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Landscape and climatic characteristics associated with human alveolar echinococcosis in France, 1982 to 2007

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Human alveolar echinococcosis (AE) is a severe hepatic disease caused by *Echinococcus multilocularis*. In France, the definitive and intermediate hosts of *E. multilocularis* (foxes and rodents, respectively) have a broader geographical distribution than that of human AE. In this two-part study, we describe the link between AE incidence in France between 1982 and 2007 and climatic and landscape characteristics. National-level analysis demonstrated a dramatic increase in AE risk in areas with very cold winters and high annual rainfall levels. Notably, 52% (207/401) of cases resided in French communes (smallest French administrative level) with a mountain climate. The mountain climate communes displayed a 133-fold (95% CI: 95–191) increase in AE risk compared with communes in which the majority of the population resides. A case–control study performed in the most affected areas confirmed the link between AE risk and climatic factors. This arm of the study also revealed that populations residing in forest or pasture areas were at high risk of developing AE. We therefore hypothesised that snow-covered ground may facilitate predators to track their prey, thus increasing *E. multilocularis* biomass in foxes. Such climatic and landscape conditions could lead to an increased risk of developing AE among humans residing in nearby areas.

Introduction

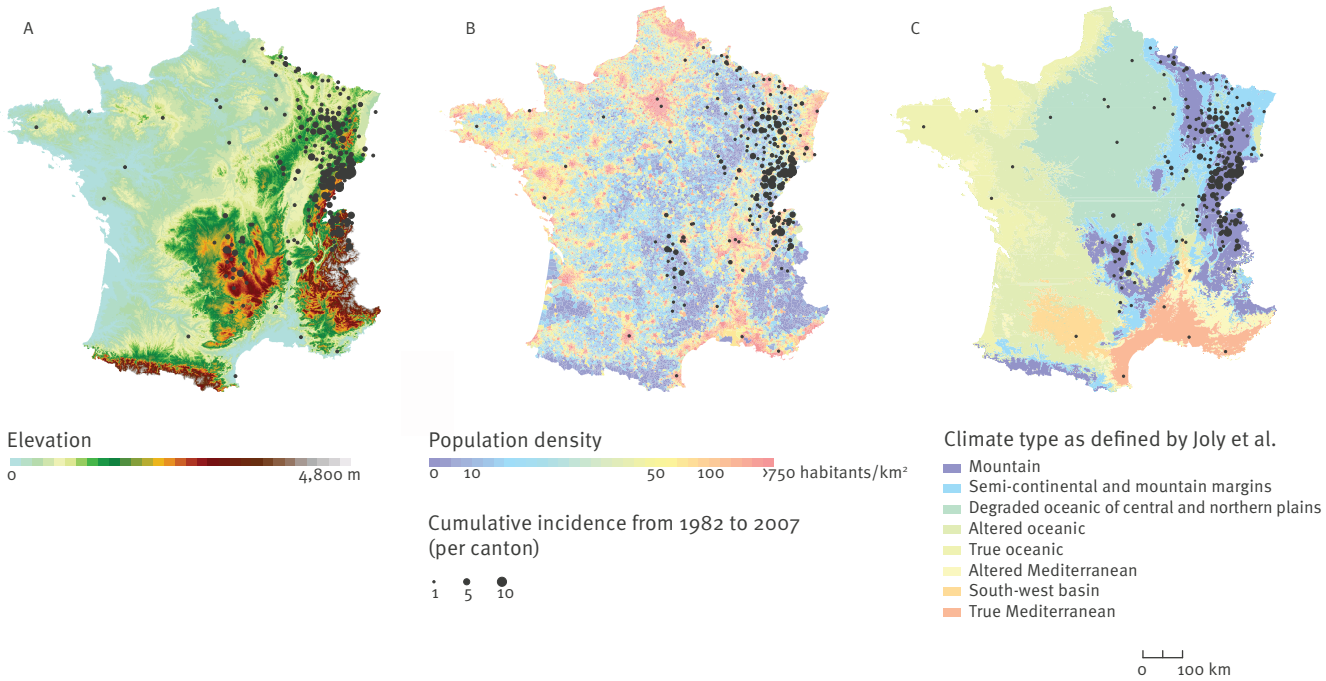
Echinococcus multilocularis is a cestode parasite that exhibits a dixenic life cycle involving circulation between canids and rodents. In France, the *E. multilocularis* sylvatic life cycle involves foxes (the main definitive host) and rodents such as *Arvicola terrestris*, *Microtus arvalis*, *M. agrestis* or *Ondatra zibethicus* (the main intermediate hosts). Humans represent an aberrant host of the parasite, although they sometimes become infected with *E. multilocularis* larvae after ingesting parasite oncospheres. When *E. multilocularis* infects humans, *E. multilocularis* metacestode cells proliferate in the liver and eventually lead to alveolar echinococcosis (AE), a rare but severe hepatic disease resembling a slow-growing liver cancer [1].

