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EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

Editorials

Why are Mexican data important?

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This issue of Eurosurveillance contains an article by a French team on the transmission of the new influenza A(H1N1) in Mexico, which uses published figures from the outbreak to estimate important parameters for transmission, among them the reproduction rate, R [1]. Such studies may have important implications for public health action in Europe.

What is R?

The growth rate of an epidemic is determined by two factors: the number of new persons infected by each case and the time from start of infectiousness in one case to start of infectiousness in the secondary cases caused by him/her. The first factor is called 'reproduction rate' and is usually denoted R. If the disease is spreading in a population that is totally susceptible the term 'basic reproduction rate' (Ro) is used. R is the product of four terms: the risk of transmission in one single contact between an infectious and a susceptible person, the frequency of such contacts in the population, the duration of infectivity of a case, and the proportion of susceptibles in the population. If R >1 this means that each case infects more than one new person, and the outbreak is likely to continue. If R < 1 the outbreak will eventually die out, even if there may be a number of cases before that. The time from infectiousness in one case to infectiousness in his/her secondary cases is called 'generation time' (Tg) and is basically a biological constant, even if its exact value depends on how it is estimated.

Values for the factors that determine R can be calculated on the basis of scientific knowledge of the disease, its context of transmission, and the immunity status of the population. However, during an epidemic an R value usually has to be derived from the analysis of the epidemic curve or by the study of transmission chains.

Several studies have now tried to estimate R (or Ro) and Tg for the new influenza A(H1N1) virus from Mexican data. In the one published in this issue of Eurosurveillance [1], the authors use one exponential fitting and one real-time estimation model to arrive at an estimate of R between 2.2 and 3.1. This is higher than the value found in an article in Science [2], which estimated Ro to be 1.4-1.6 using three models: one exponential fitting, one genetic analysis, and two standard SIR models for a confined outbreak in La Gloria. Another analysis of the minor genetic changes in the virus over time arrived at a Ro estimate of 1.16 [3].

Why is Ro important in public health?

The reproduction rate reflects effectiveness of transmission, and therefore has important implications for the efforts that public health authorities would have to make in implementing health measures aiming at containing or mitigating the outbreak.

For example, with a Ro of 1.16, preventing 14% of cases will result in eventually interrupting transmission, while with a Ro of 3.1, preventing 68% cases would be needed – assuming a total random mixing of contacts in the population.

Why are Ro estimates so different for influenza?

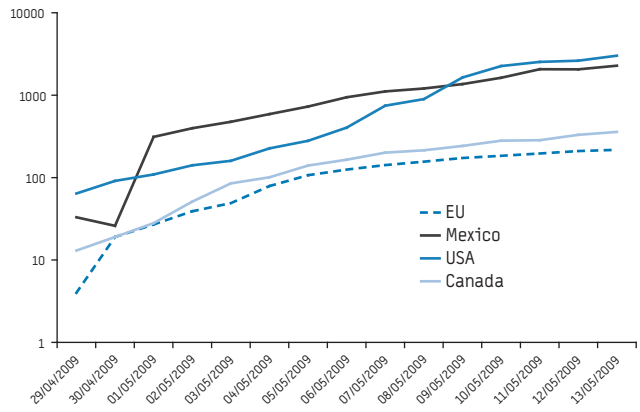
A few studies have tried to measure Ro for seasonal influenza [4], and found it to be in the order of 1.2 to 1.4. However, for most of the seasonal strains, there is already some immunity in the population from past seasons, which lowers the reproduction rate (and it should thus really not be called Ro in this situation). For any epidemic of a disease that leads to immunity after infection the initial Ro will also be higher than the actual R at any later stage, since the proportion still susceptible in the population will decrease. It should also be realised that delayed reporting of cases will affect an estimate of R; a problem that adheres to the study in this issue and the others cited above.

What influences Ro?

The risk of transmission in a contact when an infective meets a susceptible is basically a biological constant (even if it varies over the time course of the infection), as is the duration of infectiveness. However, frequency of contacts varies considerably between

Figure

Daily reported cumulative number of cases in Mexico, Canada, USA, and EU/EFTA countries, outbreak of new influenza A(H1N1), April-May 2009



populations and population groups. For example, among children in schools or day care, the contact frequency is higher than among adults [5], and it also varies by culture, by family size in a society, by types of social interaction, etc.

Why is the Ro from Mexico important?

One could question why there is so much interest around studies of R and Ro based on Mexican data. Would they apply to Europe? One could guess that contact density might be higher in a Mexican setting, but on the other hand, since the epidemic has already run its course for some time there, the proportion of non-susceptibles would be higher in Mexico and the European situation would more approach a 'true' (higher) Ro, with a totally susceptible population.

In the graph, we have just compared the daily reported cumulative number of cases in Mexico, Canada, United States, and European Union and European Free Trade Association (EU/EFTA) countries. On a semi-logarithmic scale it is evident that the slope for Europe is very much the same as for Mexico. It is difficult to estimate the time lag for Europe, but it seems that we are some 1-2 months behind. If the generation times are the same for both epidemics – which seems highly plausible – then an estimate of Ro for Mexico would apply also to Europe. A Ro just above 1 could mean that a containment strategy might be successful.

The European Centre for Disease Prevention and Control (ECDC) is continuously monitoring the situation and with more data being available every day in Europe we will obviously be able to have a better picture here soon as well. Nevertheless, the similarities of the shapes of the epidemics indicate that lessons from Mexico could apply also to Europe.

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New influenza A(H1N1) virus infections in Spain, April-May 2009

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An outbreak of infections with a new influenza A(H1N1) virus that was first detected in the United States and Mexico is currently ongoing worldwide. This report describes the initial epidemiological actions and outbreak investigation of the first 98 laboratory confirmed cases of infection with this new virus in Spain.

Background

On 25 April 2009, the World Health Organization (WHO) declared the outbreak of swine-origin influenza A(H1N1) virus infections, first reported by the United States (US) [1] and Mexico [2], as a 'Public Health Event of International Concern' (PHEIC) under the International Health Regulations (2005) [3]. The pandemic alert level was raised from level 3 to level 4 on 27 April, and to level 5 on 29 April, after verification of sustained community-level outbreaks in at least two countries from the same WHO region.

On 26 April, epidemiological and laboratory investigations on three persons returning from Mexico were initiated in Spain. On 27 April, Spain reported the first laboratory-confirmed case of the new influenza A(H1N1) virus infection in Europe, in a traveller returning from Mexico. Since then, the number of confirmed cases in Spain has risen continuously and reached a total of 98 as of 11 May 2009.

Enhanced surveillance

On 24 April, in response to alarming reports from the US of swine-origin influenza A(H1N1) virus infection in several patients [1,4] and media news of a possibly related outbreak of severe respiratory illness in Mexico, the Coordinating Centre for Health Alerts and Emergencies (CCAES) at the Spanish Ministry of Health and Social Policy, issued a national epidemiologic alert. The alert asked public health authorities at national and regional level to enhance surveillance and to report urgently any case of fever and severe respiratory illness among people with history of travel to Mexico or history of previous contact with a confirmed case of influenza virus A(H1N1) infection (Table 1).

On 25 April, following WHO's declaration of a PHEIC, the National Pandemic Influenza Preparedness and Response Plan was activated. A case definition as well as protocols for case and contact management and for infection control were developed and distributed to the National Health Service through regional health authorities and other involved institutions (Table 2).

No increase in seasonal influenza activity has been reported so far. Routine seasonal influenza surveillance will continue beyond week 20. Data analysis of mortality for all causes since 1 May has not shown an increase or change of patterns in mortality.

Since 24 April, the outbreak of new influenza A(H1N1) has been monitored by the Ministry of Health and Social Policy (Centro de Coordinación de Alertas y Emergencias Sanitarias, CCAES) jointly with the National Centre for Epidemiology (Instituto de Salud Carlos

Table 1

Timeline of key events in detection and response to the new influenza A(H1N1) virus outbreak, Spain, 24 April-11May 2009

Date	Event
24 April	Alert issued to enhance surveillance at the public health services and national health system
24 April	Information for the public and recommendations for travellers going to and returning from Mexico published on the website of the Spanish Ministry of Health and Social Policy
25 April	National pandemic influenza preparedness and response plan activated.
25 April	Case definition, case and contact management, and infection control protocols distributed
26 April	Notification of the first three cases under investigation
27 April	First laboratory-confirmed case of new influenza A(H1N1) virus infection reported.
27 April	Ministry of Health recommends avoiding non-essential travel to Mexico
27 April	World Health Organization raises pandemic alert to phase 4
29 April	World Health Organization raises pandemic alert to phase 5
29 April	First secondary case of new influenza A(H1N1) virus reported
1 May	Regional influenza laboratories to start initial testing; National reference laboratory to confirm
7 May	New case definition approved, including the United States as an affected area, reducing incubation period (seven days) and establishing fever cut off at 38ºC
11 May	First laboratory-confirmed tertiary case
11 May	Status: 98 laboratory confirmed cases of new Influenza virus A(H1N1) infection

III) and in coordination with all the Regional Surveillance and Alert Teams from the Autonomous Communities in Spain. This new influenza A(H1N1) investigation and control group also discusses and recommends prevention and control measures.

Table 2

Case definition and case classification, new influenza A(H1N1) infection, Spain, 25 April-7 May, 2009

	Incubation period 10 days
Clinical criteria	Any person with ONE of the following: • Fever (≥ 37.5 °C)* AND signs or symptoms of acute respiratory infection • Pneumonia • Death from an unexplained acute respiratory illness
Epidemiological criteria	At least ONE of the following in the 10 days* prior to disease onset: • Travel to an area where there are confirmed cases of new influenza A(H1N1) (Mexico*) • Close contact to a confirmed case of new influenza A(H1N1) virus infection • Recent history of contact with an animal with confirmed or suspected swine influenza A(H1N1) virus infection (This criterion was substituted on 27 April for: "A person employed at a laboratory and manipulating potentially contaminated samples").
Laboratory criteria	At least ONE of the following tests: • RT-PCR • Four-fold rise in new influenza A(H1N1) virus-specific neutralizing antibodies (implies the need for paired sera, at least from acute phase illness and then at convalescent stage 10-14 days later) • Viral culture
Case classification	A. Case under investigation Any person meeting clinical AND epidemiological criteria B. Probable case Any person meeting clinical AND epidemiological criteria AND with a positive influenza A infection of an unsubtypable type C. Confirmed case Any person with laboratory confirmation*

* Differences to proposed case from the European Centre for Disease Prevention and Control.

Figure 1

Geographical distribution of cases of laboratory-confirmed new influenza virus A(H1N1) infection, Spain, as of 11 May 2009

Confirmed cases of new influenza virus A(H1N1)

As of 11 May, 98 laboratory-confirmed cases of infection with the new influenza virus A(H1N1) have been reported in Spain out of 640 possible cases investigated. The geographical distribution of reported cases by region is shown in Figure 1.

Seventy-six confirmed cases (78%) acquired the infection abroad; all these cases had a history of travel to Mexico. Of the 45 cases for whom this information was available, 16 (36%) were symptomatic during the inbound flight from Mexico. Dates of return from affected areas were available for 70 confirmed cases and ranged from 20 to 29 April (Figure 2).

Information on disease onset was available for 93 cases. The first of the 93 cases reported onset of illness (any symptom) on 19 April, and the most recent case reported onset on 4 May (Figure 3).

More than 2,000 contacts have been traced and followed. Of these, 39% were household members of cases and 45% friends of cases. Twenty-one confirmed secondary cases and one tertiary case have been reported. Secondary cases were family or close contacts of cases with history of travel to Mexico. Five secondary cases were infected by primary cases that did not meet clinical criteria. The

Figure 2

Cases of laboratory-confirmed new influenza virus A(H1N1) infection, by date of travel return to Spain, as of 11 May, 2009 (n=70)

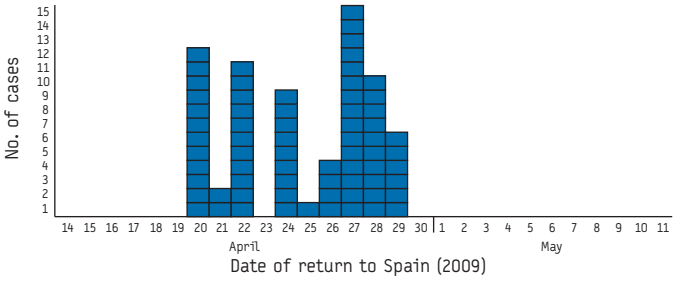
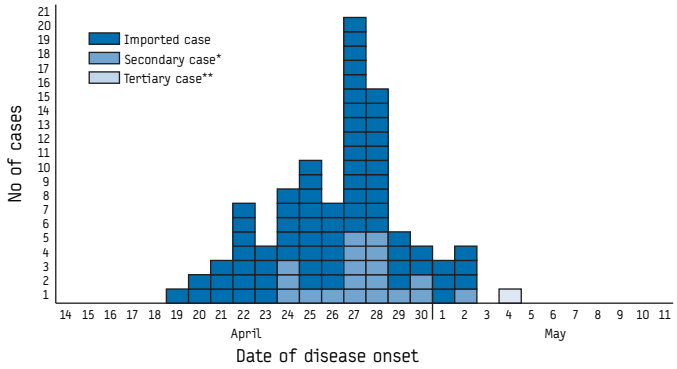


Figure 3

Cases of laboratory-confirmed new Influenza virus A(H1N1) infection, by date of disease onset, Spain, as of 11 May 2009 (n=93)



* contact of a confirmed imported case
** contact of a confirmed secondary case

tertiary case was a family contact of a secondary case. Analysis of secondary transmission is ongoing.

Four secondary cases had received prophylaxis with oseltamivir before being diagnosed as cases.

From the analysis of disease onset for primary and secondary cases, the median of the serial interval was estimated to be 3.5 days, ranging from one to six days. The estimation for the maximum incubation period ranged from one to seven days, with a median of three days.

Demographic and clinical features

Cases ranged in age from 14 to 55 years, with an average of 24 years (standard deviation (SD) 6.3) and a median of 22; 50 (51%) cases were male.

