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Effectiveness of seasonal influenza vaccination during pregnancy in preventing influenza infection in infants, England, 2013/14

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In this study we used the screening method to estimate the effectiveness of seasonal influenza vaccination during pregnancy in preventing influenza virus infection and influenza-related hospitalisation in infants under six months, in England in the 2013/14 season. Seasonal influenza vaccination in pregnancy was 71% (95% CI: 24–89%) effective in preventing infant influenza virus infection and 64% (95% CI: 6–86%) effective in preventing infant influenza hospitalisation, and should be recommended in pregnancy.

Our study assessed the effectiveness of seasonal influenza vaccination during pregnancy in preventing influenza virus infection and influenza-related hospitalisation in infants under six months of age, in England in the influenza season 2013/14. This study is the first to use the screening method to calculate such an estimate for preventing infant influenza virus infection.

Background

Influenza in infants aged under six months is responsible for a significant burden of illness, impacting on a range of health services. When comparing the incidence of cardiopulmonary-related hospitalisations during influenza seasons to the rest of the year, the average annual increase in the United States (US) was highest for children aged under six months (104 hospitalisations/10,000 children), compared to children aged between six months and 12 months and children aged between one year and three years (50/10,000 and 19/10,000 respectively) [1].

Similar age-specific hospitalisation trends have been observed in England [2]; additionally, higher rates of influenza-related paediatric intensive care unit admissions occurred among children aged under one year (0.7/100,000) compared to those aged from one to four years (0.2/100,000) and five to nine years (0.5/100,000) during the 2009 influenza A(H1N1) pandemic [3]. There are also an estimated 22.3 influenza-attributable consultations in primary care for every child aged under six months admitted to hospital, indicating the intense demands on primary care services [2]. Preventing influenza in those aged under six months is therefore an important health priority. These children are too young to receive the current seasonal influenza vaccines, which are only licensed in older children [4].

Influenza vaccination during pregnancy directly protects newborn infants from influenza virus infection through transplacental transfer of maternal antibodies [5]. Several countries including the US, Canada, the UK (UK) and other European countries, recommend seasonal influenza vaccination during pregnancy, mainly to protect pregnant women who are at increased risk of severe infection, as observed with 2009 pandemic influenza A(H1N1) [4,6,7]. While few studies in other countries have examined the effectiveness of vaccination during pregnancy in preventing infant influenza infection [8-10], there has been no previous assessment of this in England, since its introduction in 2009.

Data collection

We defined a case as an infant aged under six months, born between 1 September 2013 and 31 January 2014, with laboratory-confirmed influenza infection by RT-PCR. Cases were retrospectively identified between 30 September 2013 and 18 May 2014 (the national seasonal influenza surveillance period), from the Respiratory DataMart System (RDMS), a sentinel laboratory surveillance system which collects influenza testing data predominantly from secondary care settings in England [11]. Cases were restricted to those born between 1 September 2013 and 31 January 2014

which corresponds to the roll-out of the 2013/14 programme of influenza vaccination during pregnancy.

Cases' general practitioners were sent postal questionnaires to identify if infants were hospitalised, if each infant's mother had received influenza vaccination during pregnancy (proportion of 'cases' vaccinated, PCV) and if so, the vaccination date. Up to three postal/fax reminders were sent to general practitioners in case of non-response.

A mother was classified as fully vaccinated in pregnancy if vaccination occurred at least 14 days before the infant's birth, (considered the minimum time for the mother to develop a full immune response) or unvaccinated if mothers were vaccinated after birth or not at all. Infants whose mothers were vaccinated less than 14 days before birth or had an unknown vaccination status or vaccination date, were excluded from analysis as the mothers' immune status was uncertain.

Seasonal influenza vaccination coverage for the population of pregnant women (PPV) in England was identified through a national electronic reporting system (ImmForm) which is used nationally by general practices in England to report vaccine administration for seasonal influenza vaccination. The end of season collection reported a 99.8% response rate in 2013/14 [12]. ImmForm data were used to identify the number of pregnant women (at any stage of pregnancy) registered in primary care, and the cumulative number of these women who received seasonal influenza vaccination between 1 September 2013 and 31 January 2014, at monthly intervals from end-October. Sub-national coverage was calculated for four regions of England (London, South, Midlands and North).

Data analysis

Data analysis was undertaken using Microsoft Excel 2007 (Microsoft, Redmond, Washington) and Stata version 12.0 (StataCorp, College Station, Texas). Characteristics of cases included in the analysis were reported, including age group, sex, influenza sub-type, hospitalisation status and maternal vaccination status.

We used the screening method to estimate vaccine effectiveness (VE); this approach has been used previously to estimate VE against influenza for other groups [13-15].

Crude VE was estimated separately for all influenza cases and for hospitalised influenza cases as:

$$\mathrm{VE} = 1 - \left(\frac{PCV}{(1 - PCV)} \times \frac{(1 - PPV)}{PPV}\right)$$

Adjusted VE for all laboratory-confirmed influenza infection and hospitalised influenza cases was estimated by using the natural logarithm of PPV in each region and month of birth as an offset in a logistic regression model where the outcome was vaccination status during pregnancy for the mother of each case, therefore allowing for individually matched coverage for each case by region and month of birth. Month of birth and region were included as potential confounders as these were both related to vaccine coverage and influenza activity during the influenza season.

Results

There were 43 infants with laboratory-confirmed influenza infection reported through RDMS, born between 1 September 2013 and 31 January 2014 (Figure).

Of these 43 cases, 37 were included in the analysis. Six cases were excluded: two with no response from general practitioners, one with unknown vaccination status of the mother in the returned questionnaire, two with unknown date of maternal vaccination and one with maternal vaccination less than 14 days before birth. Of these cases, 22 were male. Median age of infants at time of influenza testing date was 13 weeks (range 2–21 weeks). Twenty-two cases tested positive for 2009 pandemic influenza A(H1N1), nine for A(H3N2), five for influenza A (unspecified) and one for influenza B.

Nationally, of 659,223 pregnant women, 262,081 (39.8%) were reported to have received seasonal influenza vaccination in pregnancy in 2013/14. Five cases' mothers were reported to have received seasonal influenza vaccination in pregnancy. The median interval between maternal vaccination and birth was six weeks (range 4–12 weeks). The crude VE for preventing all influenza cases was estimated as 76% (95% confidence interval (CI): 39–93%) and the adjusted VE was 71% (95% CI: 24–89%).

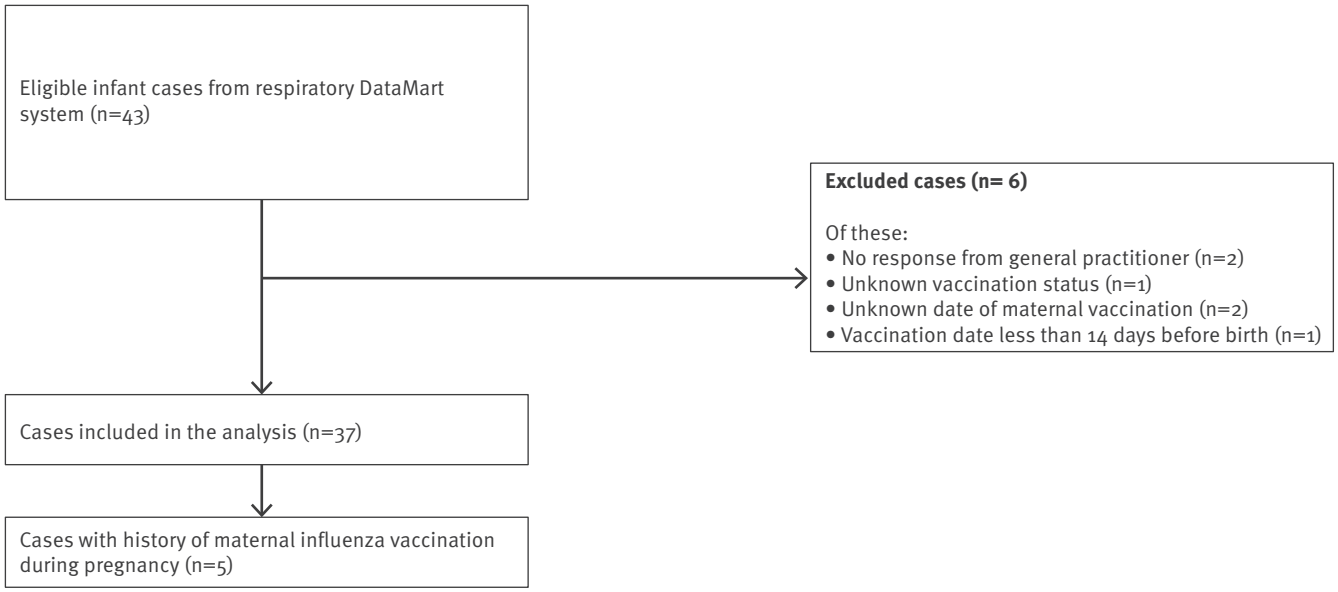
Hospitalisation data were available for 36 cases; of these, 32 cases were hospitalised, including the same five cases as before. The crude VE for hospitalised influenza cases was 72% (95% CI: 26–92%); the adjusted VE was 64% (95% CI: 6–86%).

Discussion

Our results demonstrate that vaccination during pregnancy effectively prevents laboratory-confirmed influenza infection and associated hospitalisation in infants in the first six months of life. These results are comparable with a previous randomised control trial (RCT) in Bangladesh showing that vaccination during pregnancy was 63% effective in preventing influenza in infants aged under six months [8]. Additionally, studies in the US have identified a VE of 48–91% in preventing influenza-related infant hospitalisations [9,10]. Our findings supplement the existing evidence for this intervention, underlining that previous results in earlier seasons in other settings are applicable to the UK and could have implications for other European countries also. Our VE estimate reflects a combination of both the direct effect of transplacental antibody

Figure

Recruitment of cases included in the study on effectiveness of seasonal influenza vaccination in pregnancy in preventing influenza infection in infants, England, 2013/14



transfer from mother to foetus but also a likely indirect effect from preventing influenza infection in mothers and subsequent secondary transmission to infants.

A strength of these findings, besides being the first such estimates in Europe in any season, also represent the first VE estimates for seasonal influenza vaccination during pregnancy in the 2013/14 season. In addition, to our knowledge, this is the first study to use the screening method to specifically estimate the effectiveness of vaccination during pregnancy to prevent infant influenza infection. The advantage of this approach is that routinely collected vaccine coverage and case-level data can be adjusted for key confounders to rapidly estimate and disseminate VE prior to the next influenza season. This importantly facilitates comparison of VE between different seasons as circulating influenza subtypes vary between influenza seasons and thus in the closeness of matching to the annual seasonal vaccine. In our study, the influenza subtypes identified among cases closely resembled circulating influenza in the wider population, with the 2009 pandemic influenza A(H1N1) virus predominating in 2013/14 [16].

A strength of this approach is the use of laboratory-confirmed endpoints to provide a more specific VE estimate compared to influenza-like Illness. One potential limitation is the relatively low intensity season seen in 2013/14, which resulted in relatively small numbers of cases in the study and wide CIs. Despite this, evidence of significant effectiveness was observed, although not the ability to examine sub-type specific protection. A further potential limitation of the screening method is that VE can only be adjusted for covariates measured in the population vaccine coverage data; although we were able to adjust for factors such as month, we were not able to examine the effects of trimester of vaccination, prior maternal vaccination or breastfeeding in this study. Furthermore, if unvaccinated mothers took ill infants to health services less frequently than vaccinated mothers, this may bias the VE towards a lower estimate; however such a potential bias due to differences in healthcare seeking would be less important for severe illness requiring hospitalisation. The cases included in this study represent those tested for clinical purposes. Such a selection could potentially bias VE estimates, if the decision to test was associated with the vaccination status of infants' mothers; however such an effect would be limited as clinicians are unlikely to have been aware of mothers' vaccination status.

A significant burden of influenza illness is observed in young infants when compared to children and young adults. Preventive measures are needed to reduce influenza-related morbidity among infants, and possibly alleviate pressures on health services, including primary and secondary care. Our study results suggest that seasonal influenza vaccination during pregnancy is effective in preventing laboratory-confirmed influenza illness among infants, potentially helping to address this burden of illness. Although further such studies are needed, particularly in Europe, vaccine uptake in pregnant women can be further improved and our findings can be used to support pregnant women to make informed decisions about seasonal influenza vaccination in pregnancy.

Acknowledgements

We would like to thank the laboratories participating in the Respiratory DataMart System (RDMS) and the general practitioners who responded with vaccination information, for their assistance. We are also grateful to the reviewer for their comments.

This work was undertaken as a routine public health function to monitor vaccination programmes; PHE holds permissions to collect data under Section 251 of the National Health Service Act 2006 and the 2002 Health Service (Control of Patient Information) regulations as part of monitoring the performance of the national vaccination programme.

Conflicts of interest

All authors employed by PHE which is responsible for monitoring of influenza immunisation programmes. MZ reports as a government body representative, frequent interactions with colleagues in vaccine industry to ensure good public-private liaison activities for national vaccine campaigns. MD reports personal fees from Sanofi Pasteur MSD, non-financial support from Janssen, non-financial support from the European Centre for Disease Prevention and Control (ECDC), outside the submitted work; and is an employee of public health England (PHE). PHE is a government agency that provides guidance on multiple aspects of influenza management, treatment, prevention and public health.

Authors' contributions

All authors contributed to the design of the project and writing and editing of the manuscript.

Data Collection: GD, HZ, FB

Data Analysis: GD, NA, RP

GD undertook the data analysis and had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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10 years of surveillance of human tularaemia in France

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Tularaemia has been mandatorily notifiable in France since October 2002. The surveillance aims to detect early any infection possibly due to bioterrorism and to follow up disease trends. We report the results of national surveillance from 2002 to 2012. A case is defined as a patient with clinical presentation suggestive of tularaemia and biological confirmation of infection or an epidemiological link with a biologically confirmed case. Clinical, biological and epidemiological data are collected using a standardised notification form. From 2002 to 2012, 433 cases were notified, with a median age of 49 years (range 2 to 95 years) and a male–female sex ratio of 1.8. Most frequent clinical presentations were glandular tularaemia (n=200; 46%) and ulceroglandular tularaemia (n=113; 26%). Most frequent at-risk exposures were handling hares (n=179; 41%) and outdoor leisure exposure to dust aerosols (n=217; 50%). Tick bites were reported by 82 patients (19%). Ten clusters (39 cases) were detected over the 10-year period, as well as a national outbreak during winter 2007/2008. The tularaemia surveillance system is able to detect small clusters as well as major outbreaks. Surveillance data show exposure to dust aerosols during outdoor leisure activities to be a major source of contamination in France.

Introduction

Tularaemia is a disease caused by the bacterium *Francisella tularensis*, presenting with various clinical patterns. Infection due to *F. tularensis* subspecies *holarctica* encountered in Europe is usually relatively mild and less severe than the infection due to *F. tularensis* subspecies *tularensis* present in North America [1]. The clinical presentation is directly related to the route of infection, with local symptoms at the bacterium's point of entry. The most frequent presentation is an ulcer following the inoculation of the germ, associated with local adenopathy, or an isolated increased lymph node when the inoculation lesion goes unnoticed. In case of inhalation of the bacterium, pneumonia can occur with increased lymph nodes in the mediastina. Oropharyngeal tularaemia associated with swollen ear, nose and throat lymph nodes develops after the ingestion of the bacterium, and oculoglandular tularaemia when eyelids and other periorbital structures are infected, usually through aerosols or contact with fingers carrying the bacterium.

A wide range of animals, encompassing arthropods, birds, rodents, lagomorphs, carnivores and ruminants, can carry *Francisella*, but a definitive reservoir has not been identified [2]. It is likely that different epidemiological cycles exist in different environmental settings, suggesting that the primary reservoir may vary between cycles [3]. Not all animals play a significant role in the transmission of the bacterium to humans. In France, the disease has long been known to occur in people who skin hares, and has sometimes been called hunters' wives' disease [4,5].

F. tularensis subspecies *tularensis* is considered worldwide to be a potentially weaponisable bacterium, and tularaemia fulfils the criteria for surveillance in the community in the event of deliberate release [6]. Epidemiological surveillance of human tularaemia was implemented in France in October 2002 as part of the national preparedness plan against bioterrorism. The surveillance is carried out by mandatory notification and by the analysis of strains and biological samples from patients by the national reference centre for *F. tularensis* (University Hospital of Grenoble, France). Besides detection of cases due to deliberate release of *Francisella*, the surveillance provides a precise description of cases diagnosed in France in a natural non-bioterrorist context. The purpose of this article is to present the results of national surveillance from 1 October 2002 to 31 December 2012.

Methods

In France, attending physicians and microbiologists must notify all cases of tularaemia to the regional health agencies using the standardised notification form. A case of tularaemia is defined as a patient presenting with clinical signs and symptoms suggestive of tularaemia; for a confirmed case, a positive PCR or the isolation of *F. tularensis* in a biological sample, or a seroconversion or four-fold increase of serological titre demonstrated on two samples taken with a minimum interval of two weeks; and for a probable case, a single elevated serological titre [7].