A study by the EurEchinoReg network showed that 235 of 559 (42%) European AE cases reported from 1982 to 2000 were observed in France [2]. Since 2000, the French registry of human AE cases has been maintained by the FrancEchino network. In total, 407 human cases were identified from 1982 to 2007 [3]. In France, for this period, high-risk areas included the Massif Central and north-eastern regions of the country (Figure 1), where most cases either resided in rural communes (smallest French administrative level) or resided in towns but worked as farmers or tended gardens [3].

Although behavioural [3-8] and genetic [9-11] elements have been identified as risk factors of human AE, they largely fail to explain the geographical distribution of the disease. For instance, in China, specific landscapes, such as alpine meadows, are associated with an increased prevalence of human AE [8,12]. In Europe and especially in France, an increase in the proportion of grassland is associated with vole density outbreaks.

Figure 1

Location of human alveolar echinococcosis cases with regard to elevation (A), population density (B) and climate type (C), France, 1982–2007 (n = 401)



Panel A illustrates that elevation is not the main factor associated with alveolar echinococcosis (AE) distribution. Panel B shows that cases were not located in densely inhabited areas. Panel C illustrates that AE cases were located in areas with a cold climate.

Joly et al. [21] described the types of climate as follows: Type 1 is characterised by a high number of rainy days, high cumulative precipitation levels, minimal average temperature, maximal number of days with temperatures < –5 °C, minimal number of days with temperatures >30 °C, maximal interannual variability of rainfall in July and maximal interannual variability of temperatures in January and July. Type 2 is a transition from Type 1 to Type 3. Type 2 is characterised by cold January temperatures, slightly lower precipitation levels and less frequent precipitation than Type 1, and a low ratio between autumn and summer rainfall levels. Type 3 is characterised by intermediate temperatures and low precipitation levels, especially during the summer. For Type 3, the interannual variability of rainfall is minimal, while the interannual variability of temperature is high. Other climate types were of little interest regarding AE incidence patterns and were mostly characterised by warmer winter temperatures.

For ethical reasons, case locations are shown at the canton level (fourth French administrative division).

Dietary specialisation has been described in foxes during these outbreaks leading to an increase in the burden of *E. multilocularis* in foxes [13-15]. Nevertheless, human AE case distribution does not correlate with that of grasslands, foxes or rodents. In particular, human AE is very rarely diagnosed in western France, despite the apparent presence of favourable transmission factors regarding landscapes and hosts [13]. Thus, the key environmental and geographical factors associated with human AE transmission remain poorly understood.

In this study, we hypothesised that the completion of the sylvatic life cycle and transmission of the parasite from the animal hosts to humans depend on the climatic and landscape conditions. We first assessed the association between human AE cases and environmental data at a national scale in France. In the French regions with the highest AE incidence rates, we then compared the habitat environment of AE cases with that of randomly selected residences at a local scale.

Methods

This study included all AE cases diagnosed in France from 1982 to 2007. The analyses were performed in two parts. First, at the national level, we assessed AE cumulative incidence in each commune, considering several demographic and environmental variables (e.g. elevation, landscape and climate). France is divided into five nested administrative levels (listed from largest to smallest geographical divisions: régions, départements, arrondissements, cantons and communes). Second, we conducted a case–control study in the nine most affected French départements to compare case habitats (in terms of elevation, landscape and climate) with randomly selected control habitats at various buffer sizes (i.e. circular areas centred over each habitat with a 500 m, 1000 m, 1500 m and 2000 m radius [12,16]).

