14]. Four of the 16 patients had symptoms of myocarditis, consistent with earlier studies investigating cardiac involvement in human trichinellosis [16-18]. There was evidence of ECG changes in 56% of cases in 154 cases in one study [37]. *Trichinella* larvae do not encyst in heart muscle cells: myocarditis is a consequence of the transient passage of larvae and the resulting eosinophilic infiltration in myocardial cells. In some of our cases, elevated troponin levels persisted after normalisation of CK, indicating a possible underestimation of myocarditis severity when only CK is measured. Our observation suggests that troponin measurement might be relevant as part of the initial assessment and follow-up of trichinellosis, together with conventional electrographic and ultrasound cardiac monitoring.

None of the cases had encephalitis, but some had symptoms suggestive of meningismus (photophobia, neck stiffness), although this was not confirmed as meningitis. In hindsight, the symptoms of photophobia, eye inflammation or visual disturbances could be related to ocular muscle involvement and larval invasion rather than neurological pathology.

Most patients (15/16) recovered rapidly after administration of antihelminthic treatment and/or corticoids. However, only a few randomised controlled trials and observational studies are available to guide treatment modalities. Several questions remain regarding the need for and choice or dosage of antihelminthics more than 4–6 weeks after exposure [3,38-40]. In contrast, early administration of antiparasitic treatment seems crucial to prevent or reduce trichinellosis symptoms. Recent evidence suggests that post-exposure prophylaxis within one week after infection should be strongly encouraged because the development of symptoms might be completely prevented [41].

Our report also shows the importance of obtaining a detailed food consumption history as quickly as possible when a cluster of infections with a similar clinical pattern is observed. With the combination of coordinated actions and communication, we identified a total of 16 cases, including seven who did not seek medical attention immediately. It is possible that additional cases with milder or no symptoms were missed. Although no active outreach to all potentially exposed persons was undertaken, we are confident that the large media coverage and intensive communication allowed the passive detection of most symptomatic patients. The requests for serological tests for trichinellosis at the ITM surged up to 10-fold in the immediate aftermath of the disease outbreak: none of those tests were positive.

Conclusion

Several important lessons can be learnt from this outbreak, the largest reported to date in Belgium. Although a rare disease in western Europe, *Trichinella* remains a threat to food chain safety in 2016 despite measures taken at a European level. In countries where the prevalence of *Trichinella* in wildlife or domestic pork is still high, strict application of EU regulations and adequate control in slaughterhouses might be appropriate. In case of human infections, a transnational early warning system is important to alert the appropriate authorities, who can take swift action and control further spread of an outbreak.

Clinician's awareness of the features suggestive of trichinellosis, particularly in clustered cases, can hasten early diagnosis, prevent complications and even lead to administration of effective post-exposure prophylaxis in other potentially exposed persons. The role of serial troponin measurements, in addition to CK levels, should be further explored as a marker of disease severity.

Finally, there is a need for more research and clinical trials to establish sound treatment guidelines for trichinellosis.

Conflict of interest

None declared.

Authors' contributions

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work: PM, AF, SV, CT, JN, MVE, EB, KDS, PD, JVDH, DB. Drafting the work or revising it critically for important

intellectual content: PM, AF, SV, CT, JN, MVE, EB, KDS, IG, RC, PD, JVDH, DB. Final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: PM, AF, SV, CT, JN, MVE, EB, KDS, IG, RC, PD, JVDH, DB.

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West Nile virus transmission: results from the integrated surveillance system in Italy, 2008 to 2015

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In Italy a national Plan for the surveillance of imported and autochthonous human vector-borne diseases (chikungunya, dengue, Zika virus disease and West Nile virus (WNV) disease) that integrates human and veterinary (animals and vectors) surveillance, is issued and revised annually according with the observed epidemiological changes. Here we describe results of the WNV integrated veterinary and human surveillance systems in Italy from 2008 to 2015. A real time data exchange protocol is in place between the surveillance systems to rapidly identify occurrence of human and animal cases and to define and update the map of affected areas i.e. provinces during the vector activity period from June to October. WNV continues to cause severe illnesses in Italy during every transmission season, albeit cases are sporadic and the epidemiology varies by virus lineage and geographic area. The integration of surveillance activities and a multidisciplinary approach made it possible and have been fundamental in supporting implementation of and/or strengthening preventive measures aimed at reducing the risk of transmission of WNV trough blood, tissues and organ donation and to implementing further measures for vector control.

Introduction

West Nile virus (WNV), a single stranded RNA virus of the genus Flavivirus, is mostly transmitted by mosquito bites, but also through organ transplantation, blood transfusion, in the laboratory setting and from mother to child during pregnancy, delivery, or breastfeeding [1]. The virus is maintained in a continuous vertebrate-mosquito cycle. Mosquitoes are the vectors and birds are the reservoir for West Nile virus. Humans, horses and other mammals are considered dead-end hosts and do not contribute to further spread of the disease.

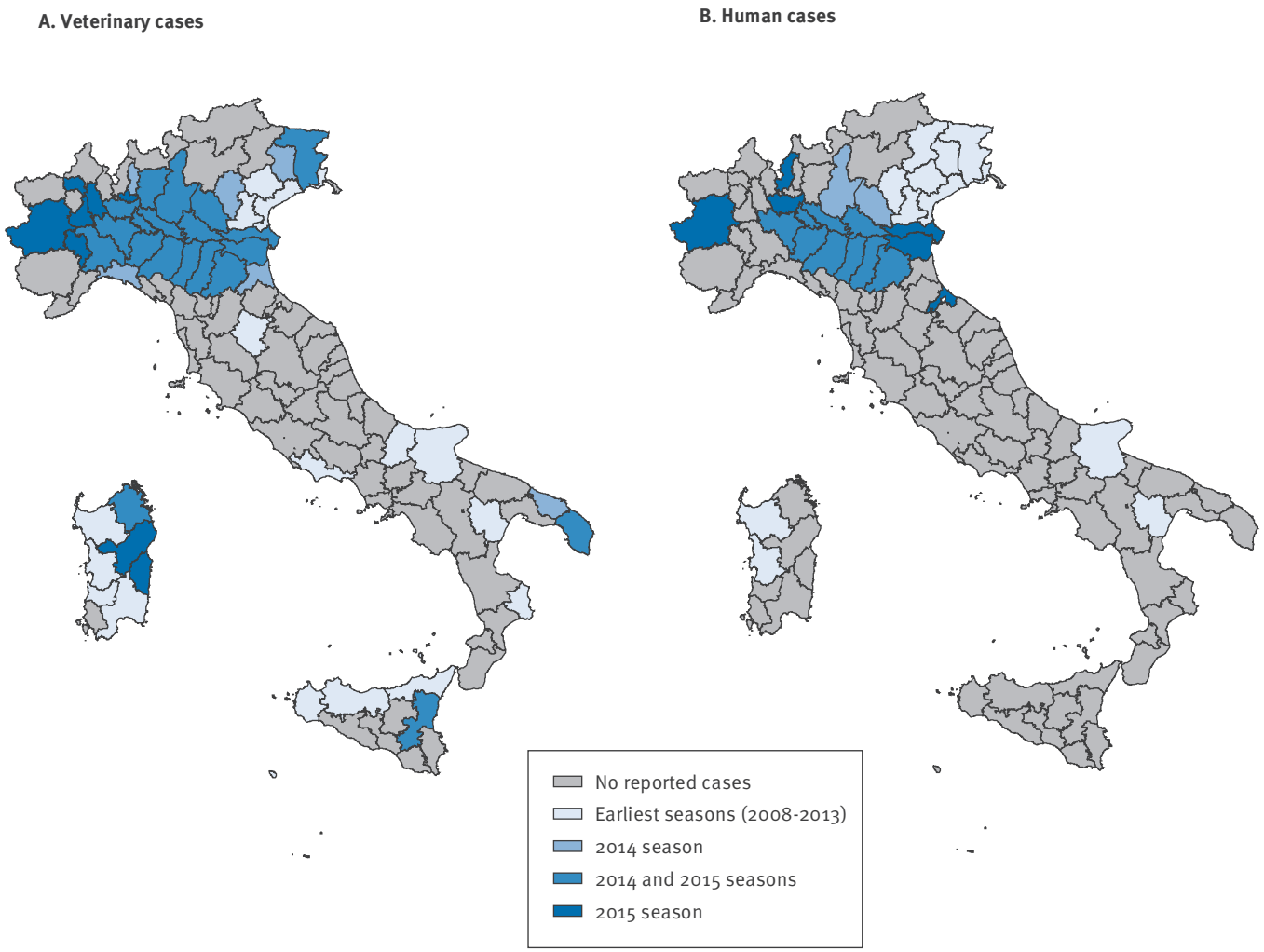
In humans, ca 80% of infections are asymptomatic, 20% of those infected may present with fever and or influenza-like symptoms, whereas less than 1% i.e. mostly the elderly and immunocompromised people, develop West Nile neuroinvasive diseases (WNND) such as encephalitis, meningo-encephalitis or meningitis that may lead to death [1,2].

In 1996, the first large human outbreak of WND in Europe was reported in Romania with 393 confirmed cases [3] and since then, the number of WNND cases reported in humans increased significantly. From 2002 to 2009, several WNV outbreaks were reported in a few European and neighbouring countries (Albania, Bosnia, Bulgaria, Croatia, FYROM, Greece, Hungary, Italy, Kosovo, Montenegro, Portugal, Romania, Russia, Serbia, Spain, Turkey, Ukraine) [4]. After 2005, an endemic transmission cycle started in some south-eastern and eastern European countries with annual outbreaks [5-8], mostly sustained by the rapid spread of WNV lineage 2 strains belonging to the Hungarian and Volgograd clade [9].