The most frequently reported symptoms were fever (96%) and cough (95%). Four cases did not have fever. Among 41 cases for whom this information was available, 17 (41%) reported diarrhoea (Table 3).

No deaths have been reported. Disease presentation has been described as a mild influenza-like illness with full recovery in all cases. Some cases were hospitalised at the beginning of the outbreak for respiratory isolation following the national pandemic preparedness plan, this procedure having no association with illness severity.

No differences in disease presentation have been described for secondary cases. No pregnancies among confirmed cases have been reported.

Information on seasonal influenza 2008-9 vaccine status is available for 52 cases (53%); of these, only five cases had history of vaccination.

Laboratory confirmation

Nose and throat swabs from cases who met clinical and epidemiological criteria were taken and referred to the national influenza reference laboratory (WHO National Influenza Centre) at the Instituto de Salud Carlos III for confirmation. Two independent assays have been used for diagnosis; a reverse transcription (RT)-nested PCR designed for typing the nucleoprotein gene and another one for subtyping the haemagglutinin gene. An alternative RT-PCR was done in case the first two PCR gave contradictory results. Amplified products were sequenced and a phylogenetic analysis was done to identify the new A (H1N1) virus. The strain identified in all cases was confirmed as genetically similar to viruses previously isolated from cases in California (A/California/04/2009).

Detailed information on co-infection with other respiratory viruses is pending. Virological studies on antiviral sensitivity and on molecular-level indicators of severity are ongoing.

Table 3

Clinical features of confirmed cases for new influenza virus A(H1N1) infection, Spain, as of 11 May 2009

Symptom	Cases with symptom/ cases for whom information is available	Percentage
Fever (≥37.5 °C)	87 / 91	96%
Cough	83 / 87	95%
Headache	27 / 44	61%
Coryza	24 / 41	59%
Sore throat	29 / 48	60%
Myalgia	29 / 49	59%
Shortness of breath	18 / 70	26%
Malaise	23 / 38	61%
Diarrhoea	17 / 41	41%
Vomiting	4 / 32	13%

Discussion

Spain was the first country in Europe to report a laboratory-confirmed case of new influenza A(H1N1) virus infection. Several factors may have contributed: intense air traffic and contacts with Mexico [5] but also a timely alert with high media coverage that raised early awareness among public health and healthcare professionals, as well as among the public.

An extremely efficient surveillance system and a sensitive case definition that was distributed early in the event made it possible to detect cases at the very beginning of the outbreak and to trace more than 2,000 close contacts. Secondary cases have been identified among close contacts of the first reported cases. However, they are still only a minor percentage of all reported cases and further spread of this new influenza virus into the community has not been documented. The last imported case had disease onset on 2 May, but the change in the case definition on 7 May including the US as an affected area may lead to notification of new imported cases.

The preliminary findings from the analysis of the first 98 laboratory-confirmed cases of the new influenza A(H1N1) virus infection in Spain indicate that symptoms in these cases appear to be similar to those of seasonal influenza. Cases observed are mainly distributed among young adults, reflecting the age structure of returning travellers from Mexico. This group has no risk factors for influenza complications and is difficult at this stage to assess the potential severity of this virus. For the time being, the impact of this outbreak on the healthcare services has been negligible.

Conclusion

The evolution of this outbreak of influenza A(H1N1) in Spain is difficult to predict. Though notification of new confirmed cases has decreased and the disease seems mild, we will continue monitoring changes in the epidemiology and/or clinical severity of new influenza A(H1N1) virus infections in Spain in order to implement appropriate prevention and control measures.

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Epidemiology of new influenza A(H1N1) in the United Kingdom, April – May 2009

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Following importations of cases from Mexico and the United States, by 11 May, United Kingdom surveillance activities had detected a total of 65 individuals with confirmed infections caused by the new influenza A(H1N1) virus. The infections were mainly in young people and younger adults and they spread within households and within schools. The illness in the United Kingdom is similar in severity to seasonal influenza and to date, besides one case of bacterial pneumonia, no clinically serious cases have occurred.

On 23 April, several cases of severe respiratory illness were confirmed as a new swine-lineage influenza A(H1N1) virus infection in the United States [1]. Genetic analysis of these viruses indicated that they were novel viruses, not detected previously in either the swine or human population in North America [2]. Coincidentally, in March and April 2009, Mexico experienced outbreaks of respiratory illness in several parts of the country. Analysis of viral isolates from affected cases in Mexico indicated that illness was associated with a novel then called "swine virus" similar to that identified in sporadic cases in the US [3]. This novel virus has since been identified in humans in Canada, Europe and elsewhere [4].

On 27 April, the first two confirmed United Kingdom cases of new influenza A(H1N1) virus infection were reported in Scotland, in a couple returning from travel to Mexico.

In response to the detection of confirmed cases of new influenza A(H1N1) in the United Kingdom, the Health Protection Agency (HPA) and the Devolved Administrations strengthened national surveillance of respiratory illness amongst travellers returning from affected areas. As part of case finding, a possible case was defined as any person with a history of acute respiratory illness and recent travel to an affected area or contact with a confirmed or probable case; a probable case was defined as a person who was a possible case and had tested positive for influenza A which was non-subtypeable and a confirmed case was an individual that tested positive for the new influenza A(H1N1) virus by specific-RT-PCR confirmed by sequence analysis.

During the period 27 April to 11 May, a total of 65 confirmed cases were detected. From the first reported cases on 27 April, initial cases were amongst travellers returning from Mexico, and then the United States, with a peak on 1 May. The first indigenously acquired infections in the United Kingdom were reported on 1 May and the proportion and number that are indigenously acquired has been reasonably stable since May 7

Cases of new influenza A(H1N1) have been identified in England (60) and Scotland (5). Of the English cases, 34 have been identified in London; six in North West and South East England; five in East of England; three in each of South West and West Midlands; two in East of Midlands and one each in North East and South East.

Of the 65 confirmed cases, 29 (45%) are female (Figure 2). Cases range in age from 5 to 73 years – with 58% of patients falling into the age range 10-29 years (Figure 2). The age distribution of indigenous cases is predominately in the 10-19 year age group (Figure 2b).

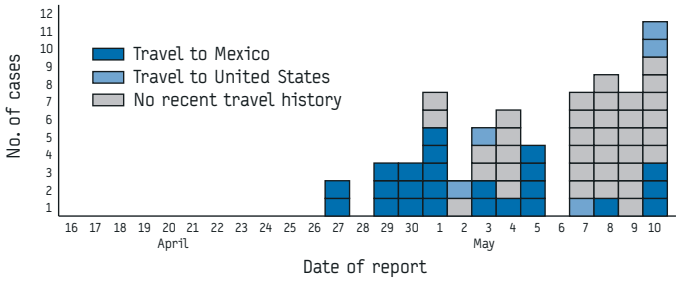
Travel history

Of the 65 cases, twenty-four reported a history of recent travel from Mexico and five from the US (one from each of California, Florida, Texas and two from New York).

The remaining 36 (56%) cases report no recent overseas travel and acquired their infection through secondary transmission in the United Kingdom. All but one of these can be linked to cases who travelled from affected areas. These indigenous cases are mainly affecting 10-19 year olds at present (Figure 2b). Of these cases, a number of secondary cases are linked to transmission in different household/close contact settings. Transmisson has also occurred in two school settings in London. An in-depth field epidemiology investigation of the school cluster is presently underway.

Figure 1

Cases of laboratory confirmed new influenza A(H1N1) by day of report and travel history, United Kingdom, 10 May 2009* (n=65)



Clinical picture

The First Few Hundred (FF100 project) aims to collect information about a limited number of the earliest laboratory confirmed cases of new influenza A(H1N1) and their close contacts [5]. This is to gain an early understanding of some of the key clinical, epidemiological, and virological parameters of the new influenza A(H1N1) virus and to facilitate real-time modelling efforts to make predictions of the future course of the United Kingdom epidemic. By 11 May, of the total of 65 confirmed cases, 53 had been reported and entered into the First Few-100 database. Cases generally presented with the most common symptoms typical of influenza – with fever (94%), sore throat (82%), headache (81%), chills (80%) and malaise (80%). Diarrhoea (28%) and arthralgia (56%) were moderately frequently reported. Five cases reported epistaxis and one a seizure. Children were more likely to have dry cough (83% vs. 55% OR = 5.7 95% CI: 0.97-34.2), malaise (89% vs. 69% OR = 8.1 95% CI 0.78-85.0) and epistaxis (24% vs. 6% OR = 4.9 95% CI: 0.46-52.4) than adults. Females were more likely to vomit than males (40% vs. 11%, OR=6.7; 95% CI: 1.1-41.1) and have diarrhoea (39% vs. 14%, OR = 4.0 95% CI: 0.8-19.8).

No case in the United Kingdom, to date has died. Amongst those patients with detailed information, three have been hospitalised – one with secondary pneumonia and two for clinical investigation. None of the cases were reported to have underlying risk factors for severe influenza or to have been vaccinated with either seasonal influenza or pneumococcal vaccine.

All of the cases except one had been treated with oseltamivir once diagnosed. Contacts are currently being actively followed up to provide information to enable estimations of epidemiological parameters such as secondary attack rate, serial interval and reproductive rate.

Conclusions

In summary, the United Kingdom continues to observe sporadic importations of new influenza A(H1N1) virus from affected areas predominately Mexico, but also now from the United States. As sustained transmission becomes established in other countries, importations from other parts of the globe to the United Kingdom will be observed. At this stage, healthy young adults and children are being proportionately more affected than other parts of the population. Based on the limited United Kingdom case series to date; the clinical presentation of cases continues to be relatively mild. Further work is on-going to describe more fully the emerging epidemiological, virological and clinical characteristics of this new influenza A(H1N1).

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Health Protection Scotland: McMenamin J, Ramsay C, Blatchford O, Goldberg D, Cowden J, Donaghy M, Eastaway A

*Authors' correction

In Figure 1 the date was corrected from 11 to 10 May. In the contributors' list the name of B. Carmen was added. These corrections were made upon the request of the authors on 18 May

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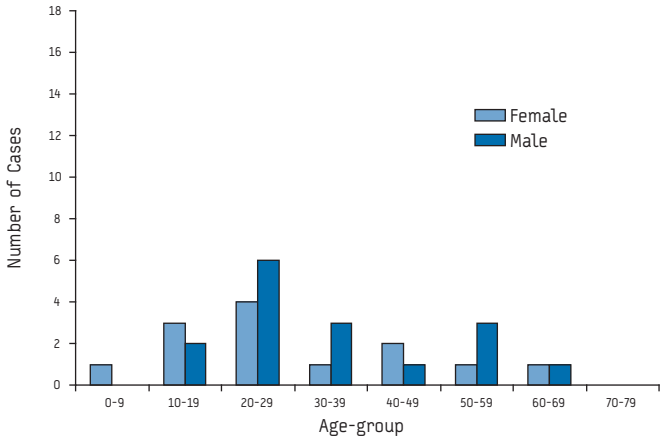
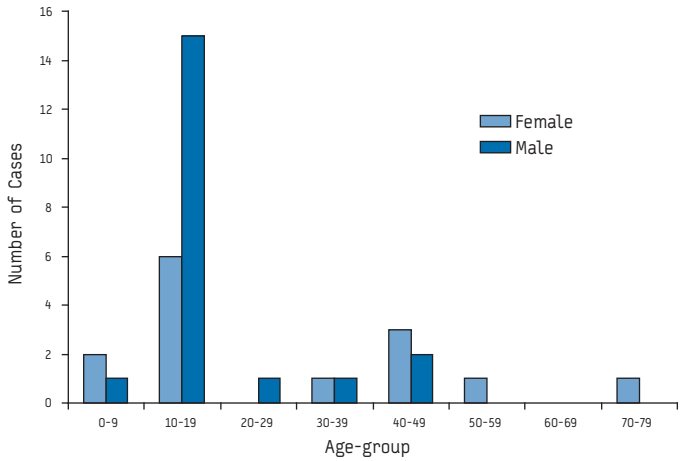
Figure 2

Cases of laboratory confirmed new influenza A(H1N1) by age-group and sex, United Kingdom, 11 May 2009 (n=65)

2a. Imported cases (n=29)



2b. Indigenous cases (n=36)



A preliminary estimation of the reproduction ratio for new influenza A(H1N1) from the outbreak in Mexico, March-April 2009

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As of 12 May 2009, 5,251 cases of the new influenza A(H1N1) have been officially reported to the World Health Organization (WHO) from 30 countries, with most of the identified cases exported from Mexico where a local epidemic has been going on for the last two months. Sustained human-to-human transmission is necessary to trigger influenza pandemic and estimating the reproduction ratio (average number of secondary cases per primary case) is necessary for forecasting the spread of infection. We use two methods to estimate the reproduction ratio from the epidemic curve in Mexico using three plausible generation intervals (the time between primary and secondary case infection). As expected, the reproduction ratio estimates were highly sensitive to assumptions regarding the generation interval, which remains to be estimated for the current epidemic. Here, we suggest that the reproduction ratio was less than 2.2 – 3.1 in Mexico, depending on the generation interval. Monitoring and updating the reproduction ratio estimate as the epidemic spreads outside Mexico into different settings should remain a priority for assessing the situation and helping to plan public health interventions.

Introduction

As of 12 May 2009, 5,251 cases of the new influenza A(H1N1) have been officially reported to the World Health Organization (WHO) from 30 countries [1,2]. Two parameters must be estimated for this new virus using mathematical and computational models: the reproduction ratio (R), which measures the average number of secondary cases per primary case; and the generation interval, which measures the average time between infection in a primary case and its secondary cases. The larger the reproduction ratio, the higher the required efficacy of public health interventions [3]. Here we use two different methods to provide preliminary estimates of R for the outbreak in Mexico.