Data acquisition

Case definition and data collection

Cases were defined as patients with compatible clinical and epidemiological histories and imaging findings or positive specific serology for AE.

Case data were obtained from the FrancEchino network registry, which is supported by the French Institute for Public Health Surveillance (Institut de Veille Sanitaire, InVS). This population-based registry actively collects French AE case data as previously described [17].

Addresses of the cases were registered in a separate anonymous database according to French regulation (Comité National pour l'Informatique et les Libertés and Comité de Protection des Personnes) in the context of biomedical research [18].

We interviewed all pathologists, parasitologists, public university hospital pharmacy staff (who are the only people allowed to deliver albendazole or mebendazole for AE treatment in France) and medicine, radiology and abdominal surgery hospital department staff who treat AE patients.

Environmental data

Demographic data were obtained from the French National Institute of Statistics and Economic Studies (INSEE) [19].

Land cover data were obtained from the European Commission programme to coordinate information on the environment (CORINE) land cover (CLC) 2006 map [20]. Pixel size was 25 m x 25 m. The 44 CLC classes are typically categorised into five groups (agricultural areas, forests and semi-natural areas, artificial surfaces, wetlands, water bodies and open spaces without vegetation). We chose to detail the group 'agricultural areas' into three subgroups (arable and permanent cultures, heterogeneous agricultural areas and pastures) because these types of landscape environments play an important role in the life cycle of foxes and voles, as observed in China and eastern France [12,13,15,16]. We also subdivided the group 'forests and semi-natural areas' into three subgroups (broad-leaf and mixed forest, coniferous forest and shrub and herbaceous vegetation). The group 'artificial surfaces' was left unmodified, while the three remaining groups were recategorised into the class 'other'. For each analysed territory (either commune or buffer around habitats), we calculated the percentage of each CLC class and the mode, i.e. the CLC class covering the largest part of the territory.

Climate data were obtained from Joly et al. [21]. Briefly, the authors provided a set of 15 raster climate maps (with a precision of 250 m x 250 m) that we used to extract 14 variables as follows: annual mean temperature, number of cold days (with minimum temperature less than –5 °C), number of warm days (with maximum temperature above 30 °C), difference in mean temperature between January and July, cumulative annual precipitation, number of rainy days in January, number of rainy days in July, difference in precipitation levels between January and the entire year, difference in precipitation levels between July and the entire year, interannual variability in temperature in January, interannual variability in temperature in July, interannual variability in precipitation in January, interannual variability in precipitation in July, (September + October) precipitation/July precipitation, and an integrative climate typology classifying French climates into eight types (Type 1: mountain; Type 2: semi-continental and mountain margins; Type 3: degraded oceanic of central and northern plains; Type 4: altered oceanic; Type 5: true oceanic; Type 6: altered Mediterranean; Type 7: south-west basin; and Type 8: true Mediterranean) (Figure 1). For each analysed territory (commune or buffer around habitats), we calculated the percentage covered by each variable modality and extracted the mode of each variable.

Elevation data were obtained from the Shuttle Radar Topography Mission (ArcGIS data and maps CD-ROM, Environmental Systems Research Institute (ESRI), Redland, CA, United States) with a precision of 100 m x 100 m.

Selection of control residences

Control residences were selected from the non-professional French telephone directory by applying a computerised algorithm based on a uniform distribution on page, column and line. We randomly selected two addresses from 10,000 inhabitants in each of the nine most affected départements, i.e. the départements with an AE incidence rate greater than the upper limit of the 95% confidence interval (CI) of the mean incidence rate.

Geographical location

Address location at the time of diagnosis was determined using Geoportail [22]. We confirmed the location data using the cadastre registry [23].

When the location of a case (or control) residence could not be accurately determined (e.g. in a hamlet with no street name and no house number), the place of residence was arbitrarily selected as the centre of the corresponding inhabited area. Therefore, the maximum imprecision for case and control residences was less than 500 m.