Figure 1

Classification of cases according to the most likely source or circumstance of infection, tularaemia cases notified in France, 2002–2012 (n=433)

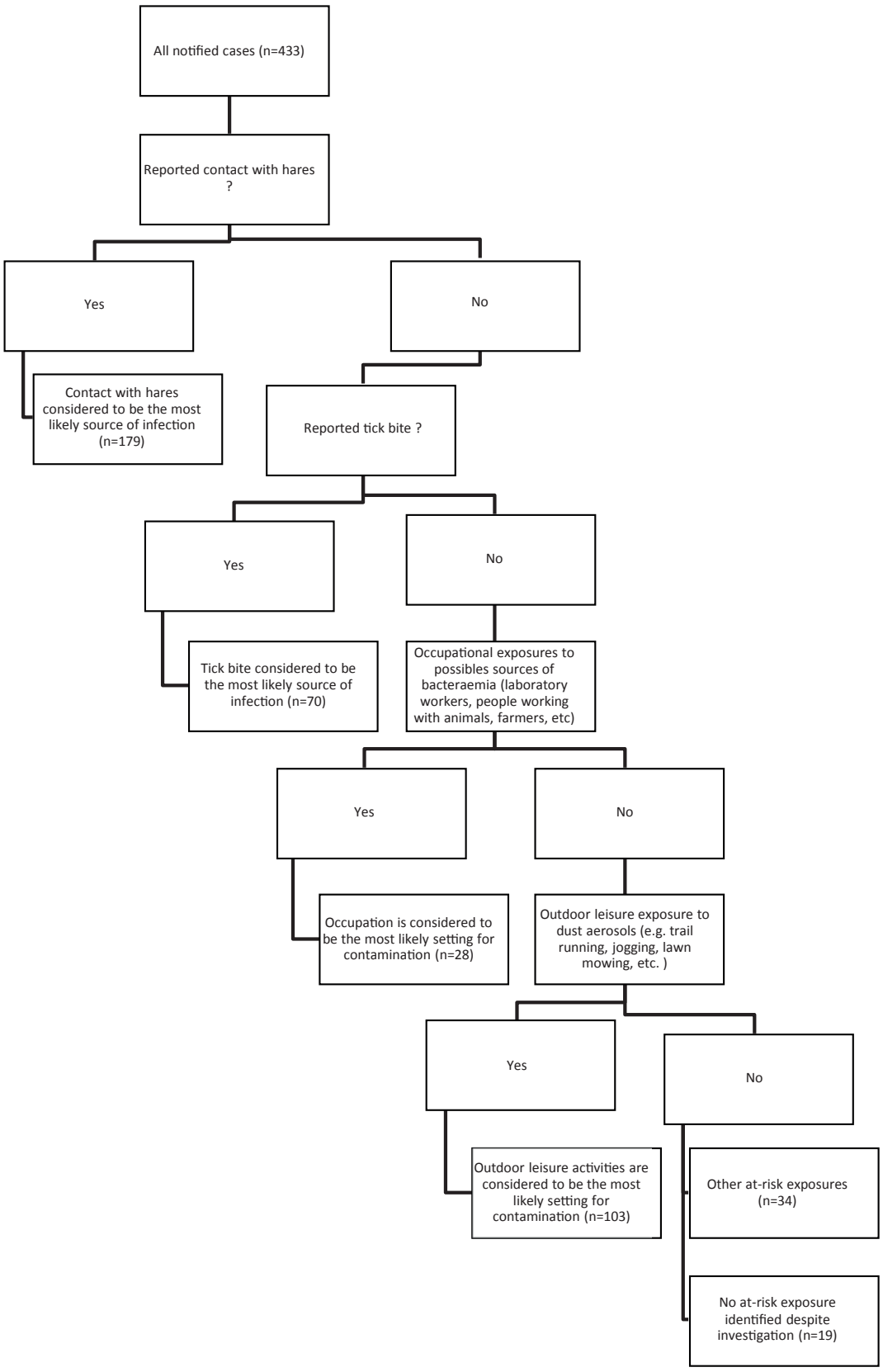
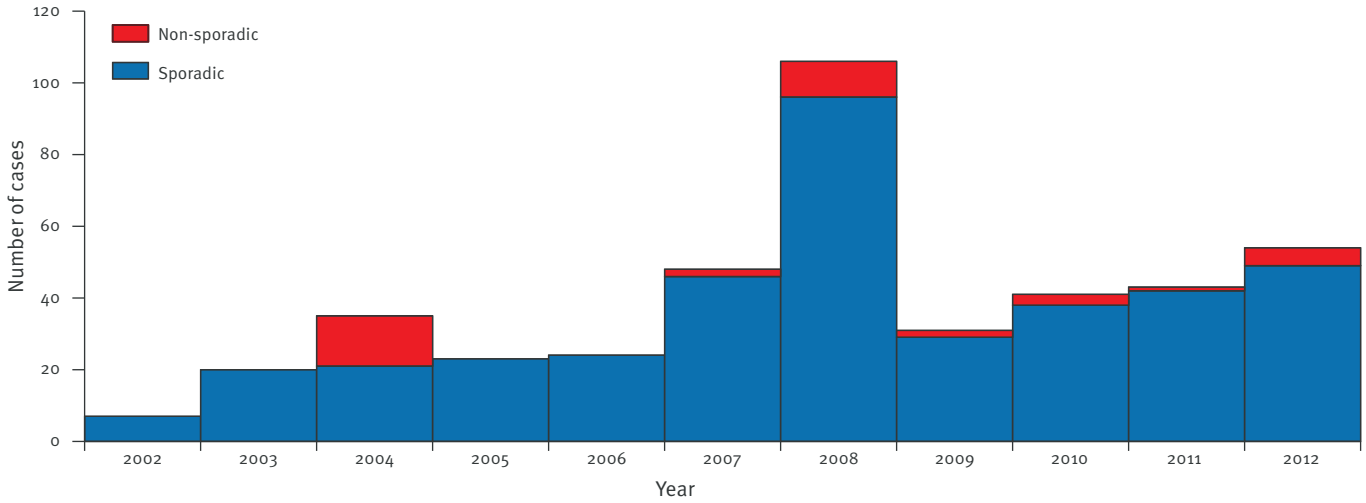


Figure 2

Number of sporadic and non-sporadic cases of tularaemia notified in France by year of notification, 2002–2012 (n=433)



The notification form used for the surveillance encompasses clinical details, diagnosis features and at-risk exposures during the month before symptom onset. Clinical presentations are determined according to reported symptoms [8]. If no at-risk exposure is mentioned on the form, a trawling questionnaire is completed with the attending physician and the patient to rule out a possible non-natural contamination. When the diagnosis is obtained by the identification of a bacterial strain, the subspecies is determined by the national reference laboratory by PCR amplification and sequencing of the 16S rRNA gene and the intergenic spacer region [9].

We attempted to attribute a most likely origin of contamination to each of the notified cases. We defined as at-risk exposures tick and mosquito bites received in Europe, direct contact with animals (including hares, rodents, wild rabbits, ruminants and crayfish), occupational exposure to animals or to an environment possibly contaminated by animals, outdoor activity exposure to dust or soil aerosols in areas where reservoir animals are present. More specifically in France, the handling, skinning or evisceration of hares have been known for years as a frequent cause of contamination [4]. By contrast, this exposure is rare in the general population, and rarer than tick bites, occupational exposure or outdoor activities. If a patient reported only one at-risk exposure, we attributed her or his most likely origin of contamination to this exposure. For patients reporting several at-risk exposures as defined above, we considered that the probability of an at-risk exposure being the source of contamination was inversely proportional to its frequency in the general population. For patients with several at-risk exposures, we attributed the most likely origin of contamination according to the following scheme (Figure 1):

- any patient infected with *F. tularensis* who reported direct contact with hares during the exposure period would have been infected through this exposure;
- a reported tick bite during the exposure period would be the contamination route of any patient unless the patient also reported direct contact with hares;
- an at-risk occupation during the exposure period would be the circumstance of contamination unless the patient also reported a direct contact with hares or a tick bite;
- leisure activities resulting in exposure to aerosols or dust in the forest during the at-risk period would be the circumstance of contamination unless the patient reported direct contact with hares, or a tick bite or an occupational exposure.

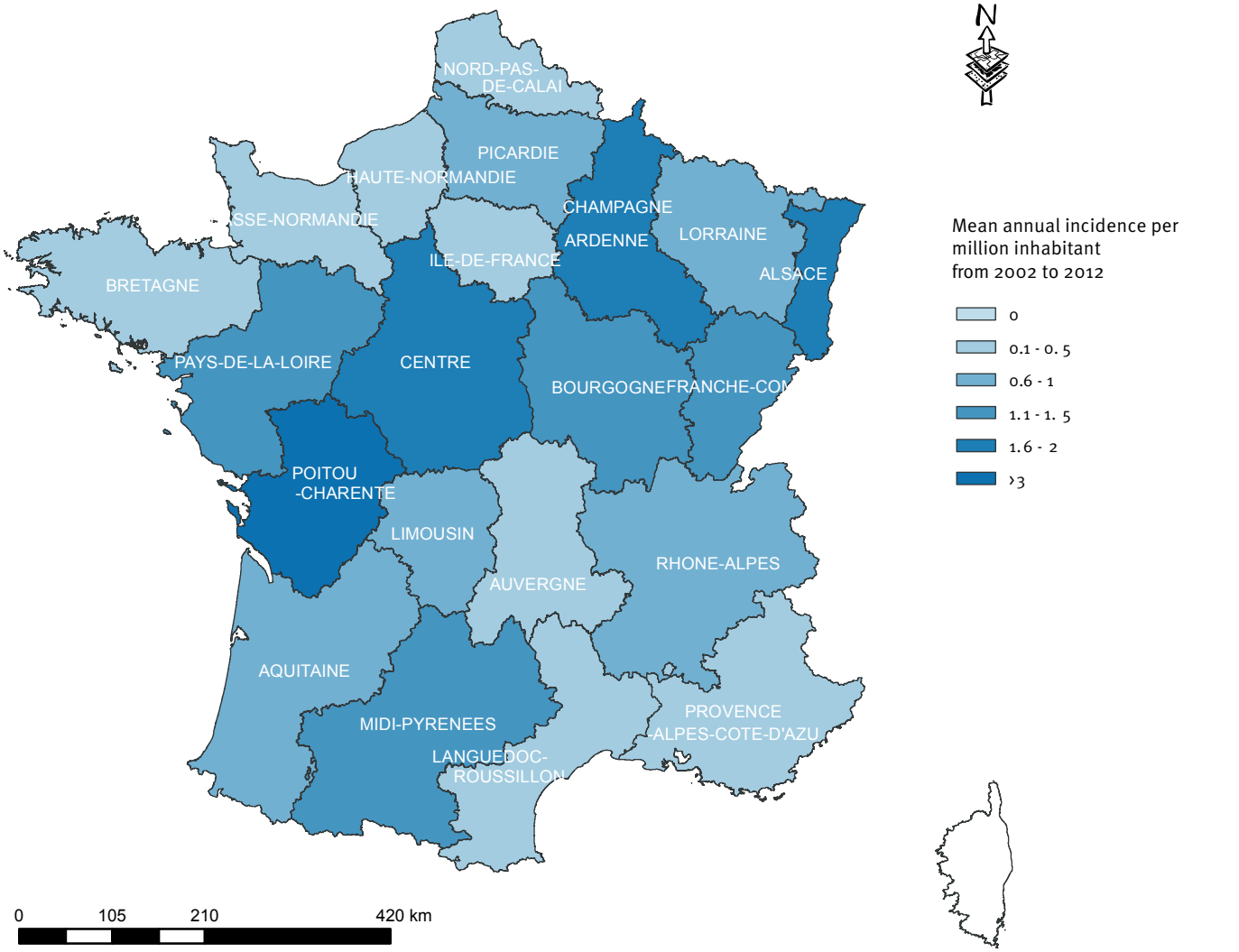
In the surveillance system, clusters of tularaemia cases are defined as more than one case reported in a single household or two or more cases in the same social circle, or as three or more cases in the same district within a 30-day period. Districts are administrative geographical areas, of which France has more than 100, including overseas territories, with populations ranging from 77,000 inhabitants to 2.5 million. All clusters are investigated using the trawling questionnaire.

All variables included in the notification form were computed using Voozanoo (EpiConcept, Paris, France) and analysed with Stata 11 (Stata Corporation, College Station, Texas, USA). Variables were compared using Pearson's chi-squared test or Student's t-test.

Surveillance data collection has been approved by the national ethics committee (CNIL), according to the French regulation on medical confidentiality.

Figure 3

Incidence of tularemia by region of residence of the cases, France 2002-2012



Results

Demographic data and seasonality

From 1 October 2002 to 31 December 2012, 433 cases of tularaemia were identified in France, making an annual mean incidence of 0.07 cases per 100,000 inhabitants (range 0.01–0.16). Of these, 395 (91%) were sporadic cases and 39 (9%) were notified as part of 10 clusters (see below) (Figure 2).

The global trend in the number of sporadic cases suggests a progressive increase in the number of notifications since surveillance began, except during winter 2007/2008, when a short sudden peak of cases was recorded (Figure 2). The peak of cases during winter 2007/2008 occurred simultaneously with an outbreak of tularaemia in hares and has been described elsewhere [10].

More cases had their onset of symptoms during autumn (September/October/November; n=135; 31%) and winter (December/January/February; n=123; 28%).

Cases were notified in all French regions except Corsica (Figure 3). The highest incidences each year were recorded in Poitou-Charentes (mean 0.32/year/100,000 inhabitants), and Alsace (mean 0.17/year/100,000).

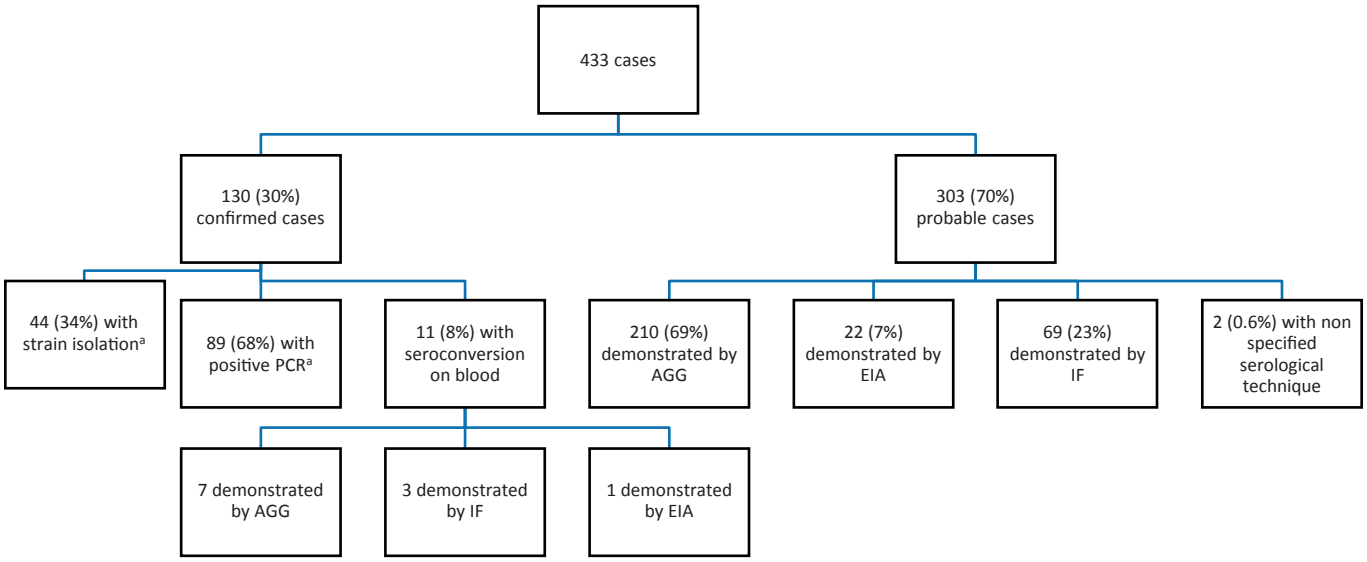
The male–female sex ratio was 1.8, and the mean age of cases was 49 years (SD=17, range 2 to 95 years).

Clinical presentation

The most frequent clinical presentations were glandular (n=200; 46%) and ulceroglandular tularaemia (n=113; 26%). Typhoidal tularaemia (n=45, 10%), pneumonic (n=42; 10%), oropharyngeal (n=25; 6%)

Figure 4

Diagnosis evidence of tularaemia infections notified in France, 2002–2012 (n=433)



[a] 14 cases had both a positive PCR and Francisella strain isolation

AGG: agglutination test; EIA: enzyme immunoassay; IF: immunofluorescence

and oculoglandular tularaemia (n=8; 2%) were less common. Information about the localisation of the lymphadenopathy was available for 210 of 313 (67%) patients presenting with glandular or ulceroglandular tularaemia. The adenopathy concerned armpit lymph nodes (LN) in 118 of 210 cases (56%), inguinal LN in 56 (27%), LN of the ear, nose and throat (ENT) area in 26 (12%), epicondylar LN in 26 (12%), mediastinal LN in 9 (4%) and popliteal LN in 5 (2%).

During the 10 years of surveillance, three patients with neurological presentations were notified. The first was a 66 year-old man who presented with encephalitis and a positive blood culture demonstrating *Francisella*. He had eaten a terrine made from hare meat a few days before the onset of disease; the remains of the terrine were found to be positive for *Francisella* on PCR. The second patient, a man aged 48 years, was admitted to an intensive care unit (ICU) with sudden brainstem encephalitis encompassing tetraplegia. Serology demonstrated a seroconversion for *Francisella* during his hospital stay. No other infectious agent that could be responsible for brainstem encephalitis was diagnosed. The patient had been bitten by a tick a few days before illness onset. The third patient was a man in his sixties with a history of pancreatic cancer and diabetes. He presented with pneumonia and encephalitis on admission, and a strain of *Francisella* was isolated from a cerebrospinal fluid (CSF) sample.

Other infrequent presentations were erythema nodosum in three patients, and a lobar pneumonia following a near-drowning in a patient who demonstrated a positive blood culture a few days after the drowning.

A total of 188 (43%) patients required hospitalisation. At the time of notification, the evolution was considered favourable for 211 cases (49%), the disease was still ongoing for 200 (46%), 20 presented with complications, and two patients had died. The complications were LN abscess in 15 cases (75%), pulmonary abscess in one case, palpebral abscess in one case, erysipelas in one case, parotiditis in one case and labyrinthitis in one case. The two deaths occurred in two male patients aged in their eighties and nineties. The first patient had a chronic history of severe cardiac arrhythmia and presented with pneumonic tularaemia with positive blood culture. The second patient presented at first with fever and had a positive blood culture of an unidentified bacterium initially assumed to be due to laboratory contamination. By the time the isolated bacterium was properly identified as *Francisella*, the patient had developed pneumonia and later presented with acute respiratory distress syndrome and septic shock.

Laboratory diagnosis

Of the 433 tularaemia cases, 130 (30%) were confirmed cases and 303 (70%) were probable cases (Figure 3). Of the 130 confirmed cases, 30 (23%) cases were diagnosed by isolation of a strain of *Francisella*, 75 (58%) by PCR, 14 (11%) by both isolation and PCR, and 11 (8%) by seroconversion. The 44 strains isolated all belonged to *F. tularensis* subspecies *holarctica* and were isolated from blood (n=19; 43%), abscess puncture (n=4; 9%), LN biopsy (n=8; 18%), skin biopsy (n=8; 18%), conjunctival swab (n=2; 5%), CSF (n=1; 2%) and undetermined samples (n=2; 5%). The 89 positive PCR were obtained from LN (n=67; 75%), skin biopsy (n=7; 8%),

Table 1

At-risk occupations of tularaemia cases notified in France, 2002–2012 (n=79)

At-risk occupation	Number (%)
Farmer/cattle breeder	38 (51)
Forest worker	12 (16)
Butcher/kitchen worker	7 (9)
Laboratory worker	4 (5)
Veterinarian/veterinary nurse	4 (5)
Fruits and vegetable producer	4 (5)
Landscaper	3 (4)
Vineyard worker	3 (4)
Petshop worker	1 (1)
Rendering plant worker	1 (1)
Horse riding teacher	1 (1)
Farm machine dealer	1 (1)
Total	**79 (100)**

Table 2

At-risk exposures reported by tularaemia cases notified in France, 2002–2012 (N=433)

At-risk exposures	Number (%)
Occupational	79 (18)
Non-occupational direct contact with animals	311 (72)
Game animals	
Hares	179 (41)
Boars	8 (2)
Roe deer	8 (2)
Breeding animals	
Rabbits	18 (4)
Cattle	2 (0.5)
Goats	2 (0.5)
Sheep	3 (0.7)
Poultry	8 (2)
Pet animals	
Dogs	16 (4)
Cats	13 (3)
Horses	5 (1)
Wildlife excluding game animals	
Rodents (excluding pet rodents)	42 (10)
Stone martens	1 (0.2)
Foxes	1 (0.2)
Non-specified animal	49 (11)
Outdoor leisure activities	217 (50)
Hunting	52 (12)
Gardening	59 (14)
Sport	51 (12)
House work/rehabilitation	11 (3)
Tick bites	82 (19)
Mosquitoes/tabanids bites	29 (7)
Total at-risk exposures	**433 (100)**

A case could report several at-risk exposures.

pharyngeal swabs (n=5; 6%), whole blood sample (n=2; 2%), abscess pus (n=2; 2%), serum sample (n=1; 1%), conjunctival swab (n=1; 1%) and undetermined samples (n=4; 4%).The 303 probable cases were diagnosed by a single elevated titre in serology using various methods (Figure 4).