Methods

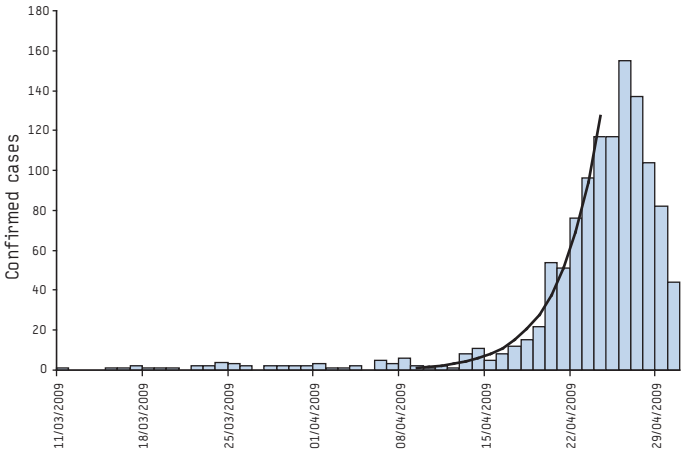
We used the daily incidence data from 11 March to 2 May 2009 as reported by the Mexican health authorities [4] (http://portal.salud.gob.mx/descargas/pdf/influenza/situacion_actual_de_la_epidemia_080509.pdf). The data consisted in 1,364 confirmed cases given as daily counts.

Two different approaches were used to estimate R:

- M1 - intrinsic growth rate [5]: the growth rate of the epidemic is estimated by Poisson regression over a given time interval and transformed to R using Laplace transform of the generation interval distribution. The assumptions are the exponential growth of the epidemic and known generation interval. After visual inspection of the epidemic curve, all periods starting before 20 April and ending after this date, more than five days long, were explored. Goodness of fit of the exponential model was judged by the deviance R–squared measure.
- M2 - real time estimation [6]: a daily reproduction ratio R(t) is determined by averaging the number of secondary cases over all possible chains of transmissions compatible with the epidemic curve. This approach assumes no imported cases, equiprobability of all chains of transmission compatible with the data and known generation interval.

Figure 1

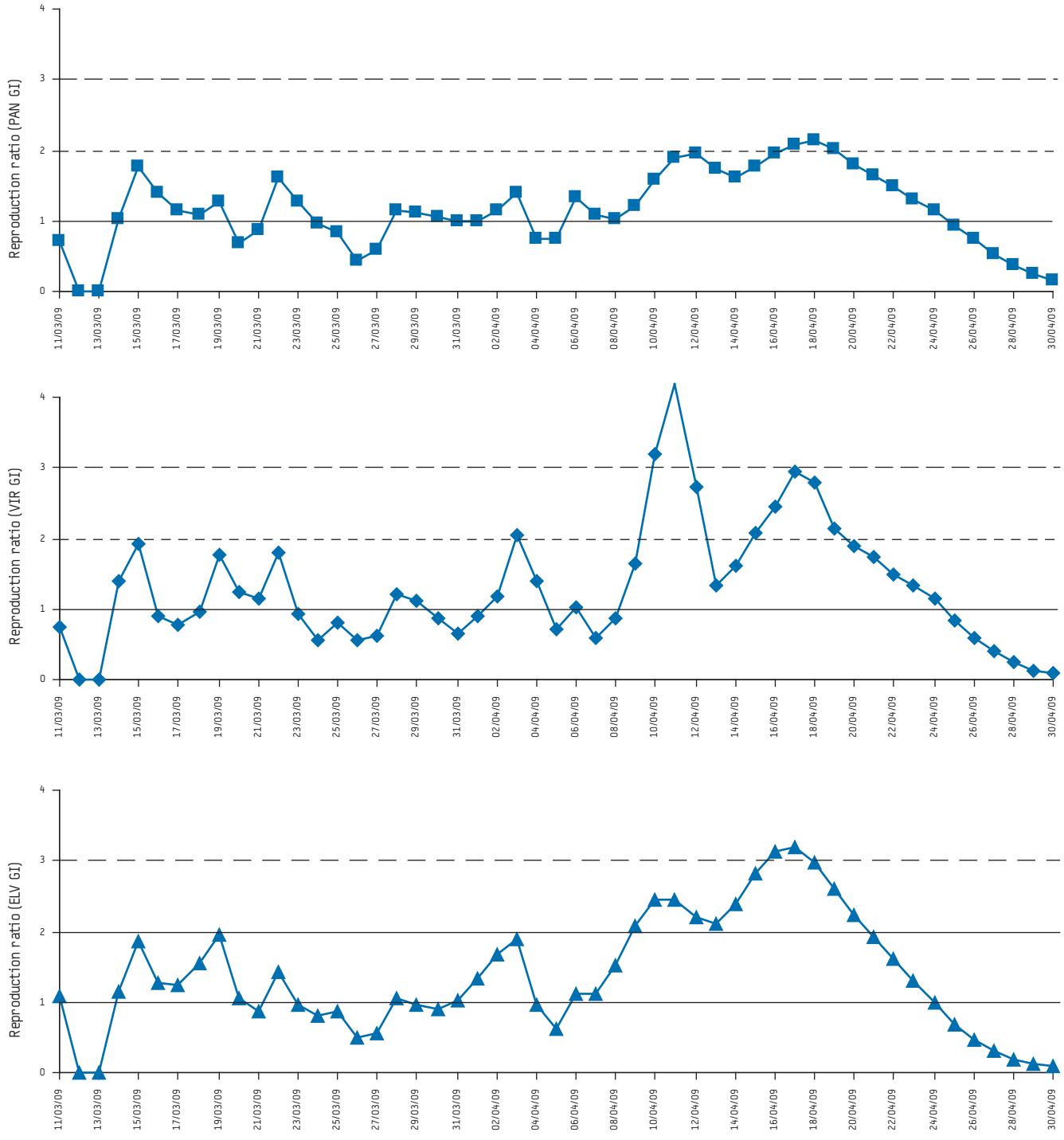
Epidemic curve of the outbreak of new influenza A(H1N1) in Mexico and fitted exponential growth over the period 9 to 24 April 2009



The two methods require full specification of the generation interval distribution. As no information regarding the actual generation interval in Mexico is available, we used three plausible candidate values of the generation interval (denoted GI) derived from different approaches: one (denoted as PAN) obtained from household studies from the 1957 and 1968 pandemics [7], one derived from viral excretion in experimental influenza infection (denoted as VIR) [8], and a hypothetical distribution introduced

Figure 2

Estimates of the daily reproduction ratio R(t) in the outbreak of new influenza A(H1N1) in Mexico, calculated with method M2 (see Methods) using three generation interval values: PAN GI (top), VIR GI (middle) and ELV GI (bottom)



in Elveback (denoted ELV) [9]. Their values with mean standard deviation (SD) were the following: PAN = 3.1 +/- 1.9 days; VIR = 2.6 +/- 1 day; ELV = 4.6 +/- 1.5 days.

Results

When using M1, the period starting on 9 April and ending on 24 April yielded the best fit for exponential growth, with daily rate r = 0.30 [CI95% 0.28-0.34] (Figure 1). The corresponding R was 2.2 [2.1, 2.4] for the PAN GI; 2.6 [2.4, 2.8] for the VIR GI; and 3.1 [2.9, 3.5] for the ELV GI. Overall, the differences in goodness of fit were small. The reproduction ratio decreased as the duration of the period used to estimate the growth rate increased: for the PAN GI, the maximum was 2.7 (8 days) and the minimum 2.0 (17 days).

With method M2, all three generation intervals led to similar profiles of R(t) with time: R(t) was around 1 up to 8 April then increased rapidly during the two following weeks (Figure 2). The magnitude of R depended on the generation interval: the maximum value was 2.1 (18 April) for the PAN GI; 4.0 (11 April) for the VIR GI; and 3.2 (17 April) for the ELV GI.

Discussion

Obtaining timely estimates of the reproduction ratio is crucial for deciding on public health interventions in case of a pandemic. In this respect, our analysis suggests that the maximum reproduction ratio was < 2.2 (for PAN GI); < 2.6 (for VIR GI) and < 3.1 (for ELV GI) during the outbreak in Mexico, subject to the following limitations.

Firstly, the epidemic curve was obtained by retrospective testing of samples, so that new cases may still be added. Indeed, for the same period (11 March to 26 April), there were 97 confirmed cases in the report published on 1 May, 682 in the 5 May report, and 803 in the 8 May report. With each new version of the epidemic curve, the reproduction ratio estimates grew smaller. The increase in the epidemic curve coincided with the setup of enhanced surveillance (starting from 16 April), suggesting improved case-finding with time. This notification/surveillance bias leads to overestimation of the reproduction ratio, as a larger number of late cases would be attributed to fewer earlier cases; on the other hand, however, the effect of public health interventions (closure of schools, restaurants and other public places, etc.) may affect the results in the opposite direction.

The assumptions required to estimate the reproduction ratio must also be taken into account. As already mentioned, the generation interval is unknown for the outbreak in Mexico, but of major importance for quantitative estimates. This illustrates the importance of estimating as soon as possible the generation time distribution to calibrate estimates of R [6]. As expected, longer generation time generally led to larger estimated R [3]. We believe the PAN GI should be favoured in the interpretation of the results, as it was determined from household data during past influenza pandemics.

A second limitation arises from arbitrary deciding which part of the epidemic curve displayed exponential growth, namely a minimum duration (five days), a starting and ending date. Stochastic variations, especially in small time series, may cause large uncertainties in the estimates [10]. Observing that the real time reproduction ratio M2, which does not rely on the exponential growth assumption, yielded smaller reproduction ratio estimates, suggests that method M1 yielded upper bound estimates.

A comprehensive analysis of all available data has independently led to the range 1.4-1.6 for the reproduction ratio [11]. At least two factors contribute to this substantially lower estimate: underreporting was explicitly taken into account and reduced the

Table

Epidemic growth rates estimated for the new influenza A(H1N1) epidemic in Mexico and corresponding reproduction ratio estimates calculated with method M1 (see Methods)

Period length (days)	Start date (m/d/y)	End date (m/d/y)	R^2	Growth rate (/day)	CI 95%	R (PAN GI)	R (VIR GI)	R (ELV GI)
5	04/19/09	04/23/09	0.8777	0.29	[0.29, 0.21]	2.2	2.5	3.0
6	04/19/09	04/24/09	0.9159	0.27	[0.27, 0.21]	2.1	2.4	2.8
7	04/16/09	04/22/09	0.9361	0.37	[0.37, 0.3]	2.6	3.1	3.9
8	04/15/09	04/22/09	0.9500	0.38	[0.38, 0.31]	2.7	3.2	4.0
9	04/15/09	04/23/09	0.9583	0.35	[0.35, 0.3]	2.5	2.9	3.6
10	04/15/09	04/24/09	0.9598	0.32	[0.32, 0.28]	2.3	2.7	3.3
11	04/14/09	04/24/09	0.9524	0.31	[0.31, 0.27]	2.3	2.6	3.2
12	04/13/09	04/24/09	0.952	0.3	[0.3, 0.26]	2.2	2.6	3.1
13	04/12/09	04/24/09	0.9537	0.3	[0.3, 0.27]	2.2	2.6	3.1
14	04/11/09	04/24/09	0.9585	0.3	[0.3, 0.27]	2.2	2.6	3.1
15	04/10/09	04/24/09	0.9619	0.31	[0.31, 0.28]	2.3	2.6	3.2
16	04/09/09	04/24/09	0.9643	0.3	[0.3, 0.28]	2.2	2.6	3.1
17	04/10/09	04/26/09	0.9564	0.26	[0.26, 0.24]	2.0	2.3	2.7
18	04/09/09	04/26/09	0.9596	0.26	[0.26, 0.24]	2.0	2.3	2.7
19	04/08/09	04/26/09	0.9544	0.26	[0.26, 0.24]	2.0	2.3	2.7
20	04/07/09	04/26/09	0.9554	0.25	[0.25, 0.24]	2.0	2.2	2.6

Note: Each line reports the best fitting period of given duration, as measured by the deviance R-squared measure.

reproduction ratio, and the generation interval, estimated from the actual epidemic, seems to have been much shorter than considered here (mean 1.9 days).

Although sensitive to all uncertainties discussed above, our early estimates show that the reproduction ratio in Mexico was in a range similar to that of past influenza pandemics [12,13].

Aknowledgements

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Sustained intensive transmission of Q fever in the south of the Netherlands, 2009

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The Netherlands is again facing a sharp increase in Q fever notifications, after the unprecedented outbreaks of 2007 and 2008. The most affected province of Noord Brabant has a high density of large dairy goat farms, and farms with abortion waves have been incriminated. Mandatory vaccination of small ruminants has started and should have an effect in 2010. A large multidisciplinary research portfolio is expected to generate better knowledge about transmission and additional control measures.

Introduction

Q fever is a zoonosis caused by the obligate intracellular bacterium *Coxiella burnetii*. Cattle, sheep and goats are the primary animal reservoir, but the causative agent has also been noted in many other animal species. Infected goats and sheep may abort, mainly in late pregnancy. The bacterium is shed in urine, faeces, milk and in especially high concentrations in placentas and birth fluids of infected animals. Bacteria are transmitted to humans mainly through the aerosol route, resulting in subclinical infection, a flu-like syndrome with abrupt onset of fever, pneumonia or hepatitis, after an incubation period of two to three weeks [1]. People with underlying conditions, especially heart valve lesions, are more susceptible to developing chronic Q fever. Endocarditis, the most common form of chronic Q fever is estimated to occur in about 1% of acute Q fever cases.

Since 1978, when Q fever in humans became a notifiable disease in the Netherlands, until 2006, the number of notifications had ranged between 1 and 32 cases annually, with an average of 17 cases per year [2]. However, in 2007, Q fever emerged as an important human and veterinary public health challenge with large epidemics in the southern part of the Netherlands [3]. In 2007, 168 human cases were notified and in 2008 exactly 1,000 human cases were registered (Figure 1). Notification criteria for acute Q fever are a clinical presentation with at least fever, or pneumonia, or hepatitis and confirmation of the diagnosis in the laboratory. Currently, the laboratory criteria are a fourfold rise in IgG antibody titre against *C. burnetii* in paired sera or the presence of IgM-antibodies against phase II antigen. Identification of *C. burnetii* in patient material with a PCR test will soon be added to the notification criteria. Notification of probable cases, defined as clinical signs with a single high antibody titre is voluntary.