Cases with insufficient address data were excluded from the buffer analysis.

Statistical analysis

Alveolar echinococcosis distribution in communes at a national scale

Global spatial clustering analysis of AE-affected communes was performed using the Moran's I coefficient.

The relationship between the AE case number of each French commune and each covariable was subjected to univariate analysis using a general linear model (GLM). Univariate quasi-Poisson models were constructed using the log population of communes as an offset and considering overdispersion patterns. The correlations between covariables were assessed using the Spearman coefficient. Only significant variables at 95% risk were retained for further analyses.

Multivariate analyses were performed according to the methodology developed by Breiman (classification and regression tree (CART) analysis) [24] to classify the communes based on AE risk. This methodology only retains the main covariates among the collinear variables, thereby generating a tree in which the terminal nodes represent classes of communes with common characteristics. The resulting classification was analysed using a quasi-Poisson GLM to estimate standardised incidence ratios.

Case–control study of habitats at a local scale

A univariate comparison of the buffers surrounding case and control habitats was performed using the Wilcoxon test for quantitative variables and the Fisher's exact test for categorical variables. Only significant variables were retained for further analyses.

Multivariable analyses were carried out using the hierarchical ascendant classification on the multiple correspondence analysis results. We determined the most homogeneous land cover and climate groups independent of the AE status of the residents within the buffers [25], using v.test to describe how each variable influences each category. The AE incidence rates of the resulting classes were then analysed using a logistic model. The associated odds ratios were then estimated.

Statistical analysis was performed using R 3.0.2 software (R foundation for statistical computing, Vienna, Austria) with the Factominer and R-PART packages. The regression models were compared using Akaike information criterion. The test results were interpreted applying a fixed threshold at $\alpha = 0.05$.

Cartography

Spatial representation of the AE data was generated using ArcGIS 9.3 software. Simple Voronoï maps were used to enhance the clarity of the buffer categories and respect ethical concerns. The commune-level results are shown aggregated at the canton level for ethical reasons.

Results

Alveolar echinococcosis distribution at a national scale

The commune of residence was identified for 389 of the 407 cases registered by the FrancEchino network between 1982 and 2007. At a national level, the communes of case residences were found to be clustered (Moran's I index = 0.6 ($Z = 26.48$, $p < 0.01$)).

Marked correlations were often found between variables, especially when belonging to the same group. For example, most correlation coefficients between demographic variables were > 0.7, most correlation coefficients between altitude variables were > 0.7, and at least one correlation coefficient was > 0.5 in-between climate variables. In contrast, the land cover variables exhibited weaker intragroup correlations (< 0.5). Additionally, the demographic variables, the altitude variables and eight of the 15 climate variables displayed overdispersion.

The GLM univariate analysis of AE-affected and non-affected communes revealed a disparity for all variables except ((September + October) precipitation/July precipitation) and two land cover classes (heterogeneous agriculture and other).

Multivariable CART analysis showed that the climate variables provided the best discrimination between AE and non-AE communes (Figure 2A, Table 1). Five classes of communes were subsequently defined. Class 1, which represented 52% (n = 201) of the cases but only 3% of the French population (1,833,904 inhabitants), comprised communes with a mountain climate (i.e. Type 1 climate, as defined by Joly et al. [21]). The standardised incidence ratio was 133 (95% CI: 95–191), compared with Class 5 (reference class). Class 5 represented 84% of the French population (46,201,895 inhabitants) but only 38 (9.8%) cases. This class comprised communes characterised by climate types other than mountain or semi-continental and a mean annual temperature above 9.4 °C. The other classes exhibited intermediate climatic conditions (Figure 2).