In Italy, the first outbreak of WNV infection was reported in 1998 in the region of Tuscany [10] and since 2001, a national veterinary surveillance plan for WNV

Figure 1

Geographical distribution of West Nile neuroinvasive disease in horses (panel A) and humans (panel B), Italy 2008–2015



based on wild bird mortality and on entomological and sentinel animal surveillance has been in place. The plan aims at monitoring areas at risk for WNV circulation, and detecting WNV seroconversion in horses in these areas [10,11]. In parallel, in 2002, human surveillance recommendations were issued by the Ministry of Health requesting all 21 Italian regions and autonomous provinces and autonomous provinces to report to the national mandatory surveillance system all hospitalised cases of aseptic meningitis and encephalitis with unknown aetiology, and cases of fever with rash in the areas where veterinary cases where identified. Moreover, health authorities were requested to actively identify cases and possible WNV seroconversion in close contacts of infected animals such as employees of stables and veterinarians or people living in the area [12].

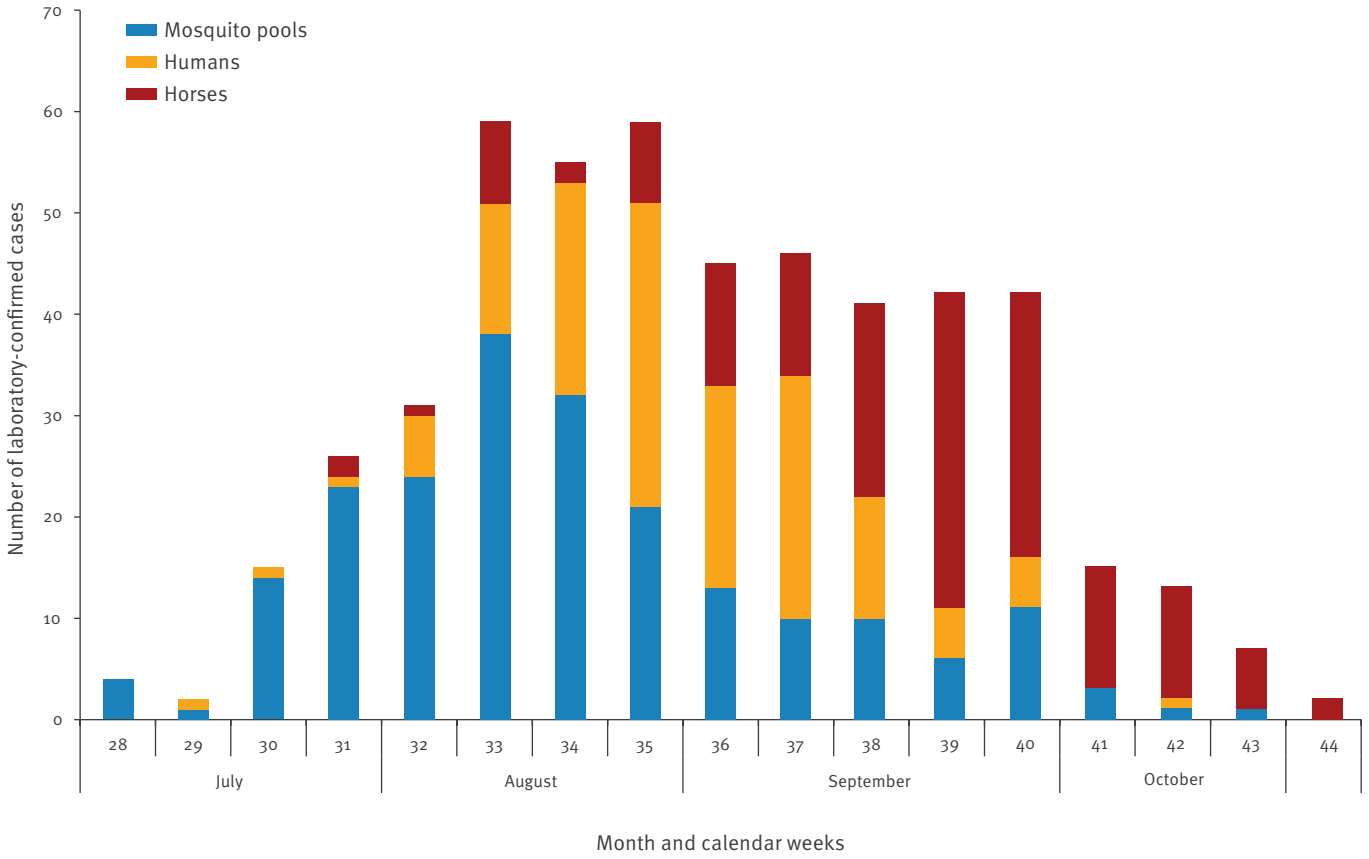
The veterinary and human surveillance systems did not detect any relevant circulation of WNV until 2008, when the virus was identified in mosquitoes, birds, horses and humans in the area surrounding the Po river delta, involving three north Italian regions [2].

Since the re-introduction of the virus in 2008, a constant and intensified WNV circulation across the whole of Italy was observed with a geographical spread of WNV to the west and south [2,13]. Moreover, from 2008 to 2011, WNV lineage 1 was responsible for reported human WNDD cases, but, since 2011, evidence of extensive circulation of lineage 2 closely related to both the Hungarian and Volgograd clades, was demonstrated [13-15]. This suggests a possible introduction of lineage 2 from central and/or eastern European countries, probably through migratory birds [16,17].

WNV has caused severe illnesses in humans in Italy every season over nearly a decade. However, cases occur sporadically and the epidemiology varies according to the virus lineage and the affected geographic area. Integrated surveillance is essential to identify outbreaks in a timely fashion and to guide prevention efforts aimed at reducing the incidence of severe cases and at reducing the probability of virus transmission via blood, tissue and organ donations.

Figure 2

West Nile virus detections in the veterinary and human surveillance by month, Italy, 2008–2015`



Here we present the evolution of the national surveillance plan in the five years following its first implementation, and briefly describe results of the WNV integrated surveillance system in Italy.

Methods

The integrated veterinary and human surveillance systems in Italy

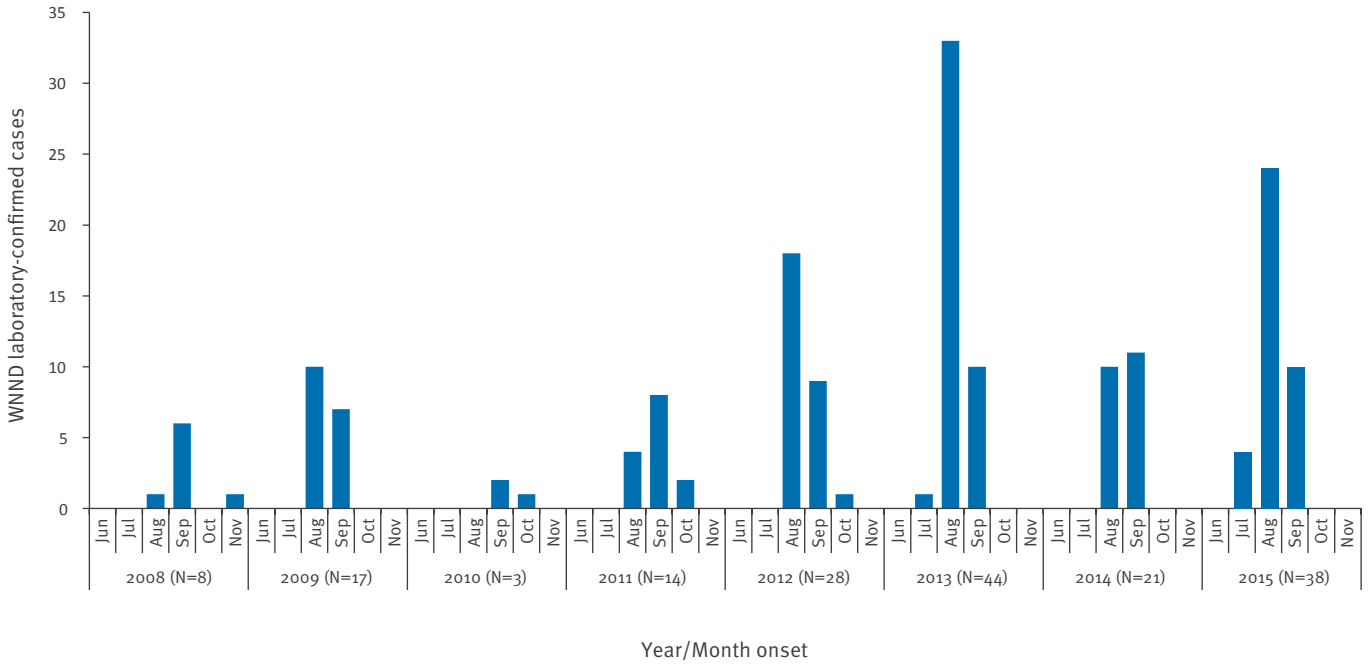
In 2008 and 2011, the national WNV veterinary surveillance plan [18] and the WNND human surveillance recommendations [19] were revised, respectively. New activities were added including the potential integration between veterinary (animals and vectors) and human surveillance. Provinces with evidence of animal and vector or human infections in the previous season have to implement active surveillance and mandatory screening of blood donations. The veterinary and human plans have been published separately and they were revised annually according to the epidemiological situation in the country. Since 2009, some north Italian regions have implemented an integrated surveillance, targeting mosquitoes, birds, and humans [15].

Pillars of the national integrated surveillance system are (i) the entomological monitoring based on mosquito collections in selected sites; (ii) the animal surveillance targeting migratory and resident birds as well as horses and poultry; (iii) the human surveillance system requesting clinicians to report all possible, probable and confirmed WNV cases, irrespective of age, using a modified European case definition which includes neurological symptoms in the clinical criteria [2].

For veterinary surveillance purposes, the Italian territory was subdivided in two distinct epidemiological territories: endemic and non-endemic. The former includes the territories where WNV was detected in the previous two years. At present these are the plain of the Po river valley, including Friuli Venezia Giulia, Emilia Romagna, Lombardy, Piedmont, Veneto regions, and the two main Italian islands: Sardinia and Sicily. The remainder of Italy is considered non-endemic. In the endemic territories, an early warning system is in place, which enables the reinforcement of the activities aiming at detecting WNV in vectors and birds [20]. In particular, the regions of the plain of the Po river i.e. Friuli Venezia Giulia, Emilia Romagna, Lombardy, Piedmont, Veneto, operate an enhanced surveillance system based on a network of fixed mosquito traps (in grids from 10 to 20 km) and on the collection of residential wild birds, mainly *Corvidae*. Timely data on viral circulation triggers preventive measures to avoid the virus transmission via blood, tissue and organ donations [15,21,22].