Current situation

From April 2009, a sharp increase in Q fever was observed again, and a total of 345 cases (including 13 probable) were notified between 1 January and 11 May 2009 (Figure 1). For 11 cases, the date of illness onset was in 2008 and one case fell ill in 2007, resulting in a total of 333 cases with confirmed or presumed illness onset in 2009. The overall male-to-female ratio for these 333 cases was 1.7:1 with a median age of 49 years (IQR 38-61 years).

The epidemic curve for 2009 shows an even steeper increase in case numbers in April-May, than in the previous two years, suggesting that an epidemic of at least the same magnitude as the one in 2008 is imminent. While most cases reside in the same region in the province of Noord-Brabant as the cases reported in 2007 and 2008 (see map in reference 3), the geographic area seems to be expanding (Figure 2).

Clinical features and diagnostics

Pneumonia is the predominant clinical presentation of the Q fever cases in the Netherlands. For those patients notified in 2008 for whom clinical details were available, 545 presented with pneumonia, 33 with hepatitis, and 115 with other febrile illness (data not yet analysed in detail). Of the 226 cases in 2009 where data regarding hospitalisation were available, 59 (26%) had been admitted to a hospital, a percentage comparable to figures in 2008, but lower than the proportion of patients hospitalised in 2007 (49%). Clinical follow-up of patients that were diagnosed with acute Q fever in 2007, shows that Q fever is not always a mild disease of short duration, as many cases still suffered from persisting fatigue several months after disease onset [4]. We have no clear information about the occurrence of other chronic sequelae, such as endocarditis at this stage.

The medical microbiology laboratories in the affected region have jointly formulated diagnostic recommendations. Cases are currently diagnosed with immunofluorescence assays (Focus

Diagnostics), in-house complement fixation tests or ELISA. Real-time polymerase chain reaction (PCR) tests were developed by eight medical microbiology laboratories and the most sensitive (98%) PCR has been selected and has proven a valuable additional tool for early diagnosis of acute Q fever in the time window before seroconversion.

Increased alertness of general practitioners together with easy availability of diagnostic services certainly has an impact on the number of notifications. The current epidemic curve based on week of notification reflects a more real time situation than in previous years, as the interval between date of illness onset and date of diagnosis has decreased from a median of 77 days in 2007 (IQR 40-121) and 29 days (IQR 19-45) in 2008 to 17 days in 2009 (IQR 12-24 days).

Separate clusters with multiple sources

It is becoming increasingly clear that the overall outbreak consists of at least 10 separate clusters with multiple sources, mainly in the province of Noord Brabant. For some clusters a clear epidemiological link could be established to small ruminant farms with clinical Q fever cases in animals presented as abortion waves. For other clusters such a link was less obvious. An example of the latter is a medium sized city (87,000 inhabitants) that experienced a second Q fever outbreak in 2009 similar to the one in 2008. In 2008, a dairy goat farm with abortions due to Q fever was suspected as the source, but in 2009 there were no veterinary notifications from the area. The 73 notified human cases residing in the city were clustered in the same part of the city as the cases that were notified in 2008. It remains unclear whether the same source is involved, whether the bacteria have persisted and survived in the local environment, whether the primary source in 2008 has resulted in secondary sources in 2009, or whether there is increased awareness among health professionals in this part of the city based on the 2008 experience.

In March 2009, the Animal Health Service reported a Q fever-positive farm in the province of Limburg with more than a thousand goats. The place also serves as a care farm for young people with mental disabilities who work there as part-time farmhands. Prompted by this notification, the municipal health service (MHS) South Limburg performed active laboratory screening by ELISA of the individuals affiliated to this goat farm. The screening, which involved a total of 96 people, has resulted in 28 notified symptomatic cases to date.

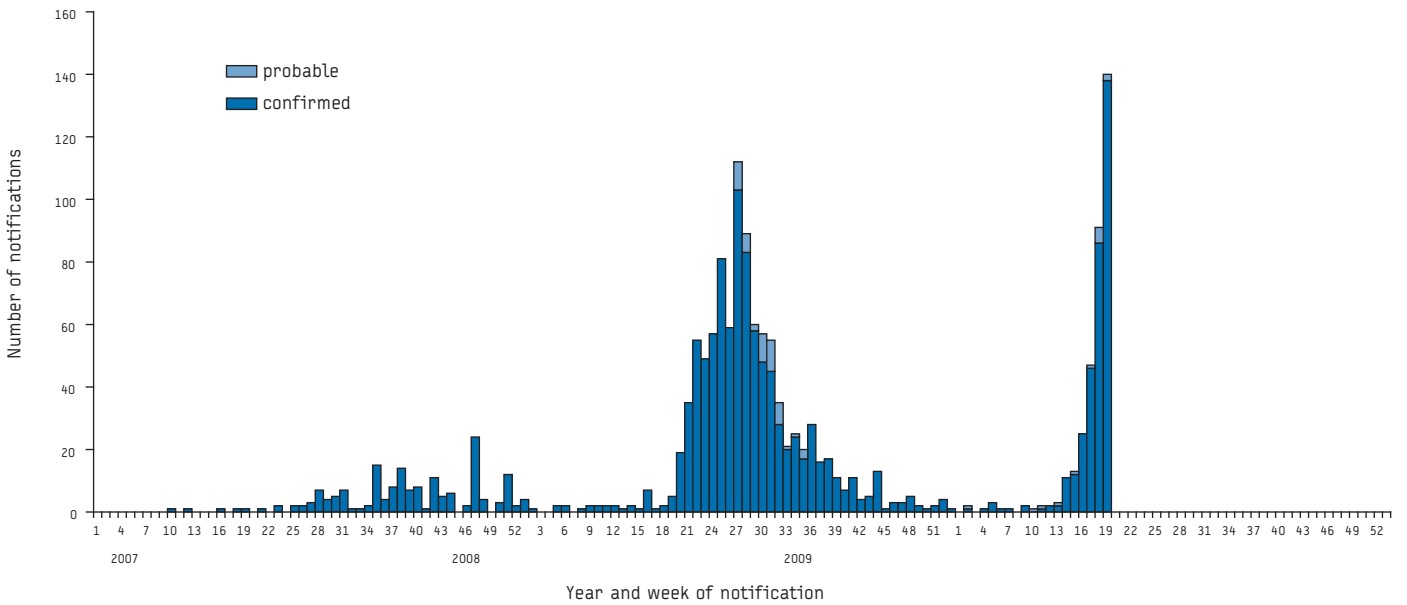
Veterinary situation

The total number of registered small ruminant farms in the Netherlands is 52,000, of which 350 are professional dairy goat farms with more than 200 adult goats and 40 are professional dairy sheep farms. In 2005, Q fever was diagnosed for the first time as a cause of abortion at a dairy goat farm, using immunohistochemistry on sections of placenta [5]. A second case was diagnosed later in 2005. In 2006, 2007 and 2008, six, seven and seven new cases at dairy goat farms were confirmed, respectively, mainly in the same area where human cases occurred. In the same period, two cases of abortion caused by *C. burnetii* were confirmed at dairy sheep farms, one in the southern and one in the northern part of the country but these two cases do not seem to be related to human cases. Analyses of abortion outbreaks showed that the average number of goats per farm was 900 of which 20% aborted, ranging from 10-60%. The average number of sheep on both infected sheep farms was 400 and the abortion rate was 5%.

Abortion outbreaks before June 2008 were reported on a voluntary basis to the Animal Health Service and also confirmed by immunohistochemistry. Since June 2008, notification of Q fever in goats and sheep is mandatory in the Netherlands. There is a legal requirement for farmers and their private veterinary surgeons to notify the occurrence of abortion in small ruminants held in deep litter houses. For large farms (>100 animals) the notification

Figure 1

Q fever notifications by week of notification, 1 January 2007 - 11 May 2009, the Netherlands (2007: n=168, 2008: n=1000, 2009 [week 1-week 19]: n=345)



criterion is an abortion wave defined as an abortion percentage higher than 5% among pregnant animals. For smaller holdings, a criterion of three or more abortions in a 30-day period is used.

From January to April 2009, this new regulation has led to notification of three dairy goat farms with clinical cases of Q fever. One farm is located in the province of Overijssel (notified in February), one in the south of the province of Limburg (notified in March), and one in the province of Noord-Brabant (notified in April).

This veterinary notification can potentially facilitate the detection of related human cases or clusters. Veterinarians, physicians and the public are informed through targeted mailings, publications and the media. The exact location of animal farms with clinical Q fever is now reported to the municipal health service. In February 2009, a nationwide stringent hygiene protocol became mandatory for all professional dairy goat and sheep farms, independent of Q fever status.

Vaccination campaigns

In the fall of 2008, a voluntary vaccination campaign was implemented in the province of Noord Brabant. In total, about 36,000 small ruminants were vaccinated in an area with a radius of 45 kilometer around Uden, a small town in the centre of the high-risk area.

Another, mandatory vaccination campaign led by the Animal Health Service (GD) started on 21 April 2009. From April to October 2009, 200,000 small ruminants will be vaccinated in an area which includes the province of Noord-Brabant and parts of the provinces of Gelderland, Utrecht and Limburg.

Ongoing research

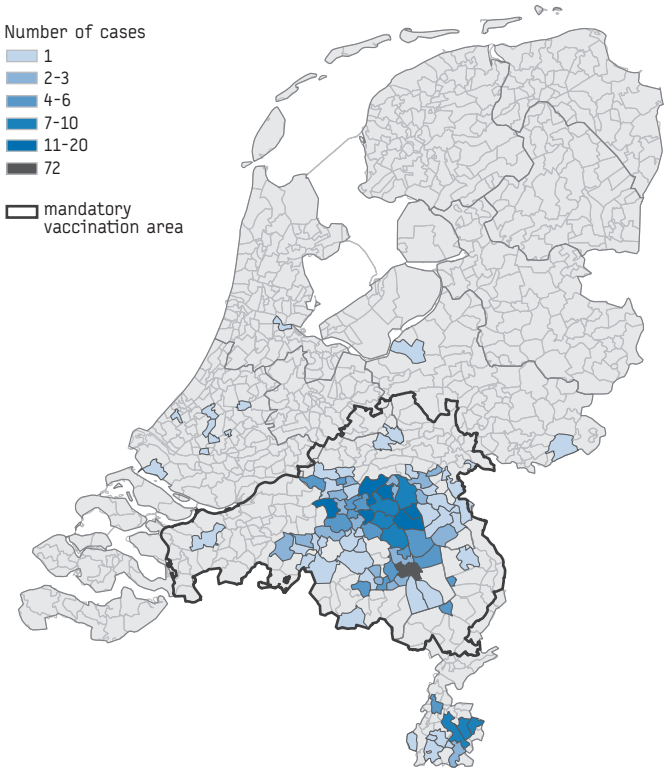
Ongoing studies address the factors involved in the 2008 epidemic at a national, regional and local level, the efficacy of the 2008 voluntary vaccination campaign in small ruminants and the nationwide occurrence of *C. burnetii* antibodies in the community and in small ruminants. From the human epidemiological perspective, a case control study is currently underway in the two main affected MHS regions of 2009, 'Hart voor Brabant' and Brabant-Southeast. Routinely collected sera of pregnant women from the affected regions over the period June 2007 to July 2008 are retrospectively screened for Q fever to study the effect of infection on pregnancy outcome (registered in a national database). An integrated human-veterinary study was started, in which small ruminant farmers and their animals will be screened for presence of *C. burnetii* antibodies. In addition, environmental samples will be obtained from a subset of these farms and the role of particulate matter in relation to *C. burnetii* transmission will be further investigated.

Conclusion

For the third consecutive year the Netherlands is facing a large outbreak of Q fever. The new upsurge in Q fever cases in 2009 is alarming. The mandatory vaccination campaign among small ruminants that was started in April 2009, if effective, is expected to reduce the occurrence of abortion waves and excretion of *Coxiella* in the lambing season 2010. There is a large portfolio of ongoing multidisciplinary research, but it will take some time before results become available that eventually will lead to the implementation of extended and improved control measures.

FIGURE 2

Notified cases of acute Q fever in the Netherlands by three-digit postal code area, 1 January – 11 May 2009 (n=344*). The black line indicates the mandatory vaccination area covering the province of Noord Brabant and parts of the provinces of Gelderland, Utrecht, and Limburg.



Source: OSIRIS notification system. Map compiled by Ben Bom, Expertise Centre for Methodology and Information Services, RIVM
* For one case the information on postal code is missing

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Anaphylaxis following unnecessary meningococcal chemoprophylaxis of a healthcare worker

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We report a case of anaphylaxis following meningococcal chemoprophylaxis of a healthcare worker, despite no history of direct contact with a patient who had meningococcal disease. The public health implications of this case are discussed.

Introduction

Chemoprophylaxis of meningococcal disease is usually recommended for close contacts, such as household members and persons directly exposed to a patient's respiratory secretions, as these have been shown to have a higher risk of invasive meningococcal disease [1]. However, chemoprophylaxis is not recommended for low risk or indirect contacts, partly because of possible adverse outcomes. These include adverse events to prophylactic antibiotics, development of antibiotic resistance and eradication of non-pathogenic *Neisseria* species which may confer protection against *Neisseria meningitidis* [2,3]. In particular, administration of prophylactic antibiotics for healthcare workers is recommended only after an unprotected exposure to respiratory secretions of an index case. We describe a case of anaphylaxis following chemoprophylaxis of a healthcare worker with no history of direct contact with a patient who had sepsis caused by *N. meningitidis*. The public health implications of this case are discussed.

Case description

In March 2009, a woman in her 40s was admitted to a hospital in the Piedmont Region, Italy, with a one-day history of fever and myalgia. On admission, the patient was unconscious, with hypotension, tachycardia, acidosis and a truncal petechial rash. Clinical and laboratory features suggested a septic shock with disseminated intravascular coagulation. Her condition rapidly worsened and death occurred two hours after admission, despite resuscitation.

As the presumptive diagnosis was meningococcal disease, pathologists collected samples of blood, cerebrospinal fluid and petechial smears during the *post mortem* examination. PCR was subsequently performed at the Istituto Superiore di Sanità Infectious Diseases Laboratory in Rome, and *N. meningitidis* serogroup B was detected in all samples.