At-risk exposures reported by cases

79 (18%) patients reported occupational activities exposing to animals or an environment possibly contaminated by animals (Table 1). A total of 82 (19%) patients reported tick bites before onset of symptoms: of these, 15(18%) lived in Alsace and 7 (8%) in Lorraine, both regions are located along the German–French border. Non-occupational direct contacts with one or more different animals were reported by 311 (72%) patients. The most frequent animals reported were hares (n=179; 41%) and rodents (n=42; 10%) (Table 2).

The most frequent at-risk exposures reported by patients were outdoor leisure exposure to dust aerosols (n=217, 50%), such as gardening, jogging or biking in the forest.

Most likely source of contamination

We determined the most likely source of contamination for the cases as described in the methods section: hare handling for 179 (41%) patients, and tick bite for 70 (16%). For other patients, the most likely circumstance of contamination was an at-risk occupation for 28 (6%) and outdoor leisure activities for 103 (24%). 34 (8%) patients had various other at-risk exposures and 19 (4%) did not report any at-risk exposures.

The sex and age distribution did not differ significantly between the four main exposure groups (hares, ticks, occupational and outdoor leisure). Systemic presentations (pneumonic tularaemia and typhoidal tularaemia) were significantly more frequent in patients exposed through outdoor leisure (n=35; 34%) or occupation (n=8; 29%) than through tick bites (n=10; 14%) or hares (n=19; 11%) ($p<0.05$) (Table 3). By contrast, glandular and ulceroglandular tularaemia were significantly more frequent in patients with tick bites as the most likely source of infection (n=60; 86%) or hares (n=185; 81%) than in patients presumably contaminated through outdoors leisure (n=56; 57%) or occupational activities (n=17; 61%) $p<0.05$).

Monthly distribution of cases by most probable source of infection is presented in Figure 5. Contamination most likely due to hares occurred during the legal hunting period (from September to February), contamination most likely related to tick bites occurred in spring and summer. By contrast, contamination most likely

Table 3

Distribution of clinical presentations, by most likely source of contamination, tularaemia cases notified in France, 2002–2012(n=380)

	Hare handling	Tick bite	At-risk occupation	Outdoor leisure activities
Glandular	94 (53%)	33 (47%)	14 (50%)	35 (34%)
Ulceroglandular	51 (28%)	27 (39%)	3 (11%)	24 (23%)
Typhoidal	10 (6%)	6 (9%)	4 (14%)	16 (16%)
Pneumonic	9 (5%)	4 (6%)	4 (14%)	19 (18%)
Oropharyngeal	12 (7%)	0 (0%)	2 (7%)	6 (6%)
Occuloglandular	3 (2%)	0 (0%)	1 (4%)	3 (3%)
Total	**179 (100%)**	**70 (100%)**	**28 (100%)**	**103 (100%)**

A case could report several at-risk exposures.

related to occupational or outdoor leisure activities occurred throughout the year.

Four patients were laboratory workers. Two of them worked in hospital laboratories and had handled biological samples of two other cases without taking appropriate precautions. The other two were working for local veterinary services and had carried out post-mortem examinations on infected hares without the necessary protection. Twelve (3%) cases were considered to be imported as they had spent the month before onset of illness in other countries where they reported at-risk exposures.

Clusters

From 2002 to 2012, 10 clusters were detected through the surveillance system: two were laboratory-acquired infections (see above), three were air-borne clusters, four were food-borne outbreaks and the origin of the last cluster is unknown.

Air-borne cluster of cases

A cluster involving 14 patients occurred among tourists staying in a rural cottage in western France in 2004 [11]. Six patients presented with pneumonia and eight with typhoidal tularaemia. The investigation concluded that there had been air-borne contamination through aerosols from a highly contaminated rural environment. A cluster of airborne tularaemia infections was notified in 2012 in two neighbours who had cleared the undergrowth together in a field on a rabbit-breeding farm. Both patients presented with pneumonia.

During the summer of 2008, 10 French tourists were hiking in Spain along the pilgrim route to Santiago de Compostela. At this time, a major tularaemia outbreak occurred in this area of Castilla y Leon [12]. Eight of the tourists reported various non-specific symptoms at the end of their travel: fever (n=5), malaise (n=8), dyspnea (n=2) and conjunctivitis (n=1). They did not receive any treatment. Five were tested by serology after they returned in France and three were positive (the two who reported dyspnea and the one who reported conjunctivitis).

Food-borne clusters

Four food-borne clusters were identified in 2007, 2008, 2009 and 2012. Three of these occurred following the handling, skinning and culinary preparation of hares rather than hare consumption. They involved, respectively, a hunter and his wife, a hunter and his neighbour, and three members of one family. The fourth food-borne cluster involved seven people who prepared and ate a hare they had found dead in the countryside for a family lunch. All seven presented with massive oedema of tongue and pharyngitis a few hours after the shared meal. The hare had been prepared following a local recipe that used the uncooked liver and blood of the animal.

Cluster with undetermined origin of contamination

A married couple were diagnosed with tularaemia by serology after returning from Italy in 2010. No at-risk exposure was retrieved from their interview and both presented with non-specific abdominal pain and digestive symptoms. Although their clinical presentation suggested food-borne contamination, it was not possible to determine the source of infection.

Diagnosis and notification delays

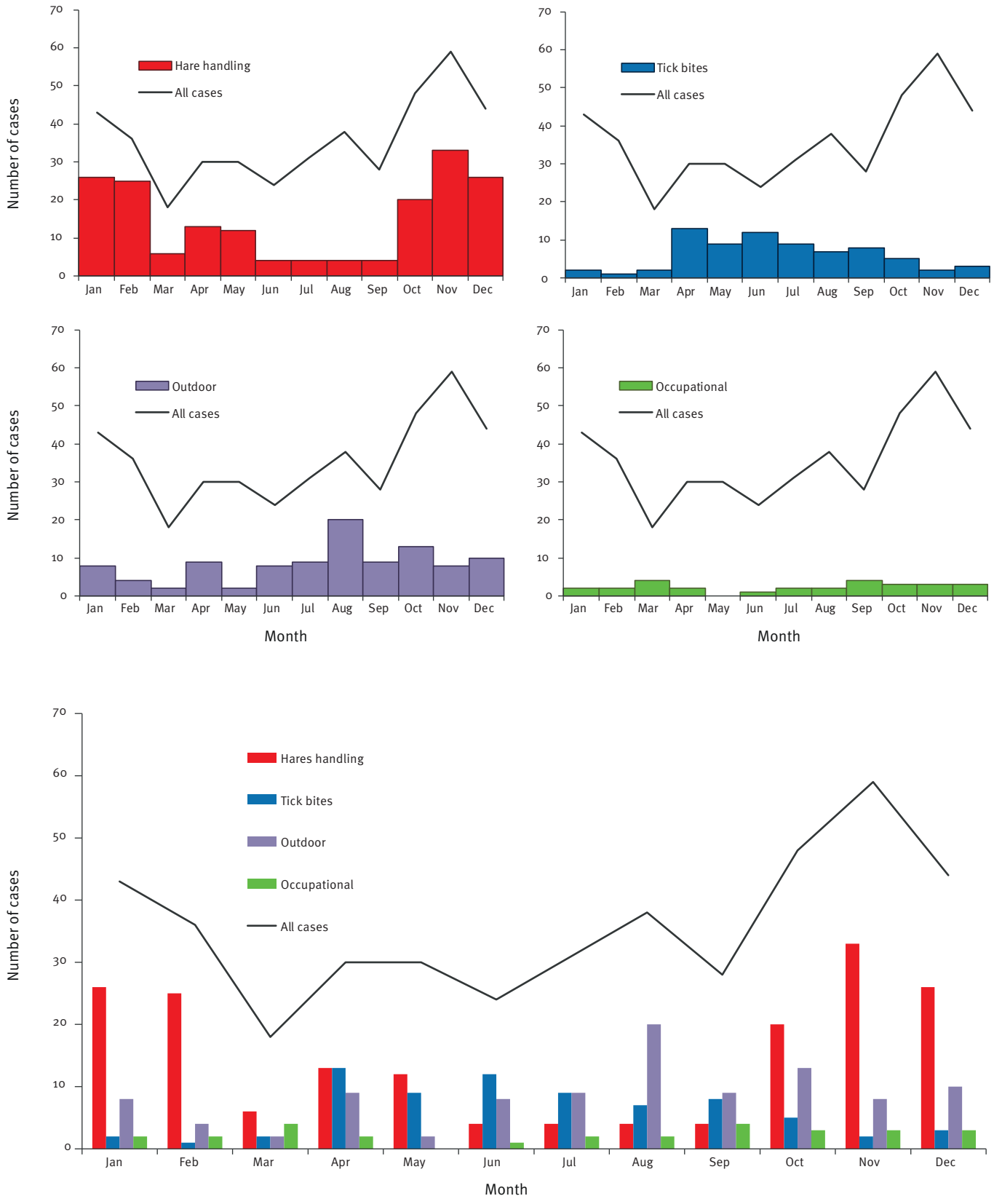
The median time from symptom onset to diagnosis was 24 days (range 1 to 254), and was 19 days from diagnosis to notification (range 0 to 470 days). However, these delays were shorter for cluster patients (respectively 11 days, p=0.003 from onset to diagnosis, and 20 days, p=0.25 from diagnosis to notification).

Discussion

Tularaemia has been mandatorily notifiable in France for 10 years, and an increasing number of cases has been reported every year since then. It is likely that the increase is due to more systematic notifications rather than to an increasing incidence of the disease, and that

Figure 5

Distribution of notified cases of tularaemia, by month of onset of symptoms, France, 2002–2012



the numbers of reported cases will continue to increase in the next few years.

Tularaemia is notifiable at the European level with a standardised case definition [13]. The case definition used in France is more specific than the European case definition, as laboratory confirmation is required to notify cases, even when an epidemiological link is demonstrated. However, if a suspected case with only an epidemiological link to a case were to be notified, we would request that the suspected case be tested for tularaemia. We are therefore confident that we do not miss cases due to our case definition. Another difference is the classification of probable versus confirmed case. Because all cases are reported to ECDC, this difference does not result in a different interpretation of data at the national or European level.

In 2011, Europe's highest annual incidences were reported in Finland (7.6/100,000 inhabitants) and Sweden (2.6/100,000) [14]. By contrast, the disease was rare in France in 2011 (0.07/100,000). The incidence in neighbouring countries the same year, such as Germany (0.17/100,000) or Italy (0.19/100,000), is higher than the national French incidence, although far lower than in Finland or Sweden. However, France's Alsace region, located along the German–French border, reported an incidence (0.17 /100,000) in 2011 comparable to the German incidence, suggesting common epidemiological patterns.

The low incidence reported in France suggests that the annual average of 40 cases notified underestimates the real number of cases. Some physicians probably do not know that the disease is notifiable because of its rarity. Moreover, because the clinical presentation may be non-specific, especially for typhoidal or pneumonic forms, some infections may not be diagnosed. Due to the absence of other independent sources of information about human tularaemia in France, the exhaustiveness of the surveillance system cannot be assessed.

The surveillance system identified 10 clusters of patients and a nationwide outbreak in 2007/2008. For all but one cluster, the investigation quickly ruled out the hypothesis of any intentional release of *Francisella*. During the winter outbreak of 2007/2008, the most frequent at-risk exposure was direct contact with hares [10]. During the same period, the surveillance of tularaemia in hares displayed a concomitant increase in this species, suggesting a causal link between the outbreak among humans and among hares or a common environmental exposure, and therefore making the hypothesis of bioterrorism unlikely. The number of cases of tularaemia in France is likely underestimated. Despite this, the results reported here confirm that the human surveillance system is able to detect small clusters as well as large outbreaks and therefore plays its role in preparedness for and early detection of bioterrorism.

The median delays from onset of symptoms to diagnosis, and from diagnosis to notification are too long and are not compatible with the necessary early detection in case of bioterrorism. However, it should be noticed that tularaemia is a rare disease in France, possibly presenting with non-specific early symptoms. Due to the absence of *F. tularensis* subspecies *tularensis* in France, most cases present with mild clinical signs, probably resulting in an increased delay before the diagnosis is suggested. Moreover, in case of bioterrorism, we would expect to detect clustered cases with severe clinical presentation and an unusual geographical distribution (urban areas), even before those cases are diagnosed with tularaemia. However, these data give important clues for improvement of the surveillance system. Special attention will be given to shortening the timeframe for diagnosis and notification in the future, especially by raising clinician and microbiologist awareness for reporting the cases to the public health authorities.

The most frequently reported at-risk exposures among cases were outdoor leisure exposure to dust aerosols. This exposure is, however, very common in the general population. More interestingly, common at-risk exposures known as main routes of infection in other European countries were far less frequent in France: mosquito bites known as a major source of infection in Sweden [15], and contact with water animals such as crayfish responsible for a major outbreak in Spain [16]. These results suggest that an aquatic ecological/epidemiological cycle of *Francisella* might be of low epidemiological importance in France. A frequent at-risk exposure among patients was direct contact with hares. Hares are known to be a major reservoir of *Francisella* and a source of infection for humans in North America and several European countries [4,17–19]. By contrast with outdoor activities, handling hares is a rare at-risk exposure in the general population but is frequent among tularaemia cases and can therefore be considered a likely cause of infection when reported. However, we may overestimate the proportion of cases attributable to hare handling since clinicians are more likely to investigate tularaemia in patients reporting hare handling than in other patients.

Tick bites were a frequent (19%) but not major at-risk exposure among French cases, compared with outdoor leisure activities (50%) or contact with hares (41%). Most cases with tick bites lived close to the German–French border in an area known to be the main focus in France for other tick-borne diseases such as tick-borne encephalitis or Lyme disease [20,21]. However, rare tick-borne tularaemia cases were identified throughout all French districts. It is also possible that cases reporting outdoors activity as their only at-risk exposure may also have been exposed to mosquito or tick bites that went unnoticed.

Our attempt to attribute a most likely source of infection based on the at-risk exposures reported by the

cases demonstrated a significant association between the clinical presentation and the likely source of infection. Systemic presentations were associated with at-risk exposures suggesting air-borne infection whereas focalised clinical presentations were associated with at-risk exposures responsible for inoculation. Moreover, the presumed sources of infection were consistent with the timeline of onset of symptoms for all cases: cases presumed to be related to contact with hares occurred during the hunting season, those presumed to have resulted from tick bites occurred in spring and summer when ticks are most active, and cases associated with occupational exposure or outdoor leisure activities were recorded throughout the year.

Only two deaths were recorded during the 10 years of surveillance. There is no follow-up of the patients after the notification, we therefore cannot be sure that other patients did not experience later unfavourable outcomes. However, because only *F. tularensis* subspecies *holarctica* is present in France, a favourable outcome is expected in most patients. Indeed, the two patients with a fatal outcome had severe underlying diseases before their tularaemia infections, and these underlying conditions may have contributed to their death.

Three patients with severe neurological presentations were notified. Central nervous system (CNS) infections due to *Francisella* have been described infrequently in the literature and most published cases presented with meningitis rather than encephalitis [22–25]. All three cases reported to the surveillance system had encephalitis with serious brain involvement. *Francisella* could be isolated from CSF in only one patient with severe underlying immunosuppressive conditions. The absence of bacterium or antibodies in the CSF of the two other patients suggest a possible immune-mediated phenomenon rather than a direct invasion of CNS.

Conclusion

Mandatory notification of tularaemia implemented in France in 2002 has demonstrated its value for the detection of clusters and outbreaks. It is very likely that the incidence is currently underestimated due to probable underdiagnosis and undernotification. Therefore, efforts should be made to increase clinician awareness of the disease and the available diagnosis tools. Currently, the main sources of infection in France are hares, outdoor activities and tick bites. Hunters should be advised to wear gloves to skin game, and people exposed to tick bites should be advised to take protective measures such as wearing long trousers for outdoors activities and to carefully examine themselves for ticks and to remove them rapidly.

Authors' contributions

AM and VV coordinate the surveillance of tularaemia, including validation of notified cases and data, coordination of investigations when needed, and analyse of surveillance data. AM and VV analysed the data presented in the manuscript. AM wrote the manuscript, VV revised the manuscript and provided comments.

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Emerging cephalosporin and multidrug-resistant gonorrhoea in Europe

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Neisseria gonorrhoeae **has consistently developed resistance to antimicrobials used therapeutically for gonorrhoea and few antimicrobials remain for effective empiric first-line therapy. Since 2009 the European gonococcal antimicrobial surveillance programme (Euro-GASP) has been running as a sentinel surveillance system across Member States of the European Union (EU) and European Economic Area (EEA) to monitor antimicrobial susceptibility in *N. gonorrhoeae*. During 2011, *N. gonorrhoeae* isolates were collected from 21 participating countries, and 7.6% and 0.5% of the examined gonococcal isolates had in vitro resistance to cefixime and ceftriaxone, respectively. The rate of ciprofloxacin and azithromycin resistance was 48.7% and 5.3%, respectively. Two (0.1%) isolates displayed high-level resistance to azithromycin, i.e. a minimum inhibitory concentration (MIC) ≥256 mg/L. The current report further highlights the public health need to implement the European response plan, including further strengthening of Euro-GASP, to control and manage the threat of multidrug resistant *N. gonorrhoeae*.**

Introduction

Neisseria gonorrhoeae has consistently developed resistance to antimicrobials used therapeutically for gonorrhoea, including penicillins, macrolides, tetracyclines and fluoroquinolones [1,2]. Surveillance of *N. gonorrhoeae* antimicrobial resistance is essential to monitor the emergence and spread of the resistance and to inform treatment guidelines. Furthermore, surveillance of antimicrobial resistance as well as treatment failures is also crucial, as reports are emerging of decreased susceptibility, in vitro resistance and clinical failure of the last line of agents for antimicrobial monotherapy: the extended-spectrum cephalosporins, cefixime (oral) and ceftriaxone (injectable) [2-6]. The European management guidelines [7] have recently been revised to recommend ceftriaxone (500 mg intramuscularly) in combination with azithromycin (2 g single oral dose) for first-line treatment of all uncomplicated gonorrhoea cases, in response to the emerging in vitro and in vivo resistance to cefixime and ceftriaxone.