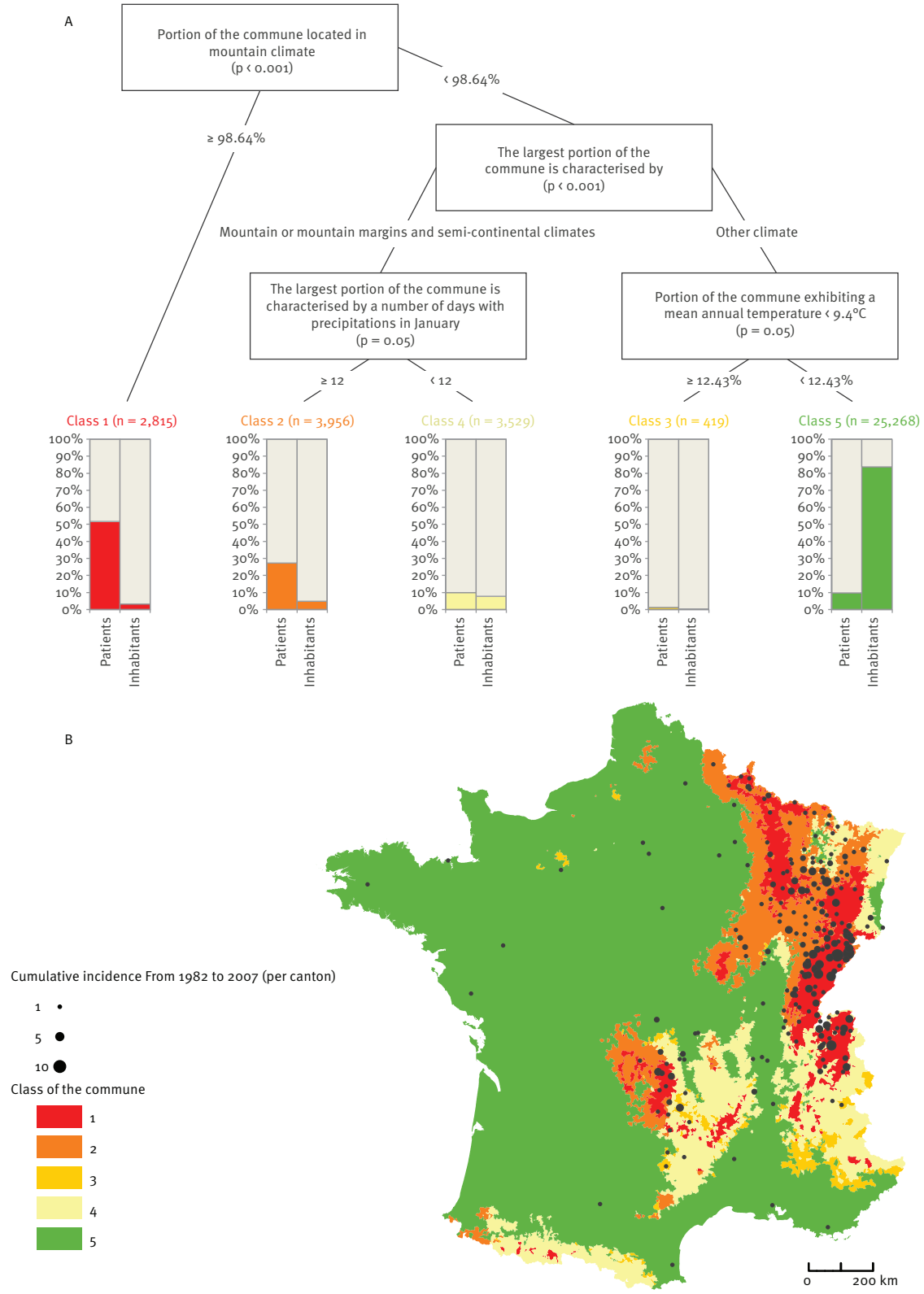
Case–control study of the respective residences in the nine most affected French départements

Of the 407 AE cases, 270 lived in the nine most affected départements. The commune of residence was identified for 266 of these cases. A precise address (i.e. hamlet or street name and number) was available for 196 of these cases. Cases with a precise address did not significantly differ from cases with non-specific addresses regarding mean age, sex ratio, clinical status or occupation (data not shown). The 74 cases without a precise address were excluded from the buffer analyses. A total of 539 control habitats were selected.

