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Surveillance report

Chikungunya virus infections among travellers returning to Spain, 2008 to 2014 2
by MD Fernandez-Garcia, M Bangert, F de Ory, A Potente, L Hernandez, F Lasala, L Herrero, F Molero, A Negredo, A Vázquez, T Minguito, P Balfagón, J de la Fuente, S Puente, E Ramírez de Arellano, M Lago, M Martinez, J Gascón, F Norman, R Lopez-Velez, E Sulleiro, D Pou, N Serre, RF Roblas, A Tenorio, L Franco, MP Sanchez-Seco

Epidemiology of pertussis in Denmark, 1995 to 2013 11
by T Dalby, PH Andersen, S Hoffmann

Research Articles

Antimicrobial resistance of Neisseria gonorrhoeae isolates in south-west Germany, 2004 to 2015: increasing minimal inhibitory concentrations of tetracycline but no resistance to third-generation cephalosporins 19
by T Regnath, T Mertes, R Ignatius

News

The European Commission Consumers, Health, Agriculture and Food Executive Agency (CHAFEA) call for tender concerning studies on vaccination closes on 13 September 26
by Eurosurveillance editorial team

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Chikungunya virus infections among travellers returning to Spain, 2008 to 2014

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Since the first documented autochthonous transmission of chikungunya virus in the Caribbean island of Saint Martin in 2013, the infection has been reported within the Caribbean region as well as North, Central and South America. The risk of autochthonous transmission of chikungunya virus becoming established in Spain may be elevated due to the large numbers of travellers returning to Spain from countries affected by the 2013 epidemic in the Caribbean and South America, as well as the existence of the *Aedes albopictus* vector in certain parts of Spain. We retrospectively analysed the laboratory diagnostic database of the National Centre for Microbiology, Institute of Health Carlos III (CNM-ISCIII) from 2008 to 2014. During the study period, 264 confirmed cases, of 1,371 suspected cases, were diagnosed at the CNM-ISCIII. In 2014 alone, there were 234 confirmed cases. The highest number of confirmed cases were reported from the Dominican Republic (n = 136), Venezuela (n = 30) and Haiti (n = 11). Six cases were viraemic in areas of Spain where the vector is present. This report highlights the need for integrated active case and vector surveillance in Spain and other parts of Europe where chikungunya virus may be introduced by returning travellers.

Introduction

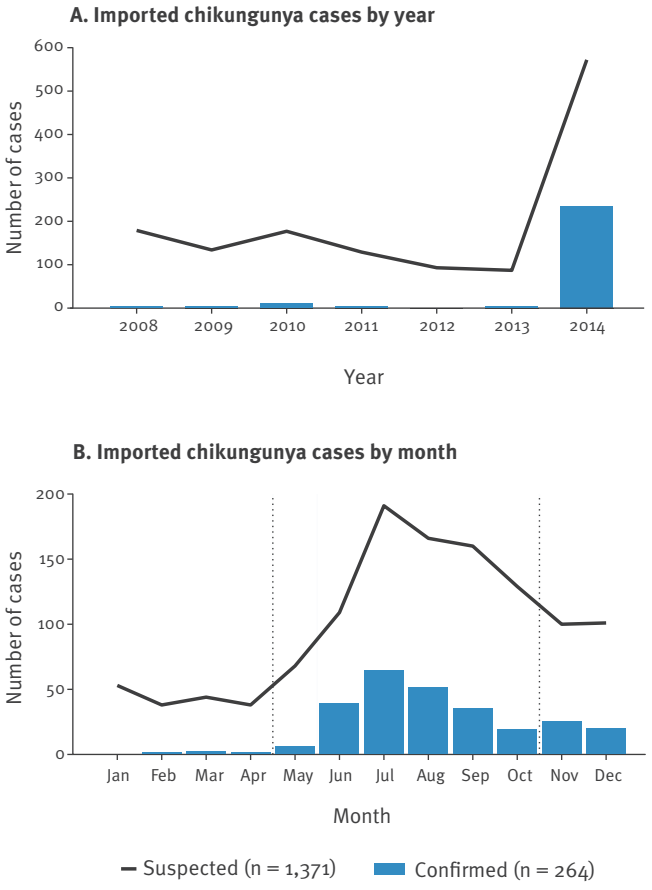
Since the first outbreak in Tanzania in 1952, chikungunya has been endemic in some parts of Africa, south-east Asia and in the Indian subcontinent [1]. In 2013, however, the first documented autochthonous transmission of chikungunya virus was reported in the Caribbean island of Saint Martin [2] and since then the infection has spread quickly to other countries and territories of the Caribbean as well as to North, Central and South America [2]. As at 31 December 2014 (the final day of our study), 24,682 confirmed autochthonous cases and 1,118,763 suspected cases of chikungunya in the Americas had been reported by the Pan American Health Organization (PAHO) [3]. A large majority of these suspected autochthonous cases (n = 802,714; 72%) were reported from the Caribbean, in particular from the Dominican Republic (539,099; 67%).

A chikungunya outbreak in 2005 in Réunion, a French overseas department and region, affected 266,000 people (ca 35% of the population), including 783 cases imported to metropolitan France [4]. During this outbreak, a mutation (A226V in the chikungunya virus E protein) that improved the replication in *Aedes albopictus* was observed, giving rise to a new virus variant whose fitness in this mosquito was increased [5].

The transmission of chikungunya virus to humans occurs mainly through bite of infected *Ae. aegypti* or *Ae. albopictus* mosquitoes, which can also transmit

Figure 1

Number of suspected and confirmed imported chikungunya cases by year and by month, Spain, 2008–14 (suspected n = 1,371; confirmed n = 264)



In panel B, the two vertical lines indicate the start and end of the active period for the chikungunya virus vector *Aedes Albopictus* in Spain (May to October).

dengue virus. In Europe, *Ae. albopictus* is established primarily around the Mediterranean basin [6,7] and has been demonstrated to be competent for chikungunya virus transmission in this region [8]. This has resulted in locally acquired infections in Italy (Emilia Romagna region, 2007) as well as in France (Var and Montpellier, 2010 and 2014 respectively) [9-11].

In Spain, despite the presence of *Ae. albopictus* in the eastern Mediterranean regions (Catalonia, Valencia, Murcia, Balearic Islands) [12] and recently in the southern region of Andalusia [13] and in the Basque Country [14], no autochthonous transmission of chikungunya virus has been reported. However, the risk of autochthonous transmission of chikungunya establishing in Spain may be elevated due to the large numbers of travellers returning to Spain from countries affected by the 2013–14 epidemic in the Caribbean and South America [2], as well as the existence of the competent vector in certain parts of Spain. In order to further assess this risk and better understand the epidemiological and laboratory characteristics of imported chikungunya cases, we retrospectively analysed the laboratory diagnostic database of the National Centre for Microbiology, Institute of Health Carlos III (CNM-ISCIII) from 2008 to 2014.

Methods

Case definition

The case definition used followed the guidelines provided by the Spanish Ministry of Health [15] and the European Centre for Disease Prevention and Control (ECDC) [16].

A suspected case was a patient meeting clinical criteria (acute onset of fever (> 38 °C) and severe arthralgia not explained by other medical conditions) and epidemiological criteria (residing in or having visited epidemic or endemic areas).

A confirmed case was a patient meeting the laboratory criteria, irrespective of the clinical presentation (with at least one of the following: virus isolation, presence of viral RNA, presence of virus-specific IgM antibodies in a single serum sample collected in acute or convalescent stage, or fourfold rise in IgG titres in samples collected at least 15 days apart) [15]. Patients with chikungunya virus or chikungunya viral RNA detected in serum were considered viraemic.

Serology and molecular analysis

The presence of IgM or IgG antibodies against chikungunya virus was detected by indirect immunofluorescence (Euroimmun, Germany).

The presence of dengue virus IgG and IgM antibodies was detected using IgM capture ELISA and IgG indirect ELISA tests [17].

The presence of chikungunya viral RNA was detected by an in-house real-time reverse transcription-PCR (RT-PCR) and confirmed by two established PCR protocols [18].

Dengue diagnostics

Dengue viral RNA was detected using RT-PCR [19]. Dengue nonstructural protein 1 antigen (NS1) was detected by enzyme immunoassay (Platelia Dengue NS1 Ag, Biorad).

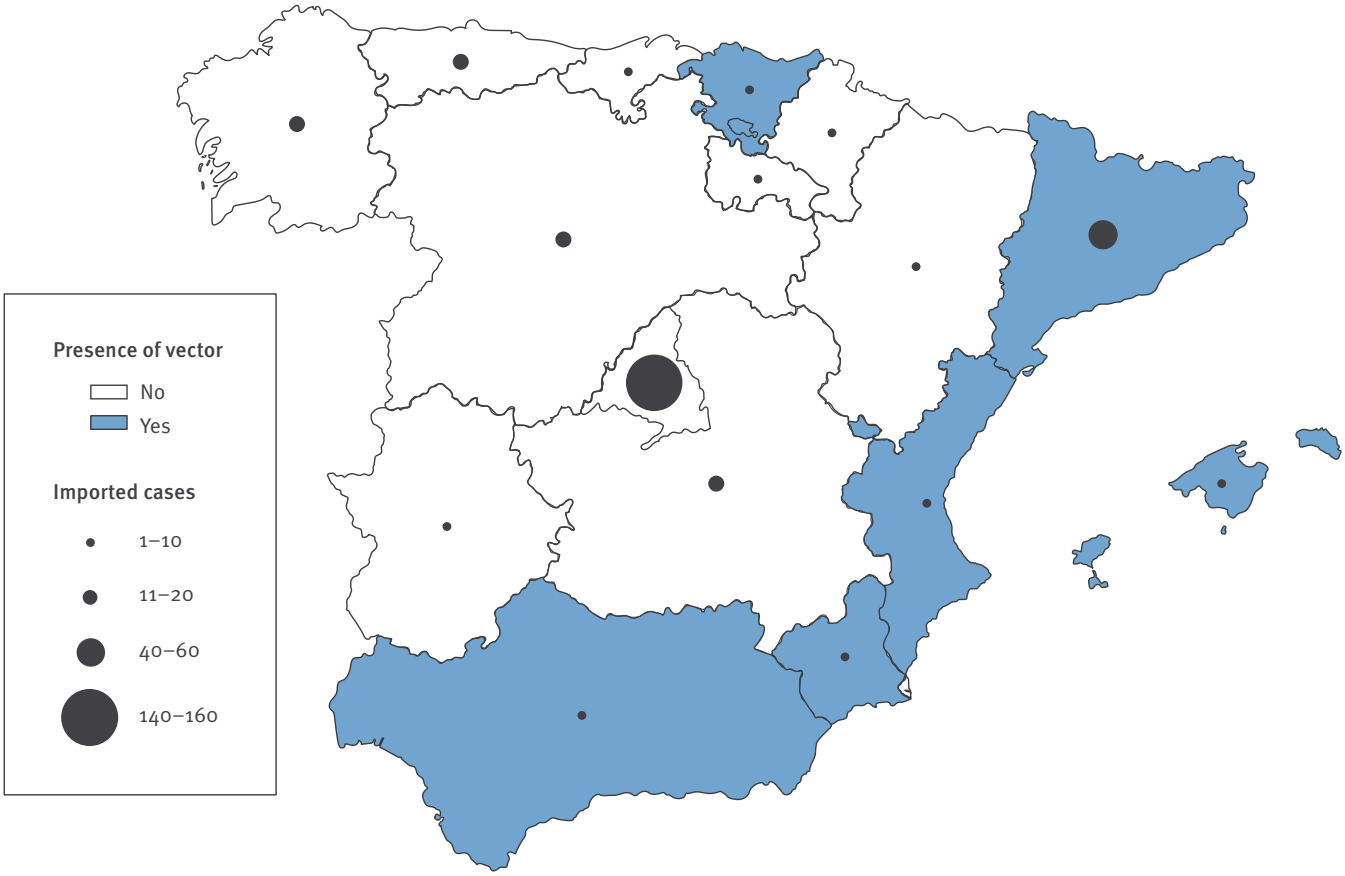
Data used

All data from samples received for diagnosis and surveillance of imported viral infections in the CNM-ISCIII between 1 January 2008 and 31 December 2014 were included in the study. Samples and data were codified with a unique ID to ensure the anonymity of patients.