Since 2009 the European gonococcal antimicrobial surveillance programme (Euro-GASP) has been implemented by the European Centre for Disease Prevention and Control (ECDC) as a sentinel surveillance system across Member States of the European Union (EU) and European Economic Area (EEA) to monitor antimicrobial susceptibility in *N. gonorrhoeae* [8]. Here we describe the spread of gonococcal isolates with in vitro resistance to cefixime and resistance to other antimicrobials surveyed across Europe, and the subsequent European response [9] to the threat of multidrug-resistant *N. gonorrhoeae* (MDR-NG) [10].

Methods

During 2011 *N. gonorrhoeae* isolates were collected from 21 participating countries (Table 1) and examined during two periods: May/June and November/December. Participating countries followed one of two paths. There was a centralised testing model [11], in which antimicrobial susceptibility testing was performed on all isolates centrally by Etest (bioMérieux, Marcy l'Etoile, France) to determine the minimum inhibitory concentration (MIC) of cefixime and ceftriaxone or agar dilution for ciprofloxacin, azithromycin, spectinomycin and gentamicin. Alternatively, decentralised testing was performed, i.e. antimicrobial susceptibility testing was performed in the participant's own national reference or local laboratory. In 2011, ten countries performed decentralised antimicrobial susceptibility testing, by Etest or agar dilution (Table 1). As well as countries fulfilling the criteria for decentralised testing [11], an external quality assessment programme and a panel of control strains [11] were established to ensure comparability of data in this hybrid testing model.

The statistical significance of any changes in the proportion of isolates with resistance to tested antimicrobials

Table 1

Resistance to cefixime, ciprofloxacin and azithromycin in *Neisseria gonorrhoeae* isolates from 21 European Union/European Economic Area countries, 2011

Country	Number of isolates tested	Resistance						Method of testing
		Cefixime		Ciprofloxacin		Azithromycin		
		Number	%	Number	%	Number	%	
Austria	106	14	13.2	72	67.9	13	12.3	Centralised
Belgium	110	1	0.9	61	55.5	4	3.6	Decentralised (AG)
Cyprus	10	1	10.0	8	80.0	1	10.0	Centralised
Denmark	125	25	20	73	58.4	15	12.0	Decentralised (Etest)
France	109	0	0.0	49	45.0	2	1.8	Decentralised (Etest)
Germany	108	11	10.2	55	50.9	1	0.9	Centralised
Greece	100	3	3.0	74	74.0	5	7.9[a]	Decentralised (Etest)
Hungary	13	1	7.7	8	61.5	0	0.0	Centralised
Ireland	64	2	3.1	9	14.1	5	7.8	Centralised
Italy	99	3	3.0	60	60.6	4	4.0	Decentralised (Etest)
Latvia	28	0	0.0	8	28.6	1	3.6	Centralised
Malta	13	1	7.7	9	69.2	0	0.0	Centralised
Netherlands	217	0	0.0	56	25.8	12	5.5	Decentralised (Etest)
Norway	77	1	1.3	25	32.5	3	3.9	Centralised
Portugal	109	0	0.0	46	46.5[b]	8	7.3	Decentralised (Etest)
Romania	26	4	15.4	21	80.8	2	7.7	Centralised
Slovakia	113	41	36.3	80	70.8	7	6.2	Centralised
Slovenia	19	7	36.8	14	73.7	1	5.3	Centralised
Spain	100	15	15.0	59	59.0	14	14.0	Decentralised (AG)
Sweden	105	8	7.6	60	57.1	1	1.0	Decentralised (Etest)
United Kingdom	251	7	2.8	75	29.9	0	0.0	Decentralised (AG)
Total	**Total: 1,902 (ciprofloxacin total 1,892, azithromycin total 1,865)**	**145**	**7.6**	**922**	**48.7**	**99**	**5.3**	

AG: agar dilution.

[a] Calculated from 63 isolates with azithromycin results.
[b] Calculated from 99 isolates with ciprofloxacin results.

between years was determined by the Z-test (chosen due to large sample size and dichotomous variables).

Results

A total of 1,902 *N. gonorrhoeae* isolates from 21 participating countries were examined in 2011, representing an increase from the 1,766 and 1,366 isolates received from 17 countries in 2010 and 2009, respectively.

The proportion of isolates that displayed in vitro resistance (formerly described as decreased susceptibility) [11] to cefixime was 7.6% (145/1,902, Tables 1 and 2) in 2011 using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint (MIC>0.12 mg/L) [12]. This is a minor decrease compared to 2010 (8.7% versus 7.6%, Z-test, p=0.25) but still significantly higher than in 2009 (5.1% versus 7.6%, Z-test, p=0.005) (Table 2). In 2010 and 2011, cefixime resistant isolates were also detected in 17 countries, compared to only 10 in 2009 (Figure). Seventeen isolates had an MIC of 0.5 mg/L in 2011, which is an increase from two and four isolates in 2010 and 2009, respectively. All isolates showing in vitro resistance to cefixime were additionally resistant to ciprofloxacin (MIC>0.5 mg/L). In 2011, the first 10 (0.5%) Euro-GASP isolates with in vitro resistance to ceftriaxone (>0.12 mg/L) were identified from the same geographical area (Austria and Germany). All ten isolates had MICs of cefixime of at least 0.12 mg/L and were also resistant to ciprofloxacin.

The rate of ciprofloxacin resistance decreased significantly from 62.7% (857/1,366) in 2009 to 48.7% (922/1,892) in 2011 (Z-test, p<0.0002), and a significant decrease from 13.2% (180/1,366) to 5.3% (99/1,865) was observed for azithromycin resistance (MIC>0.5 mg/L; Z-test, p<0.0002) (Table 2), including two (0.1%) isolates displaying high-level resistance to azithromycin (MIC≥256 mg/L) in 2011.

The modal MIC to gentamicin was 8 mg/L (MIC range: 0.5 to 16 mg/L) and no resistance to spectinomycin (MIC range: 1.5 to 64 mg/L) was demonstrated.

Table 2

Overall proportion of *Neisseria gonorrhoeae* isolates from 21 European Union/European Economic Area countries with resistance to cefixime, ciprofloxacin and azithromycin, 2009–2011

Year	Cefixime resistant			Ciprofloxacin resistant			Azithromycin resistant		
	Cefixime resistant/ total	Percentage	95% confidence intervals	Ciprofloxacin resistant/total	Percentage	95% confidence intervals	Azithromycin resistant/total	Percentage	95% confidence intervals
2009	70/1,366	5.1	4.01–6.4	857/1,366	62.7	60.2–65.3	180/1,366	13.2	11.4–15
2010	153/1,766	8.7	7.4–10.1	930/1,766	52.7	50.3–55	127/1,766	7.2	6.02–8.5
2011	145/1,902	7.6	6.5–8.9	922/1,892	48.7	46.5– 51	99/1,865	5.3	4.4–6.4

Discussion

The results from Euro-GASP have demonstrated emerging cefixime and ceftriaxone resistance across the EU/EAA region. The levels of in vitro resistance to cefixime and ceftriaxone using the EUCAST breakpoints were 7.6% and 0.5%, respectively, in 2011. For comparison, the level of in vitro resistance to cefixime was 5.1% and 8.7% in 2009 and 2010, respectively, and no isolates with in vitro resistance to ceftriaxone have been identified before 2011. However it should be noted that definitive breakpoints have not yet been established for cefixime and ceftriaxone, and several treatment failures with cefixime (200 mg×2) [13] and cefixime 400 mg [6] have previously been caused by isolates with lower cefixime MICs than the tentative EUCAST breakpoint (›0.12 mg/L). Using an MIC breakpoint of ›0.06 mg/L for cefixime resulted in much higher proportions of the Euro-GASP isolates, 22.7% (400/1,766) and 18.6% (353/1,902), displaying in vitro resistance to cefixime in 2010 and 2011 respectively. And again the same breakpoint applied to ceftriaxone gave more isolates displaying in vitro resistance: 1.4% (24/1,699, 2010) and 2.5% (47/1,902, 2011).

Even though the levels of resistance to ciprofloxacin and azithromycin decreased significantly, the resistance level is still too high for these antimicrobials to be used for empirical antimicrobial monotherapy [14], unless the susceptibility has been confirmed with antimicrobial susceptibility testing before initiating the therapy of the individual gonorrhoea cases. The wide variation in resistance rates across the different countries (e.g. 0–36.8% for cefixime in vitro resistance) represents the few isolates from some countries and the very diverse region covered by Euro-GASP.

Both gentamicin and spectinomycin are potential options for gonorrhoea treatment; however the lack of sufficient clinical efficacy and safety data and breakpoints for gentamicin [2,4,15,16], as well as the difficulties in acquiring spectinomycin in most countries, the fear of rapidly selected resistance and the reduced

Figure

Geographical distribution of gonococcal isolates with in vitro resistance to cefixime (>0.12 mg/L) in Euro-GASP participating countries, 2009–2011

effectiveness of spectinomycin at clearing pharyngeal infections [2,4,17] make these options less than satisfactory for first-line antimicrobial monotherapy. Our results clearly show that new, or combinations of current, antimicrobials are desperately needed to maintain gonorrhoea as a treatable disease, and that every effort must be made to preserve the efficacy of existing therapeutic options.

The decreasing susceptibility to the extended-spectrum cephalosporins, the increasing number of reported treatment failures to extended-spectrum cephalosporins (particularly cefixime), the associated morbidity with *N. gonorrhoeae* infection and lack of alternative treatment options have led to the development of a response plan to control and manage the threat of MDR-NG in Europe and to support Member States in EU and EEA in their national responses to MDR-NG [3,9]. Euro-GASP is a sentinel surveillance system and so is unable to detect treatment failures; the response plan [9] therefore includes a strategy for the detection and verification of treatment failures in a timely manner. Molecular tests to diagnose gonorrhoea are advantageous in that they are highly sensitive and rapid, are amenable to high-throughput and do not require an invasive specimen. However the European response plan [9] strongly emphasises that continued use of culture in sentinel sites is key to obtaining information on antimicrobial susceptibility of *N. gonorrhoeae* isolates, which is essential in order to detect emerging resistance. Even though an increase in the number of participating countries and progress in obtaining isolates for Euro-GASP in some countries have contributed to the increase in isolate numbers over the years, an absence of participation and low isolate numbers from some countries, along with differences in representativeness are limitations of Euro-GASP. Therefore as part of the European response, Euro-GASP will be strengthened to ensure a greater representation of *N. gonorrhoeae* antimicrobial resistance profiles and associated epidemiological information in Europe. Training will be provided to enable capacity building and encourage the collection of isolates from countries where no susceptibility testing is currently performed. Decentralised testing will be further promoted to improve timeliness of reporting, engage national stakeholders and facilitate the sustainability of Euro-GASP. Finally, the awareness of policy makers, clinicians, patients, and key populations will be enhanced [9]. The European response [9] aims to implement the actions as specified within the World Health Organization global action plan on antimicrobial resistance of *N. gonorrhoeae* [18].

Effective control of gonorrhoea relies entirely on appropriate treatment with antibiotics, along with effective prevention, timely diagnostics, contact tracing (including diagnostics, treatment and notification of contacts), and surveillance. Both the European response plan [9] and the revised European management guidelines [7] contribute to the fight to keep gonorrhoea a treatable infection. A further benefit of the European response [9] is that a multidisciplinary collaboration between national and international stakeholders is developed, a network that will be valuable also for future gonococcal challenges.

Authors' contributions

M Cole analysed the data and drafted the manuscript. M van de Laar and G Spiteri coordinated Euro-GASP from ECDC. The laboratories of C Ison, S Chisholm, S Hoffmann and M Unemo contributed isolate data for the study. All authors contributed to the manuscript.

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Syphilis reinfections pose problems for syphilis diagnosis in Antwerp, Belgium – 1992 to 2012

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Persons with multiple syphilis reinfections may play an important role in syphilis transmission. We analysed all syphilis tests carried out for people attending the HIV/sexually transmitted infection (STI) clinic at the Institute of Tropical Medicine, Antwerp, Belgium, from 1992 to 2012 to evaluate the extent to which syphilis reinfections were contributing to the syphilis epidemic in Antwerp. We then characterised the features of the syphilis infections in individuals with five or more episodes of syphilis. A total of 729 syphilis episodes were diagnosed in 454 persons. The majority of syphilis episodes occurred in people who had more than one episode of syphilis (445/729; 61%). A total of 10 individuals had five or more episodes of syphilis diagnosed over this period. All were men who have sex with men, HIV positive and on antiretroviral therapy. They had a total of 52 episodes of syphilis diagnosed and treated. In 38/42 of the episodes of repeat syphilis in these 10 individuals, they presented without any signs or symptoms of syphilis. Given that the majority of cases of incident syphilis in our clinic were persons with reinfections and that they frequently presented without signs of symptoms of syphilis, there is a strong case for frequent and repeated screening in all persons with a diagnosis of syphilis.

Introduction

Syphilis is a sexually transmitted infection (STI) caused by *Treponema pallidum* subspecies *pallidum*. Its protean clinical manifestations depend on the stage of disease [1]. If a person presents in the primary stage, this typically presents as a painless chancre – after an incubation period of 10–90 days. In the secondary stage, there is a diffuse rash and in tertiary syphilis, there are neurological, cardiovascular or gummatous lesions. There are no symptoms in the latent phase, which occurs between the primary/secondary and tertiary phases [1]. Since 2000, there has been a considerable increase in the incidence of syphilis in a number of high-income countries, such as Belgium, Czech Republic, Denmark, Greece, Luxembourg, the Netherlands, Norway, Slovakia, Slovenia and Spain [2]. These outbreaks have occurred predominantly in men who have sex with men (MSM), many of whom are HIV positive [2-4]. In Belgium, the incidence of syphilis was 12 cases per 100,000 population in 2012 [5].

A number of studies have pointed out the importance of core groups in the genesis of the current syphilis outbreaks in high-income countries [3,4,6-8]. In some studies, it has been argued that persons with multiple syphilis reinfections are more likely to have high numbers of sexual partners and to be involved in high-risk sexual networks. As a result they may constitute a core-within-the-core and play an especially important role in syphilis spread [8,9].

In addition, there is recent evidence that syphilis is frequently diagnosed concomitantly with hepatitis C (HCV) infection in HIV-positive MSM and might play a role in HCV transmission [5]. The current syphilis epidemic in Antwerp, which started around 2000, has been characterised by a large number of clients who have repeated episodes of syphilis (repeat syphilis) [10,11].

The diagnosis of *T. pallidum* reinfection may be more difficult than the diagnosis of the first episode of syphilis. There are three possible reasons for this. Firstly, it is difficult to distinguish syphilis reinfection from disease relapse, since the diagnosis of both of these depends on clinical findings of syphilis and a fourfold increase in non-treponemal test titres [12,13]. Secondly, syphilis infection leads to partial immunity to reinfection and thus subsequent episodes of syphilis may not present in the same way as initial episodes [9,14,15]. Thirdly, in settings where syphilis is regularly screened for in high-risk persons, syphilis is more likely to be diagnosed earlier, before the development of clinical symptoms [7,16,17]. These last two factors mean that a large proportion of repeat syphilis may be diagnosed purely on the basis of changes in titres of non-treponemal tests. Both the sensitivity and the specificity of these tests are suboptimal at various stages of syphilis [18]. As a result, the diagnosis of repeat syphilis may be both under- and over-diagnosed.

Given these diagnostic difficulties, we undertook a study of syphilis reinfections in persons attending the Institute of Tropical Medicine's (ITM) HIV/STI clinic in Antwerp, Belgium, with the following aims: to establish the proportion of syphilis episodes occurring in persons with a previous diagnosis of syphilis; to characterise the features of the syphilis infections in persons with five or more episodes of syphilis; and to discuss the optimal diagnostic and screening strategies in individuals with repeat syphilis.

Methods

We conducted a laboratory review of all syphilis tests carried out for persons attending the HIV/STI clinic at the ITM from 1 January 1992 to 31 December 2012. From 2000, people at-risk attending the clinic were routinely screened 6–12 monthly with a rapid plasma reagin (RPR) test (Becton, Dickinson and Company, United States (US)) – a non-treponemal test – and a *T. pallidum* particle agglutination (TPPA) test (Fujirebio Inc., Japan). Persons at-risk were defined as those with high-risk sexual behaviour or a history of a previous diagnosis of an STI.

An episode of syphilis was defined as an episode in which a person who had an RPR titre ≥1/8 and a ≥4-fold increase in RPR titres from a previous RPR titre and a positive TPPA test on serum. Previous RPR results were not always available and thus the definition also applied to persons who on their first visit had a positive TPPA and an RPR titre ≥1/8 and the RPR titre fell ≥4-fold following appropriate therapy. A repeat episode of syphilis, which included syphilis reinfections and reactivations, was defined as an episode in a person who had a ≥4-fold increase in RPR titre, after a previous diagnosis of syphilis who exhibited an appropriate response to therapy (≥4-fold decrease in RPR). If there were signs of primary, secondary, tertiary or neurosyphilis at the time of the repeat syphilis, then the fourfold decrease was not required.

Detailed folder reviews were carried out for all persons who had had five or more episodes of syphilis recorded over the study period. We focused on clients with five or more episodes of syphilis because we considered that these individuals may represent persons in a core sexual network that could be particularly important for syphilis transmission. Data extracted for this subgroup included demographics, clinical details including other STI diagnoses made between 1992 and 2012 and the clinical stage of syphilis at the time of diagnosis and the therapy administered.

Stages of syphilis were determined by an infectious disease specialist according to US Centers for Disease Control and Prevention surveillance definitions [19,20]. Latent syphilis was defined as syphilis characterised by RPR/TPPA seroreactivity without other evidence of disease. Persons who had latent syphilis and who acquired syphilis during the preceding year were classified as having early latent syphilis. Men who have sex with men (MSM) were defined as men who reported ever having had sex with men.

The data for the number of syphilis cases diagnosed in the same time period for the Province of Antwerp were obtained from the Belgian Scientific Institute of Public Health. Ethical approval for the study was obtained from the Institutional Review Board of the ITM.

Statistics

The chi-squared test for trend was used to assess if repeat episodes of syphilis represented an increasing proportion of the total number of syphilis cases per year. All analyses were conducted in Stata 12.0 (Stata Corp, College Station, TX, US).

Results

A total of 3,581 individuals were tested for syphilis between 1992 and 2012 in the ITM HIV/STI clinic. Some 729 episodes of syphilis were diagnosed in 454 persons, including 284 considered as first episodes and 445 as repeat syphilis (Table).

Table. Syphilis diagnoses stratified by number of epi

The syphilis episodes observed at the ITM clinic constituted 44% (729/1,662) of all episodes of syphilis diagnosed in the Province of Antwerp over the study period. Syphilis was only diagnosed in persons who were HIV positive at the time of the diagnosis or who became HIV positive at a later date. There were no cases of syphilis diagnosed from 1992 to 1994; most syphilis diagnoses occurred after 2000 (Figure 1). The majority of episodes of syphilis occurred in people who had more than one episode of syphilis (445/729; 61%) (Table). The proportion of infections due to repeat syphilis increased over the study period ($p < 0.001$) (Figure 1).