At this local scale (500 m, 1,000 m, 1,500 m and 2,000 m buffer radii as well as the communal level), the best model corresponded to a 500 m buffer radius and yielded five categories of habitat surroundings based on the Akaike information criterion (Table 2). Compared with the reference category (Category 1), only Categories 4 and 5 were associated with a significant increase in AE risk (OR: 2.40, 95% CI: 1.38–4.13 and OR: 2.72, 95% CI: 1.75–4.26, respectively) (Table 3). Category 4 consisted of habitats located in areas

Figure 2

National-level classification of French communes where residents are at high risk of developing alveolar echinococcosis, 1982–2007



Panel A demonstrates the commune class analysis results. The terminal nodes of the classification and regression tree (CART) analysis results correspond to the five classes of commune. The percentage of cases (left side) and inhabitants (right side) residing in each class are indicated. Class 5 is the reference class, where most of the French population is located. In contrast, most of the alveolar echinococcosis cases were located in Class 1 and Class 2 communes. Localisation of these classes and the cumulative cases during 1982 to 2007 are shown in panel B. For ethical reasons, case locations are shown at the canton level (fourth French administrative division).

Table 1

Classification of risk of alveolar echinococcosis in French communes at national level, 1982–2007

Commune class	Standardised incidence ratio[a] (95% CI)
Intercept	8.22×10^{-7} ($6.71 \times 10^{-7} - 1.83 \times 10^{-6}$)
1	133.26 (91.97–199.77)
2	48.47 (32.46–4.38)
3	28.52 (8.51–71.99)
4	11.07 (0.71–18.27)
5	1 (NA)

CI: confidence interval; NA: not applicable.

Each terminal node of the classification and regression tree (CART) analysis represented a class of French communes. Compared with Class 5, where most of the French population resides, persons residing in the communes of Classes 1, 2 and 3 were at significantly higher risk of contracting alveolar echinococcosis. The communes of Class 1 exhibit a mountain climate. The communes of Class 2 exhibit a rainy semi-continental climate, while the communes of Class 3 exhibit a non-mountainous, non-continental and cold climate.

[a] Standardised incidence ratio for each commune class displayed in Figure 2A.

with the greatest slope and elevation levels as well as a land cover dominated by broad-leaf and mixed forests. Category 5 included habitats characterised by pastures. Both Category 4 and Category 5 were also characterised by a very cold (≥25 days with cold temperature (less than −5 °C)) and humid (total annual precipitation ≥1,150 mm) mountain climate. The spatial distribution of these categories of habitat environments is shown in Figure 3.

Discussion

In this study, we identified a significant association between climatic factors and human cases of AE in France from 1982 to 2007 at national and local scales. The map indicating human AE cases and the principal climates in France clearly highlights this association (Figure 1). Nevertheless, we observed some discrepancies, such as a low incidence of AE in the southern mountainous areas of France despite the cold climate. When analysing the data at the national and local scales, we were able to include additional parameters such as rainfall effects and land cover composition. Applying this analysis, we also observed an almost-perfect fit between geographical and epidemiological data. Overall, our findings indicate that an increased risk of contracting AE in France is associated with residing in areas exhibiting the coldest winters, marked rainfall levels throughout the year and, to a lesser degree, forest and pasture land covers. Areas exhibiting a mountain climate that did not report cases of human AE, such as the southern Alps, were characterised by winters with relatively lower levels of rain and snow precipitation.

Interestingly, the distribution area of the principal hosts involved in the *E. multilocularis* life cycle in France extends far beyond the high-risk areas shown in our study. Indeed, foxes and *E. multilocularis*-permissive rodents are found in every rural area countrywide [13]. Therefore, climate does not act only by limiting the distribution of the intermediate and definitive host populations.