We accessed the CNM-ISCIII database, in which information was typically collected on sex, age, date of onset of symptoms, date of specimen collection, travel destination and hospitalisation. We also contacted

Figure 2

Geographical spread of confirmed imported chikungunya cases (n = 264) and presence of chikungunya virus vector *Aedes albopictus* in autonomous regions of Spain, 2008–14



local health authorities and hospitals to retrieve information on travel destination. Samples included acute and convalescent sera. We used international travel statistics obtained from a 2014 World Tourism Organization report [20] to estimate incidence rates of chikungunya virus-infected travellers returning to Spain.

Results

Infections reported and patient characteristics

During the study period (1 January 2008 to 31 December 2014), a total of 1,371 suspected chikungunya cases were identified (179 in 2008, 134 in 2009, 177 in 2010, 129 in 2011, 93 in 2012, 87 in 2013 and 572 in 2014). The most frequently reported reasons for travel were work (aid workers, missionaries, others), visiting friends and relatives and tourism. In 2008–13, the median number of suspected cases was 131 (range: 87–179). Of the 1,371 suspected cases in 2008–14, 42% (n = 572) were reported in 2014 alone.

A total of 264 (19%) suspected cases were laboratory confirmed by CNM-ISCIII during the study period. Of the confirmed cases, the majority were female (n = 159; 60%), with a median age of 43 years (range: 1–93). The number of suspected and confirmed imported cases from 2008 to 2014 in Spain is shown in Figure 1A. During 2008–13, 30 imported chikungunya cases were laboratory confirmed (6 in 2008, 4 in 2009, 11 in 2010, 5 in 2011, 1 in 2012, 3 in 2013) with a median of 5 (range: 1–11) cases identified per year, while in 2014 there were 234 confirmed imported cases. The ratio of confirmed to suspected cases shifted from 1:28 in 2008–2013 to 1:3 in 2014. The distribution of imported cases by month during our study period in Spain shows that the number of confirmed and suspected cases reached its peak during July (Figure 1B).

Travel history

The travel history of the patients is detailed in Table 1. Between 2008 and 2013, the travel destination was known for 19 of the 30 confirmed cases: 17 cases had travelled to Asia (Indonesia (n = 5), India (n = 5), Myanmar (n = 2), Thailand (n = 1), Philippines, (n = 1), other Asian countries not specified (n = 3)) and two cases had travelled to Africa (Cameroon and Equatorial Guinea). Of the 234 cases in 2014, the travel history of 220 patients (94%) was known: 154 cases (70%) reported having visited the Caribbean during the incubation period [16], 59 cases had visited Central and South America (Venezuela, Colombia, Mexico, Nicaragua, Peru, El Salvador, Panama), five had visited Africa (Angola, Madagascar, Equatorial Guinea, and

Table 1

Number of confirmed chikungunya cases (n = 264) and incidence rate of chikungunya among travellers returning to Spain, by travel destination, 2008–2014

Travel destinations – countries and territories	Mean annual number of travellers from Spain during 2008–13[a]	2008–13		2014	
		Number of cases	Incidence rate per 100,000 travellers arriving in Spain	Number of cases	Incidence rate per 100,000 travellers arriving in Spain
Americas					
Venezuela	610,333	0	0	30	4.92
Haiti	317,666	0	0	11	3.46
Dominican Republic	4,421,000	0	0	136	3.08
Guadeloupe	405,000	0	0	2	0.49
Martinique	487,000	0	0	1	0.21
Colombia	2,200,000	0	0	3	0.14
Dominica	77,250	0	0	1	1.29
Puerto Rico	3,125,750	0	0	3	0.10
Nicaragua	1,083,000	0	0	1	0.09
El Salvador	1,196,000	0	0	1	0.08
Panama	1,515,250	0	0	1	0.07
Peru	2,581,000	0	0	1	0.04
Americas (unspecified, but excluded North America)	57,849,000	0	0	21	0.04
Mexico	23,365,000	0	0	1	0.00
Africa					
Mozambique	1,911,000	0	0	1	0.05
Madagascar	218,250	0	0	1	0.46
Angola	478,000	0	0	2	0.42
Cameroon	664,666	1	0.15	0	0
Equatorial Guinea[b]	No data	1	NC	1	NC
Asia					
India	6,377,750	5	0.07	1	0.02
Asia (unspecified)	235,587,000	3	0.00	1	0
Indonesia	7,874,750	5	0.06	0	0
Myanmar	54,875	2	0.36	0	0
Thailand	21,016,750	1	0.00	0	0
Philippines	4,097,750	1	0.02	0	0
No travel destination specified					
Cases with missing data	NA	11	NA	14	NA
Total number of cases	NA	**30**	NA	**234**	NA

NA: not applicable; NC: not calculated, as no denominator data.

[a] Source: [20] for data from 2008 to 2012 and [45] for data from 2009 to 2013. At the time of analysis, traveller data were not available for 2014.

[b] Traveller data were not available for Equatorial Guinea for 2008–13.

Mozambique) and two had visited Asia (India, unspecified). No autochthonous infections acquired in Spain were identified or reported by the health authorities.

The incidence rates of chikungunya in travellers worldwide returning to Spain are shown in Table 1. The highest incidence rate in 2014 was seen in travellers returning from Venezuela, with 4.92 cases per 100,000 travellers, followed by those who had travelled to Haiti (3.46/100,000) and the Dominican Republic (3.08/100,000). In contrast, during 2008–13, the highest incidence rate was in travellers returning from Myanmar, with 0.36 cases per 100,000 travellers.

Information about reason for travel was available for a limited number of suspected cases in 2014 (n = 66). Among those 66 suspected cases, the most frequently reported reasons for travel were visiting friends and relatives (n = 27), tourism (n = 25) and work (n = 14; aid workers, missionaries, others).

Region of notification and presence of vector

Overall, 120 hospitals in all Spanish regions referred samples to the CNM-ISCIII during the study period. Of the 264 cases confirmed between 2008 and 2014, Madrid and Catalonia had the largest number of notifications (150 (57%) and 44 (17%), respectively). A total

Table 2

Indirect immunofluorescence results and interval between the dates the first and second samples were taken, for convalescent paired samples from 37 confirmed cases, Spain, 2008–14

Indirect immunofluorescence results		Number of samples	Mean time in days[a] (range) between the dates the first and second samples were taken, post symptom onset
First sample	Second sample		
IgM			
Pos	Pos	17	43 (7–79)
Neg	Pos	4	22.5 (16–29)
Neg	Neg	4	117 (53–181
IgG			
Pos	Pos	17	108 (6–210)
Neg	Pos	15	38 (16–60)
Pos	Neg	1	58[b] (NA)

NA: not applicable; Neg: negative; Pos: positive.

[a] Unless otherwise specified.

[b] The second sample was taken 58 days after symptom onset. The date the first sample was taken was not available.

of 66 of the cases (25%) were reported in regions where *Ae. albopictus* is present (Catalonia, Autonomous Community of Valencia, Basque Country, Murcia, Balearic Islands, and since 2013 southern Andalusia) (Figure 2). Of these 66 cases, six were viraemic (PCR positive) when presenting to clinicians in Spain.

In 2014, during the active period of *Ae. albopictus* in Spain (May–October), we confirmed a total of 33 imported cases in regions with vector presence, three of whom were viraemic when presenting to clinicians in Spain.

Serological diagnostics

Of the 33 confirmed imported cases in regions in which *Ae. albopictus* was present, 30 had anti-chikungunya virus IgM antibodies, suggesting recent infection.

Serology was performed on 1,147 of the 1,371 (84%) suspected imported cases. Of the 1,147 tested, 235 (20%) were positive for IgM. Of the 235 IgM-positive samples, 11 (5%) also tested positive by the molecular methods described.

From 36 IgM-positive imported cases with known date of symptoms onset, samples were collected within 0 to 37 days after onset (median: 7 days).

Paired serum samples were available for 37 confirmed cases, where the time elapsed between the first and second samples ranged from 2 to 210 days. The results of the IgM and IgG assays are shown in Table 2. The time taken for an initially positive IgM test to become negative ranged from 93 to 181 days in our study. Among cases for whom the first sample was IgG positive, some remained IgG positive for up to 210 days, when the second sample was taken. Of 37 paired serum samples, 15 demonstrated seroconversion within 14 to 60 days.

Molecular diagnostics

Molecular diagnosis was carried out for samples from 481 (35%) of the 1,371 suspected cases: viral genome was detected by RT-PCR in 39 (8%) of the 481 tested. Of the 39 patients with a chikungunya virus-positive PCR, 11 had a known date of symptom. The samples were collected 0–3 days after symptom onset except for one, which was collected after six days.

Of the 235 patients whose infection was confirmed by IgM, 86 were negative by PCR. For these 86 patients, the median time from onset of symptoms to sample collection was four days (range: 0–35).

Concurrent infection

Of the 1,371 suspected chikungunya cases reported during the study period, 817 were also tested for the presence of anti-dengue virus IgG and IgM antibodies, dengue RNA and/or dengue nonstructural protein 1 antigen (NS1). In 2014, 41% (234/572) of the suspected chikungunya cases were tested for dengue virus infection while in the rest of the study period (2008–13), 73% (583/799) of the suspected chikungunya cases were tested for dengue virus infection. Dengue RNA, NS1 and/or anti-dengue virus IgM antibodies were found in 116 of the 1,371 suspected chikungunya cases. During 2008–13, a total of 87 imported dengue cases were laboratory confirmed (9 in 2008, 13 in 2009, 28 in 2010, 9 in 2011, 12 in 2012, 16 in 2013); in 2014, there were 29 confirmed imported dengue cases. The distribution of dengue imported cases by month in 2014 showed that the number of confirmed cases reached its peak during August (data not shown). A total of 13/29 confirmed dengue cases in August 2014 reported having visited the Americas.