Clients with five episodes of syphilis

A total of 10 persons had five or more episodes of syphilis diagnosed over the study period (Figure 2). All were MSM, HIV positive and on antiretroviral therapy

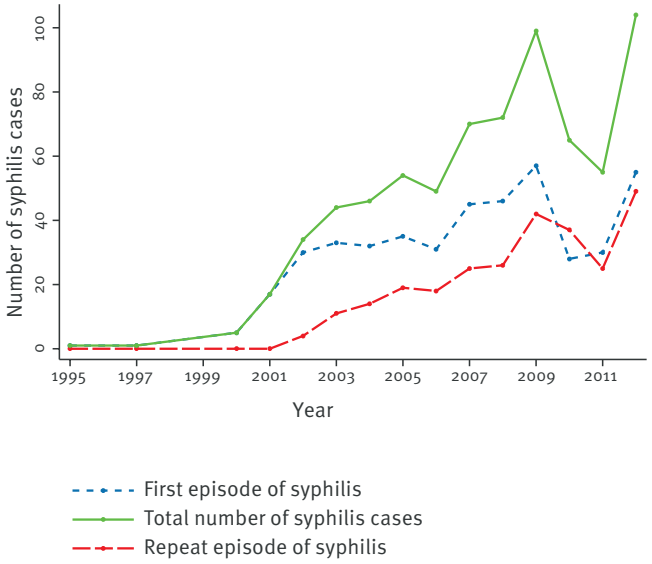
Table

Syphilis diagnoses stratified by number of episodes of syphilis per person attending Institute of Tropical Medicine's HIV/sexually transmitted infections clinic, Antwerp, Belgium, 1992–2012 (n=454)

Number of episodes of syphilis (A)	Number of persons (B)	Total number of episodes of syphilis (A × B)
1	284	284
2	100	200
3	47	141
4	13	52
5	8	40
6	2	12
Total	454	729

Figure 1

Cases of initial and repeat syphilis diagnosed per year at the Institute of Tropical Medicine, Antwerp, Belgium, 1992[a]–2012 (n=729)



[a] The graph begins in 1995 as there were no cases of syphilis diagnosed during 1992 to 1994.

with good virological and immunological responses. They had a total of 52 episodes of syphilis diagnosed and treated. The clinical stage of syphilis could be determined in all 10 first episodes of syphilis, but in only four of the 42 repeat episodes (Figure 2). All the remaining 38 repeat episodes were picked up by screening tests.

The 10 clients were diagnosed with one to eight (median: two) other STIs over the study period (Figure 2). This excluded HIV and hepatitis B, for which all 10 demonstrated evidence of previous or current infection.

The RPR titres declined by the requisite fourfold amount within a year following appropriate therapy in each of the diagnoses of syphilis, excluding the third episode in Case 9, where it took two years for this drop to occur.

The RPR/TPPA tests (both of which are routinely performed on the same serum specimen in our laboratory) were done a mean of 152 days apart (95% CI: 33–387 days).

Discussion

The incidence of syphilis continues to increase in a number of European countries [2-4,21-23] In many of these countries, the majority of these infections are occurring in MSM [2,3,23]. In our study, individuals with repeat syphilis infections constituted an increasing proportion of all syphilis infections in people visiting our clinic in Antwerp. They now constitute the majority of syphilis infections seen in our clinic.

As early as 1926, it was suggested that an increase in titre of one of the non-treponemal tests without symptoms or signs of syphilis could represent reinfection [21]. Great debate has followed about the relative importance of reinfection versus relapse in the aetiology of symptomless syphilis [24,25] and whether or not repeat syphilis presents in the same way as initial syphilis [9,25].

In the current outbreaks of syphilis in high-income countries in Europe and elsewhere, retrospective analyses of syphilis diagnoses within specific geographical areas have reached different conclusions as whether or not there are differences in how initial and repeat episodes of syphilis present. A study of MSM from San Diego, California, US, in 2004–07 found no difference [26], whereas other US studies from Florida and Seattle, Washington, in 2000–08 and 1992–2008, respectively, found that persons with repeat syphilis were more likely to present with asymptomatic disease – although in the Florida study this was only statistically significant for the HIV-infected MSM group [9,27]. In the Seattle study, repeat syphilis presented as early latent disease in 113/254 (44.5%) cases, while 309/1,191 (25.9%) cases with initial syphilis [9].

A consistent finding in studies in which individuals in a high-risk group are regularly screened for syphilis is that a relatively high proportion (with both initial onset and repeat syphilis) are found to have asymptomatic disease of recent onset. In a study that involved three-monthly RPR and TPPA screening of an HIV-positive cohort in London, England, in 2002, for example, most (26/44) new diagnoses of early syphilis were asymptomatic [15]. A study from the Netherlands in 2003 using comparable methodology had similar findings [3].

Why are repeat episodes so often asymptomatic in HIV-positive MSM?

There could be at least five explanations for the high proportion of persons identified with asymptomatic syphilis in the setting of syphilis screening programmes.

(i) False-positive test results.

There is a long list of causes of increases in the titres of the non-treponemal tests that are unrelated to syphilis [1,18]. Frequent testing may be more likely to detect these increases and misdiagnose them as asymptomatic syphilis.

(ii) Syphilis relapse.

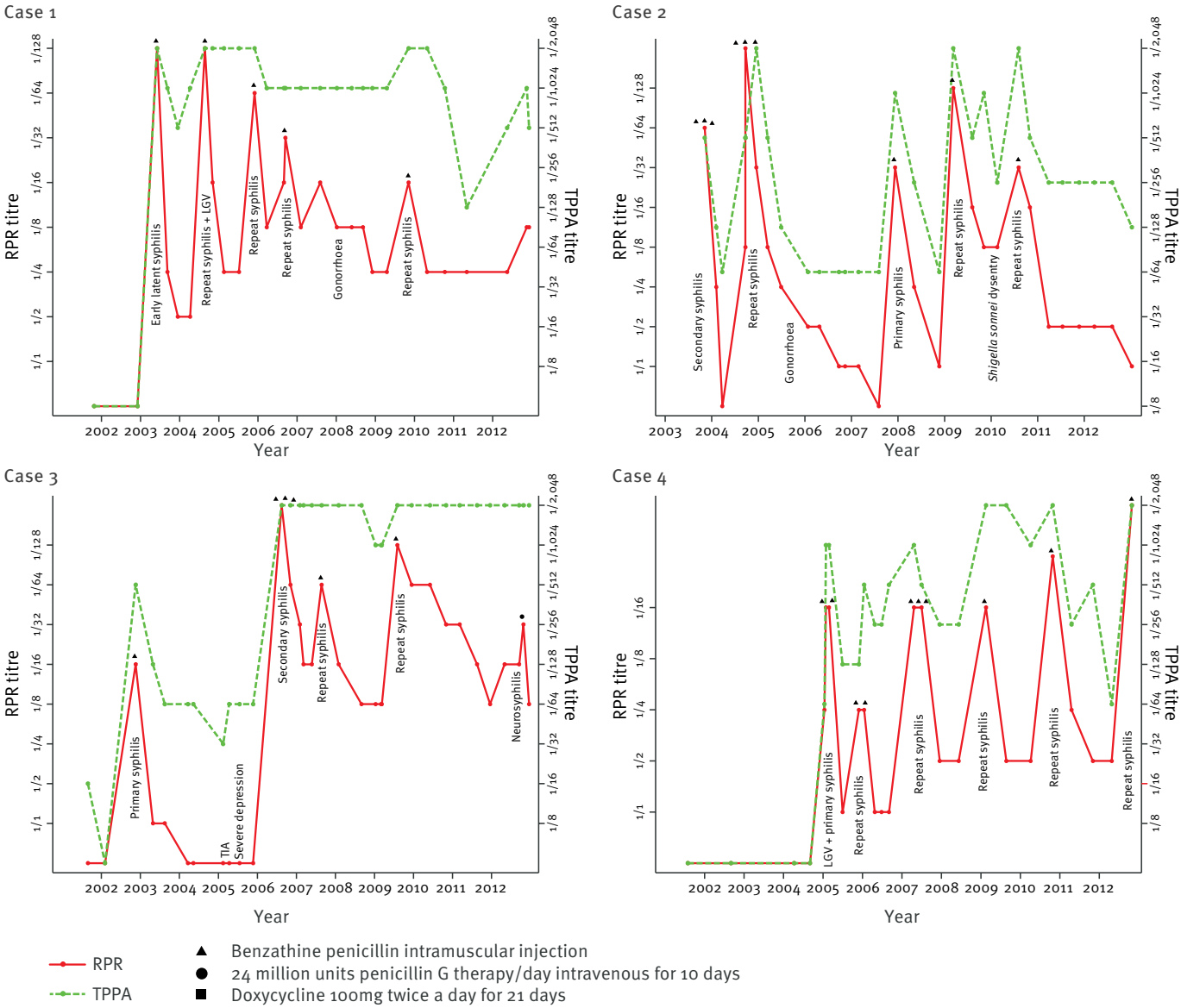
Ineffective treatment may lead to relapse [17].

(iii) Lead-time bias.

The regular sampling (up to three monthly) involved in the screening programmes would make it more likely that persons are diagnosed during the syphilis incubation period before they develop symptoms of syphilis [7,16,17].

Figure 2A

Changes in test[a] titres and diagnoses of new sexually transmitted infections in 10 cases with five or more episodes of syphilis diagnosed between 1992 and 2012, Institute of Tropical Medicine, Antwerp, Belgium



CT: *Chlamydia trachomatis*; HAV: hepatitis A virus; HCV: hepatitis C virus; LGV: lymphogranuloma venereum; RPR: rapid plasma regain; TIA: transient ischaemic attack; TPPA: *Treponema pallidum* particle agglutination.

[a] RPR and TPPA tests.

The denominators of all TPPA titres were divided by 10 due to space constraints.

(iv) Partial immunity.

Screening programmes have been taking place in high-risk populations, in which an increasing proportion of syphilis is due to reinfection [15,26]. *T. pallidum* infection induces partial immunity to reinfection in both humans and other animals, which may lead to an increased proportion of repeat infections being asymptomatic [14,15,24].

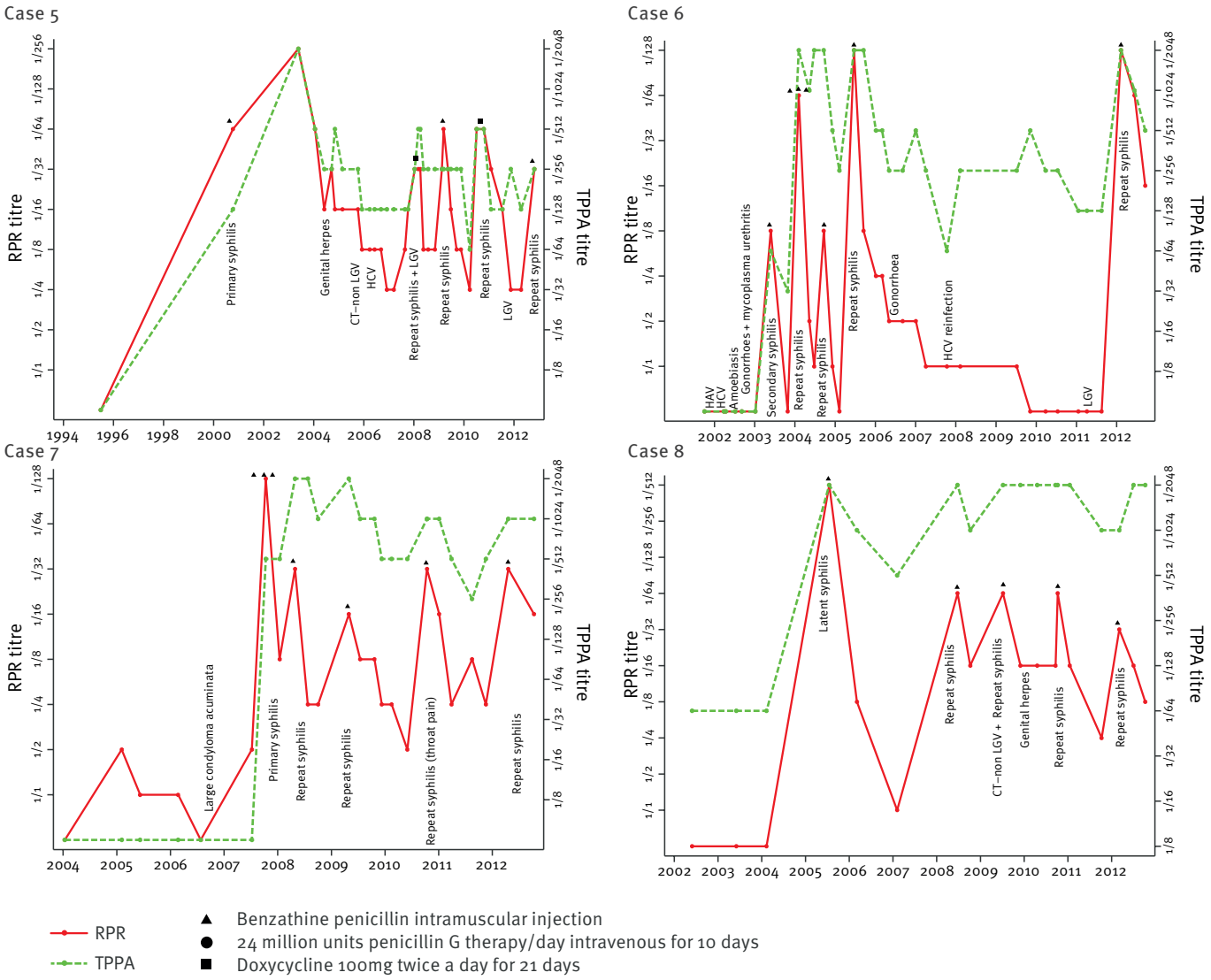
(v) Missed signs of disease.

Clinicians in screening programmes may place undue reliance on serological tests at the expense of clinical evaluations and thus be more likely to miss clinical signs of disease.

Although false-positive RPR tests and relapses can occur, we do not believe that these are dominant factors in why repeat episodes of syphilis are so often asymptomatic. The most likely causes of false-positive RPR tests in our study population, such as vaccinations and other infections [1,28] tend to cause small increases in RPR titres – not the large increases that occurred in all of the episodes seen here. Furthermore, they would not lead to the increases in TPPA titres that characterise reinfections. Since all the cases received

Figure 2B

Changes in test[a] titres and diagnoses of new sexually transmitted infections in 10 cases with five or more episodes of syphilis diagnosed between 1992 and 2012, Institute of Tropical Medicine, Antwerp, Belgium



CT: *Chlamydia trachomatis*; HAV: hepatitis A virus; HCV: hepatitis C virus; LGV: lymphogranuloma venereum; RPR: rapid plasma regain; TIA: transient ischaemic attack; TPPA: *Treponema pallidum* particle agglutination.

[a] RPR and TPPA tests.

The denominators of all TPPA titres were divided by 10 due to space constraints.

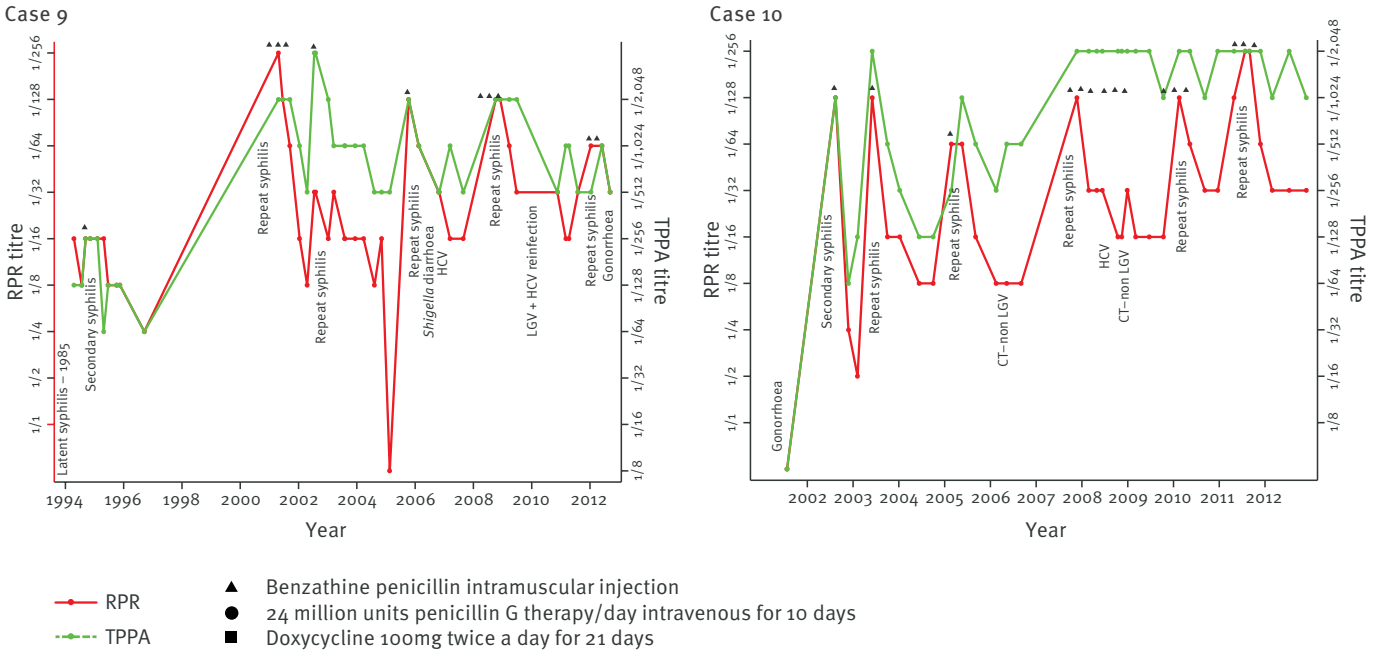
stage-appropriate, highly effective therapy under direct supervision that has an extremely low failure rate [29], we assume that relapses were very unlikely.

The lead-time bias explanation likely explains only a proportion of asymptomatic presentations. This can be inferred from comparing the incubation period of syphilis, being a mean of 21 days (range: 10–90 days) [1,12] and the time to positivity of an RPR test (ca 50% and 90% positive by three and six weeks post infection, respectively) [29] with the frequency of RPR testing (median: 114 days; 3%, 7% and 28% of tests were performed at 7, 14 and 90 days post infection, respectively). As only 28% of tests were performed within the maximum 90-day incubation period, this suggests that the lead-time bias is unable to explain more than a minority of the 91% of the repeat cases presenting asymptomatically.

Regarding clinicians missing signs of disease, we do not consider this as a likely explanation as this would not explain why only 2/10 of the initial episodes of syphilis (vs 39/42 cases of repeat syphilis) were diagnosed as asymptomatic syphilis.