In accordance with the local public health unit, the hospital management recommended treatment with a single 500 mg dose of ciprofloxacin for two doctors who had had unprotected exposure to the respiratory secretions of the patient. Chemoprophylaxis was also administered to three healthcare workers of the hospital staff. Outside the hospital setting, the contact tracing identified two household members as well as eight contacts who had been presumably exposed to the respiratory secretions of the patient and all took chemoprophylaxis. No secondary cases occurred in the following 30 days.

The following day, a healthcare worker in the same unit as one of the exposed doctors decided to take a single 500 mg dose of ciprofloxacin for fear of contracting meningococcal disease, although she had no history of direct contact with the index case. On this basis, she had not been classified by the hospital management as a close contact and thus had not been offered chemoprophylaxis. Approximately 20 minutes after taking ciprofloxacin at the workplace, she was admitted to the emergency room with pharyngeal oedema, tongue swelling and generalised skin rash. The patient recovered gradually after administration of adrenalin, antihistamines and corticosteroids. She had used ciprofloxacin in the past without any adverse reaction.

Discussion

Invasive meningococcal disease is uncommon in Italy. Approximately 180 cases (0.3 per 100,000 population) are notified annually to the infectious diseases surveillance system [4]. The highest incidence is seen among children under five years old. In the Piedmont Region, an area in north-west Italy with 4.3 million inhabitants and active laboratory-based surveillance, the incidence appears to be higher: 0.4-0.7 per 100,000 population, with a constant peak during the first year of life, ranging from five to six cases per year per 100,000 population [5].

Chemoprophylaxis is recommended in Italy only for persons with close contact to the index case up to one week before the onset of the patient's symptoms. Close contacts include: household members, contacts in child-care centres, and persons directly exposed to the patient's oral secretions [6]. Giving chemoprophylaxis to people who have not been in close contact with an index case has not proved to be effective in preventing secondary cases and is usually not recommended [3,7].

In Italy, national guidelines on meningococcal chemoprophylaxis for healthcare workers are not available. Nevertheless, the regional health authorities as well as hospitals have developed standard operating procedures, usually based on international authoritative sources, such as the Centers for Disease Control and Prevention (CDC) in Atlanta, United States (US). In accordance with CDC guidance, the operating procedures of the local health unit involved in this case recommend chemoprophylaxis for healthcare workers

after an unprotected airway exposure to infectious respiratory droplets within a distance of 1 m from a probable or confirmed case of meningococcal disease; this may happen typically during mouth-to-mouth resuscitation or management of an endotracheal tube [6,8].

A study in the United Kingdom found an attack rate of 0.8 per 100,000 healthcare workers in close contact with cases of meningococcal disease, i.e. 25 times higher than in the general population [9]. The study identified three cases of meningococcal disease in healthcare workers during a period of 15 years: all had spent at least 30 minutes in contact with the index case immediately before or after hospital admission, all had been exposed to the patients' respiratory droplets, and none had used face shields and surgical masks or taken prophylactic antibiotics.

The fluoroquinolone ciprofloxacin is often used for meningococcal chemoprophylaxis in adults because it can be given as a single oral dose, is effective in eradicating meningococcal carriage and does not interact with oral contraceptives. For the same reasons, however, unnecessary chemoprophylaxis is more likely to occur with ciprofloxacin than with other prophylactic antibiotics. Rifampicin requires a total of four doses in the course of two days and can interfere with oral contraceptives; ceftriaxone is administered as a single dose, but is not popular because it can only be administered parenterally.

Anaphylaxis following ciprofloxacin administration has been described before. In particular, three cases of anaphylactoid reactions were reported after oral administration of 500 mg ciprofloxacin to 3,200 students after two cases of meningococcal disease in the same university [10]. Limited data on the magnitude of allergic reactions following administration of drugs are available, mainly because clinical manifestations are heterogeneous (from mild to severe and potentially life-threatening) and furthermore some reactions suggesting an immunologic pathogenesis might be linked to a non-allergic mechanism. Likewise, the incidence of allergic reactions induced by oral antibiotics such as ciprofloxacin is difficult to estimate. However, according to a case/non-case study conducted on data from a passive adverse events surveillance programme, fluoroquinolones were associated with a significant increase in the reporting odds ratio of allergic reactions (2.09, 95% confidence interval (CI): 1.85-2.36) [11]. Moreover, an incidence of 5.4 (95% CI: 4.4-6.5) allergic reactions per 10,000 first administrations of ciprofloxacin has been derived from the database of a large health insurance company [12].

Another reason for concern is the potential development of antibiotic resistance. Three cases of meningococcal disease caused by a *N. meningitidis* serogroup B strain resistant to ciprofloxacin were recently reported in the United States [13]. The widespread use of fluoroquinolones, which are commonly prescribed in the United States [14], and the consequent emergence of resistant strains may explain these findings.

Finally, prophylactic antibiotics can eliminate carriage of *N. lactamica*, which leads to cross-protective immunity against *N. meningitidis* and therefore may confer protection against meningococcal disease [15,16]. This concerns young children in particular, since carriage of *N. lactamica* is inversely related to age [16].

Quantitative data about the overuse of chemoprophylaxis are scarce. A study in the United Kingdom evaluated prescribing of chemoprophylaxis for contacts of meningococcal disease by general practitioners and hospital staff [17]. Prescribing by hospital doctors was consistent with official recommendations, whereas general practitioners prescribed 118% more chemoprophylaxis than recommended. Furthermore, the highest level of unrecommeded prescriptions was observed in regions where there were both high incidence rates and high levels of publicity surrounding the cases. Most likely, this inappropriate prescribing is client-driven because meningococcal disease raises anxiety among the involved population.

Conclusions

Use of prophylactic antibiotics against meningococcal disease can lead to potentially severe adverse events, development of antibiotic resistance and eradication of non-pathogenic *Neisseria* species that may elicit cross-protective immunity. Therefore, information should be provided to the public and to healthcare workers about the potential risks from indiscriminate use of prophylactic antibiotics. Available data should be used to compare the risks related to different patterns of exposure to a case of meningococcal disease versus the possible adverse outcomes of chemoprophylaxis. To this end, thorough counselling and communication of the population-based and individual-based risk represent a key factor in the public health management of meningococcal disease cases.

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Endemic hepatitis E in two Nordic countries

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Antibodies against hepatitis E virus (anti-HEV) were found in 248 Swedish and Danish patients between 1993 and 2007. Most patients were symptomatic and tested for anti-HEV due to travel abroad. Among patients with known country of infection, most were infected in Asia, mainly on the Indian subcontinent. However, 29 patients were infected in Europe, nine of these had HEV IgM and/or HEV RNA in serum. In sera from 65 of 141 tested patients HEV RNA could be detected, and 63 strains could be typed by limited sequencing within ORF2. HEV RNA was found in sera from 71% of the patients with HEV IgM and IgG and in 18% of the patients with only detectable HEV IgG. It was also found up to three weeks after the onset of disease in 67% of the patients with known date of onset. Patients infected in Europe were infected by genotype 3, and were older than those infected by genotype 1 (mean age 55.3 vs 30 years, $p<0.001$). Since it is known that genotype 3 can infect domestic pigs, HEV strains from 18 piglets in 17 herds in Sweden and Denmark were sequenced. Phylogenetic analyses of the genotype 3 strains showed geographical clades and high similarity between strains from patients and pigs from the same area. There are thus autochthonous hepatitis E cases in Scandinavia, and there are probably many undiagnosed ones. Patients with hepatitis of unknown etiology should therefore be investigated for anti-HEV even if they have not been outside Europe, since infections acquired from pigs or other animals should be taken into consideration.

Introduction

Hepatitis E virus (HEV) is a non-enveloped positive-stranded RNA virus of 27-34 nm in diameter [1]. It is the only member of the genus Hepevirus in the family Hepeviridae. The genome is approximately 7.2 kb in length and encodes three open reading frames, from ORF1 to ORF3. ORF1 encodes for enzymes important for replication and transcription, ORF2 encodes for a capsid protein and ORF3 for a small protein of 122 or 123 amino acids that interacts with cellular proteins and contributes to viral replication. There is only one serotype but based on genetic diversity HEV strains are classified into four genotypes designated with Arabic numerals 1 to 4. The genotypes are further divided into up to seven subtypes designated with Roman characters a – g, each with distinct geographical distribution [2]. Genotypes 1 and 2 only infect humans, mainly in Asia, and Africa, where they are endemic and may cause large outbreaks. Genotype 2 has been found causing outbreaks in Mexico and Africa. Strains of the other two genotypes, 3 and 4, have been shown to infect not only humans, but also domestic pigs, wild boars, deer, and other mammals. These two genotypes have not been reported to cause outbreaks. In endemic countries, as India, genotype 1 infects humans, while HEV isolates from swine belong to genotype 3 or 4 [3]. However, genotype 3 strains have also been isolated from sporadic human cases of hepatitis E, and from domesticated pigs in several European countries, in the United States (US) and in Japan, while genotype 4 strains have been found in humans and pigs exclusively in Asia, as China, Taiwan, Japan and Vietnam [4-7].

Hepatitis E is transmitted mainly by the faecal-oral route, usually through contaminated drinking water. Usually, the infection is self-limited, although some persons develop fulminant hepatitis. In pregnant females the illness is particularly severe with up to 20% fatality rate in the third trimester, but it may be even higher in patients with underlying chronic liver disease [8,9]. Chronic hepatitis E infections have also been described in transplant patients on immunosuppressive treatment [10].

Hepatitis E was previously considered to mainly affect the inhabitants of or travellers to Asia and Africa, due to high endemicity in these parts of the world. However, in recent years there have been several reports on autochtonous hepatitis E cases in Europe, including United Kingdom (UK), the Netherlands and France [6,7,11,12], and also in the US, New Zealand and Japan [4,13,14]. There have also been increasing numbers of reports on high seroprevalence in Europe and the US. Antibodies againts HEV (anti-HEV) were found in 17% of blood donors in the UK and in France, in 21-33% of blood donors and 50% of farmers in Denmark and 5 to 9% of the general population and 13% of veterinarians in Sweden [11,15-18]. These data indicate that there is a high prevalence of hepatitis E infections also in Europe, albeit most infections are subclinical and most of them may be zoonotic. The study presented here was performed to investigate which genotypes of HEV were imported to Denmark and Sweden between 1993 and 2007, and to find out if there were any endemic HEV strains and,

if so, to determine their relation to HEV strains obtained from pigs in these countries.

Materials and methods

Identification of human cases with hepatitis E

Patients with a recent travel history and with clinical signs of hepatitis not caused by hepatitis A, B, C or D virus were investigated for hepatitis E at the Swedish Institute for Infectious Disease Control (Smittskyddsinstitutet, SMI), Solna, Sweden. Sera from these patients were tested for HEV IgG and IgM by a kit using two recombinant HEV antigens corresponding to structural region of the HEV (Diagnostic Biotechnology, Singapore). Between 1993 and 2006, all samples were also tested for HEV IgG and IgM by using the until then commercially available ELISA kits from Abbott Laboratories (Abbott Laboratories, Chicago, IL). All reactive sera were tested for HEV RNA by PCR and the amplified fragments were sequenced.

Identification of pigs infected by HEV

HEV RNA was isolated and sequenced from 18 HEV strains from pigs. The strains were obtained from two HEV prevalence studies (Breum, unpublished; Widén et al., unpublished). Eight strains were from piglets from seven Danish pig herds and 10 strains were from piglets from 10 Swedish herds.

RNA extraction

HEV RNA was extracted from 200 ul serum from humans or faecal suspension from pigs using QIAamp UltraSense Virus Kit (Qiagen, GmbH, Germany) as described by the manufacturer. Five ul RNA were used for cDNA synthesis in 20 ul mix containing 5 ul 5X First Strand buffer (Invitrogen, Life Technologies, Carlsbad, CA), 10 mM DTT (Invitrogen, Life Technologies, Carlsbad, CA), 0.5 mM dNTP (Thermo Scientific, Abgene®, Epsom, UK), 100 U Superscript II Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA), 0.5 ul RNasin (Promega, Madison, US) and 0.1 U random hexamere primers (Roche Diagnostics, GmbH, Germany). Reverse transcription was performed at room temperature for 15 minutes and then at 42°C for two hours.

Nested PCR in the RdRp domain of ORF1 region

A nested PCR was carried out in a 50 ul reaction with 5 ul cDNA, 0.06 ul of 0.2 mM of each primers ISP-4232 and EAP-4576 [19], 5 ul 10X Taq.buffer general, 2 mM MgCl2 (Applied Biosystems, Roche Molecular Systems, New Jersey, US), 0.2 mM dNTP (Thermo Scientific, Abgene®, Epsom, UK) and 4 U Taq polymerase (Thermo Scientific, Abgene®, Epsom, UK). The PCR reaction was carried out for 40 cycles with denaturation at 94°C for 20s, annealing at 60°C for 30s and extension at 72°C for 60s. The second round reaction was carried out similarly but with 5 ul first round product instead of cDNA, 2.5 mM MgCl and 0.06 ul of 0.2 mM of each primer ISP-4232 and IAP-4561 [19].

Nested PCR in the ORF2 region

Two different nested PCRs for amplification of the ORF2 region were performed. PCR:1 was carried out in a 50 ul reaction with 10 ul cDNA, 0.1 ul of 0.2 mM primer HE110 [14], 0.119 ul of 0.2 mM primer HE041 (14), 5 ul 10X Taq.buffer general, 2.5 mM MgCl2, 0.2 mM dNTP, 6 U Taq polymerase. The PCR reaction was carried out for 40 cycles of denaturation at 94°C for 20s, annealing at 56°C for 20s and extension at 72°C for 60s. The second round reaction was carried out with 5 ul PCR product, primers HE110 and HE3159 [20] with reagents and cycling as in the first round.