Of the 116 dengue cases, five were also confirmed as positive for chikungunya virus, one in 2010 and four in 2014. Of these five, three had anti-dengue virus and

Table 3

Dengue virus and chikungunya virus laboratory results from coinfected confirmed imported cases, Spain, 2008–14 (n = 5)

Case	Confirmed dengue	Confirmed chikungunya			Travel destination	Year
		IgM	IgM plus PCR	PCR		
1	IgM positive	Pos	Neg	Neg	Venezuela	2014
2		Pos	Neg	Neg	Philippines	2010
3		Pos	Neg	Neg	Dominican Republic	2014
4		Neg	Neg	Pos	Dominican Republic	2014
5	IgM and PCR positive	Neg	Neg	Pos	Venezuela	2014

Neg: negative; Pos: positive.

anti-chikungunya virus IgM, one had anti-dengue virus IgM and chikungunya viral RNA, and one had dengue viral RNA and chikungunya viral RNA. The coinfected patients in 2014 had returned from Venezuela (n = 2) and the Dominican Republic (n = 2). The patient coinfected in 2010 had returned from the Philippines (Table 3).

Discussion

We have described a 7.8 fold increase in the number of imported chikungunya virus infections from 2008–13 (30 cases) to 2014 (234 cases) in Spain, with the number in 2014 being the highest recorded in the country. Every year since 2006, imported chikungunya cases have been identified among travellers returning to Spain. During 2006 to 2007, 29 laboratory-confirmed imported cases were diagnosed among a cohort of 308 travellers with symptoms compatible with acute or recent chikungunya virus infection on their return to Spain [21]. The majority of these cases (n = 20) had visited India or the Indian Ocean Islands. Similarly, during 2008 to 2013, most imported cases (17/19) arrived from Asia. As a result of an ongoing outbreak in the Americas, with more than 780,000 suspected cases by 31 October 2014 [22], however, the majority of cases imported to Spain in 2014 were from the Caribbean and northern parts of South America [23], demonstrating the potential of this large chikungunya outbreak to affect Spain.

The Caribbean is a popular travel destination for Spanish travellers during the spring and summer months. It is also a destination for migrants living in Spain, who travel back home to visit friends and relatives, as shown by the large proportion of chikungunya cases in our study who had visited friends and relatives (27/66). Spain has a dynamic population of Latin American and Caribbean immigrants with permanent residency in Spain who frequently travel to their country of origin. Immigrants from the Caribbean, especially the Dominican Republic, have represented the one of the largest proportion of immigrants in Spain in recent decades and a continued growth of Dominican immigration is predicted for the future [24]. This correlates with the high number of confirmed chikungunya cases from the Dominican Republic (136/264) seen in our study. Furthermore, migrants visiting friends and relatives are less likely than tourists to seek travel health advice, and therefore to take preventive measures during their stay, representing an important gateway for the entry of chikungunya virus in Europe [25]. This challenge requires appropriate countermeasures such as targeted guidance to these groups of travellers at risk and early screening.

During 2015, however, the virus spread from the Caribbean to South and Central America, and more countries have become affected. The virus spread in Colombia, Venezuela, Brazil and other South American countries during the summer of 2015. In Brazil, where in 2014 a total of 3,657 cases distributed in eight municipalities were reported, in 2015 the number of cases increased more than fivefold, to 20,661 cases in 84 municipalities, mainly in north-east and south-west states [26].

Appropriate surveillance and investigation of imported chikungunya cases could also aid in understanding better the epidemiological and virological dynamics of the outbreak in the Caribbean and Latin American countries. Returning travellers can serve as indirect sentinels to monitor the geographical spread of the outbreak in the Americas. For example, in our study 52% of imported cases came from the Dominican Republic, in line with PAHO´s report in October 2014 that showed 62% of cases in the Americas stem from this country [22]. Similarly, in late 2014, 13% of chikungunya virus-infected travellers were returning from Venezuela, around the time the country was facing a large re-emergence of vector-borne diseases [27]. If we look in 2015 for chikungunya cases in Spain imported from Venezuela, the percentage would probably be larger due to the large epidemic there.

Sharing of data gained through analysis of returning travellers could support countries where no or scarce data on chikungunya have been reported. In our study, for example, we saw an imported case from Mozambique, a country that only recently demonstrated circulation of chikungunya virus [28]. We also saw cases in travellers returning from Colombia, El Salvador and Nicaragua, which have reported local

transmission, and from Panama, which had reported imported cases in 2014 [3,29]. While this can serve as an indication, using travel information alone is not sufficient to definitively determine the country source of infection as travel is seasonal to most destinations and the epidemic may have been introduced before infected travellers returned.

Although the chikungunya outbreak in the Caribbean has led to an increase in the number of cases in Spain, enhanced surveillance may have also contributed to the rise. Chikungunya was classified a notifiable disease in 2013 by the Consejo Interterritorial del Sistema Nacional de Salud and became law in 2015, after our study period [30,31]. This may have led to greater awareness among patients and physicians, resulting in better ascertainment of suspected cases. Before the outbreak in the Caribbean, 1 in 28 suspected chikungunya cases were confirmed positive in the ISCIII whereas in 2014, the ratio was 1 in 3 (confirmed vs suspected cases). In 2008–13, of the 799 cases notified only 30 were confirmed, whereas in 2014, 234 of 572 notified cases were confirmed. Similarly, in south-eastern France, an increase in the number of suspected cases was noted in 2010 due to enhanced surveillance for chikungunya implemented after autochthonous chikungunya transmission was reported [10].

Although autochthonous transmission of chikungunya has been documented in south-eastern France in 2010 and 2014 [8,10] as well as in Italy in 2007 [9], Spain has not reported local transmission of chikungunya virus, despite the presence of *Ae. albopictus* mosquitoes in the country [32]. Chikungunya virus strains with the A226V viral mutation have not yet been described in America, as the circulating genotype is the Asian one and not the East/Central/South African (ECSA) genotype, in which the mutation arose [5]. This mutation should be considered in risk assessments of local transmission in Spain. Our data show that most travellers returned to Spain during the warm months of June to September, coinciding with the period of activity for *Ae. albopictus* (May–October). In 2014, the highest number of confirmed imported cases of chikungunya were in areas in which the vector was present (n = 66; Catalonia, Autonomous Community of Valencia, Murcia, Basque Country, Balearic Islands and Andalusia). Importantly, six of these 66 confirmed cases were positive by PCR. Such viraemic travellers are potential disseminators of the virus through bites of vectors, or by donating blood [33]. The ability of infected vectors to effectively overwinter until the next hot season further adds to the importance of imported chikungunya [34]. Considering that autochthonous transmission in France in 2010 occurred following importation of only two cases of chikungunya, the high number of infected travellers returning to Spain highlights the increased risk of autochthonous transmission becoming established. It is important to note that, to date, countries affected by localised outbreaks in the EU have been able to contain further spread of the disease [10,35].

Serological diagnosis can be performed by detection of specific IgM antibodies in serum from four to five days after the onset of symptoms, or a fourfold rise in chikungunya- specific IgG antibody titre in a paired serum sample. Chikungunya-specific IgM can persist for months, in particular in patients with persistent arthralgia [36]. The reported sensitivity of the commercial Immunofluorescence test used for detection of chikungunya-specific IgM has been 98.3%, with a sensitivity of 96.9% [37]. The specificity and sensitivity for the detection of IgG is 100.0% and 95.4%, respectively [37]. In our study, we observed that IgM can be detected from a median of seven days after onset of symptoms and that IgG can be detected from 16 days after the first sample is taken. This highlights that if specimens are collected very early in the course of the illness and tested only for IgM antibodies, serological diagnostic testing may not detect cases. In convalescent samples, we observed that IgM can persist up to 79 days. This should be taken into consideration when sampling and testing for chikungunya virus in returning travellers. Further study is also merited as complete case information for this analysis was available only for a limited subset of cases.

As chikungunya virus infection has similar symptoms as dengue and both viruses can circulate in the same area, chikungunya fever has often been mistaken for dengue. Confirmed dengue imported cases among travellers seen in our study support the suspicion of dual endemic circulation of dengue and chikungunya viruses in the Caribbean. Coinfections have been reported in Saint Martin, where 2.8% of chikungunya cases were reported as dengue coinfections in December 2013 to January 2014 [38]. In 2010, south-eastern France reported the concomitant emergence of dengue and chikungunya viruses, with two autochthonous infections reported for each [10,35]. Here we report five coinfections, four occurring in 2014 and one in 2010. Coinfections in travellers returning to Europe from the Indian Ocean Region in 2006 have been reported [39]. In addition, imported cases of Mayaro virus infection, an American alphavirus, were described in Germany in 2013 [40], France in 2010 [41] and the Netherlands in 2008 [42]. An imported case of O'nyong nyong virus infection, an African alphavirus, was described in Germany in 2013 [43]. Differential diagnosis of alphaviruses is therefore important and physicians should familiarise themselves with their clinical presentation.

The importance of coinfection is further highlighted as *Ae. albopictus* is also a recognised vector with competence and capacity to transmit dengue virus. With the increasing spread of *Ae. albopictus* in southern Europe, the risk of the establishment of these two arboviruses in Europe has also increased [44]. Continued awareness of other emerging diseases is needed to ensure rapid detection and control. The implementation of a strategic national surveillance system adapted to the early detection of both chikungunya and dengue,

combined with vector monitoring systems, is urgently needed.

Considering the intense international traffic between Spain and countries affected by the 2013–14 chikungunya outbreak, as well as immigration and the distribution of competent vectors, chikungunya virus is becoming a threat to Spain and neighbouring countries. This report highlights the need for integrated active case and vector surveillance in Spain and other parts of Europe where the virus may be introduced by returning travellers. We furthermore highlight our experiences with diagnosing samples from returning travellers and how they can be used to indirectly monitor the spread of the outbreak in the Americas. An outbreak of chikungunya in Spain could have a considerable impact on public health, the safety of blood donation supplies and on tourism. With the 2013–14 outbreak in the Americas, the number of chikungunya cases among travellers returning to Spain from affected areas is likely to continue to increase, especially in the summer season in Spain. Mediterranean countries should strengthen preparedness for the re-emergence and/or reintroduction of chikungunya virus and other *Aedes*-transmitted diseases, especially in regions where *Ae. albopictus* is present or could become established.

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Conflict of interest

None declared

Authors' contributions

MDF, MB, LF, MPSS and AT: conceived and design the study; AP, AN, AV,LH, PM, FL, LHR, PB, TM, JdlF, MM and ES: performed molecular and serological diagnosis; MDF, MB, LF, MPSS, AT, FdO, NS, JG, SP, ML, RLV, FN and RFR: contributed to acquisition, analysis and interpretation of data; MDF, MB and LF: drafted the article. All authors revised it critically and approval the final version of the article.

LF and MPSS are senior authors.