Figure 2C

Changes in test[a] titres and diagnoses of new sexually transmitted infections in 10 cases with five or more episodes of syphilis diagnosed between 1992 and 2012, Institute of Tropical Medicine, Antwerp, Belgium



CT: *Chlamydia trachomatis*; HAV: hepatitis A virus; HCV: hepatitis C virus; LGV: lymphogranuloma venereum; RPR: rapid plasma regain; TIA: transient ischaemic attack; TPPA: *Treponema pallidum* particle agglutination.

[a] RPR and TPPA tests.

The denominators of all TPPA titres were divided by 10 due to space constraints.

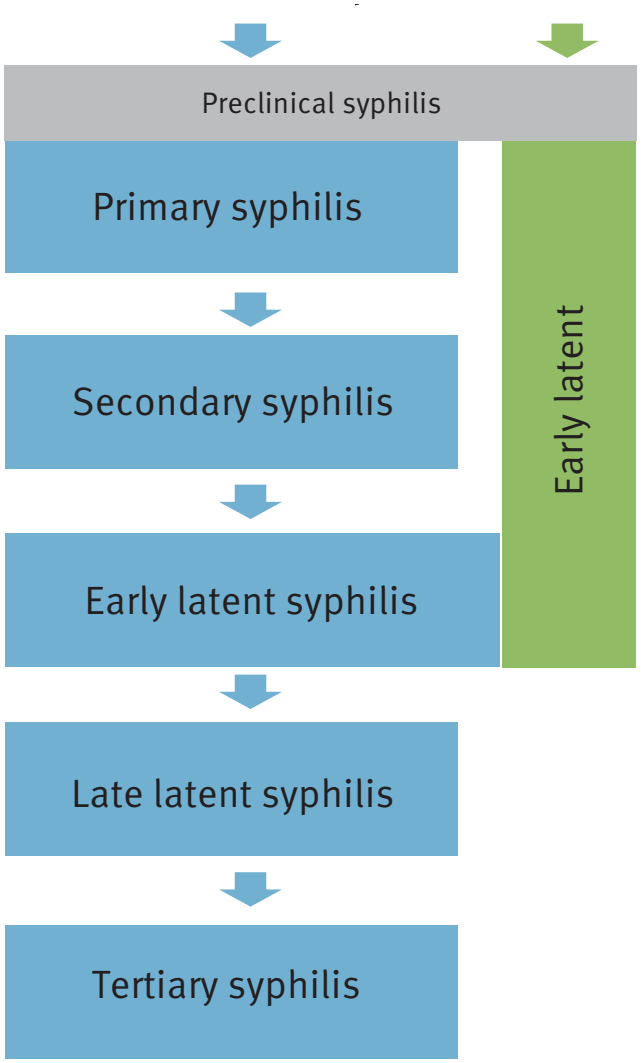
These considerations suggest that lead-time bias and partial immunity may both play a role in the way that repeat syphilis presents asymptomatically. Although our findings are based on small numbers, may not be generalisable to other populations undergoing screening and require replication, they have important implications. They suggest that persons with multiple episodes of syphilis may be more likely to present with asymptomatic disease. Because untreated syphilis may remain infectious for more than a year [29], this could result in these individuals having an even larger role in syphilis transmission than their central position in high-risk sexual networks would have led to. This makes a good case for screening these individuals frequently on a long-term basis. Although further study is required, it may be prudent to screen all individuals with a repeat syphilis diagnosis three to six monthly or, failing that, screen them six monthly with a more sensitive test such as an IgM test. A study from 1999 to 2008 in Zurich, Switzerland, found that an IgM enzyme-linked immunosorbent assay was negative in considerably fewer cases of primary syphilis cases compared with the RPR test (4% vs 42%) [30].

It should be noted that persons with asymptomatic syphilis are usually defined as having latent syphilis and there is good evidence to suggest that this category of syphilis is less infectious [28]. There are, however, a number of reasons why it is inappropriate to classify the asymptomatic syphilis diagnosed in our cases as latent syphilis. Latency in a number of articles and textbooks is conceived as being the stage following primary/secondary syphilis [1,12,31]. In the individuals with reinfections analysed in our study, however, syphilis was either diagnosed before the primary/secondary stages could manifest (during the incubation period) or it presented without signs of primary/secondary disease (true asymptomatic). Thus, we propose that latency be reconceptualised from being a category that follows primary /secondary disease to one which, in addition, can occur before or contemporaneous to primary/secondary disease (Figure 3). Lead-time bias will also mean that some persons will be diagnosed so early that it is not possible to predict what stage they would have presented with if they had not been treated. Further research is necessary to ascertain how infectious individuals with repeat episodes of syphilis are.

More attention needs to be directed to this emerging issue of repeat syphilis. Not only may it play an important role in syphilis transmission but individuals with repeat syphilis may also merit a specific, still to be defined, clinical work-up. This could include a lumbar puncture to exclude relapse from infection arising from a neurological reservoir of *T. pallidium* and additional confirmatory tests (including TPPA tests). The transient ischaemic attack and severe depression that Case 3

Figure 3

Adjusted conceptual framework of the natural history of untreated syphilis



In addition to the traditional framework (depicted in blue), syphilis can be diagnosed at a preclinical stage in the setting of screening (grey) or directly as an early latent stage without having passed through the primary/secondary stages (green). A higher proportion of clients with repeat syphilis may present directly as early latent syphilis.

was diagnosed with between numerous episodes of syphilis may reflect such a neurosyphilis (Figure 2). Although speculative, lumbar puncture at this stage may have led to his neurosyphilis being treated earlier and may have prevented further relapses of syphilis. Optimal therapy for repeat syphilis has also not been completely defined thus far. Finally, interventions that are able to address the risky behaviours underpinning repeat syphilis infections in HIV-positive MSM are urgently required.

Acknowledgements

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Conflicts of interest

None declared.

Authors' contributions

Conceived and coordinated the study: Chris Kenyon. Carried out the data analyses and folder reviews: Chris Kenyon. Drafted the manuscript: Chris Kenyon, Emmanuel Bottieau. Contributed to the manuscript: Lut Lynen, Eric Florence, Marc Vandenbruaene, Severine Caluwaerts, Ludwig Apers, Patrick Soentjens, Marjan Van Esbroeck.

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Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011

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Farm animals are a potential reservoir for human *Clostridium difficile* infection (CDI), particularly PCR ribotype 078 which is frequently found in animals and humans. Here, whole genome single-nucleotide polymorphism (SNP) analysis was used to study the evolutionary relatedness of *C. difficile* 078 isolated from humans and animals on Dutch pig farms. All sequenced genomes were surveyed for potential antimicrobial resistance determinants and linked to an antimicrobial resistance phenotype. We sequenced the whole genome of 65 *C. difficile* 078 isolates collected between 2002 and 2011 from pigs (n=19), asymptomatic farmers (n=15) and hospitalised patients (n=31) in the Netherlands. The collection included 12 pairs of human and pig isolates from 2011 collected at 12 different pig farms. A mutation rate of 1.1 SNPs per genome per year was determined for *C. difficile* 078. Importantly, we demonstrate that farmers and pigs were colonised with identical (no SNP differences) and nearly identical (less than two SNP differences) *C. difficile* clones. Identical tetracycline and streptomycin resistance determinants were present in human and animal *C. difficile* 078 isolates. Our observation that farmers and pigs share identical *C. difficile* strains suggests transmission between these populations, although we cannot exclude the possibility of transmission from a common environmental source.

Introduction

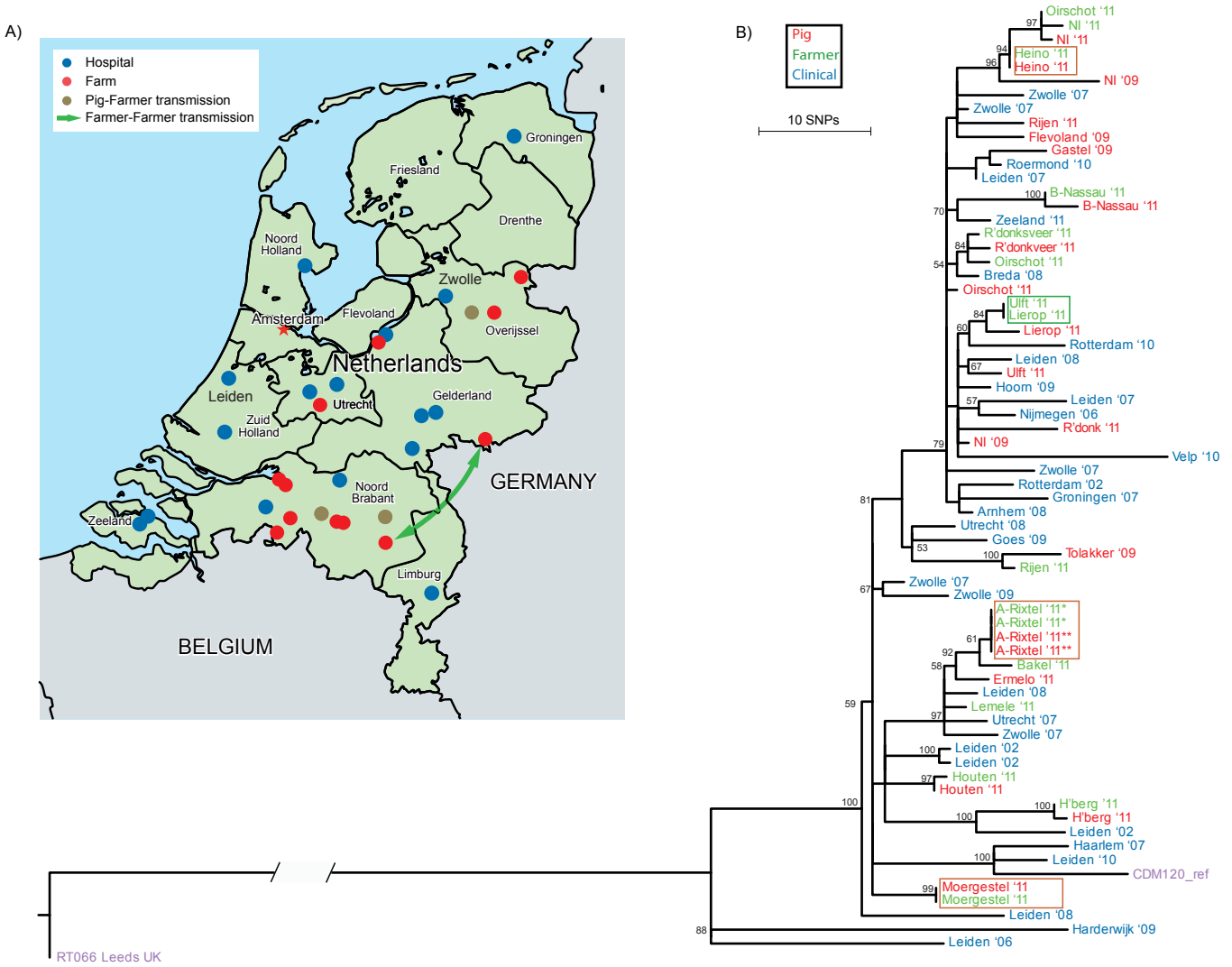
In the past decade *Clostridium difficile* has emerged rapidly to become the most common cause of antibiotic-associated diarrhoea in healthcare facilities worldwide. Antibiotic treatment, advanced age and hospitalisation are the major risk factors for developing *C. difficile* infection (CDI) leading to diarrhoea, pseudomembranous colitis or death [1,2]. CDI is increasingly recognised in the community setting [3-6] where exposure to antibiotics is an important risk factor [5], while the use of proton pump inhibitors [4], outpatient healthcare exposure [7], obesity and inflammatory bowel disease (IBD) [8] are potential risk factors. *C. difficile* virulence is primarily mediated by two potent enterotoxins, TcdA and TcdB, which are encoded in a pathogenicity locus (PaLoc) [9-11]. The binary toxin may contribute to the virulence of *C. difficile* as well [12], but its role in CDI is still under debate [13-16]. *C. difficile* produces highly resistant and infectious spores, which can survive in the environment for a long time and facilitate environmental transmission within the healthcare setting [17].

Symptomatic individuals are an important source of *C. difficile* transmission in a hospital setting, and patient isolation and antibiotic stewardship have been proven to be effective infection control measures [18,19]. The role of asymptomatic carriers as donors of transmission may also be significant [20-23], and diverse novel subtypes are continuously introduced in the healthcare system, highlighting a link to a large and diverse community reservoir [24]. Interestingly, *C. difficile* PCR ribotype 078, which is commonly found in the healthcare system of various European countries [25], is more often associated with community-acquired CDI [26]. Notably, this variant is the most common type found in pigs [27-30] and other farm animals [31-33].

Several studies have reported an overlap between *C. difficile* genotypes isolated from humans and animals [27,34-38] using conventional typing methods such as PCR ribotyping, multilocus sequence typing (MLST), and multilocus variable-number tandem repeat analysis (MLVA). However, these methods do not have the discriminatory power to distinguish between closely related strains as is required for transmission tracking. In this study, we used whole genome sequencing and

Figure 1

Transmission events and phylogeny of *Clostridium difficile* 078, the Netherlands 2002–11 (n=65)



A. Distribution of Dutch hospitals and pig farms included in this study. Only pig farms with a known location were plotted. Blue dots represent the hospitals (n=16) where isolates from hospitalised patients were obtained, red dots represent pig farms (n=12) where isolates from farmers and pigs were obtained. Brown dots represent the pig farms where pigs and farmers had identical C. difficile isolates. The green arrow indicates a potential (long-range) transmission event between two farms.

B. Phylogenetic tree revealing likely transmission between pigs and humans. Shown is the reconstructed phylogenetic tree based on 774 core genome single-nucleotide polymorphisms (SNPs). Samples are colour-coded according to their source: pig (red), farmer (green) and clinical isolate (blue). Identical genotypes with an epidemiological link (i.e. same location/farm) are marked with brown boxes. Long-range transmission events (i.e. different locations) are marked with a green box. The tip labels are coded with the city name followed by two numbers that represent year of isolation ('08 ≙ 2008). The CDM120 genome (purple) is used for the reference-based mapping, RT066 (purple) is used as an out-group to root the tree. The scale indicates the branch length that correspond to 10 SNP differences. The numbers for the internal nodes show the support from 100 non-parametric bootstraps of a maximum likelihood reconstruction (only bootstrap values>50 are shown).

phylogenetic analysis to track single clones in human and animal populations to demonstrate potential inter-species transmission.

Methods

Collection of *Clostridium difficile* isolates

In total, the genomes of 65 isolates designated PCR ribotype 078 were sequenced and analysed. Of these 65 isolates, 34 were derived from healthy humans (n=15) and pigs (n=19) on 19 Dutch pig farms (farm isolates) and 31 from hospitalised patients in various Dutch hospitals. Of the farm isolates, 24 isolates were paired by farm (i.e. 12 pairs of human and pig isolates from 12 farms), whereas the remaining 10 (from three farmers and seven pigs) were not paired. The majority of the farm isolates were collected in 2011 by the Institute for Risk Assessment Sciences of the Utrecht

University as part of another study [34]. Thirty-one randomly selected clinical isolates originating from various Dutch hospitals between 2002 and 2011 were obtained from the Dutch National *C. difficile* reference laboratory at Leiden University Medical Center. In addition, one PCR ribotype 066 strain was included; this strain was obtained from our Leeds-Leiden/European Centre for Disease Prevention and Control (ECDC) reference strain collection [39]. Details of all sequenced isolates are listed in Table 1, including the European Nucleotide Archive (ENA) sample accession numbers. Two isolates were sequenced in duplicate.

Bacterial culture and genomic DNA preparation

C. difficile was cultured on blood agar plates (BioMérieux, the Netherlands), inoculated into liquid medium (brain–heart infusion (BHI) broth supplemented with yeast extract and cysteine) and grown over night (ca 16 hours) anaerobically at 37 °C. Cells were pelleted, washed with phosphate-buffered saline (PBS), and genomic DNA preparation was performed using a phenol–chloroform extraction as previously described [40].

Whole genome sequencing

Paired-end multiplex libraries were created as previously described [41]. Sequencing was performed on an Illumina HiSeq 2000 platform, with a read length of 100 bp.

In silico MLST

The alleles for the seven housekeeping genes used for *C. difficile* MLST [42] (http://pubmlst.org/cdifficile/), adk, atpA, dxr, glyA, recA, sodA, and tpi, were analysed in silico to determine the sequence type (ST). All sequenced genomes were aligned with the CDM120 genome using the multiple sequence alignment editor Seaview [43], after which each individual MLST allele was analysed for sequence variation.

SNP calling and recombination detection

Illumina sequence data were mapped to the *C. difficile* 078 reference genome, M120, (European Molecular Biology Laboratory (EMBL) accession number: FN665653) as paired-end reads using SMALT software (http://smalt.sourceforge.net/), and SNPs were identified as previously described [41]. A potential confounder within the downstream phylogenetic analysis is the effect of homologous recombination, which has the potential to interfere with the phylogenetic signal within the dataset. To alleviate this problem we used the approach developed by Croucher et al. [40] to identify regions in the genome of each isolate where there was evidence of recombination. We then removed those sites from our alignments used in downstream analyses.

Phylogeny and detection of non-phylogenetic SNPs

Phylogenetic trees were constructed using RAxML [44] with a general time reversible (GTR) model with a gamma correction for among-site rate variation combined with 100 random bootstrap replicates (default). Finally, metadata (source, year of isolation, geographical location) was transferred to the reconstructed tree.

Mutation rate estimation

The mutation rate across the population was estimated using the Bayesian evolutionary analysis sampling trees (BEAST) software v1.7.5 [45]. BEAST operates by utilising an explicit model of evolution to compute the mutation rate on each branch of a phylogenetic tree. This enables the translation of evolutionary time into calendar units: days or years. In order to ensure that the dataset was converging consistently, three independent Markov chain Monte Carlo (MCMC) chains were run, each of 100,000,000 states. From these, we removed an initial 10% as a burn-in (10,000,000 states) for each chain and joined the chains using LogCombiner (part of the BEAST suite), taking a sample every 10,000 states.

Genome-wide scan for antimicrobial resistance determinants

De novo assembly was performed for each sequenced genome using the Velvet assembler [46]. The assembled contigs were then ordered against the reference genome M120 using ABACAS [47], which was required for downstream analysis using Artemis Comparison Tool (ACT) [48]. The ordered contigs were used to perform BLAST homology searches for transposons and antimicrobial resistance determinants. The results of this analysis and the discovery of novel potential transposons were visualised using ACT [48]. In addition, the presence of antimicrobial resistance determinants located on the identified transposons were confirmed using the ResFinder 2.1 server [49], with an 98% threshold for identity.