Figure 3

Human West Nile neuroinvasive disease cases by month of symptom onset and year, Italy 2008–2015 (n=173)



WNND: West Nile neuroinvasive diseases.

Data collected through the Veterinary Plan are registered using an information system (SISMAN) that records and manages laboratory results and publishes weekly and daily reports describing the outcomes of the surveillance activities. A web-based geographic information system (WebGIS) was developed for displaying thematic maps and to help the veterinary services to explore the area surrounding the outbreak, and to create buffers around the reported cases.

Human cases are notified by regional and local authorities to the Ministry of Health and to the Istituto Superiore di Sanità (ISS, national public health institute) using a specific password-protected web-based system, which permits to report probable and confirmed cases, adding available epidemiological (including the province of exposure), clinical and laboratory information. The web-based system is accessible also to the National Blood Center (NBC) and to the National Transplant Center (NTC), which implement precautionary measures on blood donation and transplant activities also on the basis of data on WNV human cases.

In order to rapidly identify, define and update the map of the affected areas i.e. provinces during the vector activity period, a real-time data exchange protocol is in place between the two systems.

Definitions and identification of at risk areas in Italy

The national plan for human surveillance defines as 'affected areas' all the provinces (Nomenclature of Units for Territorial Statistics (NUTS)-3) [23] where laboratory-confirmed WNV infections in animals, vectors or humans, irrespective of age, were notified in the previous years or during the surveillance period. The 'surveillance period' covers the months between the 1 June and 30 October, which is considered the period with the highest vector activity. Identification of an affected area immediately triggers the definition of the 'surveillance area' for the whole region (NUTS-2 level) where the affected area is located. In the surveillance area, passive human surveillance has to be set up, and physicians are requested to report all possible, probable and confirmed WNND cases. In the affected areas, the NBC and the NTC immediately activate the WNV screening, by Nucleic Acid Amplification Test (NAT), of all blood, blood component, and organ donations until the end of the vector season in order to avoid WNV transmission [24].

Data analysis

In our analysis we included autochthonous confirmed cases of WNND, West Nile fever and infections detected in blood and in organ donors; we also analysed data on vectors and birds. Annual incidence rates in human in the period from 2008 to 2015 were calculated using annual resident province population. All population data were obtained from the Italian National Institute of Statistics (ISTAT) [8]. The statistical analysis was carried out using STATA software version 11.2 (Stata Corporation, College Station, TX, US). Maps were produced using Epi Info version 7 (CDC, Atlanta, GA, US).

Table 1

Veterinary surveillance for West Nile virus in birds, mosquitoes, horses and chickens, Italy by year, 2008–2015

Years	Mosquitoes pool		Birds		Horses		Chickens	
	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
2008	152	8	490	45	1,532	563	76	1
2009	217	20	3,753	22	4,430	223	2,517	0
2010	1,068	13	4,182	3	2,728	128	1,213	5
2011	2,113	8	3,026	11	3,424	197	2,505	34
2012	2,366	14	2,260	21	2,081	63	2,461	6
2013	2,324	146	3,761	79	2,735	50	2,363	0
2014	5,834	125	5,368	48	5,882	27	1,974	7
2015	2,300	101	2,147	67	1,313	30	24	1
Total	**16,374**	**435**	**24,987**	**296**	**24,125**	**1,281**	**13,133**	54

Results

In Italy from 2008 to 2015, the circulation of WNV was reported in mosquitoes, birds and horses in the territory of 14 regions, with 173 indigenous cases of human WNND notified in eight regions (Apulia, Basilicata, Emilia-Romagna, Friuli Venezia Giulia, Lombardy, Piedmont, Sardinia, Veneto). Figure 1 shows the geographical distribution of human and equine neuroinvasive cases detected in Italy from 2008 to 2015.

Results of the veterinary surveillance from 2008 to 2015 are reported in Table 1. Since 2008, more than 16,000 mosquito pools have been tested, with 435 positive results, in eight regions and 30 provinces where subsequently human WNND cases were reported. Positive mosquito pools without consecutive or previous detection of WNND in humans were detected in 2008 in the province of Brescia, Lombardy region (n=1); in 2011 in the province of Messina, Sicily region (n=1) and in 2014 in the province of Genoa province, Liguria region and in the province of Alessandria province, Piedmont region (n=2, respectively).

In the provinces where there were also human cases, viral circulation in mosquitoes preceded human WNV (WNND and West Nile fever) cases with a mean of 22 days (range 0–58), except for few provinces (2011: n=1, 2012: n=2, 2013 and 2015: n=3, respectively) where the identification of human cases anticipated the evidence of viral circulation in vectors (Figure 2).

Also from 2008 to 2015, more than 24,000 residential birds were examined, with 296 positive results. WNV was found in birds in six regions. In the 15 provinces where there were human cases, positive birds were identified with a mean of 35 days (range 1–315 days) before the appearance of human cases.

During 2008 to 2010, all isolated viruses belonged to WNV-lineage 1, from 2011 to 2012 lineage 1 and 2 co-circulated with a higher proportion of lineage 1 and from 2013 to 2015 lineage 2 prevailed over lineage 1 with evidence of lineage 1 circulation only in one province each season.

Human cases

The national incidence of WNND peaked in 2013 (1.66/1,000,0000 inhabitants) and 2015 (1.34/1,000,0000). From 2008 to 2011, the annual incidence was relatively low (median: 0.41/1,000,000; range: 0.11–0.64). From 2012 to 2015, the national incidence of WNND increased by threefold (median: 1.20/1,000,0000; range: 0.78–1.66). The increase in disease incidence was initially concentrated in northern Italy moving over time towards the south, mainly to areas in central and to some areas in southern Italy. However, 91% (157/173) of the WNND cases detected during the entire study period were reported from three regions (Emilia-Romagna, Lombardy and Veneto) in the Po river plain area (Table 2) with the Emilia-Romagna and Veneto regions reporting the highest incidence (1.60 and 1.46/1,000,000 respectively).

The distribution of human WNND cases by month and year of symptom onset is reported in Figure 3.

The median age of cases was 73 years (range: 10–90 years) during the entire surveillance period, varying from a minimum of 67 years (range: 41–68 years) in 2010 to 77 years (range: 42–89 years) in 2013. Most of the reported cases (69%, 120/173) were male. All WNND cases were hospitalised: 82 presented as encephalitis, 44 as meningo-encephalitis, 31 as meningitis, seven as polyradiculoneuritis, one as facial paralysis and eight as other neurological symptoms: meningeal symptoms (n=2), extrapyramidal syndrome (n=1), confusion (n=1), headache (n=1), ataxic paraparesis (n=1), neuropathy of lower limbs (n=1), symptoms were not specified for one case. Eighteen of the 173 WNND cases died (2009: n=3; 2011: n=5; 2012: n=1; 2013: n=7; 2014 and 2015: n=1 each), corresponding to an overall case fatality rate of 10%. The median age of WNND fatal cases was 82 years (range: 34–89 years), 14 of 18 were male. All fatal cases were reported to have chronic conditions before symptom onset and 10 presented with encephalitis, six with meningo-encephalitis and two with meningitis.

In the entire period, 69 confirmed cases of WNV fever were reported to the national surveillance system from

Table 2

Incidence of human West Nile neuroinvasive disease cases per 1,000,000 inhabitants by province and year, Italy 2008–2015 (n=173)

Region	Province	2008		2009		2010		2011		2012		2013		2014		2015	
		N.	*Incidence*	*N.*	*Incidence*	*N.*	*Incidence*	*N.*	*Incidence*	*N.*	*Incidence*	*N.*	*Incidence*	*N.*	*Incidence*	*N.*	*Incidence*
Piedmont	Torino	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	1	0.44
Lombardy	Brescia	0	NA	0	NA	0	NA	0	NA	0	NA	2	1.60	1	0.79	0	NA
	Cremona	0	NA	0	NA	0	NA	0	NA	0	NA	1	2.76	3	8.28	4	11.06
	Lodi	0	NA	0	NA	0	NA	0	NA	0	NA	1	4.43	2	8.73	3	13.07
	Mantova	0	NA	2	4.88	0	NA	0	NA	0	NA	6	14.59	2	4.82	3	7.23
	Milano	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	4	1.25
	Pavia	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	5	9.12	5	9.11
Veneto	Belluno	0	NA	0	NA	0	NA	1	4.68	0	NA	0	NA	0	NA	0	NA
	Padova	0	NA	0	NA	0	NA	0	NA	0	NA	1	1.08	0	NA	0	NA
	Rovigo	3	12.18	5	20.23	0	NA	0	NA	0	NA	5	20.61	0	NA	1	4.12
	Treviso	0	NA	0	NA	0	NA	6	6.75	6	6.85	4	4.54	0	NA	0	NA
	Venezia	1	1.18	1	1.17	2	2.33	1	1.16	15	17.72	2	2.36	0	NA	0	NA
	Verona	0	NA	0	NA	0	NA	0	NA	0	NA	1	1.10	1	1.08	0	NA
	Vicenza	1	1.17	0	NA	1	1.15	0	NA	0	NA	0	NA	0	NA	0	NA
Friuli Venezia Giulia	Gorizia	0	NA	0	NA	0	NA	0	NA	1	7.15	0	NA	0	NA	0	NA
	Pordenone	0	NA	0	NA	0	NA	0	NA	2	6.44	0	NA	0	NA	0	NA
	Udine	0	NA	0	NA	0	NA	2	3.69	1	1.87	0	NA	0	NA	0	NA
Emilia-Romagna	Bologna	1	1.04	2	2.05	0	NA	0	NA	0	NA	1	1.01	1	1.00	2	1.99
	Ferrara	2	5.62	5	13.97	0	NA	0	NA	0	NA	5	14.18	0	NA	1	2.82
	Modena	0	NA	2	2.91	0	NA	0	NA	0	NA	7	10.17	2	2.85	8	11.39
	Parma	0	NA	0	NA	0	NA	0	NA	0	NA	1	2.32	1	2.26	3	6.74
	Piacenza	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	2	6.93	1	3.47
	Reggio nell'Emilia	0	NA	0	NA	0	NA	0	NA	0	NA	6	11.48	1	1.87	1	1.88
	Rimini	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	1	2.98
Apulia	Foggia	0	NA	0	NA	0	NA	0	NA	0	NA	1	1.59	0	NA	0	NA
Basilicata	Matera	0	NA	0	NA	0	NA	0	NA	1	5.00	0	NA	0	NA	0	NA
Sardinia	Oristano	0	NA	0	NA	0	NA	3	18.05	2	12.22	0	NA	0	NA	0	NA
	Sassari	0	NA	0	NA	0	NA	1	2.97	0	NA	0	NA	0	NA	0	NA

NA: not applicable.

four Italian regions (Emilia-Romagna n=31, Lombardy n=3, Marche n=1, and Veneto n=34).