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Epidemiology of pertussis in Denmark, 1995 to 2013

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We describe incidence and age distribution of laboratory-confirmed pertussis in Denmark from 1995 to 2013. Notification has been mandatory since 2007. Since 1997, an acellular monocomponent vaccine has been used. The latest epidemic occurred in 2002 with an incidence of 36 per 100,000; since 1995, only six infant deaths have been recorded. The inter-epidemic incidence lies below 10 per 100,000. In 1995, the mean age of confirmed cases was 9.2 years (95% confidence interval (CI): 7.9–10.5; median: 5.1), this gradually increased to 23.9 years in 2013 (95% CI: 22.0–25.8; median: 15.7). In 1995, 14% of laboratory-confirmed cases were 20 years and older, 43% in 2013. In the study period, the highest incidence among children was among those younger than one year with incidences between 84 and 331 per 100,000 in inter-epidemic periods (mean: 161/100,000) and 435 for the epidemic in 2002. After introduction of a preschool booster in 2003, the highest incidence among children one year and older changed gradually from three to five-year-olds in 2003 to 12 to 14-year-olds in 2013. In 2013, PCR was the primary method used for laboratory-diagnosis of pertussis in Denmark, while serology was the method with the highest percentage of positive results.

Introduction

Pertussis (whooping cough) is a highly contagious respiratory tract infection caused by the bacterium *Bordetella pertussis*. In the early 20th century before the introduction of vaccines, pertussis was the cause of extensive morbidity and numerous infant deaths. In Denmark during 1900 to 1959, 19–53% of all infants contracted pertussis, and up until the 1930s, ca 10% of cases had a fatal outcome [1]. With the introduction of vaccines in the mid-19th century the incidence of pertussis decreased markedly all over the world, but more than 50 years into the vaccine era, pertussis is still prevalent and causes substantial outbreaks, even in countries with a long history of vaccination. In 2008, pertussis was the cause of an estimated 195,000 deaths worldwide among children younger than five years, primarily in the developing world [2]. In Denmark during 1920 to 1929, 2,569 infants died from pertussis, but in the years 1995 to 2013, only six deaths from pertussis were recorded and all were infants younger than two months, i.e. too young to have started vaccination [3,4].

In countries where vaccines have almost eliminated infant deaths due to pertussis, the knowledge about pertussis in the general population has also declined. Thus, many falsely believe pertussis to be solely a childhood infection, and a common misperception is that pertussis vaccination will protect you for life. In reality, immunity after pertussis vaccination or even after an episode of pertussis is only short-lived, at an estimated four to 12 years or four to 20 years, respectively [5], or five to 10 years as a rule of thumb. Consequently, all age groups in the population can contract pertussis, and adult pertussis is not as rare as commonly thought. Several reports have shown that the key sources of pertussis infection for vulnerable non-vaccinated infants are parents or other adults in close contact (reviewed in [6]). Since pertussis in adults can present as just a prolonged cough without the characteristic whoop or the post-tussive vomiting [7-9], it is important to realise that pertussis is a disease of all age groups. With increased knowledge, novel vaccination strategies and improved diagnostic methods, the transmission of pertussis to non-vaccinated infants can hopefully be diminished. This paper summarises the epidemiology of laboratory-confirmed pertussis in Denmark during the period from 1995 to 2013 as well as the distribution of laboratory-methods used across the whole of Denmark in 2013 for the diagnosis of pertussis.

Background on pertussis vaccination and diagnostics in Denmark

Vaccination against pertussis was introduced in Denmark in 1961 as a whole-cell (wP) vaccine [10]. In 1997, this vaccine was replaced by an acellular vaccine (aP) with pertussis toxoid (PTx) as the sole pertussis antigenic component (Table 1) and in 2003, a preschool booster at five years of age was introduced. The aP

Figure 1

Incidence of laboratory-confirmed pertussis per 100,000 population, Denmark, 1995–2013 (n = 13,269)

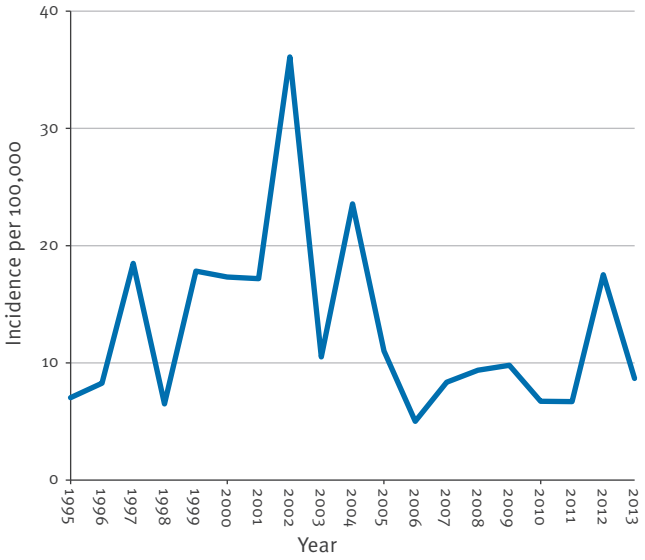
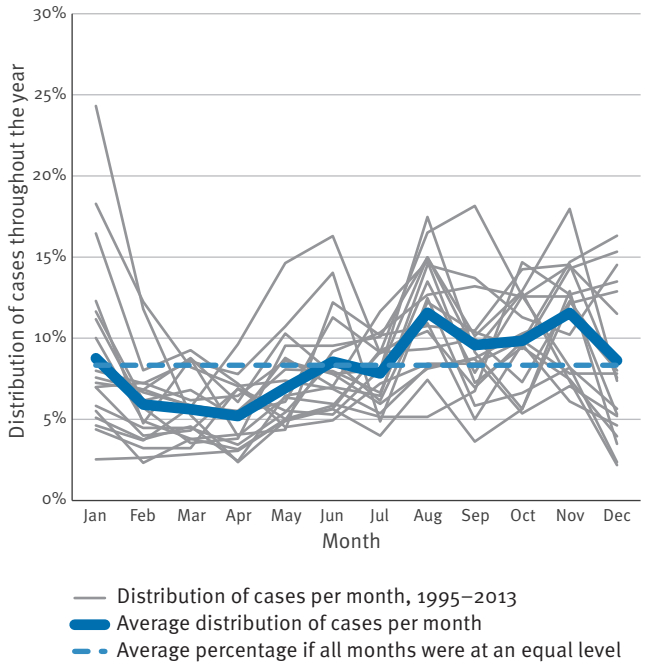


Figure 2

Seasonality of laboratory-confirmed pertussis, Denmark, 1995–2013 (n = 13,269)



vaccines used in Denmark are unique compared with other aPs around the world in that (i) they contain high amounts of PTx at 40 µg for the infant series and 20 µg for the preschool booster, and (ii) the toxoid is prepared by hydrogen peroxide detoxification of the pertussis toxin, rather than the formaldehyde and/or glutaraldehyde inactivation used by many other manufacturers (reviewed in [11]). Detoxification by hydrogen peroxide has been shown to result in a lower degree of epitope impairment of the toxin compared with formaldehyde, and the generated immune response may therefore be more effective [12,13]. Moreover, the Danish aP vaccines elicit a stronger antibody response against pertussis toxin than a number of other aPs [11,14,15].

Laboratory diagnosis of pertussis in Denmark was initially done by culture of the bacterium, and this was only done at the clinical microbiological laboratory at Statens Serum Institut (SSI). In 1998, PCR was introduced at SSI [16] and from 2002, public regional clinical microbiology laboratories gradually began using either culture or PCR. Subsequently, the proportion of pertussis diagnostics in Denmark performed at SSI decreased gradually from 100% in 1995 to 98% in 2002, 64% in 2006 and to 32% in 2013. For regional clinical microbiology laboratories not performing pertussis diagnostics, samples were, and still are, sent either to SSI or to one of the other regional clinical microbiology laboratories. Thus, the diagnostics for pertussis covered the whole of Denmark in the whole period from 1995 to 2013. In 2013, seven clinical microbiology laboratories in Denmark including SSI were performing diagnostic tests for pertussis. All were employing PCR, and the only laboratory also maintaining the culture of *B. pertussis* was SSI. At SSI, culture is performed on Regan-Lowe agar, and for the whole study period, the PCR was performed as an initial IS481/IS1001 PCR followed by a confirmatory ptxP PCR. Details on the PCR methods used at the regional laboratories are not available. In 2010, serology was introduced at SSI as an in-house IgG anti-PT ELISA with a cut-off at 75 IU/mL. The test is only considered valid for individuals eight years and older in order to avoid interference from antibodies elicited by the preschool booster normally given at five years of age [17,18]. Positive results from serology-confirmed children are only included in the database if the vaccination registry proves that the latest vaccination happened more than two years previously and if the child is older than six months; such cases are very few (16 during the study period).

Culture of *B. pertussis* has a specificity of 100%. However, the sensitivity is low compared with PCR and serology [19-21]. This is mainly due to the difficulty in obtaining a nasopharyngeal sample of sufficient quality, the low chance of retrieving viable *B. pertussis* [22], the long incubation time necessary for culture and the fact that culture is only reliable in the first two weeks of symptoms [23]. PCR is useful in the first three weeks of symptoms and is also dependent on a correctly obtained nasopharyngeal sample [23,24].

Serology is useful for diagnosis of patients having had symptoms for more than two weeks [23,25] and serology is therefore particularly efficient in diagnosing pertussis among adults, who can experience mild symptoms and therefore may be more likely to seek medical attention at a later stage of the disease, after some weeks with symptoms [7,26,27].