Antibiotic resistance

The minimum inhibitory concentration (MIC) for tetracycline was determined using E-test (BioMérieux, the Netherlands) on *Brucella* plates (Mediaproducts BV, the Netherlands) under anaerobic conditions at 37 °C. Streptomycin resistance was tested by disk diffusion method, using Sensi-Neotabs 500 µg disks (Rosco, Denmark). Results were interpreted using the tetracycline breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI) [50] and streptomycin breakpoints from Corver et al. [51].

Results

Mutation rate of *Clostridium difficile* 078 from the Netherlands

We performed whole genome sequencing on 65 *C. difficile* 078 strains isolated between 2002 and 2011 from various sources (animal or human) and locations in the Netherlands (Figure 1A; Table 1). The human isolates

(n = 46) were obtained either from hospitalised patients suffering from CDI (n = 31) or from asymptomatic colonised humans working on Dutch pig farms (n = 15). *C. difficile* 078 was also isolated from asymptomatic pigs (n = 19). In total, 12 pairs of pig/farmer isolates were included, collected at the same time from the same farms where the sampled farmers resided and worked.

We initially compared the genotypes of the *C. difficile* 078 isolates with MLST, the traditional gold standard for epidemiological typing of bacterial pathogens. MLST analysis was done using the DNA sequences of seven housekeeping genes [42], which were extracted from the whole-genome dataset. The concatenated sequence length of the MLST loci (3,501 nt) represents ca 0.09% of the whole genome. Our results demonstrated that all of the *C. difficile* 078 isolates belonged to ST11, and did therefore not provide a degree of resolution that could be used to track and understand the spread of this organism (data not shown).

To increase the discriminatory power of the analysis, we mapped the whole genome data for each sequenced isolate to the *C. difficile* 078 reference genome M120 [52] and identified all SNPs. Using this approach we identified 3,927 SNPs within the non-repetitive genome (95.2% of the entire genome). Of these, 3,153 SNPs were identified as acquired through horizontal gene transfer or homologous recombination. These SNPs were removed as they disrupt the true phylogeny, leaving a clonal frame of 774 phylogenetically informative SNPs for further downstream analysis. Of these, 373 SNPs were found only in the *C. difficile* 066 isolate (ST11), a close relative of *C. difficile* 078 [39], which was used to root the phylogenetic tree. A population-specific mutation rate of *C. difficile* 078 was estimated, using the isolation dates of our sequenced samples for calibrating the time scale of the phylogenetic tree. Based on our collection, the mutation rate for the *C. difficile* 078 lineage was estimated to be 2.72 x 10^{-7} substitutions per site per year (95% confidence interval (CI): 1.43 x 10^{-7}–3.99 x 10^{-7}) which is equivalent to 1.1 SNP per genome per year (95% CI: 0.6–1.6) when multiplied by the number of sites present in the *C. difficile* 078 genome. This mutation rate is comparable to published estimates for *C. difficile* 027 [53] and genomes obtained from a selection of 24 distinct STs [54].

Identical genotypes in humans and farm animals

In order to study potential transmission of *C. difficile* 078 between farm animals and humans, we compared 12 pairs of farmer and pig strains by whole genome SNP typing (Table 2). Interestingly, three farmer/pig pairs, collected at three farms located in Heino, Aarle-Rixtel and Moergestel, shared identical genotypes, i.e. had no SNP differences (Table 2). In addition, two pairs collected at farms in Hardenberg and Houten were separated by only one SNP difference. In all probability, one SNP difference is indicative of a very recent potential transmission event (less than one year earlier). Consequently, using one SNP difference as a threshold for defining suspected transmission on farms, the number of potential transmission events between farmers and animals increased to five, representing five of the 12 sequenced farmer/pig pairs. Of the remaining seven paired samples, only two differed more than 10 SNPs, whereas five had three (n = 3), four (n = 1) or seven (n = 1) SNP differences. The paired animal and human samples with only three to four SNP differences could suggest that a potential transmission event occurred a few years before, and from that moment, the bacterium evolved separately inside different hosts. The paired isolates with more than 10 SNPs difference were genetically so diverse that direct transmission was ruled out.

Population structure of *Clostridium difficile* 078 in the Netherlands

To study the closely related paired farm isolates in a broader evolutionary context, we compared the 12 pairs with 41 additional *C. difficile* samples that were epidemiologically unrelated to the farm isolates and collected over a longer period of time. These 41 samples included 10 individual (i.e. unpaired) farm isolates (from three farmers and seven pigs) collected between 2009 and 2011, and 31 independent (i.e. non-outbreak) clinical isolates obtained from hospitalised patients suffering from CDI collected at various Dutch hospitals between 2002 and 2011. According to the definitions described by Kuijper et al. [55], the majority of these clinical isolates (n = 23) were defined as healthcare-associated cases, while two cases were defined as community-associated; for six clinical isolates the onset was unknown (Table 1).

A maximum likelihood phylogeny was generated using the 401 phylogenetic SNPs identified in the genomes of the 65 sequenced isolates (Figure 1B). In total, 61 distinct SNP genotypes were observed among the 65 *C. difficile* 078 isolates. Two isolates (Oirschot '11 and Leiden '06) at the periphery of the phylogenetic tree differed by 49 SNPs, which gave an indication of the extent of variation present in the phylogeny. Interestingly, the inferred phylogeny of Dutch *C. difficile* 078 revealed a general lack of clustering related to strain source (i.e. swine, farmer or clinical), as demonstrated by the mingling of strain sources in the phylogenetic tree (Figure 1B). Isolates from the same source group did not form distinct clusters, while several distinct heterogeneous groups were observed that included isolates obtained from diverse sources. This was especially apparent in the cluster consisting of a clinical isolate (Breda '08), a pig isolate (R'donksv.'11) and two farmer isolates (Oirschot '11 and R'donksv.'11) that were all collected in the same region (Noord Brabant) of the Netherlands (Figure 2). Interestingly, only four SNP differences separated the clinical isolate (Breda '08) from the nearest farm isolate (R'donksv.'11). Given the three year window in which these isolates were collected and the estimated mutation rate of 1.1 SNP difference per

Table 1A

Clostridium difficile type 078 isolates used in this study, the Netherlands, 2002–11 (n=65)

R_L#T[a]	Year	City	RT	Isolate	Source	Related isolates	Association	ENA ID[b]
8080_2#24	2006	Leiden	078	6072310	Clinic	Non-outbreak	Healthcare	ERS138026
8080_2#25	2006	Nijmegen	078	6086336	Clinic	Non-outbreak	Healthcare	ERS138027
8080_2#26	2007	Leiden	078	7001233	Clinic	Non-outbreak	Healthcare	ERS138028
8080_2#27	2007	Groningen	078	7004578	Clinic	Non-outbreak	Unknown	ERS138029
8080_2#28	2007	Utrecht	078	7005405	Clinic	Non-outbreak	Unknown	ERS138030
8080_2#29	2007	Zwolle	078	7021455	Clinic	Non-outbreak	Healthcare	ERS138031
8080_2#30	2007	Zwolle	078	7044912	Clinic	Non-outbreak	Community	ERS138032
8080_2#31	2007	Zwolle	078	7066827	Clinic	Non-outbreak	Community	ERS138033
8080_2#32	2007	Zwolle	078	7071308	Clinic	Non-outbreak	Healthcare	ERS138034
8080_2#33	2007	Zwolle	078	7086074	Clinic	Non-outbreak	Healthcare	ERS138035
8080_2#34	2007	Leiden	078	7091952	Clinic	Non-outbreak	Healthcare	ERS138036
8080_2#35	2008	Leiden	078	8011061	Clinic	Non-outbreak	Healthcare	ERS138037
8080_2#36	2008	Utrecht	078	8013820	Clinic	Non-outbreak	Healthcare	ERS138038
8080_2#37	2008	Leiden	078	8051728	Clinic	Non-outbreak	Healthcare	ERS138039
8080_2#38	2008	Leiden	078	8055344	Clinic	Non-outbreak	Healthcare	ERS138040
11250_1#22	2008	Arnhem	078	8056692	Clinic	Non-outbreak	Unknown	ERS362924
8080_2#40	2008	Breda	078	8091554	Clinic	Non-outbreak	Healthcare	ERS138042
8080_2#41	2009	Harderwijk	078	9012668	Clinic	Non-outbreak	Healthcare	ERS138043
8080_2#42	2009	Goes	078	9019497	Clinic	Non-outbreak	Unknown	ERS138044
8080_2#43	2009	Hoorn	078	9077637	Clinic	Non-outbreak	Healthcare	ERS138045
8080_2#44	2010	Roermond	078	10005075	Clinic	Non-outbreak	Healthcare	ERS138046
8080_2#45	2010	Rotterdam	078	10015222	Clinic	Non-outbreak	Healthcare	ERS138047
8080_2#46	2010	Velp	078	10080193	Clinic	Non-outbreak	Healthcare	ERS138048
8080_2#47	2011	Zeeland	078	11012929	Clinic	Non-outbreak	Healthcare	ERS138049
8080_2#49	2009	Zwolle	078	1103	Clinic	Non-outbreak	Unknown	ERS138051
8080_2#58	NI	Leeds	066	066 (root)[c]	Clinic	Reference collection	Unknown	ERS138052
8080_2#61	2002	Rotterdam	078	126065	Clinic	Non-outbreak	Unknown	ERS138053
8080_2#62	2002	Leiden	078	126819	Clinic	Non-outbreak	Healthcare	ERS138054
8080_2#63	2002	Leiden	078	126938	Clinic	Non-outbreak	Healthcare	ERS138055
8080_2#64	2002	Leiden	078	129820	Clinic	Non-outbreak	Healthcare	ERS138056
8080_2#71	2010	Leiden	078	53737	Clinic	Non-outbreak	Healthcare	ERS138060
8080_2#72	2007	Haarlem	078	47337	Clinic	Non-outbreak	Healthcare	ERS138063
8080_2#50	2009	Gastel	078	P29	Pig	Un-paired	Farm	ERS138064
8080_2#51	2009	NI	078	P60	Pig	Un-paired	Farm	ERS138065
8080_2#52	2009	Flevoland	078	P27	Pig	Un-paired	Farm	ERS138066
8080_2#53	2009	NI	078	P70	Pig	Un-paired	Farm	ERS138069
8080_2#54	2009	Tolakker	078	P52	Pig	Un-paired	Farm	ERS138070
8080_2#67	2011	Aarle-Rixtel	078	H205[d]	Farmer	Pair 1	Farm	ERS138073
8080_2#68	2011	Aarle-Rixtel	078	B37_2[e]	Pig	Pair 1	Farm	ERS138074
9221_6#55	2011	NI	078	H102	Farmer	Pair 12	Farm	ERS199786
9221_6#56	2011	Raamsdonksveer	078	B31_3	Pig	Pair 9	Farm	ERS199787
9221_6#57	2011	Heino	078	B17_3	Pig	Pair 4	Farm	ERS199788
9221_6#58	2011	Ulft	078	H121	Farmer	Pair 11	Farm	ERS199789
9221_6#59	2011	Rijen	078	B27_7	Pig	Pair 10	Farm	ERS199790
9221_6#60	2011	Baarle-Nassau	078	H230	Farmer	Pair 2	Farm	ERS199791
9221_6#61	2011	Oirschot	078	H189	Farmer	Pair 8	Farm	ERS199792
9221_6#62	2011	Lierop	078	B23_6	Pig	Pair 6	Farm	ERS199793

NI: not identified; RT: ribotyope.

[a] R_L#T, run, lane and tag number.

[b] European Nucleotide Archive sample submission number.

[c] Included as root sequence.

[d,e] Sequenced in duplicate.

Table 1B

Clostridium difficile type 078 isolates used in this study, the Netherlands, 2002–11 (n=65)

R_L#T[a]	Year	City	RT	Isolate	Source	Related isolates	Association	ENA ID[b]
9221_6#63	2011	Aarle-Rixtel	078	H205[d]	Farmer	Duplicate	Farm	ERS199794
9221_6#64	2011	Hardenberg	078	B15_1	Pig	Pair 3	Farm	ERS199795
9221_6#65	2011	Oirschot	078	H21	Farmer	Un-paired	Farm	ERS199796
9221_6#66	2011	Oirschot	078	B30_5	Pig	Pair 8	Farm	ERS199797
9221_6#67	2011	Rijen	078	H122	Farmer	Pair 10	Farm	ERS199798
9221_6#68	2011	Hardenberg	078	H95	Farmer	Pair 3	Farm	ERS199799
9221_6#69	2011	Raamsdonk	078	B28_1	Pig	Un-paired	Farm	ERS199800
9221_6#70	2011	Bakel	078	H214	Farmer	Un-paired	Farm	ERS199801
9221_6#71	2011	Raamsdonksveer	078	H158	Farmer	Pair 9	Farm	ERS199802
9221_6#72	2011	Heino	078	H88	Farmer	Pair 4	Farm	ERS199803
9221_6#73	2011	Lemele	078	H111	Farmer	Un-paired	Farm	ERS199804
9221_6#74	2011	Baarle-Nassau	078	B39_4	Pig	Pair 2	Farm	ERS199805
9221_6#75	2011	Moergestel	078	B4_2	Pig	Pair 7	Farm	ERS199806
9221_6#76	2011	NI	078	B20_1	Pig	Pair 12	Farm	ERS199807
9221_6#77	2011	Moergestel	078	H16	Farmer	Pair 7	Farm	ERS199808
9221_6#78	2011	Aarle-Rixtel	078	B37_3[e]	Pig	Duplicate	Farm	ERS199809
9221_6#79	2011	Ermelo	078	B1_5	Pig	Un-paired	Farm	ERS199810
9221_6#80	2011	Lierop	078	H170	Farmer	Pair 6	Farm	ERS199811
9221_6#81	2011	Ulft	078	B22_6	Pig	Pair 11	Farm	ERS199812
9221_6#82	2011	Houten	078	B29_10	Pig	Pair 5	Farm	ERS199813
9221_6#83	2011	Houten	078	H141	Farmer	Pair 5	Farm	ERS199814

NI: not identified; RT: ribotyope.

[a] R_L#T, run, lane and tag number.

[b] European Nucleotide Archive sample submission number.

[c] Included as root sequence.

[d,e] Sequenced in duplicate.

genome per year (95% CI: 0.6–1.6), one would expect to observe two to four SNP differences (prediction interval: 1.8–4.8) between these isolates in case of transmission during this time. Therefore, the observed four SNP difference in this cluster suggests a possible transmission link between farm and clinical isolates.

The phylogenetic tree also demonstrated a general lack of geographic clustering (Figure 1B). This is particularly evident for the isolates from Leiden and Zwolle that were dispersed throughout the phylogeny. This observation suggested that related *C. difficile* 078 strains were widely distributed across the country and were frequently transmitted between locations. Interestingly, the analysis revealed two farmers with no obvious epidemiological link that were colonised with identical *C. difficile* 078 isolates (Figure 1B; green box). The farms were located at Lierop and Ulft (ca 100 km apart), emphasising the lack of geographic signal in these results.

Tetracycline and streptomycin resistance determinants are shared between *Clostridium difficile* 078 strains from humans and pig

C. difficile genomes carry a broad array of mobile genetic elements that are not included in our phylogenetic SNP analysis but often encode clinically relevant phenotypes such as antimicrobial resistance (AMR). We assembled and analysed the *C. difficile* 078 genomes to identify potential mobile elements containing AMR determinants and then mapped these onto the phylogenetic tree. We observed the presence of a mobile element with high homology (92.7%) to a previously described transposon Tn*6190* [51] and a novel potential transposon that was designated as Tn*6235* in this study (data not shown). These transposons potentially confer resistance to various antibiotics, including tetracycline, and each transposon grouped into distinct phylogenetic clusters (Figure 3).

Mobile element Tn*6190*, harbouring tetracycline resistance determinant tetM (EMBL accession number: EU182585.1; 98.9% identity), was present in 24 of the sequenced genomes that were obtained from diverse hosts. The majority of these 24 genomes grouped together in a monophylogenetic cluster (Figure 3; orange dots). Tetracycline susceptibility testing confirmed that the presence of tetM correlated with tetracycline resistance (Figure 3 orange branches; Table 3). The novel mobile element Tn*6235* was present in its full length (ca 40 kb; 100% homology) in 10 sequenced *C. difficile* 078 genomes that formed a

Table 2

Single nucleotide polymorphism differences between paired farm isolates of *Clostridium difficile* 078, the Netherlands 2009–11 (n=24)

Pair number	R_L#T[a]	Location of farm	RT	Source	ID	SNP differences
1	8080_268_6#78	Aarle-Rixtel	078	Pig	B37_2	0
	8080_2#67		078	Farmer	H205	
2	9221_6#74	Baarle-Nassau	078	Pig	B39_4	3
	9221_6#60		078	Farmer	H230	
3	9221_6#64	Hardenberg	078	Pig	B15_1	1
	9221_6#68		078	Farmer	H95	
4	9221_6#57	Heino	078	Pig	B17_3	0
	9221_6#72		078	Farmer	H88	
5	9221_6#82	Houten	078	Pig	B29_10	1
	9221_6#83		078	Farmer	H141	
6	9221_6#62	Lierop	078	Pig	B23_6	4
	9221_6#80		078	Farmer	H170	
7	9221_6#75	Moergestel	078	Pig	B4_2	0
	9221_6#77		078	Farmer	H16	
8	9221_6#66	Oirschot	078	Pig	B30_5	10
	9221_6#61		078	Farmer	H189	
9	9221_6#56	Raamsdonksveer	078	Pig	B31_3	3
	9221_6#71		078	Farmer	H158	
10	9221_6#59	Rijen	078	Pig	B27_7	19
	9221_6#67		078	Farmer	H122	
11	9221_6#81	Ulft	078	Pig	B22_6	7
	9221_6#58		078	Farmer	H121	
12	9221_6#76	NI	078	Pig	B20_1	3
	9221_6#55		078	Farmer	H102	

ID: sample identifier. RT: ribotyope.

[a] R_L#T, run, lane and tag number.

distinct monophylogenetic cluster with strains from various sources (Figure 3; purple dots). Blast homology searches of this genomic region revealed an open reading frame with homology (100% identity) to a putative aminoglycoside 3'-phosphotransferase aphA1 (M26832) which may confer streptomycin resistance. Streptomycin susceptibility testing confirmed that all 10 isolates present in the monophylogenetic cluster were streptomycin-resistant (Figure 3 purple branches; Table 3).

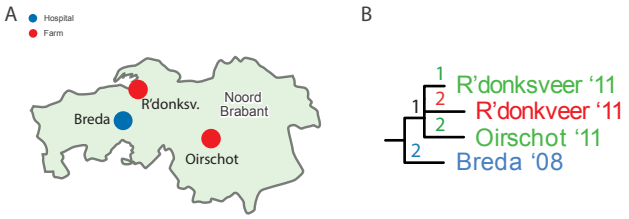
Discussion

We used whole genome sequencing and phylogenetic SNP analysis to study the overlap of *C. difficile* 078 genotypes in animals and humans. In three cases, Dutch farmers were colonised with identical *C. difficile* 078 clones as pigs kept on the same farms. We have also shown that the presence of clonal strains in pigs and farmers was common, as demonstrated by the number of farmer/pig pairs (five of 12) where clonality (defined as ≤1 SNP difference) was observed.