Table 1

Age and sex distribution of patients from Sweden and Denmark (1993-2007) with serological markers against hepatitis E virus (HEV)

	Number of patients with anti-HEV IgM					Number of patients with anti-HEV IgG only					
	Sweden		Denmark			Sweden		Denmark			
Age	M	F	M	F	Sub-total	M	F	M	F	Sub-total	Total
0-9	0	0	2	0	2	0	1	2	0	3	5
10-19	8	1	3	1	13	2	2	3	0	7	20
20-29	13	8	8	6	35	8	4	9	2	23	58
30-39	4	4	6	2	16	7	3	24	7	41	57
40-49	4	0	2	1	7	9	4	14	3	30	37
50-59	0	1	1	1	3	5	5	11	10	31	34
60-69	2	3	3	0	8	2	5	9	2	18	26
>70	1	0	0	0	1	3	3	1	3	10	11
Total	**32**	**17**	**25**	**11**	**85**	**36**	**27**	**73**	**27**	**163**	**248**

M = male, F = female

Table 2

Hepatitis E virus (HEV) RNA detection in serum samples from Swedish and Danish patients (1993-2007) with anti-HEV IgM and IgG or anti-HEV IgG only

Patient origin	N	Anti-HEV IgM + IgG	Anti-HEV IgG only	HEV RNA positive (%)	HEV RNA in IgM + IgG positive sera	HEV RNA in IgG only positive sera	Number of sequenced strains
Sweden	82	44	38	44 (57 %)	38 (86 %)	9 (24 %)	44
Denmark	59	36	23	21 (36 %)	19 (53 %)	2 (9 %)	21
Total	**141**	**80 (57 %)**	**61 (43 %)**	**65 (46 %)**	**57 (71 %)**	**11 (18 %)**	**63 (97 %)**

Table 3

Presence of hepatitis E virus (HEV) RNA in serum of patients from Swedish and Denish patients (1993-2007) in relation to onset of disease when this information was known

Number of weeks after onset of disease	Number of samples	HEV RNA detection in ORF 1/number tested (%)	HEV RNA detection in ORF 2/number tested (%)
1	53	35 (66 %)	30 (57%)
2	6	6 (100%)	3 (50 %)
3	3	2 (67%)	1 (33 %)
4	8	2 (25 %)	3 (38 %)
5	4	2 (50%)	2 (50%)
>6	10	1 (10%)	0
Total	**84**	**48 (57 %)**	**39 (46 %)**

PCR:2 was carried out with with 5 ul cDNA, and primers HE3156 and HE3157 [20]. Two microliters of this product were further amplified with primers HE3158 and HE3159 [20].

Sequencing the ORF2 region

The amplified products were purified using the EZNA Cycle Pure Kit (Omega Bio-Tek, GA, US) according to the manufacturers instructions. The sequencing reaction was made with BigDye Terminator Cycle Sequencing Ready reaction kit version 3.1 (Applied Biosystem, CA, US). The ABI PRISM 3100 genetic analyser (Applied Biosystems, CA, US) was used for electrophoresis and data collection.

Phylogenetic analysis

The sequences obtained were analysed in the programs SeqMan and Sequencing Analysis. Eighty-four analysed sequences were aligned with the corresponding region of 554 sequences obtained from GeneBank. The phylogenetic analysis was carried out with the

Table 4

Reported country of infection and infecting hepatitis E virus (HEV) genotype of Swedish and Danish patients (1993-2007) with anti-HEV IgM and IgG or with only detectable anti-HEV IgG

Country/region of infection	Number of samples	Number of samples from patients with anti-HEV IgM	Number of samples from patients with anti-HEV IgG only	HEV RNA positive samples/ tested from patients with anti-HEV IgM	HEV RNA positive samples/tested from patients with anti-HEV IgG only	Geno-type 1	Geno-type 3
Europe							
Sweden	8	1	7	1/1	1/5	0	2
Sweden*	1	1	0	0	1/1	1	0
Denmark	7	2	5	2/2	0/1	0	2
Bulgaria	1	0	1	0	0/1	0	0
Canary Islands	2	0	2	0	0/1	0	0
Spain**	2	2	0	1/1	0	0	1
Italy	2	0	2	0	0/1	0	0
Majorca	1	1	0	1/1	0	0	1
Greece	1	0	1	0	0	0	0
Serbia	2	1	1	1/1	0	0	1
Poland	1	0	1	0	1/1	0	0
Russia	1	0	1	0	0	0	0
Subtotal	29	8	21	6/6	3/11	1	7
Asia	2	1	1	0/1	0/1	0	0
Afghanistan	5	3	2	1/3	0/2	1	0
Bangladesh	16	12	4	8/11	1/3	9	0
India	34	24	10	19/22	4/9	21	0
Nepal	5	4	1	4/4	0	4	0
Pakistan	15	9	6	8/9	2/3	10	0
Thailand	6	0	6	0/0	0/3	0	0
Singapore	1	0	1	0	0	0	0
Indonesia	1	0	1	0	0	0	0
Subtotal	85	53	32	40/50	7/21	45	0
Middle East							
Syria	1	0	1	0	0/1	0	0
Turkey	1	0	1	0	0/1	0	0
Iraq	1	1	0	0/1	0	0	0
Iran	1	0	1	0	0	0	0
Subtotal	4	1	3	0/1	0/2	0	0
Africa							
Tanzania	1	0	1	0	1/1	1	0
Somalia	1	1	0	0/1	0	0	0
Ethiopia	1	0	1	0	0	0	0
Egypt	1	0	1	0	0	0	0
Subtotal	4	1	3	0/1	1/1	1	0
South America							
Dominican Republic	2	1	1	0	0/1	0	0
Brazil	1	0	1	0	0	0	0
Country not reported	122	21	101	8/22	1/23	9	0
Total	**248**	**85**	**163**	**54/80**	**11/61**	**56**	**7**

* Contact case to an infected relative from Pakistan
** The numbers for Spain exclude Canary Islands and Majorca which are listed separately

PHYLIP package version 3.65 [21]. Evolutionary distances were using the F84 algorithm in the DNADIST program with transition/transversion ratio of 4.29. Phylogenetic trees were constructed using UPGMA and Neighbor-joining method in the NEIGHBOR program in the PHYLIP package. The trees were visualized using the program Tree View, version 1.6.6. Bootstrap analysis of 1,000 replicas was perfomed with the programs SEQBOOT and CONSENSE in the PHYLIP package.

Results

There was no significant difference in age and sex distribution between the patients from Sweden compared with those from Denmark (Table 1). Anti-HEV IgM and IgG was found in 85 patients, 57 (67%) of those were males. There was also a predominance of males, 109/163 (67%), among patients in whom only anti-HEV IgG without detectable IgM was found (Table 1). The mean age of the patients with anti-HEV IgM was 31.5 years, while those with only detectable anti-HEV IgG were older with a mean age of 43.6 years.

HEV RNA could be detected in serum from 65 of 141 tested anti-HEV positive patients (Table 2). The PCR in the ORF1 region was more sensitive and could amplify 63 of the strains, while 51 of the strains were amplified in the ORF2 region. HEV strains could be amplified in 68% of the sera from patients with IgM anti-HEV, as well as in 18% of sera from patients with detectable anti-HEV IgG only (Table 2).

The time of onset of disease in relation to the time of sample collection was known for 84 patients (Table 3). All six patients sampled 2-3 weeks after onset had detectable HEV RNA in serum when ORF 1 was amplified, while only three of these patients had detectable HEV RNA when ORF2 was amplified. In two patients HEV RNA was detected as long as five weeks after onset of illness.

The countries of infection, known for 126 (51%) of the patients, were mainly in Asia with India, Pakistan and Bangladesh as dominating countries (Table 4). Twenty-nine patients (23%) were infected in Europe, while the rest were infected in the Middle East, Africa or South America (Table 4). Six of the patients infected in Europe were injecting drug users (IDUs) and one case in Sweden was a contact of an HEV-infected relative from Pakistan [22].

The PCR amplified regions could be sequenced for 63 of the 65 PCR amplified isolates and 56 patients were found infected with genotype 1, while seven were infected with genotype 3 (Table 4). Those with genotype 1 had all been infected in Asia and Africa, apart from the Swedish contact of a case from Pakistan, while all those with genotype 3 were infected in Europe.

There was a predominance of males in both groups, with five males among the seven patients infected by genotype 3 and 43 males among the 56 patients infected by genotype 1. The patients infected by genotype 3 were older than those with genotype 1. The mean age of patients infected with genotype 3 was 55.3 years, while the mean age of those with genotype 1 was 30 years ($p<0.001$; unpaired t-test).

HEV RNA could be detected in six faecal samples from six out of 10 piglets tested (in six out of 10 Swedish breeding herds) and in eight samples from piglets originating from seven Danish breeding herds. All piglets were found infected with genotype 3.

In the phylogenetic analysis all isolates could be allocated to either genotype 1 or 3 (Figure 1). It was also found that genotype 3 could be subdivided into two major clades, here tentatively designated 3-I and 3-II (Figures 1 and 2b). Subtypes 3a, c, and d clustered in clade 3-I, while strains of subtype 3e, g, and f were found in clade 3-II (Figures 1 and 2b). This sequenced region of ORF2 was not available in GenBank for subtypes 3h and 3j. The 371 nucleotides of the genomic region coding for the methyltransferase was available for these subtypes and from three genotype 3f strains and was compared with the corresponding region of the genotype 3 strain from a Swedish pig (accession number EU360977). The nucleotide sequence of the Swedish pig strain diverged by 81–84% from subtype 3h and j in this region, whereas it was 88-89% similar to subtype 3f. Sequences of the same subtypes were similar in 88-90% to each other and in 84-86% to the sequences of the other subtypes. Based on this comparison, the Swedish strains found in clade 3-II may belong to subtype 3f.

Even if most genotype 1 sequences available in GenBank originate from India and Nepal, there was a geographical clustering with these strains and 1a, 1b and 1c strains from China, Japan and Kyrgyzstan forming one cluster, while another cluster was formed by 1d and 1e strains from Africa (Figure 2a). In our study, the majority of patients infected on the Indian subcontinent were infected by 1a. The sequences from isolates from India and Pakistan were similar to strains available in GenBank from these countries and from Nepal, while those from patients infected in Bangladesh were found on a separate branch. However, one strain from a patient infected with 1a in Tanzania was more similar to strains from India than to strains from Africa, and was thus an exception.

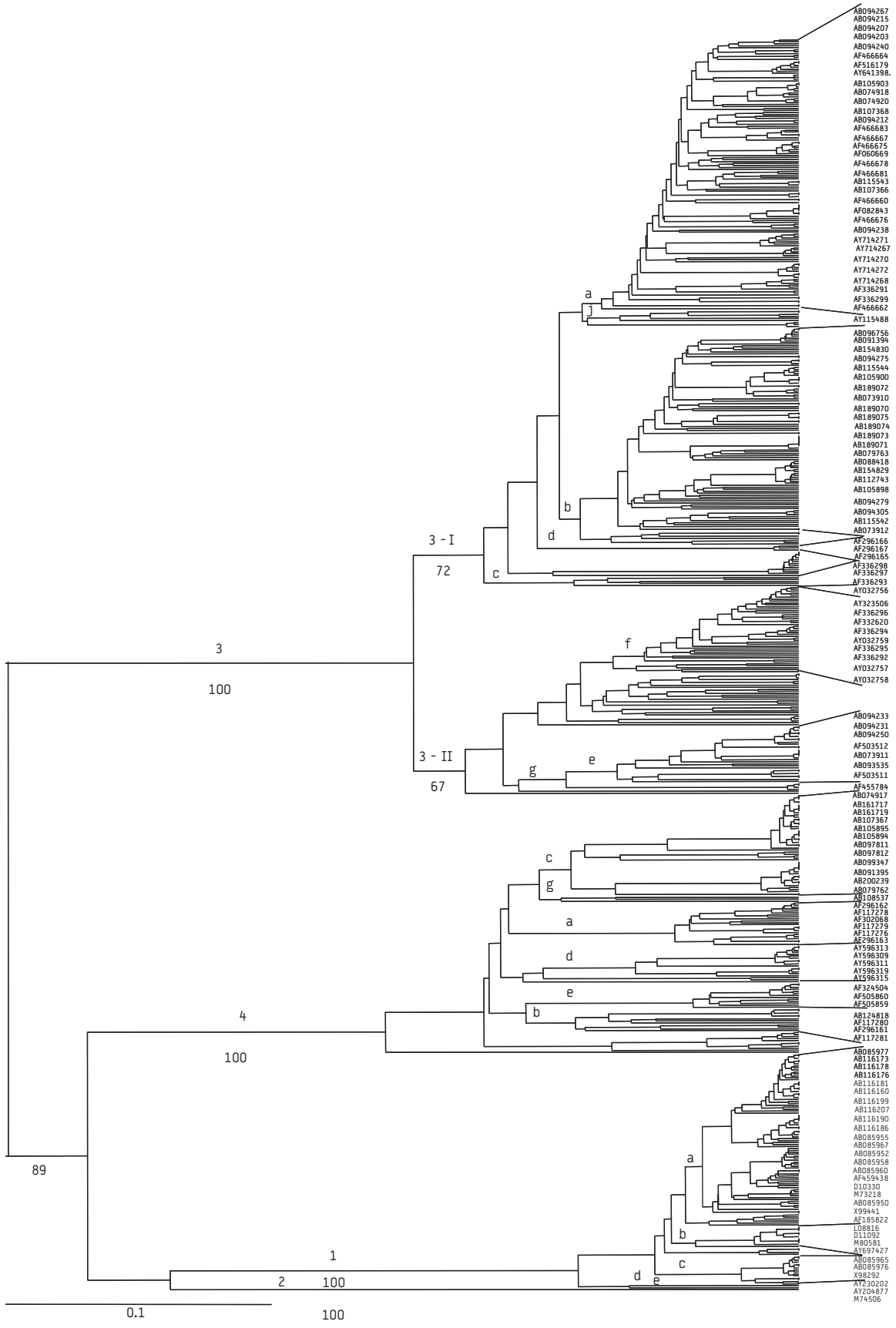
The strains found in clade 3-I were from Asia, mainly Japan, South Korea and China, and the US. Two strains in our study were found in this clade, one was from a Swedish pig herd the other was from a woman infected in Serbia/Montenegro and was similar to a Japanese strain (AB094212). All other genotype 3 isolates in this study were found in clade 3-II and clustered according to geographical origin (Figure 2b). There were two major subclusters within 3-II one was formed by 3f strains from Europe the other by subtype 3e and 3g strains from Japan, Mongolia and Kyrgyzstan. There was geographical clustering also within the clade formed by European isolates. One branch was formed by strains from Spain and France, one with strains from the Netherlands and France and one with Swedish and Danish strains intermixed with three strains from Spain (Figure 2b). The isolates from one Swede and one Dane infected in Spain were similar to strains from Spanish pigs. The strains from individuals infected in Sweden or Denmark were all similar to strains from Swedish and Danish pigs (Figure 2b). Pig strains from two Swedish breeding herds were found similar to Japanese and Mongolian strains within clade 3-II.