Figure 3

Age-distribution of laboratory-confirmed cases of pertussis, Denmark, 1995–2013 (n = 13,269)

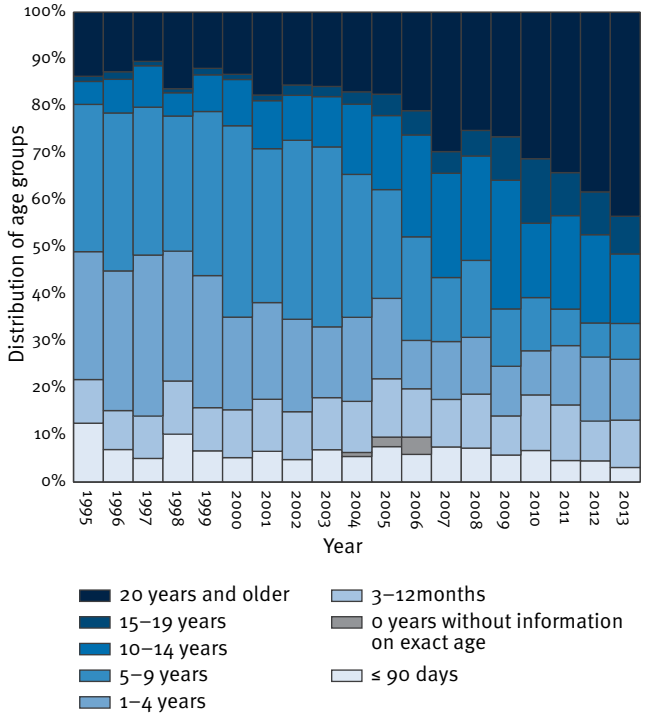
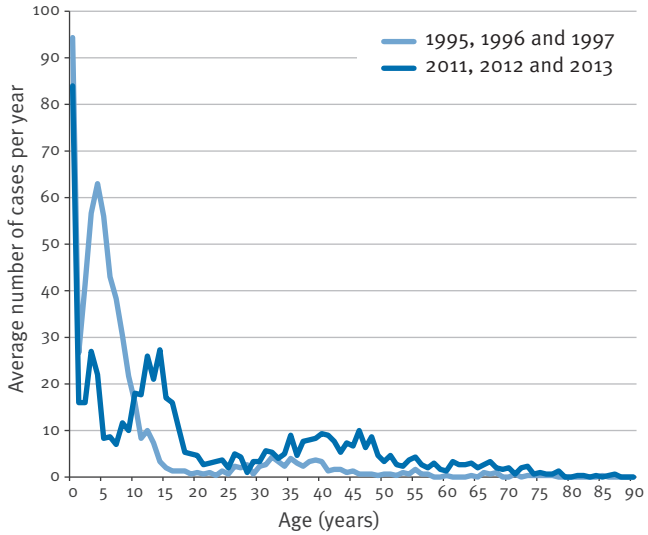


Figure 4

Average number of laboratory-confirmed pertussis for two periods by patient age, Denmark, 1995–97 (n = 1,776) and 2011–13 (n = 1,836)



Initially, since only the laboratory at SSI performed diagnostics of pertussis, data on all laboratory-confirmed cases were registered at SSI. When regional clinical microbiology laboratories gradually began using PCR or culture for pertussis starting in 2002, they all voluntarily submitted their data on confirmed pertussis cases to SSI for surveillance purposes. This voluntary reporting was ongoing at full coverage until 2007 when it became mandatory to report all laboratory-confirmed cases of pertussis to SSI. The national database of laboratory-confirmed pertussis at SSI therefore covers the whole of the country since 1995, i.e. from before the mandatory reporting was initiated. Recently, a national database (The Danish Microbiology DataBase (MiBa)) has also been established which automatically receives electronic real-time copies of all test reports, whether positive or negative, from all Danish departments of clinical microbiology, making it possible to investigate the use of laboratory methods across the country [28,29]. In addition, cases of confirmed pertussis among children younger than two years need to be notified on a paper form. The national surveillance of pertussis in Denmark covers only laboratory-confirmed cases. Cases based on the clinical picture alone or based on an epidemiological link are not registered.

Deaths from pertussis were registered as part of a routine used in the period from 1995 to 2013 that systematically requested information from the notifying clinician on possible sequelae, including death, for all notified cases in children younger than two years.

During the whole period from 1995 to 2013, the national vaccination uptake remained at a high level, averaging 89% for the third primary vaccination for the birth cohorts 2003 to 2013. However, the method of calculation changed from using an administrative method between 1995 and 2005 to using a register-based approach (retrospectively) since 2006. In the period from 1995 to 2005, uptake of all three infant doses of the combination vaccine containing the acellular pertussis component, DTP3/DTaP, was in the range of 90–99% at 12 to 23 months of age. For the birth cohorts 2006 to 2013, DTaP uptake was in the range of 85–91%, with an increasing trend [30]. Regarding the five-year booster vaccination, coverage for the birth cohorts 2000 to 2008 was in the range of 81–87%, also with an increasing trend [31].

Methods

Data from the Danish national database on laboratory-confirmed pertussis was analysed for the period from 1995 to 2013. Except for a few cases registered before the reporting of cases became mandatory in 2007 (68 cases in the period from 2002 to 2004 without date of birth, age or date of diagnosis, 20 cases in 2006 with age but without date of birth or date of diagnosis, 120 cases in the period from 2004 to 2006 with age and date of diagnosis but without date of birth), all entries include the Danish personal identification number (CPR number), date of birth, date of sampling for the test that confirmed the diagnosis and name of the laboratory that performed the diagnostic test. Information on diagnostic method was unavailable for entries before 2010.

Table 1

Historic overview of pertussis vaccines and schedules used in Denmark

Change	Vaccines	Schedule
1961	**wP** (+DT)	5, 6, 7 and 15 months
1969	wP	**5 and 9 weeks, 10 months**
1997	**aP primary series, 40µg PT *(DTaP-IPV)***	3, 5 and 12 months
2002	aP primary series, 40µg PT *(DTaP-IPV/**PRP-T**)*	3, 5 and 12 months
2003	aP primary series, 40µg PT *(DTaP-IPV/PRP-T)*	3, 5 and 12 months
	aP booster, 20µg PT *(TdaP)*	**5 years**
2004	aP primary series, 40µg PT *(DTaP-IPV/PRP-T)*	3, 5 and 12 months
	aP booster, 20µg PT *(TdaP-**IPV**)*	5 years

aP: acellular pertussis vaccine containing pertussis toxoid (PT) as the sole pertussis antigenic component; wP: whole-cell pertussis vaccine.

The composition of the vaccines is marked in italics according to international nomenclature. Changes are marked in bold letters.

Incidence was calculated based on annual population data from StatBankDenmark [32].

Data from the clinical microbiology laboratories in Denmark (through MiBa) [28] was analysed for samples submitted for pertussis diagnostics in the period from 1 January to 31 December 2013. In some instances, two simultaneous samples are sent from the same patient, and we therefore filtered the data so that samples taken on the same day and with the same result were treated as a single sample in the analysis.

We also analysed data from the national Danish database on notified cases of pertussis among children under the age of two years for the period from 1995 to 2013. This database also contains information on the patients' vaccination status.

Results

Annual incidence

Since 1995, the annual total number of laboratory-confirmed cases of pertussis in Denmark has ranged from 272 to 1,272, with an epidemic peak in 2002 at 1,938 cases. With a population ranging from 5.2 million in 1995 to 5.6 million in 2013 , this corresponds to annual incidences between 5 and 36 per 100,000. Apart from a peak in 2012 with 978 cases corresponding to an incidence of 18 per 100,000, the level has been stable since 2005 at a mean value of 451 cases annually, an incidence of 8 per 100,000 (Figure 1). At present, in early 2016, it has thus been 14 years since an epidemic of pertussis has occurred in Denmark. It is evident that pertussis in Denmark is not as seasonal as many other respiratory tract infections. When looking at the combined data for the whole period, there was however a tendency towards a lower occurrence in the months of February to April and a higher occurrence in the months of August to November (Figure 2).

Age distribution

The age distribution of cases shows that more and more adult cases are found (Figure 3 and Table 2). In 1995, 80% of all cases were found among children younger than 10 years but in 2013, this figure had decreased to 34%. Similarly, in 1995, cases among adults 20 years and older accounted for 14% of all cases but this figure increased to 43% in 2013. The median age of laboratory-confirmed pertussis gradually increased from 5.1 years in 1995 (interquartile range (IQR): 1.5–8.7) to 15.7 years in 2013 (IQR: 4.8–41.5). Moreover, due to the introduction of the preschool booster in 2003 the largest age group among older children diagnosed with pertussis changed from the 3–5-year-olds between 1995 and 1997 to the 12–14-year-olds between 2011 and 2013 (Figure 4). This age-specific peak shifted gradually after the booster was introduced (data not shown here, but described previously [11]). We chose the first three and the last three years in the study period for Figure 4 because the total number of cases for the two periods were comparable.

Infant pertussis and vaccinations

For the whole study period 1995 to 2013, the proportion of infant pertussis (in those younger than 12 months) among notified laboratory-confirmed cases younger than two years averaged 83%, ranging from 77% to 90%. The average proportion of unvaccinated cases among all cases younger than two years of age was 46%, ranging from 25% to 58%. Conversely, the average proportion of fully (three doses) vaccinated pertussis cases was 10%, ranging from 3% to 15%, indicating vaccine failures even within the first two years of life.

Diagnostic methods

Laboratory data from MiBa on samples submitted for pertussis diagnostics in the whole of Denmark for the year 2013 (n = 4,569) showed that 79.6% of the samples were submitted for PCR (n = 3,639), 17.8% were submitted for serology (n = 811) and 2.6% were submitted for culture (n = 119). Seven laboratories performed diagnostics for pertussis. All seven used PCR, while only one laboratory (SSI) also performed culture and serology. When looking at samples found positive for pertussis (n = 520), 72% were found by PCR (n = 374), 28% were found by serology (n = 143) and 0.6% were found by culture (n = 3). The number of positive samples was higher than the reported number of cases, as two or more samples were often submitted from the same patient. The proportions of positive results for each of the three methods were 10% for PCR (374/3,639), 18% for serology (143/811) and 2.5% for culture (3/119). The proportion of positive results (confirmed pertussis) for PCR methods in the seven individual laboratories ranged from 6.5% to 13.7%. Sera submitted as part of

Table 2

Number and (incidence) of laboratory-confirmed cases of pertussis, Denmark, 1995–2013 (n = 13,269)

Age	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
≤ 90 d[a]	46	30	49	35	63	48	60	91	38	68	45	16	34	37	31	25	17	44	15
0 years total	80 (115)	66 (94)	137 (202)	74 (109)	150 (227)	142 (214)	162 (241)	285 (435)	99 (154)	215 (330)	131 (202)	54 (84)	80 (123)	96 (149)	76 (116)	69 (109)	61 (96)	127 (214)	64 (110)
1–4 years	100 (38)	129 (47)	334 (120)	95 (34)	266 (96)	182 (66)	189 (70)	375 (139)	83 (31)	224 (84)	102 (39)	28 (11)	56 (22)	62 (24)	57 (22)	35 (13)	47 (18)	133 (51)	63 (25)
5–9 years	115 (39)	146 (48)	307 (98)	99 (30)	331 (99)	376 (110)	301 (86)	726 (207)	211 (60)	381 (109)	138 (40)	60 (18)	62 (18)	84 (25)	66 (20)	42 (13)	29 (9)	71 (22)	37 (11)
10–14 years	18 (7)	31 (11)	86 (31)	17 (6)	74 (26)	91 (30)	94 (30)	182 (57)	59 (18)	187 (55)	94 (27)	59 (17)	101 (29)	114 (32)	148 (42)	59 (17)	74 (22)	184 (54)	72 (21)
15–19 years	4 (1)	7 (2)	9 (3)	3 (1)	13 (5)	10 (4)	11 (4)	42 (15)	12 (4)	33 (11)	27 (9)	14 (4)	21 (7)	28 (8)	50 (15)	51 (15)	34 (10)	89 (25)	39 (11)
20–29 years	8 (1)	12 (2)	18 (2)	14 (2)	19 (3)	24 (3)	20 (3)	46 (7)	17 (3)	32 (5)	10 (2)	11 (2)	30 (5)	26 (4)	22 (3)	25 (4)	20 (3)	54 (8)	25 (4)
30–39 years	25 (3)	23 (3)	46 (6)	25 (3)	49 (6)	51 (6)	85 (10)	129 (16)	32 (4)	80 (10)	37 (5)	18 (2)	33 (4)	40 (5)	32 (4)	24 (3)	32 (4)	99 (14)	52 (7)
40–49 years	11 (1)	12 (2)	15 (2)	8 (1)	22 (3)	18 (2)	25 (3)	61 (8)	18 (2)	49 (6)	27 (3)	11 (1)	42 (5)	29 (4)	55 (7)	34 (4)	39 (5)	115 (14)	71 (9)
≥ 50 years	6 (0.4)	8 (0.5)	23 (1)	9 (1)	23 (1)	29 (2)	32 (2)	59 (3)	20 (1)	51 (3)	30 (2)	17 (1)	30 (2)	34 (2)	34 (2)	33 (2)	36 (2)	106 (5)	63 (3)
Unknown	0	0	0	0	0	0	0	33	15	20	0	0	0	0	0	0	0	0	0
Total	367 (7)	434 (8)	975 (18)	344 (6)	947 (18)	923 (17)	919 (17)	1,938 (36)	566 (11)	1,272 (24)	596 (11)	272 (5)	455 (8)	513 (9)	540 (10)	372 (7)	372 (7)	978 (18)	486 (9)

Incidences are shown in brackets.