The clonal *C. difficile* 078 strains in farmers and farm animals that were identified indicate that interspecies transmission has occurred, although we cannot

Figure 2

Phylogenetic cluster showing relatedness of *Clostridium difficile* clinical, pig and farmer isolates, the Netherlands, 2008–11 (n=4)

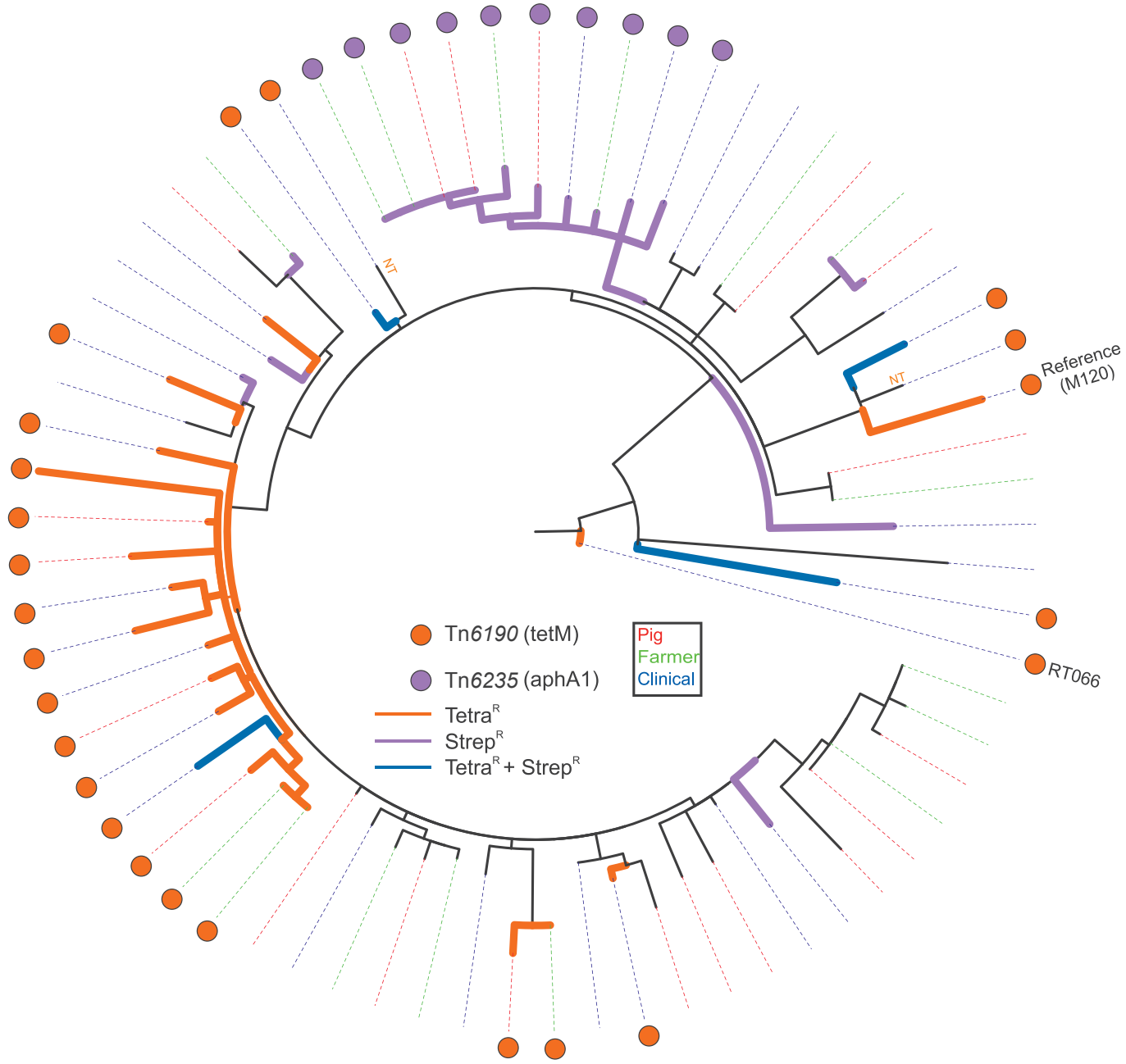


A. Geographical map showing the location of the isolates present in the phylogenetic cluster shown in panel B. Blue dot represents a hospital (Breda), red dots represent the two pig farms (R'donksv. and Oirschot).

B. Zoom-in on a phylogenetic cluster containing highly related isolates from different sources (swine, farmer and clinical isolates). The numbers on the tree branches represent the number of single-nucleotide polymorphisms differences in the cluster, the tip labels are coded with city (Breda) followed by two numbers that represent year of isolation ('08 ≙ 2008).

Figure 3

Phylogeny of *Clostridium difficile* 078 isolates showing the presence of antimicrobial resistance determinants, the Netherlands, 2002–11 (n=65[a])



NT = not phenotypically tested.

Circular representation of the C. difficile 078 phylogeny with coloured dots representing the distribution of antimicrobial resistance (AMR) determinants. The legend shows the identified transposons together with the AMR determinants (between brackets) located on the transposon. The coloured dotted lines represent the source of the respective isolates (swine, farmer and clinical isolate). The presence of Tn*6190* (tetM) is associated with tetracycline resistance; 078 isolates phenotypically tested as tetracycline-resistant are indicated with orange tree branches, streptomycin-resistant isolates are indicated with purple tree branches, isolates resistant to both tetracycline and streptomycin are indicated with blue tree branches.

[a] Two isolates were sequenced in duplicate. Ne RT 066 sequence was included as root sequence. In total, 68 sequences are shown.

Table 3

Results of Antimicrobial susceptibility testing

Isolate	Source	Strepto-mycin	Tn*6235*	Tetra-cycline	Tn*6190*
6072310	Clinic		Absent		Present
6086336	Clinic		Absent		Present
7001233	Clinic	NT	Absent		Absent
7004578	Clinic		Absent		Present
7005405	Clinic		Present		Absent
7021455	Clinic		Absent		Present
7044912	Clinic		Present		Absent
7066827	Clinic		Absent		Present
7071308	Clinic		Absent		Absent
7086074	Clinic		Absent		Absent
7091952	Clinic		Absent		Present
8011061	Clinic		Absent		Absent
8013820	Clinic		Absent		Absent
8051728	Clinic		Absent		Present
8055344	Clinic		Present		Absent
8056692	Clinic		Absent		Absent
8091554	Clinic		Absent		Absent
9012668	Clinic		Absent		Absent
9019497	Clinic		Absent		Absent
9077637	Clinic		Absent		Present
10005075	Clinic		Absent		Present
10015222	Clinic		Absent		Present
10080193	Clinic		Absent		Present
11012929	Clinic		Absent		Absent
1103	Clinic	NT	Absent	NT	Present
P29	Pig	NT	Absent		Absent
P60	Pig	NT	Absent		Present
P27	Pig		Absent		Absent
P70	Pig	NT	Absent		Absent
P52	Pig	NT	Absent		Absent
RT066	Clinic		Absent		Present
126065	Clinic	NT	Absent	NT	Absent
126819	Clinic	NT	Absent	NT	Absent
126938	Clinic	NT	Absent	NT	Absent

Isolate	Source	Strepto-mycin	Tn*6235*	Tetra-cycline	Tn*6190*
129280	Clinic		Absent		Absent
H205	Farmer		Present		Absent
B37.3	Pig		Present		Absent
53737	Clinic	NT	Absent	NT	Present
47337	Clinic		Absent		Present
H102	Farmer		Absent		Absent
B31.3	Pig		Absent		Absent
B17.3	Pig		Absent		Absent
H121	Farmer		Absent		Present
B27.7	Pig		Absent		Absent
H230	Farmer		Absent		Present
H189	Farmer		Absent		Absent
B23.6	Pig		Absent		Present
H205	Farmer		Present		Absent
B15.1	Pig		Absent		Absent
H21	Farmer		Absent		Absent
B30.5	Pig		Absent		Absent
H122	Farmer		Absent		Absent
H95	Pig		Absent		Absent
B28.1	Pig		Absent		Present
H214	Farmer		Present		Absent
H158	Farmer		Absent		Absent
H88	Farmer		Absent		Absent
H111	Farmer		Present		Absent
B39.4	Pig		Absent		Present
B4.2	Pig		Absent		Absent
B20.1	Pig		Absent		Absent
H16	Farmer		Absent		Absent
B37.3	Pig		Present		Absent
B1.5	Pig		Present		Absent
H170	Farmer		Absent		Present
B22.6	Pig		Absent		Present
B29.10	Pig		Absent		Absent
H141	Farmer		Absent		Absent

NT: not available for testing; shown are the distribution of the mobile elements Tn*6190* and Tn*6235* among the 078 genomes.
Green: sensitive (S); orange: intermediate (I); red: resistant (R).

Minimum inhibitory concentration cut-off levels used:

Antibiotic	S	I	R
Tetracycline (µg/mL)	‹4	8	≥16
Streptomycin (mm)	≥15	NA	‹15

mm: zone diameter breakpoint in mm; NA: not applicable.

exclude the possibility that they shared a common (environmental) exposure source, e.g. acquisition of spores from a shared common environmental source. However, we believe that direct transmission is plausible. Firstly, the faecal–oral route is the main route of *C. difficile* transmission, and farmers have a high probability of exposure to pig faeces. Secondly, genomes with zero SNP differences were isolated from farmers and pigs. If acquisition of identical *C. difficile* strains in humans and animals was a result of transmission from a common source, then either it must have been a very recent environmental transmission event or it did not evolve inside either host after the exposure. Finally, the possibility of an intermediate host can be excluded

for clonal cases because circulation via an intermediate host for a certain period is likely to result in SNP differences. If the cases of clonal *C. difficile* 078 strains in farmers and farm animals are indeed a result of direct interspecies transmission, it would be informative to know the direction of this transmission.

The faecal–oral route of acquisition makes it logical for the direction to be from pig to human. In addition, the high carriage rates of *C. difficile* among farmers [34,56] also suggest movement from pigs to farmers. This was further supported by the identical antibiotic resistance determinants (tetracycline and streptomycin) shared between animal and human strains, an observation that is in line with previous studies [35,57,58]. The independent insertion of Tn*6235* or Tn*6190* at the same locations in the *C. difficile* 078 genomes (data not shown) in combination with phylogenetic clustering of these isolates, suggest that Tn*6235* and Tn*6190* were introduced once in a progenitor genome that has since then spread in both human and animal hosts. Interestingly, tetracycline is not frequently used in the Dutch healthcare system, whereas it is still the preferred pharmacotherapeutic group for the veterinary industry in the Netherlands [59]. This suggests that tetracycline resistance could be arising in *C. difficile* isolates from pigs and passed on to the human population. Future, more systematic studies should provide more direct evidence for the direction of transmission.

In addition to the contribution of farm animals as a reservoir for human CDI, we want to emphasise that more than half (58%) of our sequenced farmer/pig pairs were not clonal. Two of the twelve pairs had a SNP difference above 10 SNPs. This suggests that exposure to multiple sources other than farm animals may be responsible for colonisation of the farmers and their pigs. *C. difficile* can be found almost anywhere in the environment (soil, water, and potentially food) making it hard to pinpoint which alternative reservoirs are significant contributors to the spread of *C. difficile* 078 in the community. Currently, several potential (environmental) vectors of transmission have been identified, including but not limited to birds, insects, pets and rodents such as rats and mice [60-64]. Our analysis also revealed two farmers who were not geographically linked but were colonised with identical *C. difficile* 078 isolates. These had been isolated ca 100 km apart from each other, which could suggest exposure to a common environmental source. Another possible explanation could be transport of pigs between the two involved farms that resulted in indirect transmission.

We further analysed the farm isolates in a broader context of clinical isolates with no obvious epidemiological links to the farms. Our analysis demonstrated that all sources, farmer, pig and clinical, were distributed throughout the entire phylogenetic tree and no single clusters per source were identified. These observations are in line with previous research on mixed human and animal *C. difficile* populations [35,65]. Both studies showed that animal isolates did not constitute a distinct lineage from human isolates. A possible explanation for this observation is that *C. difficile* 078 strains may have frequently been transmitted between sources, rather than persisting exclusively in one host. Consequently, the clonal strains in farmers and farm animals we identified may be part of a larger network that could have links with the healthcare system. The heterogeneous phylogenetic cluster with limited SNP diversity shown in Figure 2 is an example of potentially linked clinical and farm isolates. Additional patient data for the clinical isolate Breda '08 showed that, although symptoms started five days before hospitalisation (suspected community onset), the patient was living in a long-term healthcare facility and therefore constituted a healthcare-associated case.

The strength of this study is that we applied for the first time the highly discriminatory method whole genome SNP typing to study the relatedness of *C. difficile* 078 isolates obtained from farmers and farm animals. A limitation of this study is the small number of clinical samples that were community-associated; such samples may have allowed us to demonstrate more links between farm animals, farmers and the wider community. In addition, the bacterial strain cohort was restricted to isolates obtained in one country, the Netherlands.

The recent trends in epidemiological data show that *C. difficile* 078 is an important type found in the Dutch healthcare system and its prevalence has remained stable between 2009 and 2013 (data not shown). Besides symptomatic patients, other sources play a major role in the spread of *C. difficile* within the healthcare system, for instance asymptomatic carriers visiting a healthcare facility [24]. Asymptomatic carriage can be common among hospitalised patients [20,21], although future large studies are needed to determine the precise scale of onward transmission by these carriers. The reservoirs from which these carriers in the community are colonised remain to be elucidated. Importantly, it is becoming clear now that the community reservoir for human CDI is much more diverse and larger than previously expected [24,66]. Here, we demonstrate that transmission from pigs to farmers is one of the potential routes by which *C. difficile* is entering the human population, and that these isolates also carry antimicrobial resistance determinants that might be a result of selection in response to antibiotic exposure in pigs.

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Conflict of interest

None declared.

Authors' contributions

CWK, JC, EJK, and TDL designed the study. SD, ECK, and LL collected strains and epidemiological data. CWK, IMJG, and ECK did laboratory work and DNA extraction. DH did whole-genome sequencing. CWK, TRC, AM, and HPB did bioinformatic analysis. CWK, TRC, AM, and TDL analysed data. CWK, TRC, AM, JC, EJK and TDL interpreted data. CWK prepared the figures. CWK, TRC,JC, EJK, and TDL wrote the report.

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The '2014 European guideline on the management of syphilis' has now been published

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On 27 October 2014, the evidence-based '2014 European guideline on the management of syphilis' was published online [1]. This guideline, a thoroughly updated version of the 2008 version [2], provides up-to-date guidance regarding the diagnosis and management of syphilis in Europe. It includes important updates such as:

- recommendations for broader use of validated and quality assured PCRs and new treponemal tests;
- more flexible options for serologic screening, that is, traditional sequence algorithm (non-treponemal test as initial test), reverse sequence algorithm (treponemal test as initial test), or a combined version (both non-treponemal and treponemal tests for ideal initial testing);
- discussions regarding advantages and disadvantages with different screening algorithms, including obtaining false-negative and false-positive specimens in the serologic diagnosis;
- recommending long-acting penicillin G (benzathine penicillin G) as the only first-line therapy regimen in early syphilis and in late latent syphilis, that is, procaine penicillin is no longer any first-line therapy option in any phase of syphilis.

Further details regarding recommended diagnostics, when to use and how to interpret the results of different tests, recommended management of neurosyphilis and cardiovascular, ocular, auricular and congenital syphilis, alternative treatment regimens, contact tracing, management of sexual contacts, and follow-up including test-of-cure, are also available in the newly launched guideline [1].

Syphilis, classified as acquired or congenital, with the aetiological agent *Treponema pallidum* subspecies *pallidum* remains a major public health concern globally. In 2008, the World Health Organization (WHO) estimated 10.6 million cases among adults worldwide (0.2 million in the WHO European region) [3]. In the European Union and European Economic Areas, 20,802 syphilis cases were reported (5.1 [range: 1—8.5] per 100,000 population) in 2012 [4]. The majority (>80%) of syphilis cases were reported in patients ≥25 years of age and half (48%) of the cases were reported in men who have sex with men (MSM). After a long-term decreasing trend, overall rates are now relatively stable but several countries have reported dramatic increases during last decade, mainly among MSM. Furthermore, 91 cases of congenital syphilis cases were reported in 11 countries (23 reporting countries) in 2012 [4].

As long as appropriate testing of suspicious syphilis cases and/or screening are performed, syphilis is relatively easy to detect by adequate serological tests, supplemented with a validated and quality assured PCR in particularly early primary syphilis. However, all laboratory results should be considered together with clinical data and sexual risk anamnesis. Syphilis is also easy to treat with benzathine penicillin G. Suboptimal supply of benzathine penicillin G in several European countries could threaten the management of syphilis, including the initiative to eradicate congenital syphilis, in Europe.

* Author's correction:

On 18 November 2014, the number of cases of congenital syphilis reported in 2012 was changed from 101 to 91 at the request of the authors.

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The Innovative Medicines Initiative launches call on Ebola and other filoviral haemorrhagic fevers

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On 6 November 2014, the Innovative Medicines Initiative 2 Joint Undertaking (IMI2 JU) [1] launched its second Call for Proposals in the framework of the IMI2 'Ebola and other filoviral haemorrhagic fevers programme (the Ebola+ programme)' [2]. Submissions for the Call can be made as of 22 November and closes on 1 December, and has an indicative budget of € 140 million.

The following topics are covered in the Call for proposals:

Topic 1: Vaccine development Phase I, II, and III
Topic 2: Manufacturing capability
Topic 3: Stability of vaccines during transport and storage
Topic 4: Deployment and compliance of vaccination regimens
Topic 5: Rapid diagnostic tests

The Call and its topics will be further explained in a 17 November webinar.

The IMI is a public-private partnership, between the European Union and the European pharmaceutical industry, represented by the European Federation of Pharmaceutical Industries and Associations (EFPIA) in the life sciences area and launched in the year 2008. For the period 2014 to 2024, its budget is €3.3 billion. It has 46 projects running, some of which focus on challenges in drug development such as drug and vaccine safety, antimicrobial resistance and creating European platform to discover novel medicines. Others projects target specific health issues, e.g. diabetes, lung disease, oncology, inflammation and infection, and tuberculosis.

For more information about the Call and the IMI, read here: http://www.imi.europa.eu/sites/default/files/uploads/documents/IMI2Call2/IMI2_Call2_Text_FINAL.pdf.

References

1. The Council Regulation (EU) No 557/2014 of 6 May 2014 establishing the Innovative Medicines Initiative 2 Joint Undertaking. Official Journal L 169, 7.6.2014, p. 54–76 . Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.L_.2014.169.01.0054.01.ENG
2. Innovative Medicines Initiative. Internet. Available from: http://www.imi.europa.eu/content/imi-2-call-2-0