Situation in 2015

In Italy in 2015, since the beginning of the surveillance period, for the first time since 2008, four cases, two from Emilia-Romagna and Lombardy region, respectively, all had symptom onset in the last week of July (calendar week 31). The last human cases reported had onset of symptoms or were detected in mid-September and the integrated surveillance detected signals of WNV circulation (veterinary cases), for the first time ever, in a northern-western region (Piedmont), where also one WNND case was reported. Moreover, virus circulation was detected in animals for the first time in the southern Italian regions Apulia (1 horse) and Sicily (1 chicken), where no human cases were reported.

In 2015, a total of 316,614 WNV NAT screening tests were conducted in blood donors in the affected provinces and 13 asymptomatic donors, six in Emilia Romagna and seven in Lombardy were identified. No donor or organ transplant recipients were positive for WNV among the 168 tested.

Discussion and conclusions

In Italy from 2008 to 2015, the circulation of WNV was reported in mosquitoes, birds and horses in the territory of 14 regions [10], with 173 auchtotonous cases of human WNND notified. [11]. A peak of cases was reported in 2013, a second peak was observed in 2015. From 2008 to 2015, the Italian contribution to the European case load increased substantially i.e. from a minimum of 11% (14/128) in 2011 to a maximum of 56% (60/108) in 2015 [3,9]. Moreover, in this period, an expansion of the Italian affected areas from the north-east to the north-west and south was observed.

From 2008 to 2010, only circulation of lineage 1 was reported. The complexity of the epidemiological scenario increased in 2011 with the detection of the novel lineage 2 which overcame lineage 1 from 2013 to 2015 and was responsible for both human and animal cases [12]. It is not clear why there was more WNV activity

in the past 4 years of surveillance (2012–2015) compared to the earlier surveillance period (2008–2011). However, virus lineage and virulence, weather conditions, bird population size and immunity, vector density, and human behaviour, are all factors that play a role in determining if, when and where human outbreaks may occur.

In Europe, since 2008, WNV has spread into areas not previously affected, including Greece [25], Portugal [26], Turkey [27], and many eastern European countries (Albania, Bosnia and Herzegovina, Bulgaria, Croatia, the former Yugoslav Republic of Macedonia, Kosovo under UN Security Council Resolution 1244, Montenegro, Serbia) [28-30]. In the same period the disease has been reported in Hungary, Israel, Italy, Romania, Russia, Spain and Ukraine [2,29].

The fact that the WNV has become endemic in Italy has brought the local and national Italian authorities to strengthen the WNV surveillance system and for this reason, probably, Italy is the country with the highest number of reported cases in EU, while other EU countries have different epidemiological situations, with different surveillance systems and objectives [31].

Surveillance of WNV circulation requires an interdisciplinary approach given the complexity of the viral biological cycle. For this reason, the integration of entomological, veterinary and human surveillance systems is an essential tool for public health. In fact, the veterinary and entomological surveillance activities are crucial for estimating the public health risk associated with WNV, and for the effective and timely control of the disease in humans. In Italy, a serious effort has been made to strengthen the integration of human with veterinary and entomological surveillance, reaching tangible results in the prevention of the disease [3,7]. In addition, since 2014, some Italian regions have explored the feasibility of using vector and animal surveillance data to trigger blood and organ donor safety measures. Encouraging results were obtained in 2014, during a season with high level of transmission. In 2015 however, veterinary surveillance did not signal WNV circulation before the occurrence of human cases in all affected provinces. Still, when province borders are not taken strictly into account and the surrounding territories are considered, the entomological and veterinary surveillance was able to detect the virus circulation also in 2015. Veterinary surveillance identified WNV circulation in some regions (Apulia, Sardinia, Sicily) without any human cases. This could be related both to the under ascertainment or under notification of human cases to the surveillance system or to limited circulation of the virus between bird and mosquito populations in rural areas.

In conclusion, an integrated human, animal and vector surveillance is crucial to timely set up preventive measures, such as the early detection of infected blood donors.

The integration of surveillance activities and the multidisciplinary approach in Italy might be a good practice to be implemented also in other affected countries for the identification of viral circulation. They have been fundamental to implement and/or strengthen preventive measures aimed at reducing the risk of transmission of the WNV to humans.

The work that Italy has being doing in the past 8 years of surveillance is constantly enriched by an intense research activity and also by the development of innovative tools for the quantification of health risk in order to implement prevention measures and effective control.

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Conflict of interest

None declared

Authors' contributions

CR, CN, AB, coordinated the human surveillance for WNV infections at national level, GV coordinated the activities of the national reference laboratory for WNV; SP coordinated the surveillance for WNV in blood donors, LL coordinated the surveillance for WNV in organ donors, PC and FM coordinated the veterinary surveillance of WNV; RC, PA, RB, MT, AP, FR, GP, MC, AL coordinated the surveillance activities for WNV infections at regional level; CR, CN, AB analysed the data and wrote the manuscript. All authors contributed to the discussion and reviewed the manuscript. All authors saw, commented upon and approved the final version of the paper.

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National survey of colistin resistance among carbapenemase-producing *Enterobacteriaceae* and outbreak caused by colistin-resistant OXA-48-producing *Klebsiella pneumoniae*, France, 2014

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From January 2014 to December 2014, 972 consecutive non-replicate carbapenemase-producing *Enterobacteriaceae* isolates from colonised or infected patients were collected at the Associated French National Reference Centre as part of the French national survey on antimicrobial resistance. It included 577 *Klebsiella* spp. (59%), 236 *Escherichia coli* (24%), 108 *Enterobacter* spp. (11%), 50 *Citrobacter* spp. (5%), and a single *Salmonella* spp. isolate (0.1%). Of 561 *K. pneumoniae* isolates, 35 were found to be resistant to colistin (6.2%). PFGE analysis revealed a clonal outbreak involving 15 *K. pneumoniae* isolates belonging to sequence type ST11, recovered in a single hospital in the Picardie region in northern France. Those clonally related isolates showed variable levels of resistance to colistin, ranging from 4 to 64 mg/L. They harboured the bla_{OXA-48} carbapenemase gene and the $bla_{CTX-M-15}$ extended-spectrum beta-lactamase gene. Among the 91 *Enterobacter cloacae* isolates, seven were resistant to colistin and produced different types of carbapenemases. Surprisingly, none of the *E. coli* and *Citrobacter* spp. isolates showed resistance to colistin. This national survey including carbapenemase-producing isolates recovered in 2014 reported a high rate of colistin resistance in *K. pneumoniae* and *E. cloacae* (6.2% and 7.7%, respectively) in France.

Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) resistant to colistin are increasingly reported. They represent an additional link in the development of pan-drug resistance. However, the epidemiology of colistin resistance among enterobacterial isolates is currently almost unknown in most parts of the world. In Italy, an increase in carbapenemase-producing *Enterobacteriaceae* has been noted in the past years, but the situation remains unknown in France [1]. The lack of information about the prevalence of colistin resistance among multidrug-resistant enterobacterial isolates derives from several reasons: (i) so far, there has been limited interest in that field, (ii) methods used for determination of colistin susceptibility are not adequate, and (iii) the lack of well-defined breakpoints does not allow precise determination of prevalence. However, the recent identification of a plasmid-borne polymyxin resistance determinant (MCR-1) raised a very serious concern in that resistance to colistin might widely disseminate [2].

The aim of this study was to evaluate retrospectively the prevalence of colistin resistance among a collection of CPE strains recovered in France during a period of one year and to analyse the phenotypic, genotypic features and clonality of the colistin-resistant isolates.