[a] Incidence could not be calculated for infants ≤ 90 days since populations statistics are not available for age categories shorter than one year.

a diagnostic serology package for atypical pneumonia at SSI were positive for pertussis in 11% (39 of 368) of the samples, while sera submitted solely for diagnosis of pertussis at SSI were positive in 23 of the samples (104/443).

As mentioned previously, serology is particularly efficient when diagnosing pertussis among adults and although the method is still new in Denmark, it has already had an impact. It is moreover obvious, that the method is increasingly useful with increasing age of the patient. In fact, in 2012 and 2013, 27% of all laboratory-confirmed cases in Denmark for the age group 8–19 years were confirmed by serology. For the age group 20–49 years, this figure was 38%. When looking only at cases 50 years and older, 49% of these were confirmed by serology (Table 3).

Discussion

Many countries have seen a resurgence of pertussis in recent years [33], but this has not been seen in Denmark where the latest epidemic occurred in 2002. The peak in 2012 coincided with peaks in many other European countries [34] and the high incidence in Denmark was therefore most probably influenced by the high incidence in neighbouring countries.

Unfortunately, the incidence in Denmark is difficult to compare to other countries as there are substantial differences in awareness, diagnostic practices, notification systems, population densities, communities that refuse vaccination etc., as illustrated in a number of publications [35-37]. Surveillance data from some of Denmark's neighbouring countries exemplify this; the incidences per 100,000 in the years 2010 to 2013 were in the ranges 2 to 3 in Sweden, 52 to 90 in Norway, 4 to 10 in Finland, 0.8 to 18 in England and 21 to 82 in the Netherlands [38-42]. Australia had incidences in the range of 54 to 173 per 100,000 in the same period [43]. All these countries should be comparable in terms of general health and vaccination coverage.

The proportion of confirmed pertussis found among adults in Denmark in relation to the total number of confirmed cases has gradually increased, and almost half of all the cases in 2013 were adults. This shift in age groups has been seen in many other countries around the world [33,44-47] and is thought to be due to several factors, primarily improved awareness and improved diagnostic methods [48]. Moreover, the introduction of a five-year booster vaccination in 2003 shifted the peaks of age-specific incidences among children when comparing the period 1995 to 1997 with the period 2011 to 2013. Cases among unvaccinated infants can be used as an indicator for the actual occurrence of pertussis since the attention on infants suspected of pertussis will presumably always be high. The number of pertussis cases among up to 90 days-old infants has decreased from a mean inter-epidemic level of 47 per year in the period 1995 to 2001 to 29 in the period 2007 to 2013 (27 cases when discounting

Table 3

Number of laboratory-confirmed pertussis cases with information on the diagnostic method used for confirmation of pertussis, Denmark, 2010–13 (n = 13,269)

Age group	Method	2010		2011		2012		2013	
		n	%	n	%	n	%	n	%
0 years	Culture	4	6	3	5	7	6	0	0
	PCR	64	93	58	95	120	94	64	100
	Serology	1	1	0	0	0	0	0	0
1–7 years	Culture	2	4	2	3	3	2	1	1
	PCR	46	94	56	92	166	95	73	91
	Serology	1	2	3	5	5	3	6	8
8–19 years	Culture	8	6	4	3	4	1	2	2
	PCR	118	86	94	76	216	71	96	73
	Serology	12	9	25	20	83	27	33	25
20–29 years	Culture	3	4	3	3	6	2	0	0
	PCR	51	61	66	73	152	57	98	66
	Serology	29	35	22	24	110	41	50	34
≥ 50 years	Culture	2	6	3	8	3	3	0	0
	PCR	20	61	18	50	54	51	30	48
	Serology	11	33	15	42	49	46	33	52
Total	Culture	19	5	15	4	23	2	3	1
	PCR	299	80	292	78	708	72	361	74
	Serology	54	15	65	17	247	25	122	25

Percentages show the contribution of each method for each year.

the peak in 2012). This could indicate that the true level of pertussis in Denmark has declined since the 1990s.

When comparing data across the study period, we assume that a gradual increase should have been observed, reflecting improved laboratory methods after the introduction of PCR in 1998 and serology in 2010. PCR has in fact been found to be five times more sensitive than culture [49], and a part of the increase from 1998 to 2002 is probably due to the introduction of PCR. However, the levels after 2004 were comparable to the levels before 1999. The enhanced awareness on adolescent and adult pertussis should also have had an impact. However, this has not been the case, and only six infant deaths from pertussis were registered in Denmark between 1995 and 2013, emphasising that the disease burden of pertussis is indeed low. The baseline level between peaks even decreased from 947, 923 and 919 cases, respectively, in the three years 1999, 2000 and 2001 to 455, 513, 540, 372 and 372 cases, respectively, in the five years from 2007 to 2011. A possible explanation could be that the introduction of the aP vaccine in 1997 and the preschool booster in 2003 lead to improved immunity in the population compared with the wP era. This is, however, purely hypothetical.

Analysis of the methods used for pertussis diagnostics in Denmark in 2013 shows that PCR was the most frequently used method while serology had the highest percentage of positive results. However, as PCR samples are often analysed routinely for a larger panel of infections, the proportion of positive results for the different assays should be compared with caution. Culture was rarely used and only few of the samples were positive. The diminished use of culture poses a problem in the sense that the circulating *B. pertussis* strains are not monitored. Recovery of isolates from PCR samples could perhaps compensate for this [50].

In the coming years, we expect to see a further increase in the numbers and proportion of confirmed pertussis among Danish adults. This change is not expected to be due to an increase in the true incidence, but rather to a virtuous circle of increased use of serology that will increase awareness of adult pertussis, leading again to increased test activity.

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Conflict of interest

None declared.

Authors' contributions

All authors have contributed to the writing of the manuscript. Tine Dalby: Collection and analysis of data on laboratory-confirmed pertussis, main writer of the manuscript. Peter Henrik Andersen: Analysis of data regarding vaccine coverage and cases among small children. Steen Hoffmann: Critical review and important feedback on the manuscript.

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Research article

Antimicrobial resistance of *Neisseria gonorrhoeae* isolates in south-west Germany, 2004 to 2015: increasing minimal inhibitory concentrations of tetracycline but no resistance to third-generation cephalosporins

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Increasing antimicrobial resistance of *Neisseria gonorrhoeae*, particularly to third-generation cephalosporins, has been reported in many countries. We examined the susceptibility (determined by Etest and evaluated using the breakpoints of the European Committee on Antimicrobial Susceptibility Testing) of 434 *N. gonorrhoeae* isolates collected from 107 female and 327 male patients in Stuttgart, south-west Germany, between 2004 and 2015. During the study period, high proportions of isolates were resistant to ciprofloxacin (70.3%), tetracycline (48.4%; increasing from 27.5% in 2004/2005 to 57.7% in 2014/2015; p = 0.0002) and penicillin (25.6%). The proportion of isolates resistant to azithromycin was low (5.5%) but tended to increase (p = 0.08). No resistance and stable minimum inhibitory concentrations were found for cefixime, ceftriaxone, and spectinomycin. High-level resistance was found for ciprofloxacin (39.6%) and tetracycline (20.0%) but not for azithromycin; 16.3% of the isolates produced betalactamase. Thus, cephalosporins can still be used for the treatment of gonorrhoea in the study area. To avoid further increasing resistance to azithromycin, its usage should be limited to patients allergic to cephalosporins, or (in combination with cephalosporins) to patients for whom no susceptibility testing could be performed or those co-infected with chlamydiae.

Introduction

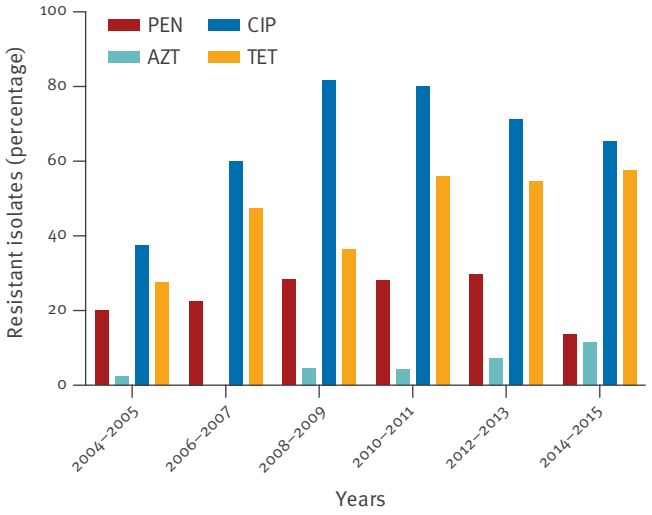
Numbers of gonorrhoea cases have increased, and the World Health Organization has estimated 106 million new cases in adults worldwide for 2008, which was 21% higher than numbers for 2005 [1]. At the same time, rising rates of antimicrobial resistance of its causative agent, *Neisseria gonorrhoeae*, have been reported in many parts of the world including Europe, even against the third-generation cephalosporins, cefixime and ceftriaxone [2,3]. For this reason, cefixime alone is no longer recommended as single-drug treatment for gonorrhoea in Europe or the United States [4,5].

The European Gonococcal Antimicrobial Surveillance Programme (EURO-GASP) was established by 12 European countries in 2004 in response to the emerging antimicrobial resistance of *N. gonorrhoeae*, as part of the European Surveillance of Sexually Transmitted Infections Project [6]. In 2016, EURO-GASP has participation from laboratories from 21 European Union/European Economic Area countries, which regularly report gonorrhoea susceptibility testing results and epidemiological surveillance data, and submit gonococcal isolates for centralised testing or participate in decentralised testing. Since 2009, EURO-GASP has been coordinated by the European Centre for Disease Prevention and Control (ECDC). Resistance data have been published regularly and in a timely manner [7,8], but the numbers of isolates tested per country are relatively low (between 10 and 251 in 2011 [8]) and therefore most likely not representative of the epidemiological situation of the individual countries.