Our results show that AE high-risk areas are much more associated with winter temperatures and high precipitation levels than summer climatic conditions. Indeed, residing in the majority of French territories with an oceanic climate, cool summer temperatures and temperate winters was not associated with high human incidence rates of the disease. This association between AE incidence and residing in areas where winters are cold and humid corroborates previous studies conducted in China [26] and France [27]. Furthermore, several studies have highlighted a link between climatic conditions and *E. multilocularis* biomass in foxes. In Germany, foxes living in agricultural regions with high levels of precipitation harboured the greatest parasite burden [28,29]. Furthermore, the degree of fox infection was negatively associated with annual temperatures in the German federal state of Saxony-Anhalt [30]. The mechanism by which cold and humid winters enable successful completion of the parasite life cycle remains to be explained. One hypothesis demonstrated in Alaska suggests that snow acts as a parasite life cycle facilitator [31]. The overall distribution of AE observed throughout the northern hemisphere supports this hypothesis [2,32]. Regular snowy periods during the winter may greatly affect the predator–prey relationship by assisting foxes to capture rodents and thus increasing the degree of fox infection during late winter and early spring [33]. Climatic conditions may also support the conservation of *E. multilocularis* eggs in the environment. Although cold temperatures do not affect egg viability, hot and dry episodes during the summer can easily destroy the eggs [34]. Therefore, regions exhibiting cold winters, cool summers and a humid climate throughout the year may best support the *E. multilocularis* life cycle. The relatively low incidence of human AE in the southern Alps and eastern Pyrenees may be due to the relatively hot and dry summers, despite the cold winters exhibited in these regions. Additionally, these mountain regions are fragmented with deep valleys, which reduce the amount of contact between different fox populations. This may support autochthonous AE foci, as shown in northern Italy [35], or impede infected foxes from importing the parasite following the extinction of local *E. multilocularis* populations. In southern France, only the western part of the Pyrenean mountains and a few patches in the southern Alps and Massif Central exhibit climatic conditions conducive to AE transmission. In these areas, AE transmission may not be perennial because the foci are relatively small and there is limited host exchange between the foci and other permissive areas [36].

Table 2

Classification of land cover and climate in buffers of a 500 m radius around habitats in the nine départements in France most affected by alveolar echinococcosis, 1982–2007

Variable	v.test[a]
Category 1 (48 patients, 193 controls)	
Mean annual temp ≥ 9.4 and < 10.4 °C	**17.147148**
Number of days with cold temp (< −5 °C) ≥ 14 and < 25	**13.488042**
Mean elevation < 304 m	**12.684608**
Number of rainy or snowy days in January ≥ 13	9.087546
Total annual precipitation ≥ 940 and < 1,150 mm	8.942823
Total annual precipitation ≥ 800 and < 940 mm	8.914855
Range elevation < 61 m	7.851899
Number of rainy days in July ≥ 9	7.56979
Mountain margins and semi-continental climate (type 2)	6.171585
Difference between July and January mean temp. ≥ 14.7 and < 15.7 °C	6.088195
Category 2 (4 patients, 16 controls)	
Total annual precipitation ≥ 710 and < 800 mm	9.863617
Difference between July and January mean temp. ≥ 14.7 and < 15.7 °C	7.757912
Number of rainy days in July ≥ 6 and < 7	6.857535
Degraded oceanic climate (type 3)	6.857535
Altered oceanic climate	6.6954
Category 3 (45 patients, 179 controls)	
Difference between July and January mean temp. ≥ 16.9 °C	**16.971824**
Mean annual temp. ≥ 10.4 and < 11.4 °C	**14.648773**
Number of days with hot temp. (> 30 °C) ≥ 15 and < 23	**14.494255**
Number of rainy days in July ≥ 8 and < 9	**12.257087**
Number of rainy or snowy days in January ≥ 9 and < 11	6.288545
Number of days with cold temp. (< −5 °C) ≥ 14 and < 25	5.616076
Category 4 (31 patients, 52 controls)	
Range elevation ≥ 124 and < 230 m	**13.688276**
Mean slope ≥ 12.5%	**13.031426**
Range slope ≥ 29.9	**10.958377**
Range elevation ≥ 230 m	9.745691
Number of days with cold temp. (< −5 °C) ≥ 25	8.943433
Range slope ≥ 19.3 and < 29.9	8.873805
Total annual precipitation ≥ 1,150 mm	8.865216
Broad-leaf and mixed forest	8.100202
Mean slope ≥ 6.7 and < 12.5	7.5422
Mountain climate (type 1)	7.313873
Category 5 (67 patients, 99 controls)	
Mean annual temp. < 9.4 °C	**20.812435**
Number of days with cold temp. (< −5 °C) ≥ 25	**13.021636**
Difference between July and January mean temp. ≥ 15.7 and < 16.9 °C	**10.621712**
Mountain climate (type 1)	9.925993
Number of days with hot temp. (> 30 °C) < 4	9.340607
Mean elevation ≥ 740 m	9.256912
Number of days with hot temp. (> 30 °C) ≥ 4 and < 9	8.237715
Number of rainy days in July ≥ 9	7.154073
Total annual precipitation ≥ 1,150 mm	6.75911
Pastures	5.155408

Entries In bold are variables with a v.test > 10.