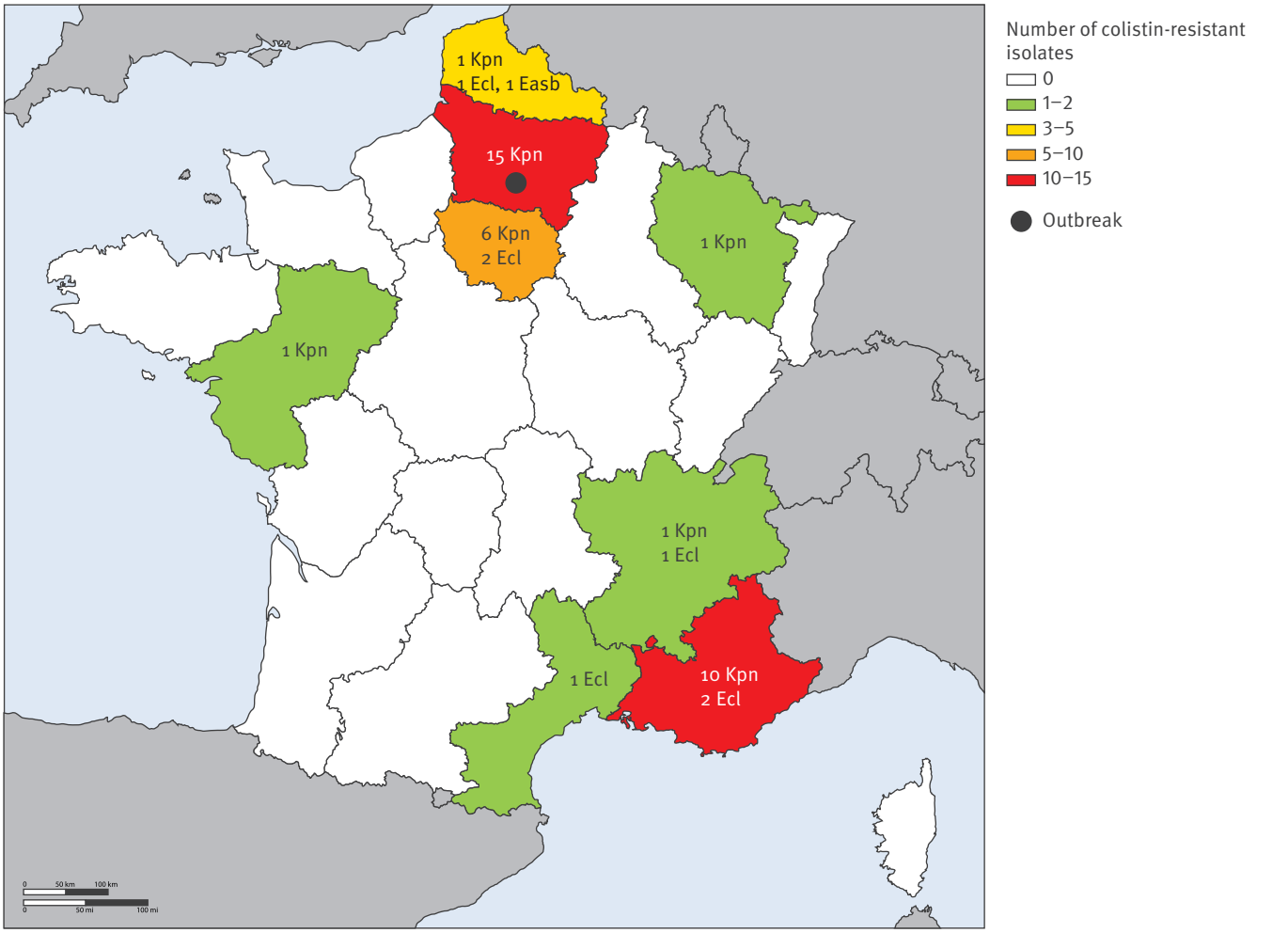
Methods

Carbapenemase-producing *Enterobacteriaceae* isolates

From January to December 2014, 972 consecutive non-duplicate isolates of carbapenemase-producing *Enterobacteriaceae* were isolated in private laboratories and hospitals in France either by screening for colonisation or by analysing clinical samples in the context of infections. They were recovered from rectal swabs or stools (n = 625), urine samples (n = 250), respiratory

Figure 1

Geographic distribution of colistin-resistant *Enterobacteriaceae* isolates, France, January–December 2014 (n = 43)



Easb: *Enterobacter asburiae*; Ecl: *Enterobacter cloacae*; Kpn: *Klebsiella pneumoniae*.

tract samples (n = 35), blood samples (n = 22), wounds (n = 24), catheter (n = 7), vaginal swabs (n = 3) and other specimens (n = 6). Those isolates were sent to the Associated French National Reference Centre for characterisation of resistance mechanisms to carbapenems as part of the French antibiotic resistance survey. The 972 carbapenemase-producing *Enterobacteriaceae* isolates included 577 isolates of *Klebsiella* spp. (59%), 236 isolates of *Escherichia coli* (24%), 108 isolates of *Enterobacter* spp. (11%), 50 isolates of *Citrobacter* spp. (5%), and a single isolate of *Salmonella* spp. (0.1%). Species that are naturally resistant to colistin (*Proteus* spp., *Morganella morganii*, *Providencia* spp., and *Serratia* spp.) had been excluded before the initiation of this study. Only a single isolate per patient was included in the study. All isolates were identified using the Microflex bench-top MALDI-TOF mass spectrometer (Bruker, Champs-sur-Marne, France).

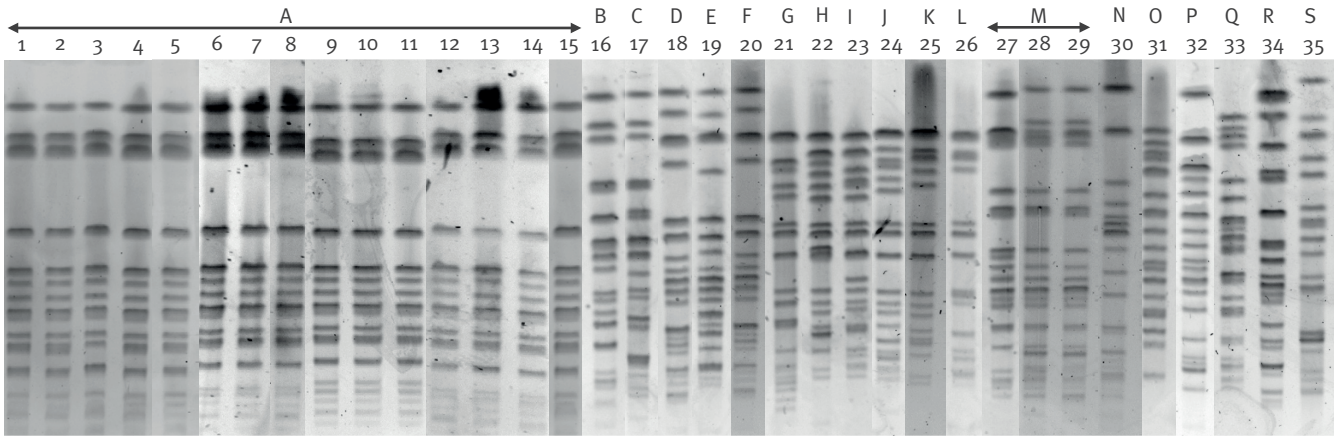
Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) of colistin (CS) were determined using broth microdilution method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [3]. As recommended, *E. coli* ATCC 25922 was used as quality control strain.

For the colistin-resistant isolates, susceptibility to other classes of antibiotics was also tested. Susceptibility to imipenem, ertapenem, and tigecycline was tested by broth microdilution method according to CLSI guidelines, whereas susceptibility to the other antibiotics was tested by the standardised agar disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4]. The antibiotics tested using disk diffusion method were: amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), cefotaxime (CTX), cefoxitin (FOX), ceftazidime (CAZ), cefepime (FEP), temocillin (TEM), ciprofloxacin (CIP), gentamicin (GM), amikacin

Figure 2

PFGE patterns of *Xba*I-digested chromosomal DNA of colistin-resistant *Klebsiella pneumoniae* isolates, France, January–December 2014 (n = 35)



The numbers correspond to the isolates from Table 2 and the letters indicate the PFGE type.

(AK), trimethoprim-sulfamethoxazole (SXT) and fosfomycin (FOS).

The MIC results for colistin and the disk diffusion diameters were interpreted according to susceptibility breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4].

Molecular characterisation

The *mgrB* genes of *K. pneumoniae* and *Enterobacter* spp. isolates were amplified using specific primers (Table 1), knowing that the MgrB protein is a negative regulator of the PhoPQ two-component system and that alterations in the *mgrB* gene are commonly involved in acquisition of colistin resistance in *K. pneumoniae* [5-7]. The plasmid-mediated *mcr-1* gene encoding colistin resistance was sought as described previously [2]. Detection of extended-spectrum beta-lactamases (ESBL) and carbapenemases genes was performed with specific primers as described previously [8]. Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, US). The nucleotide and deduced protein sequences were analysed at the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov) by the Basic Local Alignment Search Tool (BLAST) programme.

Genotyping

Genotyping was performed to evaluate the clonal relationship of the colistin-resistant *K. pneumoniae* and *E. cloacae* isolates by pulsed-field gel elctrophoresis (PFGE) with *Xba*I-digested genomic DNA and interpreted according to Tenover criteria [9]. Multilocus sequence typing (MLST) for *K. pneumoniae* was performed using the simplified protocol at the Institut Pasteur website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) [10].