As cases of gonorrhoea or antimicrobial resistance patterns of *N. gonorrhoeae* isolates are not subject to reporting in Germany, data regarding current antimicrobial susceptibility and its development over time are scarce. Only three individual studies have addressed this issue in the past 10 years. Abraham et al. analysed 50 isolates collected between 2001 and 2010 in

Figure 1

Percentages of *Neisseria gonorrhoeae* isolates resistant to penicillin, azithromycin, ciprofloxacin, or tetracycline, south-west Germany, 2004–2015



AZT: azithromoycin; CIP: ciprofloxacin; PEN: penicillin; TET: tetracycline.

Dresden, Saxony [9], while Horn and colleagues have reported the results of a nationwide surveillance study conducted by the Paul-Ehrlich-Society of Chemotherapy in 2010/2011 in which 213 isolates submitted by 23 laboratories were analysed [10]. Additionally, minimum inhibitory concentrations (MICs) of selected antibiotics for 65 *N. gonorrhoeae* isolates collected in 2004/2005 in southern Germany have been reported [11]. None of these studies has reported cephalosporin-resistant *N. gonorrhoeae* isolates. Data from EURO-GASP, however, have provided evidence for the presence of cephalosporin-resistant *N. gonorrhoeae* isolates in Germany, too [7,8]; in fact, an Austrian patient with a cefixime-resistant *N. gonorrhoeae* isolate acquired his infection in Munich, south Germany [12].

To gain more information on the antimicrobial susceptibility of *N. gonorrhoeae* in Germany and elucidate possible changes in antimicrobial resistance occurring over time, we analysed the susceptibility patterns of all *N. gonorrhoeae* isolates identified and tested in our laboratory between 2004 and 2015 (n = 434). Since age and sex have been identified as risk factors for harbouring antimicrobial-resistant *N. gonorrhoeae* isolates [13,14], we additionally analysed our data regarding these parameters. Unfortunately, the study design chosen did not provide information regarding other possible risk factors, e.g. working as professional sex worker or being a man who has sex with men (MSM).

Methods

Bacterial isolates

Between July 2004 and March 2015, 434 bacterial isolates were grown from swabs obtained from patients aged 16 years and over living in the greater Stuttgart area (a radius of around 50 km from Stuttgart city centre). Although difficult to estimate because *N. gonorrhoeae* is not mandatorily notifiable, the number of isolates tested may correspond to 30–70% of all isolates detected in that area within the period of time indicated. The samples were mainly submitted by private practitioners (primarily urologists, internists, dermatologists, and gynaecologists). For 284 (65.4%) patients, further samples (swabs or urine) had been submitted for the detection of *Chlamydia trachomatis* by PCR. Based on these laboratory results, 36 (12.7%) patients were co-infected with *C. trachomatis* while 248 (87.3%) were *C. trachomatis*-negative.

Gram-negative, oxidase-positive diplococci were identified biochemically as *N. gonorrhoeae* by using the API or the Vitek 2 system (both bioMerieux, Marcy-l Etoile, France). Isolates were subjected immediately to antimicrobial susceptibility testing and subsequently stored at –70 °C for future analyses.

Susceptibility testing

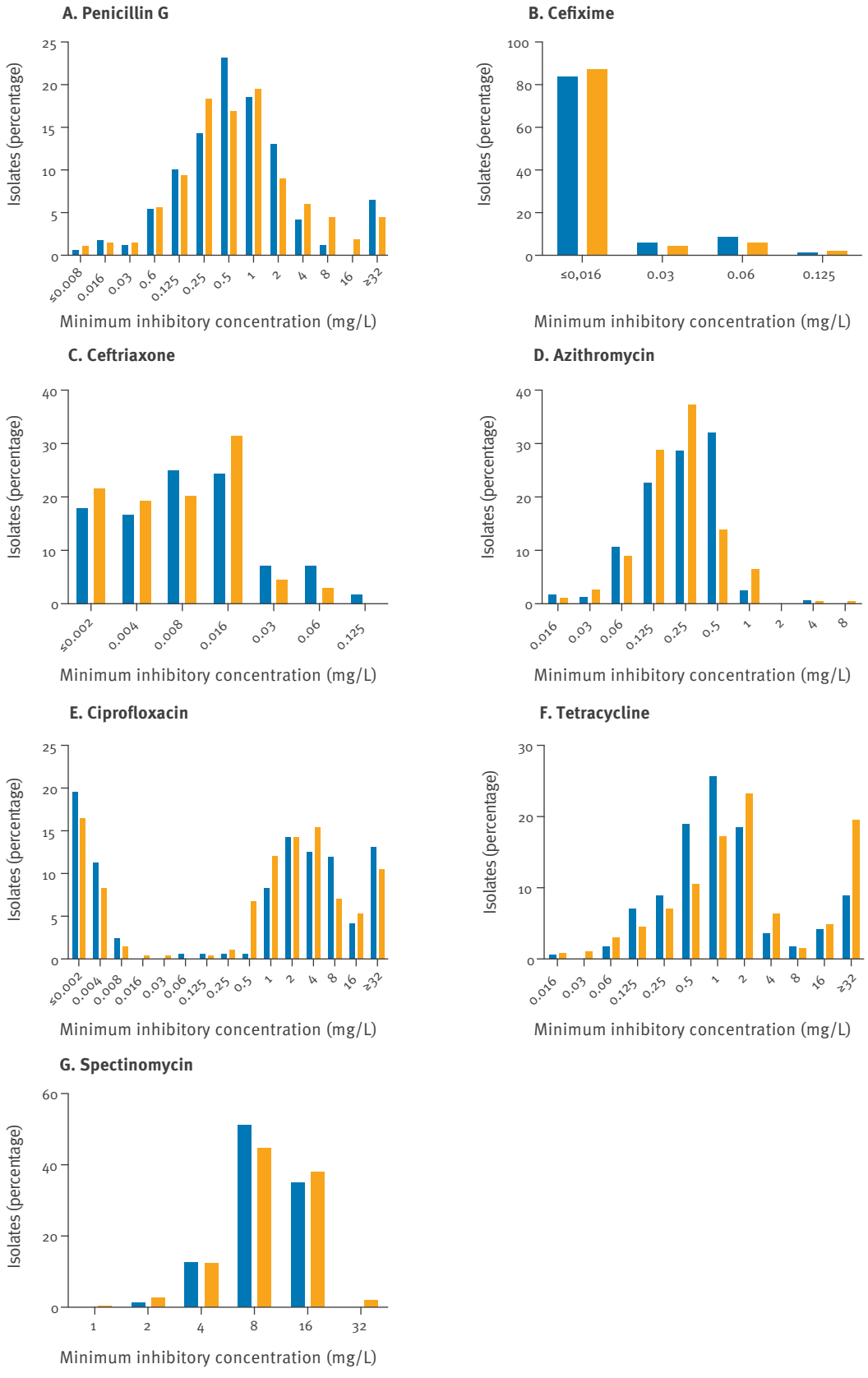
Briefly, bacterial inoculums were prepared in sterile saline at 0.5 McFarland standard and tested against the antibiotics indicated by using the Etest system (AB Biodisk, Solna, Sweden) on Mueller-Hinton chocolate agar, except for testing of azithromycin, for which a chocolate GC II agar with IsoVitaleX was used (both, BD Diagnostic Systems, Heidelberg, Germany) [11]. Plates were incubated at 35–36.5 °C and 5% CO2 for 20–24 hours. Betalactamase production was assessed by using nitrocefin disks (BD Diagnostic Systems). The ATCC *N. gonorrhoeae* strain 49226 was used as an internal control. Susceptibility was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) breakpoints [15,16].

Statistical analyses

Data were statistically analysed by using GraphPad Prism version 6.0a for Mac OS X. Data on antibiotic resistance, patients' sex, or specialisation of referring practitioners were analysed using the two-tailed Fisher's exact test. Data regarding age were analysed using the Mann–Whitney U-test. To elucidate potential changes in the antibiotic susceptibility in *N. gonorrhoeae* isolates over time, data also were analysed for two different periods: July 2004 to December 2009 and January 2010 to March 2015, as well as for two-year periods. Differences were considered statistically significant at $p < 0.05$. Trends were defined as p-values between 0.05 and 0.1.

Figure 2

Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates to (A) penicillin G, (B) cefixime, (C) ceftriaxone, (D) azithromycin, (E) ciprofloxacin, (F) tetracycline and (G) spectinomycin, south-west Germany, July 2004 to December 2015 (n=168) vs January 2010 to March 2015 (n=266)



Blue bars (n=168) represent isolates from July 2004 to December 2009. Yellow bars (n=266) represent isolates from January 2010 to March 2015.

Table 1

Antimicrobial activities of selected antibiotics against *Neisseria gonorrhoeae* isolates, Stuttgart, 2004–2015 (n=434)

	MIC (mg/L)			% of isolates (S/I/R*)	
Antimicrobial agent	**MIC_{50}**	**MIC_{90}**	**Range**	**EUCAST** [8]	**CLSI** [9]
Penicillin G	0.5	8	0.002 to >32	9.4/65.0/25.6	9.4/65.0/25.6
Cefixime	0.016	0.03	<0.016 to 0.125	100/NM/0	100/ NM /0
Ceftriaxone[a]	0.008	0.03	<0.002 to 0.125	100/NM/0	100/NM/0
Azithromycin	0.25	0.5	0.016 to 128	73.5/21.0/5.5	n.d.
Ciprofloxacin	2	32	<0.002 to >32	29.5/0.2/70.3	29.7/5.8/64.5
Tetracycline	2	32	0.016 to >256	31.1/20.5/48.4	17.3/34.3/48.4
Spectinomycin	8	16	1 to 32	100/NM/0	100/NM/0

CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing; I: intermediate; MIC: minimum inhibitory concentrations; n.d.: not determined (no breakpoints available); NM: no MICs for intermediate susceptibility defined; R: resistant; S: susceptible.

[a] Data available for 432 isolates.

Results

Resistance patterns

The median age of the patients was 33 years (range: 16–76), 107 (24.7%) were female and 327 (75.3%) were male. Analysis of antimicrobial susceptibility of the isolates to standard antibiotics revealed high proportions of intermediate or resistant isolates for penicillin (90.6%), ciprofloxacin (70.5%), and tetracycline (68.9%) with lower proportions for azithromycin (26.5%; Table 1). No resistance to cefixime, ceftriaxone, or spectinomycin was detected. Thirty-one isolates (7.1%) were susceptible to all antimicrobials tested.

High-level plasmid-mediated resistance to tetracycline (≥16 mg/L) was seen in 87 (20.0%) isolates whereas 172 (39.6%) isolates expressed high-level resistance to ciprofloxacin (≥4 mg/L). Seventy of 430 (16.3%) for which data on betalactamase production was available were betalactamase positive and thus expressed high-level plasmid-mediated resistance to penicillin. High-level resistance to azithromycin (≥256 mg/L) was not detected.