Discussion

Hepatitis E is not considered a major public health problem in non-endemic countries. This study confirms that most cases of hepatitis E in Scandinavia are imported from Asia. However, several cases have been infected in Europe, which is generally regarded as a non-endemic region. There have been rather few reported cases of autochthonous hepatitis E in European countries to date [11,19,21], although several reports have shown a seroprevalence ranging from 5 to 33% in the adult population in Europe, Japan and the US [11,15-18]. This indicates that hepatitis E is not uncommon in these countries, although most infections are subclinical or inapparent.

Figure 1

Phylogenetic tree based on 279 nucleotides of the capsid region of ORF 2 in 638 hepatitis E virus (HEV) strains



The branches with strains of known subtypes are marked with the subtype designation. The accession numbers of the strains with known subtypes according to Lu et al. 2006 [2] are given at the nodes with lines separating strains belonging to different subtypes. The figures at the internal nodes are boot strap values of 1,000 replicas.

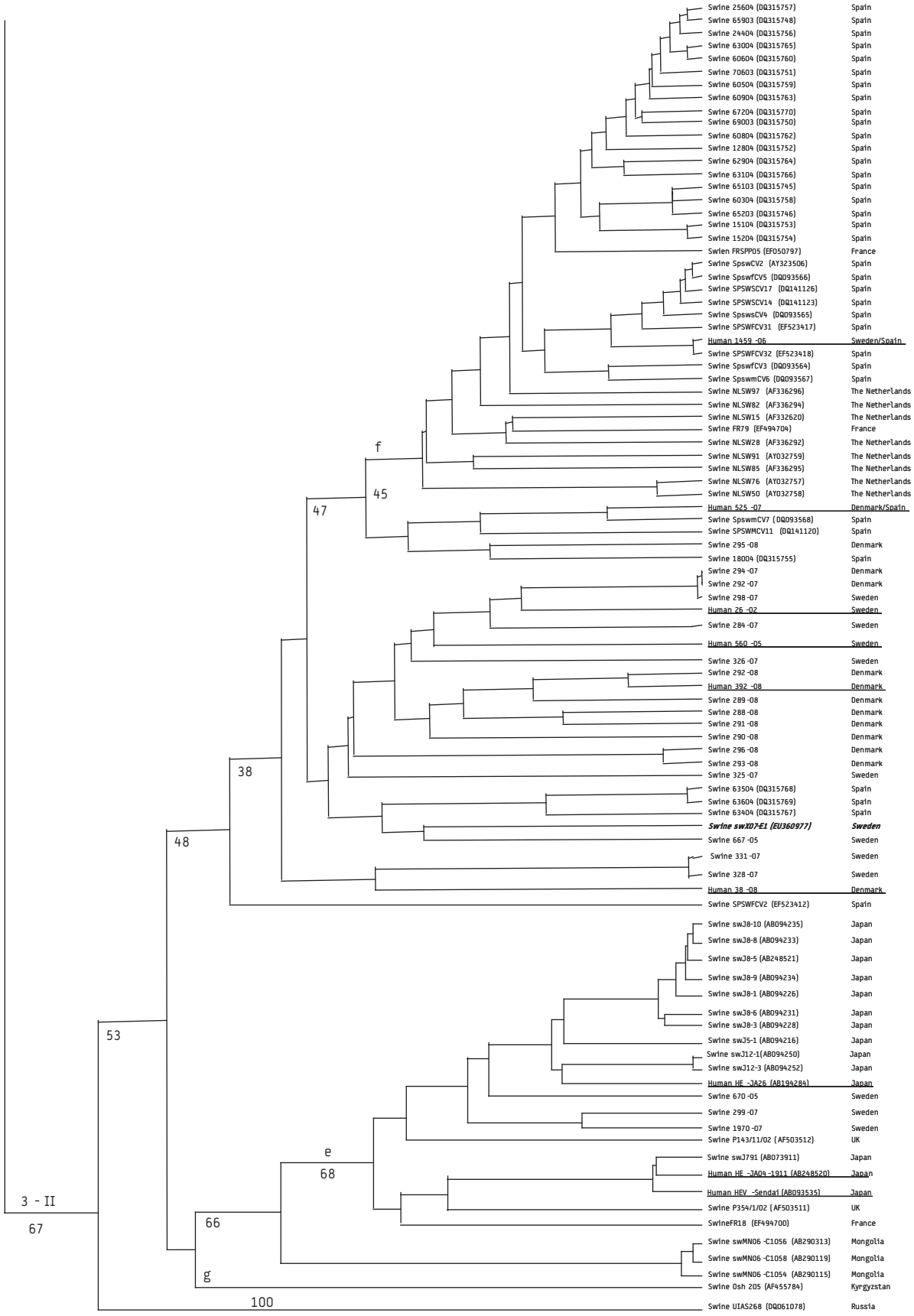
Figure 2a

Branch formed by genotype 1 strains of the phylogenetic tree shown in Figure 1

The strains described in this study are shown in bold. The figures at the internal nodes are boot strap values of 1,000 replicas.

FIGURE 2B

Branch formed by the genotype 3 strains forming branch 3-II shown in Figure 1



The human genotype 3 strains are underlined; those described in this study are in addition shown in bold. The HEV sequences from domestic pigs described in this study are shown in bold italic. The figures at the internal nodes are boot strap values of 1,000 replicas.

In our study HEV RNA was detected in 67% of sera sampled within three weeks after onset of illness from patients with this information known. This is in accordance with an HEV RNA detection rate of 56-59% in sera sampled 15 to 20 days after onset in Chinese patients with hepatitis E [23]. However, in our study HEV RNA was also detected in 18% of sera from patients with anti-HEV IgG only, which is an unexpectedly high frequency and has not been described earlier, since the presence of IgG in the absence of detectable IgM is considered a marker of past infection. Anti-HEV IgG may persist for several years after infection, but whether lifelong immunity is conferred remains uncertain [24]. There is only one serotype of HEV, but it is not known if reinfections induce IgG response only or if also the IgM levels become elevated. It is also not known if there is a viremic phase during a reinfection when the level of IgG is low and the immune response has been elicited towards another HEV genotype. Since most of the patients in this study were from Scandinavia and it is known that there is a rather high seroprevalence against HEV in Sweden and Denmark [16-18] it is possible that individuals with low level antibodies towards genotype 3 when infected with genotype 1 developed disease and viraemia with anti-HEV IgG elevation only.

In this study the only case infected by genotype 1 in Europe was epidemiologically linked to a case from Pakistan. All other patients infected in Europe were infected with genotype 3 strains. These individuals were mainly males and were 20–25 years older than the cases infected by genotype 1. This is in accordance with previous recent reports from the UK, France and Germany showing that genotype 3 is the autochthonous genotype of HEV, which gives disease mainly in males over the age of 50 [15,25].

Since autochthonous hepatitis E in humans in Europe has been caused by strains with 99–100% identity to European swine HEV [26], the suspected route of infection is through direct contact with pigs or other infected mammals or by foodborne transmission. Foodborne transmission was described in Japan in patients infected after consumption of undercooked pig liver or meat from wild boar or deer [27-29]. This route of infection may occur also in Europe since HEV has been detected in commercial pig liver sold in groceries and there is a high HEV seroprevalence in many pig herds [30,31]. Phylogenetic analysis of the genotype 3 strains revealed that most Asian and American strains belong to one major clade and that the European strains belong to another clade. There were also geographical clades of the genotype 3 strains, and strains from patients infected in Sweden and Denmark were similar to strains from Swedish and Danish piglets, while patients infected in Spain had genotype 3 strains similar to those of Spanish pigs. This pattern has previously not been described and enables a possible identification of the country of origin of the strain infecting the patient. This in turn may help to trace the source of infection and to identify a possible food item from that country.

Antibodies to HEV have been shown to be prevalent among blood donors and apart from the faecal-oral and foodborne route HEV may be transmitted also through blood or blood products as has been reported from Japan, Saudi Arabia, France, and the UK [32-34]. HEV has also been reported to be transmitted by organ transplantations [35]. Some organ-recipients have developed chronic hepatitis E infection [10]. In our study the viraemia lasted for a relatively long period in most patients. Thus, transmission of HEV by blood or blood products may theoretically also occur in Sweden. Bloodborne transmission may also occur through injecting drug use. In our study six of the patients were IDUs among those with HEV IgG but no detectable HEV IgM or RNA. More than 60% of Swedish IDUs have anti-HEV [17], which further supports the conclusion that hepatitis E may be transmitted parenterally in this cohort of individuals. The high seroprevalence indicates that most probably IDUs are frequently reinfected with HEV. The IDUs in our study were investigated for HEV infection due to elevated transaminases. However, genotype 3 reinfections have been shown not to induce elevation of liver enzymes or detectable HEV IgM among patients on hemodialysis in Japan [36], but HEV RNA was not looked for in these patients. It is thus not known if reinfections with genotype 3 cause viraemia. Lack of HEV RNA in the sera from the anti-HEV IgG positive IDUs may either indicate that reinfection with genotype 3 does not give rise to viraemia, or that a continuous low-level exposure to HEV keeps the immune status at a level preventing reinfection with HEV, or that there is a long lasting immunity with detectable HEV IgG.

Hepatitis E in developed countries has a natural history that differs from classical hepatitis E in endemic areas. In the study presented here we have shown that HEV genotype 3 strains are indigenous in Sweden and Denmark, with high similarity between strains infecting humans and pigs. Prospective studies are needed to define the incidence of autochthonous infections in Scandinavia. It is also important to determine whether and how the spread occurs from pigs to humans and if there are other animal sources for zoonotic transmission of HEV, since genotype 3 appears to be a primarily animal virus that crosses the species barrier. In conclusion, hepatitis E should thus be considered in the diagnosis of patients with acute hepatitis, regardless of travel history.

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Can the Swedish new variant of Chlamydia trachomatis (nvCT) be detected by UK NEQAS participants from seventeen European countries and five additional countries/regions in 2009?

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In 2006, a new variant of *Chlamydia trachomatis* (nvCT) was reported in Sweden. The nvCT contains a deletion that includes the targets for the *C. trachomatis* genetic diagnostic single-target systems from Roche Diagnostics and Abbott Laboratories. Roche and Abbott have now developed certified dual-target assays that can detect the nvCT. This study examined the nucleic acid amplification tests (NAATs) currently used (in 2009) for *C. trachomatis* detection in laboratories from 17 European countries and five countries/regions outside Europe that are participating in the United Kingdom (UK) National External Quality Assessment Service (NEQAS). It further examined changes in these laboratories' testing strategy during the period from 2006 to 2009, and their performance regarding nvCT detection. A UK NEQAS blinded nvCT specimen was distributed to all 283 participating laboratories, which were asked to analyse the specimen according to their routine *C. trachomatis* diagnostic protocols for endocervical swabs. BD ProbeTec was the most commonly used NAAT, followed by Cobas Amplicor, Cobas TaqMan, and Aptima. From 2006 to 2009, the use of Cobas Amplicor, which does not detect the nvCT, decreased, but it was still used by 22% (n=57) of responding participants in 59% of the countries, 54 of these 57 used it as first assay. Virtually all of the other participants detected the nvCT correctly. Laboratories using commercial or *in house* NAATs that do not detect the nvCT are encouraged to carefully monitor their *C. trachomatis* incidence, participate in effective internal and external quality assurance and controls schemes, and to consider changing their testing system.

Introduction

In most middle- and high-resource settings nucleic acid amplification tests (NAATs) are the most commonly used tests for rapid, highly sensitive and specific detection of *Chlamydia trachomatis*.

In 2006, a new variant of *C. trachomatis* (nvCT), which contains a 377 bp deletion in the cryptic plasmid, was reported in Sweden [1,2]. This deletion includes the genetic targets for commercially available single-target systems that were at the time used worldwide, namely the Amplicor *C. trachomatis/Neisseria gonorrhoeae* (CT/NG) test, the Cobas Amplicor CT/NG test, and the Cobas TaqMan CT/NG test (Roche Diagnostics), as well as the RealTime CT/NG test (Abbott Laboratories). Subsequently, nvCT was identified in high proportions (10-65%) in most counties across Sweden. The affected NAATs were used in two thirds of the Swedish counties, and many thousands of false negative samples were reported [3-5]. Previous studies, using *ompA* gene sequencing and a new multilocus sequence typing (MLST), showed that the nvCT seems to be of clonal nature, belonging to genotype E and displaying a unique MLST sequence type [3,5]. Other commercial genetic diagnostic systems that are internationally available, such as a) the BD ProbeTec ET (Becton Dickinson), b) the Aptima CT and Aptima Combo 2 (Gen-Probe), c) the artus *C. trachomatis* PCR Kit (Qiagen), d) the artus *C. trachomatis* Plus PCR Kit (Qiagen), and e) the CHLAMYDIA tr. Q - PCR Alert Kit (Nanogen), were able to identify the nvCT; these NAATs target(s) are a) the cryptic plasmid (outside the deletion), b) specific 23S and 16S *rRNA* sequences, c) the *ompA* gene, d) both the *ompA* gene and the cryptic plasmid (outside the deletion), and e) the cryptic plasmid (outside the deletion), respectively.

Both Abbott Laboratories and Roche Diagnostics have now designed new sensitive and specific dual-target assays, namely the Abbott RealTime CT/NG (Abbott; new version, CE mark-certified in January 2008) that targets another sequence of the cryptic plasmid in addition to the sequence affected by the nvCT deletion, and the Cobas TaqMan CT v2.0 (Roche; CE mark-certified in June 2008) that detects the chromosomal *ompA* gene in addition to the sequence affected by the nvCT deletion [4]. Despite active surveillance and a number of studies performed in many countries [6], only sporadic cases of nvCT have so far been reported outside Scandinavia, e.g. in France [7], Ireland [8], and Scotland [9].