Results

Klebsiella pneumoniae

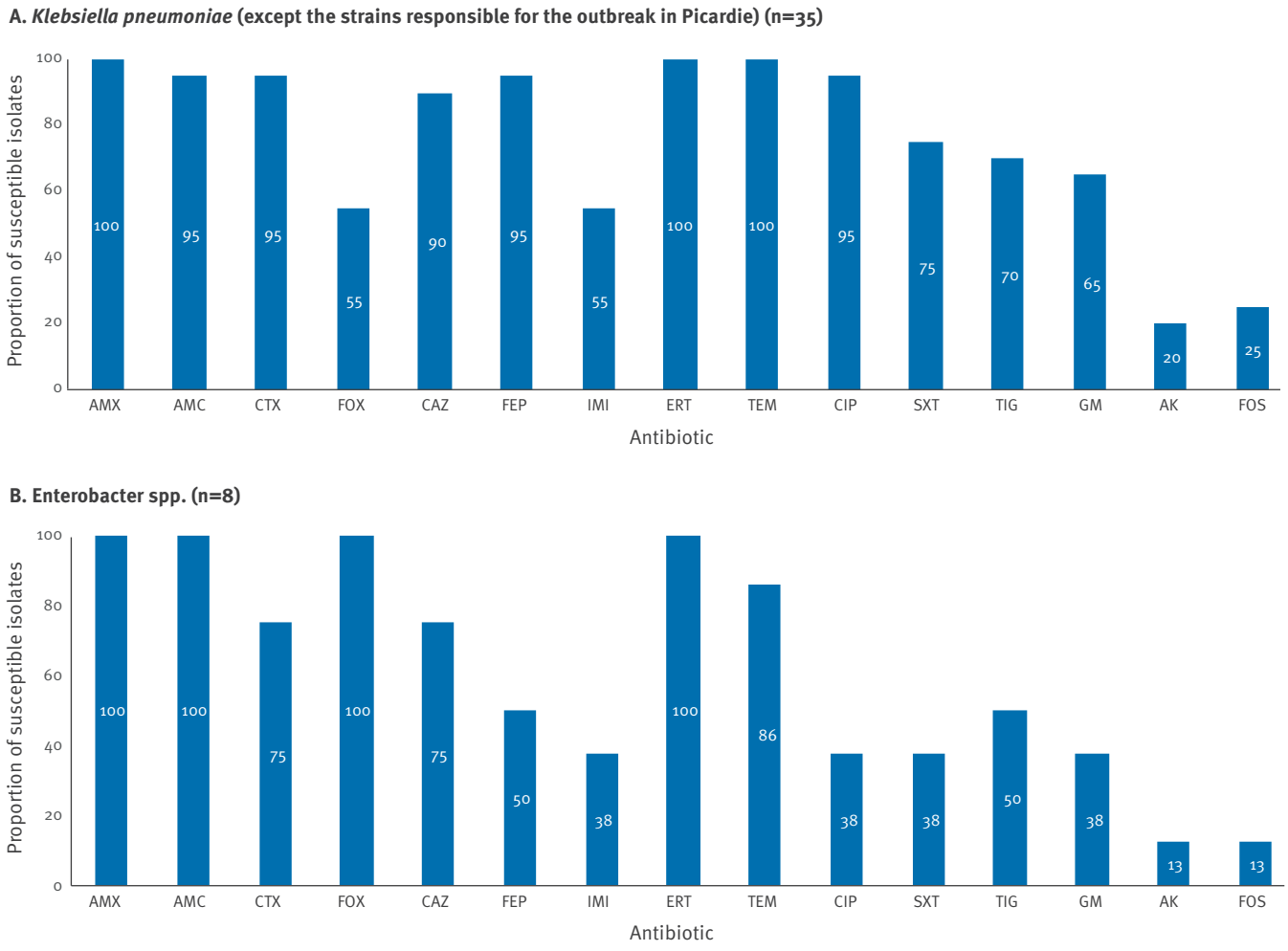
Of 561 *K. pneumoniae* isolates, 35 were found to be resistant to colistin (6%). Fifteen of the 35 colistin-resistant *K. pneumoniae* isolates were recovered from a single hospital in the Picardie region, northern France (Figure 1). We could not obtain the exact dates of their isolations due to the retrospective nature of the study. These isolates had mostly been recovered from rectal swab specimens, but also from a catheter, a urinary sample, a wound exudate and a respiratory specimen (isolates 1 to 15, Table 2). PFGE analysis revealed that the 15 isolates were clonally related (Figure 2, Table 2). The clone was of the ST11 type, and was susceptible only to cefoxitin, amikacin and fosfomycin (Table 2). A single isolate among these 15 was susceptible to tigecycline. The 15 isolates harboured both the *bla*OXA-48 carbapenemase gene, and the *bla*CTX-M-15 extended-spectrum beta-lactamase (ESBL) gene, and the MICs for colistin ranged from 4 to 64 mg/L (Table 2).

The other 20 colistin-resistant *K. pneumoniae* strains were mostly recovered from the regions Ile-de-France (n = 6) and Provence-Alpes-Côte d'Azur (n = 10) (Figure 1). These strains presented high MIC values for colistin ranging from 16 to >128 mg/L (isolates 16 to 35, Table 2). They produced either the carbapenemases OXA-48 (15/20), KPC-2 (3/20), NDM-1 (1/20), or both OXA-48 and NDM-1 together (1/20) (Table 2). Overall, 14 of the 20 isolates produced the ESBL CTX-M-15. PFGE analysis identified 18 clonal patterns among the 20 isolates (n = 3 for clone M) (Figure 2, Table 2), and MLST assigned the isolates to eight sequence types (STs) (Table 2).

Sequencing of the *mgrB* gene of those *K. pneumoniae* isolates revealed various *mgrB* alterations and none

Figure 3

Antimicrobial non-susceptibilities among colistin-resistant isolates, France, January–December 2014



AK: amikacin; AMC: amoxicillin/clavulanic acid; AMX: amoxicillin; CAZ: ceftazidime; CIP: ciprofloxacin; CTX: cefotaxime; ERT: ertapenem; FEP: cefepime; FOS: fosfomycin; FOX: cefoxitin; GM: gentamicin; IMI: imipenem; SXT: trimethoprim-sulfamethoxazole; TEM: temocillin; TIG: tigecycline.

of the strains harboured the plasmid-encoded *mcr-1* gene.

Antimicrobial susceptibility data for the colistin-resistant *K. pneumoniae* isolates not involved in the outbreak revealed that most isolates (19/20) were non-susceptible to third- and fourth-generation cephalosporins (Figure 3A). They were also frequently resistant to ciprofloxacin (19/20), trimethoprim-sulfamethoxazole (15/20) and tigecycline (14/20). They were less often resistant to gentamicin and cefoxitin (13/20 and 11/20, respectively). Amikacin and fosfomycin remained the most active agents against colistin-resistant *K. pneumoniae* (16/20 and 15/20 were susceptible, respectively) (Figure 3A).

Enterobacter spp.

Among the 91 *Enterobacter cloacae* isolates, seven were resistant to colistin (7.7%). They showed high MIC values for colistin (ranging from 16 to >128 mg/L) (isolates 36 to 42, Table 2). They produced the carbapenemases OXA-48 (4/7), VIM-1 (1/7), IMP-1 (1/7), or both OXA-48 and VIM-1 together (1/7) (Table 2). In total, three of seven strains were CTX-M producers, with two isolates producing CTX-M-15 and a single isolate producing CTX-M-2. The colistin-resistant *E. cloacae* isolates were recovered in different geographical regions in France (Figure 1, Table 2), and results of the PFGE analysis revealed that they were not clonally related (data not shown).

The single carbapenem-resistant *E. asburiae* strain was resistant to colistin. It had an MIC of colistin above 128 mg/L and produced the VIM-1 carbapenemase (isolate 43, Table 2).

Table 1

Oligonucleotides used as primers in this study, France, January–December 2014

Oligonucleotides	Sequence (5'-3')	Reference
Kpn mgrB ext F	TTA AGA AGG CCG TGC TAT CC	[7]
Kpn mgrB ext R	AAG GCG TTC ATT CTA CCA CC	[7]
Kpn mgrB int F	CGG TGG GTT TTA CTG ATA GTC	This study
Kpn mgrB int R	GAA CAT CCT GGT CGC ACA TT	This study
Ent mgrB ext F	CGG TTT ACT CTA TGA AAC AAG TGC	This study
Ent mgrB ext R	GCG AAG GAA GGA AAT CAC CT	This study

All *Enterobacter* spp. isolates had a wild-type *mgrB* gene, leaving unexplained the colistin resistance mechanism (*E. cloacae* and *E. asburiae*) (Table 2).

Of the eight colistin-resistant *Enterobacter spp.* isolates, four were non-susceptible to cefepime and tigecycline, and three were non-susceptible to ciprofloxacin, trimethoprim-sulfamethoxazole and gentamicin (Figure 3B). Amikacin and fosfomycin were the most active agents against colistin-resistant *E. cloacae* (all seven isolates were susceptible) (Figure 3B).

Other species

None of the *E. coli* (n = 236) and *Citrobacter* spp. (n = 50) isolates were resistant to colistin.

Discussion

We describe here a clonal outbreak involving 15 *K. pneumoniae* isolates recovered from a single hospital in the Picardie region in northern France. This outbreak was caused by a colistin-resistant OXA-48 and CTX-M-15-producing *K. pneumoniae* of ST11 type that was susceptible only to cefoxitin, amikacin and fosfomycin. Surprisingly, those clonally related isolates had variable MIC values for colistin ranging from 4 to 64 mg/L. An ST11 clone co-producing OXA-48 and CTX-M-15 was responsible for a large outbreak involving 44 patients in a hospital in Madrid, Spain, from 2009 to 2014 but only 3.4% of the isolates were resistant to colistin [11].

Several outbreaks of colistin-resistant KPC-producing *K. pneumoniae* (mainly attributed to the international epidemic clone type ST258) have been reported across Europe, in Greece [12,13], Hungary [14], Italy [15-17] and the Netherlands [18]. A single outbreak of colistin-resistant VIM-1-producing *K. pneumoniae* has also been described in Spain [19].

We report also 20 colistin-resistant *K. pneumoniae* strains recovered from the regions Ile-de-France and Provence-Alpes-Côte d'Azur. These strains belonged to 10 sequence types (n = 2 ST147, n = 3 ST258, n = 6 ST101, n = 3 ST307) and PFGE analysis identified 18 patterns among the 20 isolates. All three KPC-producing *K. pneumoniae* isolates belonged to ST258, the most common clone for KPC-producing isolates [20]. The OXA-48-producing *K. pneumoniae* isolates belonged to nine sequence types with six strains that were ST101, the most common clone identified among OXA-48-positive *K. pneumoniae* [21].

Sequencing of the *mgrB* gene revealed *mgrB* alterations which are likely to be responsible for colistin resistance as described previously [5-7]. Interestingly, the three strains belonging to the single clone M recovered in the Provence-Alpes Côtes-d'Azur region had different mechanisms of *mgrB* inactivation (Table 2). The occurrence of such different mechanisms of colistin resistance among clonally related isolates indicates that it is not the product of clonal dissemination of a single colistin-resistant *K. pneumoniae* strain, but rather clonal dissemination of a carbapenemase-producing isolate, which has acquired colistin resistance thereafter.

The rates of colistin resistance among the carbapenemase-producing isolates were 7.7% for *Enterobacter* spp. and 3.6% for *K. pneumoniae* isolates (excluding the isolates responsible for the outbreak in the Picardie region). The resistance rate observed among the carbapenemase-producing *K. pneumoniae* isolates was much lower than the high rates reported in the neighbouring countries of southern Europe such as Spain (20%) [19] and Italy (43%) [1].