N. gonorrhoeae isolates from female patients (n=107) were more resistant to penicillin (33.6%, 95% CI: 25.4–43.0% vs 23.2%, 95% CI: 19.0–28.1%; p=0.041) and ciprofloxacin (78.5%, 95% CI: 69.7–85.3% vs 68.2% 95% CI: 63.0–73.0; p=0.0499) than isolates from male patients (n=327). There was no difference regarding resistance to azithromycin or tetracycline (data not shown). Patients older than 25 years (n=317) and those aged 25 years or younger (n=117) did not differ regarding resistance to penicillin (p=0.536), azithromycin (p=0.481), ciprofloxacin (p=0.478) or tetracycline (p=0.450).

Comparison of antimicrobial resistance 2004–2009 vs 2010–2015

Demographic data of the two subpopulations, patients whose isolates were analysed in 2004–2009 and those whose isolates were analysed 2010–2015, were comparable (Table 2). Applying the EUCAST definitions for resistance [15], the proportion of isolates resistant to tetracycline significantly increased and in fact almost doubled (2004–2009, 30.4%; 2010–2015, 55.6%; Table 3). There also was a trend (p=0.084) towards increasing resistance to azithromycin (2004–2009, 3.0%; 2010–2015, 7.1%; Table 3) while the resistance to penicillin or ciprofloxacin did not change significantly within the study period. A more detailed analysis by plotting the data for two-year periods confirmed this overall pattern for the whole study period (tetracycline, p=0.006; penicillin, p=0.411; ciprofloxacin, p=0.844; azithromycin, p=0.133; Figure 1); notably, resistance to ciprofloxacin increased between 2004/05 and 2008/09 but decreased thereafter (p<0.0001, each).

Analysis of the individual MICs of the seven antibiotics tested revealed some minor changes for most antibiotics (Figure 2). In contrast, proportions of isolates with MICs of 0.5 or 1 mg/L to tetracycline decreased considerably while those of isolates with MICs of 2, 4 or ≥32 mg/L increased (Figure 2F). Likewise, the increase in isolates with MICs>0.5 mg/L to azithromycin became evident but the proportions of isolates with MICs of 0.125 and 0.25 mg/L were rather higher in the second period than before (Figure 2D). The unique pattern of ciprofloxacin revealed two groups of isolates, i.e. one expressing very low MICs and the other one with MICs≥0.5 mg/L while there were almost no isolates with MICs at the level of the EUCAST breakpoint concentration, 0.06 mg/L, or slightly higher or lower (Figure 2E). Notably, there was a stable pattern for both cefixime and ceftriaxone, excluding also potential shifts of MICs at lower concentrations (Figure 2B and C), which might go undetected when only the breakpoints are considered.

Discussion

Although we were unable to detect cephalosporin-resistant *N. gonorrhoeae* isolates within the study

Table 2

Demographic data from *Neisseria gonorrhoeae*-positive patients, south-west Germany, 2004–2009 vs 2010–2015 (n=414)

Characteristics	July 2004 to December 2009	January 2010 to March 2015	p-value
Number of isolates	168	266	
Sex			0.909
Female	42 (25.0%)	65 (24.4%)	
Male	126 (75.0%)	201 (75.6)	
Age in years (median; range)	32; 17-68	34; 16-76	0.718
Specialisation of senders			
Urology	78 (47.0%)	149 (56.0%)	0.076
Gynaecology	37 (22.3%)	61 (22.9%)	0.906
Internal medicine	20 (12.0%)	22 (8.3%)	0.235
Dermatology	16 (9.6%)	20 (7.5%)	0.479
Others or no specialisation	15 (9.0%)	14 (5.3%)	0.167

Table 3

Resistance of *Neisseria gonorrhoeae* isolates, south-west Germany, 2004–2009 vs 2010–2015 (n=434)

	2004–2009 (n=168)		2010–2015 (n=266)		
Antibiotic agent	**No**	**% (95% CI)** [a]	**No**	**% (95% CI)**	**p**
Penicillin G (>1 mg/L)	42	25.0 (19.0-32.1)	69	25.9 (21.0-31.5)	0.910
Azithromycin (>0.5 mg/L)	5	3.0 (1.1-7.0)	19	7.1 (4.6-10.9)	0.084
Ciprofloxacin (>0.06 mg/L)	111	66.1 (58.6-72.8)	194	72.9 (67.3-77.9)	0.133
Tetracycline (>1 mg/L)	62	36.9 (30.0-44.4)	148	55.6 (49.6-61.5)	0.0002

CI: confidence interval.

[a] Percentage of resistant isolates defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints plus 95% CI.

period, cefixime resistance has been reported for many European countries including Germany [8]. Thus, our data, although in line with two recently published German studies [9,10], cannot be considered representative for the situation in Germany, but they indicate that the true overall cefixime resistance rate in Germany may be considerably lower than the 10.2% reported for 2011 by EURO-GASP [8]. In fact, all laboratory results may strongly depend on the area where the samples were collected and the numbers of patients included from high-risk groups. Our key finding in this context, however, is the absence of an increase in MICs to cephalosporins from the period 2004–2009 to the period 2010–2015 (Figure 1). Thus, a possible rise in cephalosporin-resistance in the future may not be the result of previous and current (mis-)usage of cefixime in the local population, but rather due to the introduction of new resistant strains into that population.

The EUCAST breakpoint of cefixime may be too high, as *N. gonorrhoeae* isolates with this or lower MICs have been described as the cause of infections that did not resolve under cefixime standard therapy [8]. Considering a breakpoint of 0.06 mg/L, seven isolates (1.6%) tested by us with a MIC of 0.125 mg/L were resistant to cefixime.

We detected azithromycin resistance in 5.5% of the isolates and none of the isolates expressed high-level resistance; both findings correspond to the data recently published by Horn et al. [10]. In contrast, an azithromycin resistance rate of 0.9% has been reported for Germany by EURO-GASP [8]. Notably, we observed a strong trend towards more isolates with MICs › 0.5 mg/L of azithromycin from the period 2004–2009 to 2010–2015 (p=0.084), although in Europe, resistance significantly decreased between 2009 and 2011 [8]. Still, increasing azithromycin high-level resistance of *N. gonorrhoeae* isolates and a concomitant decline in isolates expressing decreased susceptibility have been shown for Scotland from 2004 to 2007 [17] from where it appears to have spread to England and Wales [18]. Only single high-level resistant isolates have been shown for other European countries, including Italy [19], Sweden [20], France [21] and Ireland [22]. To prevent further increasing resistance to this valuable drug, azithromycin usage should be limited to patients who need to be treated with this drug, i.e. patients with allergy to cephalosporins or patients with simultaneous chlamydial infection (in which case azithromycin should be given together with cephalosporins) and those for whom no drug susceptibility testing of the causative agents can be performed.

The highest resistance rate was observed for ciprofloxacin (70.3%). This is in accordance with the data published by Horn et al. [10] while resistance rates of around 50% have been reported by others for Germany [8,9]. Resistance rates for ciprofloxacin are also high in most other European countries [8], and this might be a sign of a still high fluoroquinolone usage by practitioners for the empirical treatment of gonorrhoeae, as ciprofloxacin resistance of *N. gonorrhoeae* has been shown to decline after cessation of ciprofloxacin usage [23]. It remains unclear at the moment, however, whether the observed increase and decrease of ciprofloxacin resistance correlates with changes in ciprofloxacin usage within the study period. In contrast to the other antimicrobials tested, MICs to ciprofloxacin showed a bimodal distribution similar to what has been shown for *N. gonorrhoeae* isolates from Japan [24].

Tetracycline resistance also was high (as previously observed by others [10]), and increased significantly within the study period (from 27.5% in 2004/2005 to 57.7% in 2014/2015). This might reflect its wide usage. Doxycyline was the second most commonly prescribed antimicrobial drug in German outpatient departments in 2011 [25]. When we compared the antimicrobial susceptibility for 2004–2009 vs 2010–2015, considerably more isolates were included in the latter than in the former period, although the second period was three months shorter than the earlier one. Even if it must remain speculative at the moment, this finding might indicate a true increase in cases of gonorrhoea in the past years, similar to what has been reported for England and Sweden [26,27], and thus may correspond to the trend reported for syphilis in Germany [28]. A reintroduction of the previous obligation to report all cases of gonorrhoea in Germany, currently under discussion, may answer this question in the future. An increasing gonorrhoea incidence, however, also might at least partially result from the previously discussed ciprofloxacin usage for empirical treatment of the infection in the presence of high resistance rates to this drug [29].

We did not detect spectinomycin resistance. This might be due to its long-lasting non-usage, since its usage leads to increasing resistance rates [30]. Thus, this drug could potentially be used for the empirical treatment of gonorrhoea. Known limitations, however, are its unavailability in many European countries including Germany and its reduced effectiveness in the treatment of pharyngeal gonorrhoea [31].

In contrast to previous publications [13,14], we did not observe age-related differences in susceptibility rates, and isolates from female patients showed higher resistance rates than those from male patients. This might be due to differences in study populations. One limitation of our study in this context is the fact that we do not have information regarding patients who may belong to high-risk populations. For instance, it has been shown that *N. gonorrhoeae* isolates from MSM are more resistant to antimicrobials than those collected from men who have sex with women [32,33]. Likewise, infected commercial sex workers are more likely to harbour resistant *N. gonorrhoeae* isolates than other patients [32,34]. In contrast, a recent EURO-GASP study has demonstrated decreasing cefixime MICs for *N. gonorrhoeae* of MSM for the period 2009–2011, and highest MICs for isolates from men who have sex with women [13]. Furthermore, around 75% of the bacterial isolates analysed in the present study were collected from men; thus, our data may be more valid for male than for female patients.

In conclusion, the current resistance situation of *N. gonorrhoeae* isolates in south-west Germany may be less dramatic than in other parts of Germany or other European countries. High resistance rates to some antimicrobials and changes of susceptibility over time, however, call for a more stringent monitoring system, e.g. the obligation of every laboratory to report all *N. gonorrhoeae* isolates together with their susceptibility testing results.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

TR designed the study, analysed the data, and wrote the manuscript; TM designed the study, contributed bacterial isolates, and wrote the manuscript; RI analysed the data and wrote the manuscript.

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The European Commission Consumers, Health, Agriculture and Food Executive Agency (CHAFEA) call for tender concerning studies on vaccination closes on 13 September

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The European Commission Consumers, Health, Agriculture and Food Executive Agency (CHAFEA) call for tender to carry out two studies on the added value of strategic and life-course approach to vaccination, and on shortcomings related to low vaccination in healthcare workers, respectively, will close on 13 September 2016.

According to the tender document 'each study has to include a representative number of Member States with a broad coverage as regards the variety of the governmental or constitutional structure/political concept, available legislative and managerial frameworks for programme coordination, vaccination programme performance, available programme capacities (infrastructure, human resources) within the EU.'

The contract, designed to carry out the two studies, is for EUR 220,000.000 and the duration is for 11 months.

Questions and answers for the tender were last updated on 31 August 2016.

Read more about the tender here.