The aims of this report were to describe the NAATs currently used (in 2009) for *C. trachomatis* detection in laboratories from European countries (n=17) and countries/regions outside Europe (n=5) that are participating in the United Kingdom (UK) National External Quality Assessment Service (NEQAS). It further aimed to identify changes in these laboratories' testing strategy during the

period from 2006 to 2009, and to highlight their performance regarding detection of the nvCT.

Materials and Methods

The UK NEQAS distributes clinically relevant and educational specimens for external quality assessment (EQA). In the UK NEQAS scheme for *C. trachomatis* detection ('Molecular'), at present there are 283 participating laboratories (274 laboratories from 17 European countries and nine laboratories from five countries/regions outside Europe). However, most of the participating laboratories are in the UK (see Table 1). For surveillance and educational purposes, a blinded EQA specimen (Specimen 9119 in UK NEQAS Distribution 2402, issued in January 2009, as well as blinded specimens of three wildtype *C. trachomatis* strains) containing the nvCT, 1.67-3x10^4 elementary bodies per ml of reconstituted lyophilised specimen, was prepared as previously described [10]. Vacuum integrity and moisture content (<2%) of the freeze-dried specimen were validated and approved before distribution to all 283 participants. The laboratories were requested to reconstitute the specimen in molecular grade water and analyse the specimen according to their routine protocols for detecting *C. trachomatis* from an endocervical swab.

Results

Nucleic acid amplification tests (NAATs) used in 2009 for C. trachomatis diagnostics and changes in testing strategy during 2006-2009

Of the 283 laboratories participating in the scheme, 261 (92.2%) returned results on the nvCT specimen. In 2009, BD ProbeTec was the most commonly used main NAAT (39.5% of laboratories), followed by Cobas Amplicor (20.7%), Cobas TaqMan (16.1%), and Aptima (5.7%) (Table 1).

During the period from 2006 to 2009, the use of Cobas Amplicor decreased. However, it was still used as main NAAT in 2009 by 54 participants in 13 (59.1%) of the countries. In contrast, the numbers of laboratories using Cobas TaqMan, Abbott, and Nanogen Q-PCR have increased (Figure 1).

Detection of the Swedish new variant of C. trachomatis (nvCT)

The reporting laboratories used more than seven different commercial assays, *in house* single-target (n=7) or multi-target (n=3) real-time PCR assays, or did not specify their method (n=8). Twelve of the laboratories used two different assays (Table 2). However, specific testing algorithms used for routine diagnostics in these laboratories were not accessible.

Table 1

Countries and laboratories, including the main diagnostic assay used, participating in the UK NEQAS scheme for molecular detection of *Chlamydia trachomatis* in 2009

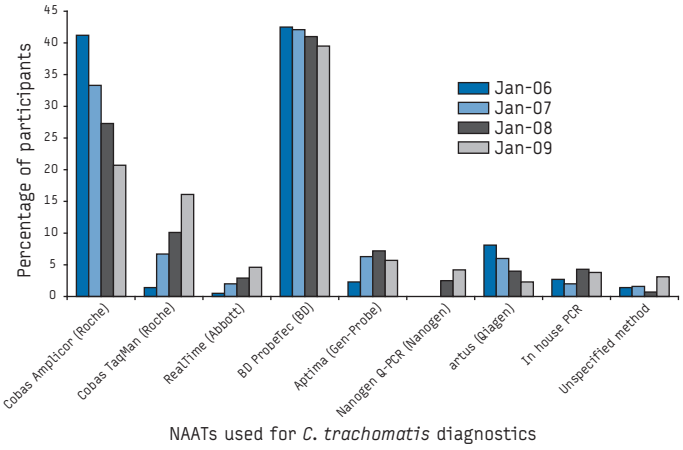
Country	No. of participating laboratories	Cobas Amplicor (Roche)[a]	Cobas TaqMan v2.0 (Roche)	Abbott RealTime (Abbott)	BD ProbeTec (Becton Dickinson)	Aptima Combo 2 (Gen-Probe)	Nanogen C. tr. Q-PCR Alert (Nanogen)	artus (Qiagen)[b]	In house single-target real-time PCR[c]	In house multi-target real-time PCR[c]	Unspecified method	Not returning results
Austria	5	2	-	-	1	-	-	1	-	-	1	-
Belgium	5	1	-	-	2	-	-	2	-	-	-	-
Croatia	1	1	-	-	-	-	-	-	-	-	-	-
Denmark	4	-	1	-	1	-	-	-	1	-	-	1
Finland	5	-	2	-	1	-	-	-	-	-	-	2
Germany	1	-	-	-	-	-	-	-	-	-	-	1
Greece	1	-	-	-	-	-	-	-	-	-	-	1
Hong Kong	2	1	-	-	-	-	-	-	-	-	-	1
Ireland	10	3	2	1	1	1	-	-	-	1	-	1
Israel	3	3	-	-	-	-	-	-	-	-	-	-
Italy	42	6	3	3	7	-	7	2	2	-	4	8
Kuwait	1	-	-	-	-	-	-	-	-	-	-	1
Macao	1	1	-	-	-	-	-	-	-	-	-	-
Malta	1	1	-	-	-	-	-	-	-	-	-	-
Netherlands	7	2	-	-	3	1	-	-	1	-	-	-
Norway	5	-	2	-	2	-	-	-	-	-	1	-
Portugal	5	-	-	1	2	-	1	-	-	-	1	-
Slovenia	2	-	1	-	-	-	-	-	1	-	-	-
South Africa	2	1	-	-	-	-	-	-	-	-	-	1
Sweden	8	-	1	1	4	-	-	-	-	-	-	2
Switzerland	14	10	-	2	1	-	-	-	1	-	-	-
United Kingdom	158	22	30	4	78	13	3	1	1	2	1	3
Total	283	54	42	12	103	15	11	6	7	3	8	22

[a] A few laboratories used Amplicor CT/NG (Roche). However, it was not possible to determine the exact number.
[b] Both artus *C. trachomatis* PCR Kit (*ompA* gene; Qiagen) and artus *C. trachomatis* Plus PCR Kit (*ompA* gene and cryptic plasmid; Qiagen) were used. However, it was not possible to determine how many laboratories used which kit.
[c] Details about *in house* assays were often reported and could not be accessed retrospectively.

Eighty percent (n=209) of the laboratories correctly reported the presence of *C. trachomatis* in the nvCT specimen (Figure 2). The majority (94%, 51/54) of the laboratories using Cobas Amplicor as their first assay reported a false negative result, as expected. However, one laboratory using Cobas Amplicor, an assay that can not detect the nvCT, reported a false positive result. Furthermore, two additional laboratories reported an equivocal result: They used Cobas Amplicor, which was negative, but to confirm their results used Aptima and Cobas TaqMan, which detected the nvCT correctly. The reasons for using this double testing strategy were not available. Presumably it does not reflect their routine diagnostics of all *C. trachomatis* samples. Furthermore, one laboratory using the Abbott system reported a negative result. All remaining laboratories reported a positive result (Figure 2).

Figure 1

Diagnostic assays (main NAAT) used by participating laboratories in the UK NEQAS scheme for molecular detection of *Chlamydia trachomatis* from 2006 to 2009*



*The total number of participating laboratories and laboratories returning results (in parenthesis) was 221 (100%), 263 (95.8%), 278 (100%), and 283 (92.2%), in 2006, 2007, 2008, and 2009, respectively.
NAAT: nucleic acid amplification test; NEQAS: National External Quality Assessment Service.

Discussion and conclusions

This report highlights the NAATs currently used (in 2009) for *C. trachomatis* detection in laboratories from 22 countries participating in the UK NEQAS scheme, alterations in their testing strategy during the period from 2006 to 2009, and their performance regarding detection of the nvCT.

Most of the laboratories (94%) using Cobas Amplicor, the second most common assay, as their first assay, reported an expected false negative result for the nvCT. However, two laboratories reported an equivocal result, i.e. negative with the Cobas Amplicor, but positive with an additional assay that detected the nvCT. One laboratory using the Cobas Amplicor assay reported a false positive result. This result suggests incorrect reporting either of the type of assay that was used or of the result, misinterpretation of the results, mix-up of specimens or contamination with other *C. trachomatis* strain or PCR amplicon.

One laboratory that was using the Abbott system and should have detected the nvCT, reported a negative result. A possible explanation could be that the older RealTime CT/NG test, the single-target assay that does not detect the nvCT, was used instead of the new Abbott RealTime CT/NG dual-target test. It is unlikely to reflect a sensitivity issue because the nvCT specimen contained a high number of elementary bodies per ml.

All other assays including the new Abbott RealTime CT and Roche Cobas TaqMan v2.0 performed well.

Laboratories that are still using Amplicor CT/NG, Cobas Amplicor CT/NG, and *in house* NAATs targeting the nvCT deletion in the cryptic plasmid are encouraged to monitor their C. trachomatis incidence in order to quickly identify unexplained significant declines in the normal or estimated local incidence and to alert reference centres about it. In addition, they are strongly encouraged to consider the feasibility of changing to a diagnostic method that can detect the nvCT, because using an additional NAAT on all negative samples is not feasible in the longer term.

Ideally, clinicians submitting samples to these laboratories should be objectively informed about the problem to diagnose the nvCT. An unexplained significant decline in incidence may be due to the emergence of nvCT. However, as other undetected mutants may emerge, monitoring of the incidence rate and participation of all laboratories in effective internal and external quality assurance and controls schemes are crucial.

Table 2

Combination of assays used in laboratories reporting using more than one assay for molecular detection of *Chlamydia trachomatis* in the UK NEQAS scheme in 2009

First assay	Second assay	No. of laboratories
Cobas Amplicor (Roche)	Cobas TaqMan v2.0 (Roche)	3
Cobas Amplicor (Roche)	Aptima Combo 2 (Gen-Probe)	1
Cobas TaqMan v2.0 (Roche)	Cobas Amplicor (Roche)	1
Cobas TaqMan v2.0 (Roche)	Aptima Combo 2 (Gen-Probe)	1
Cobas TaqMan v2.0 (Roche)	Nanogen C. tr. Q-PCR Alert (Nanogen)	1
BD ProbeTec (Becton Dickinson)	Cobas Amplicor (Roche)	1
BD ProbeTec (Becton Dickinson)	Aptima Combo 2 (Gen-Probe)	1
BD ProbeTec (Becton Dickinson)	In house single-target real-time PCR	1
In house single-target real-time PCR	artus (Qiagen)	1
Unspecified assay	Cobas Amplicor (Roche)	1

Based on the present study, it is obvious that a substantial number of laboratories in many European countries can still not detect the nvCT. However, the study only included laboratories participating in the UK-NEQAS scheme and thus gives a far from complete picture regarding the situation in the whole of Europe. The coverage in many participating countries was limited and it cannot be excluded that by selecting laboratories that are members of EQAS such as UK NEQAS a bias for high performance centres is introduced. Furthermore, no countries in eastern Europe were represented. In several of these countries, there are many shortcomings in the diagnosis of *C. trachomatis* and use of internationally available commercial NAATs is rare [11,12]. Some of the nationally produced and *in house* NAATs that are in use for diagnosis of *C. trachomatis* [11] may have their target in the nvCT plasmid deletion.

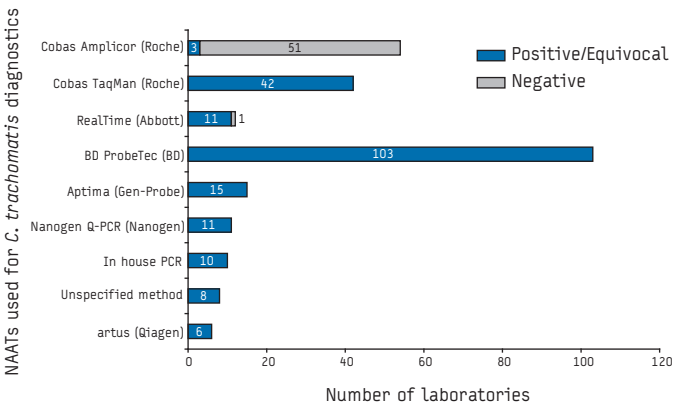
Even if the nvCT so far has been mainly detected in the Scandinavian countries, regular national and international surveillance, evaluation of the *C. trachomatis* diagnostic assays that are used, participation in external quality assessments including different diagnostic methods, and general evaluation of diagnostic guidelines are crucial. It cannot be excluded that the nvCT or other undetected mutants, e.g. *C. trachomatis* variants that do not contain the cryptic plasmid [13], are in a stage of early transmission in several countries. These mutants have a diagnostic selective advantage, can spread rapidly due to an accumulation of undetected and untreated cases that escape contact tracing, and may even possess biological advantages.

In comparison with wildtype *C. trachomatis* strains, no significant differences in symptoms and signs, sequelae, antimicrobial susceptibility, bacterial growth characteristics, cells/DNA load in NAAT samples have been associated with nvCT [3,4,14]. However, the incidence of nvCT in many Swedish counties has remained high and is even increasing in several counties using BD ProbeTec, an assay targeting a sequence outside the nvCT deletion. It has still not been ruled out whether the nvCT possesses particularly strong survival capabilities or other biological advantages over wildtype *C. trachomatis* strains. Further studies will soon be reported, which undertake a comprehensive phenotypic and genetic characterisation of the nvCT strain, estimate statistically the time point of emergence of the nvCT in certain Swedish counties, and follow the transmission of the nvCT in several Swedish counties, using Roche/Abbott and BD ProbeTec.

In general, more frequent and comprehensive internal and external quality assessment and quality assurance of different diagnostic methods may be required for many infectious agents worldwide, not just for *C. trachomatis*. The distributed control samples included in these exercises should reflect not only currently transmitted strains, but also temporally, geographically and genetically diverse strains. Ideally, most NAATs would use several species-specific targets in multicopy essential genes, giving diagnostic assays high sensitivity, specificity, and preventing false negative results due to different types of mutations.

FIGURE 2

Results and diagnostic method for detection of the new variant of *Chlamydia trachomatis* (nvCT) from 261 NEQAS laboratories in 22 countries in 2009



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