None of the 236 carbapenemase-producing *E. coli* isolates were colistin-resistant or carried the *mcr-1* gene. This is surprising considering that a recent report of the French antimicrobial resistance Resapath surveillance network identified the plasmid-borne *mcr-1* gene in 21% of ESBL-producing *E. coli* isolates recovered from faeces of veal calves in France between 2005 and mid-2014 [22]. The plasmid-borne *mcr-1* colistin resistance gene has also been found in many neighbouring countries of France, for example among ESBL-producing *Enterobacteriaceae* isolates recovered from river water and imported vegetable samples in Switzerland [23], in *E. coli* isolates recovered from calves and piglets in Belgium [24], in swine and human wound infections in Germany [25], and in food and human bloodstream infections in Denmark [26]. The *mcr-1* gene was also detected in *Salmonella enterica* from food samples in Portugal [27] and France [28]. An *E. coli* isolate co-harbouring the *bla*VIM-1 carbapenemase gene and the *mcr-1* gene was described in Switzerland [29] and an isolate co-producing NDM-9 and MCR-1 was reported from China [30]. We believe that the plasmid carrying the *mcr-1* gene might be currently more prevalent among ESBL-producing isolates than among carbapenemase-producing isolates in human samples, which would explain why we did not identify this gene in our collection of carbapenemase-producing isolates.

Amikacin and fosfomycin were most effective against the colistin and carbapenem-resistant *K. pneumoniae* (susceptibility rates of 80% and 75%, respectively) and

Table 2a

Characteristics of the colistin-resistant *Klebsiella pneumoniae* and *Enterobacter* spp. clinical isolates, France, January–December 2014 (n = 43)

Isolate	Site of isolation	Origin	MIC CS[a]	*mgrB* genotype	Carbapenemase	Associated beta-lactamase	Co-resistances[b]	ST	PFGE
1	?	Picardie	32	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT	11	A
2	Catheter	Picardie	8	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
3	Rectal swab	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
4	Rectal swab	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
5	Rectal swab	Picardie	64	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
6	Urine	Picardie	32	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
7	Rectal swab	Picardie	64	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
8	Wound	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
9	Respiratory	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
10	Rectal swab	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
11	Rectal swab	Picardie	8	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
12	Rectal swab	Picardie	64	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
13	Rectal swab	Picardie	8	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
14	Rectal swab	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
15	Rectal swab	Picardie	4	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
16	Rectal swab	Nord-Pas-de-Calais	128	IS*1R* in promoter region (between nt −45 and −46)	OXA-48	CTX-M-15	CIP GM SXT TIG	147	B
17	Rectal swab	Ile-de-France	128	*mgrB* WT	NDM	CTX-M-15	CIP GM SXT TIG	147	C
18	Rectal swab	PACA	>128	IS*Kpn26*-like in coding region (between nt +74 and +75)	KPC	-	CIP AK SXT TIG	258	D
19	Rectal swab	Rhône-Alpes	128	MgrB truncated (27 amino acids)	KPC	-	CIP AK SXT TIG	258	E
20	Blood	PACA	16	Full gene deletion[c]	KPC	-	CIP AK SXT TIG	258	F
21	Rectal swab	Lorraine	64	Single nucleotide deletion (nt 74)	OXA-48	CTX-M-15	CIP GM	101	G
22	Rectal swab	Ile-de-France	32	Single nucleotide deletion (nt 23)	OXA-48	CTX-M-15	CIP GM	101	H
23	Rectal swab	Ile-de-France	64	IS*1R* in promoter region (between nt −36 and −37)	OXA-48 + NDM	CTX-M-15	CIP GM	101	I
24	Urine	PACA	64	IS*1R* in promoter region (between nt −45 and −46)	OXA-48	CTX-M-15	CIP GM SXT TIG	101	J
25	Abcess	Ile-de-France	32	MgrB M27K	OXA-48	CTX-M-15	CIP GM	101	K
26	Urine	Pays de la Loire	128	Duplication of 19 nucleotides	OXA-48	CTX-M-15	CIP SXT FOS	101	L
27	Urine	PACA	64	IS*1R* in coding region (between nt +21 and +22)	OXA-48	CTX-M-15	CIP GM SXT TIG	307	M
28	Rectal swab	PACA	64	*mgrB* WT	OXA-48	CTX-M-15	CIP GM SXT TIG FOS	307	M
29	Rectal swab	PACA	64	IS*5*-like in coding region (between nt +74 and +75)	OXA-48	CTX-M-15	CIP GM SXT TIG	307	M
30	Urine	PACA	>128	Full gene deletion[b]	OXA-48	-	CIP GM AK SXT TIG FOS	611	N
31	Rectal swab	Ile-de-France	32	IS*Kpn14*-like in promoter region (between nt −45 and −46)	OXA-48	-	CIP GM SXT TIG	23	O

Isolates 1-35: *Klebsiella pneumoniae*; 36–42: *Enterobacter* cloacae; 43: *Enterobacter* asburiae.

AK: amikacin; CIP: ciprofloxacin; CS: colistin; FOS: fosfomycin; GM: gentamicin; MIC: minimum inhibitory concentration; NA: not applicable; nt: nucleotide; PACA: Provence-Alpes-Côte-d'Azur; PFGE: pulsed-field gel electrophoresis; ST: sequence type; SXT: trimethoprim-sulfamethoxazole; TIG: tigecycline; WT: wildtype.

[a] MIC of colistin determined by broth microdilution method.

[b] Resistant or intermediate susceptibility to antibiotic.

[c] Full gene deletion: no PCR product was detected with external or internal primers.

Table 2b

Characteristics of the colistin-resistant *Klebsiella pneumoniae* and *Enterobacter* spp. clinical isolates, France, January–December 2014 (n = 43)

Isolate	Site of isolation	Origin	MIC CS[a]	*mgrB* genotype	Carbapenemase	Associated beta-lactamase	Co-resistances[b]	ST	PFGE
32	Rectal swab	PACA	32	IS*102*-like in coding region (between nt+36 and+37)	OXA-48	CTX-M-15	CIP GM SXT TIG	20	P
33	Respiratory	Ile-de-France	32	IS*1R* in promoter region (between nt −61 and −62)	OXA-48	CTX-M-15	CIP SXT TIG FOS		Q
34	Blood	PACA	>128	*mgrB* WT	OXA-48	CTX-M-15	CIP SXT TIG	39	R
35	Rectal swab	PACA	32	MgrB truncated (32 amino acids)	OXA-48	-	FOS	13	S
36	Rectal swab	Nord-Pas-de-Calais	64	*mgrB* WT	OXA-48+VIM	CTX-M-15	CIP GM SXT	NA	T
37	Stools	Languedoc-Roussillon	64	*mgrB* WT	OXA-48	CTX-M-15	GM AK	NA	U
38	Respiratory	Ile-de-France	32	*mgrB* WT	VIM	-	SXT TIG	NA	V
39	Rectal swab	PACA	>128	*mgrB* WT	OXA-48	-	CIP GM SXT TIG	NA	W
40	Rectal swab	Ile-de-France	16	*mgrB* WT	OXA-48	-	FOS	NA	X
41	Rectal swab	PACA	>128	*mgrB* WT	IMP	CTX-M-2	No	NA	Y
42	Respiratory	Rhône-Alpes	>128	*mgrB* WT	OXA-48	-	TIG	NA	Z
43	Urine	Nord-Pas-de-Calais	>128	*mgrB* WT	VIM-1	-	CIP TIG	NA	α

Isolates 1-35: *Klebsiella pneumoniae*; 36–42: *Enterobacter* cloacae; 43: *Enterobacter* asburiae.

AK: amikacin; CIP: ciprofloxacin; CS: colistin; FOS: fosfomycin; GM: gentamicin; MIC: minimum inhibitory concentration; NA: not applicable; nt: nucleotide; PACA: Provence-Alpes-Côte-d'Azur; PFGE: pulsed-field gel electrophoresis; ST: sequence type; SXT: trimethoprim-sulfamethoxazole; TIG: tigecycline; WT: wildtype.

[a] MIC of colistin determined by broth microdilution method.

[b] Resistant or intermediate susceptibility to antibiotic.

E. cloacae isolates (susceptibility rates of 87%). The rate of tigecycline non-susceptibility was high (70% for *K. pneumoniae* and 50% for *Enterobacter* spp.), probably because of a strong selective pressure by this last-line antibiotic.

Conclusion

This national survey on carbapenemase-producing isolates recovered in 2014 discovered a high rate of colistin resistance in *K. pneumoniae* and *E. cloacae* (6.2% and 7.7%, respectively) in France. These resistance rates remain much lower than those observed in other European countries such as Greece, Italy and Spain. No plasmid-encoded *mcr-1* gene was identified here. Therefore it seems that it is still possible to control the spread of those multidrug-resistant isolates based on accurate identification of colistin resistance and isolation of plasmid-encoded MCR-1 producers. Amikacin and fosfomycin remained the antibiotic agents most effective against those isolates which were resistant to polymyxins and produced a carbapenemase.

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Conflict of interest

None declared.

Authors' contributions

AJ, LP, and PN contributed to the design of the study. AJ performed the experiments. AJ, LP, and PN analysed the data. AJ, LP, LD, and PN contributed to the writing of the manuscript.

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Towards a consensus on genotyping schemes for surveillance and outbreak investigations of *Cryptosporidium*, Berlin, June 2016

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This report outlines the evidence and main conclusions presented at an expert workshop on *Cryptosporidium* genotyping held on 16 and 17 June 2016, hosted by the Robert Koch Institute, Berlin, and funded by EU COST Action FA1408 "A European Network for Foodborne Parasites: Euro-FBP" (http://www.euro-fbp.org).

The consultation brought together 23 scientists and experts in public and animal health from 12 European countries and the United States (US) to discuss how *Cryptosporidium* spp. surveillance and outbreak investigations could benefit from a harmonised approach to intra-species differentiation of the two main human pathogens, *C. parvum* and *C. hominis*. These are major zoonotic and anthroponotic causes of gastroenteritis, respectively. There is currently no standardised genotyping scheme for these protozoan parasites.

The workshop was organised in two parts: firstly, specialists described the current state of knowledge and need, and secondly, four working groups considered different aspects of the development, implementation and maintenance of *Cryptosporidium* genotyping schemes.

An overview of genotyping *Cryptosporidium* for public health purposes

Laetitia Kortbeek (National Institute for Public Health and the Environment, the Netherlands) described the diagnosis of *Cryptosporidium* and the usefulness of genotyping for epidemiology. Although cryptosporidiosis cases are notifiable in some European Union (EU) countries, testing and diagnostic practices are variable. Improved understanding of the epidemiology, sources and transmission of cryptosporidiosis is needed, but surveillance is also highly variable and the quality of the data provided to the European Centre for Disease Prevention and Control (ECDC) hinders comparisons between countries [1]. Improved diagnosis and basic surveillance across the EU would provide the means to estimate and compare the prevalence of cryptosporidiosis and detect changing trends in transmission.

The complexity of *Cryptosporidium* transmission was highlighted using data from the Netherlands, where a proportion of *Cryptosporidium*-positive stools are genotyped to identify species. In the second half of 2012, an excess of cases, mainly due to *C. hominis*, triggered an alert to other EU countries via ECDC's Epidemic Intelligence Information System for Food and Waterborne Diseases (EPIS); the United Kingdom (UK) and Germany also reported an increase [2]. An ongoing case–control study in the Netherlands failed to reveal an endemic source. In the following year, *C. parvum* predominated and risk factors for infection included the use of inland bathing waters and animal contact (not unexpected for *C. parvum*). More discriminatory genotyping of isolates could contribute to the identification of parasite sources and routes of transmission. As a first step, partial sequencing of a gene encoding a highly variable surface antigen (gp60) has shown that *C. hominis* allele IbA10G2 is highly prevalent throughout Europe, whereas *C. parvum* has greater diversity at this locus [3]. There is no specific licensed treatment in the EU for cryptosporidiosis, so understanding the epidemiology and improving the ability to identify sources through genotyping are important for the interruption of transmission routes and subsequent disease reduction.

The confusing world of *Cryptosporidium* typing

Giovanni Widmer (Tufts University, US) described how consideration of the reproductive biology and genetics

of the parasite and analysis of metadata from studies that used the same genotyping markers have provided further clarification of *Cryptosporidium* diversity, especially within *C. parvum*. The lifecycle involves asexual and sexual reproductive stages, requiring a multilocus scheme to account for sexual recombination within genetically diverse populations. Therefore, it is important to select markers that are sufficiently distant or located on different chromosomes, to ensure they are not in linkage. Excluding markers that provide redundant information reduces wastage and increases efficiency. As part of the marker selection process, ordination methods such as principal coordinates analysis and rank abundance plots can be used to estimate objectively how informative individual genetic markers and their combinations are. Because of the multivariate nature of multilocus data, ordination methods are ideal to visualise genetic similarity among isolates [4] and infer the likely source of an outbreak. In silico analysis of existing data can be used to improve and harmonise current genotyping approaches for surveillance and outbreak investigations.

Human epidemiology and food-borne outbreaks

Rachel Chalmers (National Cryptosporidium Reference Unit, UK) showed how supplementing epidemiological and environmental data with *Cryptosporidium* species and gp60 allele identification has strengthened the statistical evidence of association with food exposures in outbreaks. In May 2012, an excess of 300 cases of *C. parvum* was linked to the consumption of pre-cut mixed salad leaves, spinach and tomatoes [5]. The odds of association with eating pre-cut mixed salad leaves were increased when the case definition was restricted to those infected with gp60 allele IIaA15G2R1. In 2015, *C. hominis* infections exceeded expected numbers by more than 900 cases in late summer/early autumn, triggering an EPIS alert, with a similar increase reported by the Netherlands. Hypothesis-generating questionnaires revealed no sufficiently common exposures or risk factors to allow a case–control study. Isolates with the gp60 allele IbA10G2 predominated. Not only is this allele highly prevalent among *C. hominis* isolates from northern Europe, but there is also limited heterogeneity at other loci, highlighting the limitation of multilocus genotyping as an epidemiological tool for this species [3]. Suitable samples [6] with the IbA10G2 allele were further analysed by whole genome sequencing. Very few differences were seen in pairwise comparisons, with at most 50 single nucleotide polymorphisms (SNPs) observed in the ca 9.2 Mbp genome; the significance of these extremely small differences is currently unknown. In contrast, a *C. parvum* outbreak of more than 300 cases at the end of 2015 was defined by an unusual gp60 allele, IIdA24G1, recognised initially by the Scottish Parasite Diagnostic and Reference Laboratory, highlighting the value of genotyping routinely and including the data in national surveillance. A case–control study revealed food-linked exposures and the outbreak remains under investigation at the time of writing, demonstrating the difficulties in food chain investigations.

Zoonotic transmission

Karin Troell (National Veterinary Institute, Sweden) illustrated the importance of applying One Health approaches to the investigation of *Cryptosporidium* as a zoonosis. In Sweden, samples are tested from any likely host animal that is linked to a human cryptosporidiosis case, for example from household cats when *C. felis* has been detected in a patient [7]. This has led to collaborative studies on other, less common, species causing human infections. These findings reinforce the need for clinical diagnostics to detect not only *C. parvum* and *C. hominis*.

The most common zoonotic species in humans, *C. parvum*, has an unusual epidemiology in cattle in Sweden, where some studies have shown low prevalence even in young calves. This is in contrast to other countries where *C. parvum* is the main cause of cryptosporidiosis in pre-weaned calves [8]. Despite this, one of the most common *C. parvum* gp60 alleles in cattle, IIaA16G1R1, is also frequent in humans in Sweden. To support epidemiological investigations, a multilocus sequencing tool based on nine SNP markers across five chromosomes has been evaluated in a multiplex PCR on numerous samples; high discriminatory power and evidence of transmission between calves and humans in Sweden was shown.However, further studies of the population structure of *C. parvum* are needed across Europe to assess the broader applicability of this scheme.

How diversity relates to transmission to humans

Simone Cacciò (National Institute of Health, Italy) described the apparent geographic diversity of *C. parvum* in Ireland, Italy, and Scotland, as revealed by multilocus analyses. Studies so far indicate that in those countries, *C. parvum* populations from humans and livestock may have become isolated from each other, to the extent that the opportunity for genetic interchange appears limited [9]. To investigate the degree of genetic isolation, further studies are needed across Europe that include the major hosts for *C. parvum*. One study showed that in the UK, a high proportion of *C. hominis* isolates were indistinguishable at multiple loci, contrasting with those from Uganda, where a more diverse population structure was found [10]. Therefore, conclusions from one location may not be widely applicable and information is specific to host populations, whether these are defined geographically or demographically. A European-wide project (COMPARE; http://www.compare-europe.eu/) aims to increase the number of whole genome sequences for *Cryptosporidium* and to develop bioinformatic pipelines that would further the understanding of the population biology and determinants of virulence of the parasite. Information from COMPARE will undoubtedly benefit typing scheme development.

Four working groups considered how the evidence presented could be used to develop, implement and maintain suitable genotyping resources for *Cryptosporidium*.

Are the genetic and population structures of *Cryptosporidium* amenable to developing a genotyping scheme?

One working group considered whether reliable predictions of transmission can be made by combining genotyping with epidemiological and clinical data, considering that genetic diversity and population structures differ for *C. parvum* and *C. hominis*. It concluded that data are currently unavailable for much of Europe and are often not comparable because of lack of standardisation, indicating the need for further studies. Sampling frames need to follow the One Health concept, including both human and animal samples. Comparative analysis of increasingly available genome sequence data can provide a solid basis for marker selection. An evaluation process should be defined and applied to those markers already used.

What needs to be done to develop a standardised, multilocus genotyping scheme?

Another working group considered the development of separate multilocus schemes for *C. parvum* and *C. hominis* to provide robust, cost-effective assays, suitable for specialist and reference laboratories. Fragment sizing of regions containing tandem nucleotide repeats was considered alongside in-house sequencing. The decision whether to choose fragment sizing or sequencing will depend on the best workflow for individual laboratories, but markers that provide the same results with either method would be desirable. Sequence data from gp60 remains important. The most suitable markers need to be identified through a structured and objective process, ideally starting from whole-genome comparisons. Well-defined panels of samples are needed for biological and statistical evaluation of individual markers and their combinations, before progressing to inter-laboratory trials. DNA standards should be available. A web-based database needs be developed to contextualise metadata and genetic identification of isolates.

A multilocus genotyping scheme as a component of epidemic preparedness and response

A third working group considered multilocus genotyping as a component of a resilient response for health protection, highlighting that any scheme should be informative for epidemiological investigations and the detection and management of outbreaks, and that genotyping results should be incorporated into the collection of high quality epidemiological data. Differentiating between what is 'nice to know' and 'essential to know' is important: at present, there is more to be gained from genotyping *C. parvum,* as a high proportion of *C. hominis* cases in Europe have the gp60 allele IbA10G2, which is associated with low diversity at other markers. If genotyping all cases cannot be justified, selection will depend on outbreak size and available information and is probably best delivered as a test done in specialist or reference laboratories. Simulated outbreak exercises should be undertaken.

Sustainability of a standardised, multilocus genotyping scheme

The final working group discussed the elements needed to sustain a standardised scheme, including validation, external quality control (EQA), and inclusion of future developments, for example identification of new informative markers. A good mechanism for EQA should be established using an independent provider, also providing training modules and DNA standards. Central, ongoing collection of a minimum set of metadata are needed to facilitate surveillance of genotypes and meaningful comparisons and interpretation; this may be possible through the *Cryptosporidium* database at http://CryptoDB.org. Nomenclature for multilocus genotypes needs to be adopted for effective interdisciplinary communication.

Conclusions

Increased standardisation of diagnostic practices for *Cryptosporidium* is fundamental to the meaningful interpretation of surveillance data and distribution of species and genotypes. A robust, standardised, multilocus genotyping scheme should be developed, using a defined process to replace or supplement the multitude of genotyping methods used. Although further genotyping of *C. parvum* would be highly informative, this procedure may not always be warranted for the genetically more conserved *C. hominis* in Europe. A web-based database, enabling interpretation of genotype occurrence and distribution trends in an epidemiological context, is required. Genotype data should be incorporated into national surveillance programmes, and a standardised nomenclature provided for effective communication with public health professionals.

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Conflict of interest

None declared.

Authors' contributions

RC wrote the first draft of the manuscript, based on the meeting notes and interpretation recorded by participants GR and LC. SC critically reviewed the paper and gave input to the content, which was incorporated in the report. Both authors read and approved the final manuscript.

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