[a] v.test describes how the variable influences a category. It specifies how much the proportion of the modality of a variable within the category differs from the proportion in other categories. A variable modality was considered to be specific for a category when |v.test| > 3 with p < 0.01. For more readability, we only show the variables with the most influence producing a positive impact on the category.

Table 3

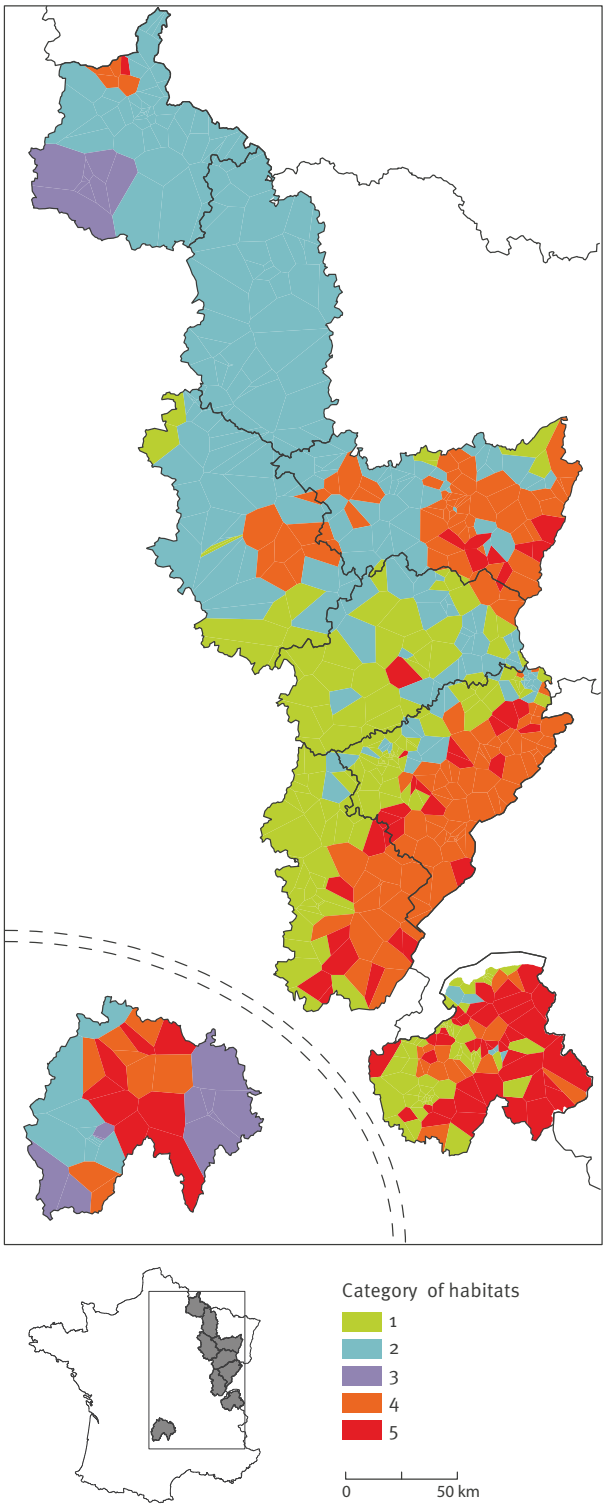
Odds ratios associated with land cover and climate classification of the 500 m-radius buffers in the nine French départements most affected by alveolar echinococcosis, 1982–2007

Category of habitat surroundings	Number of patients/ controls	Odds ratio (95% CI)
1	48/193	1 (NA)
2	4/16	1.01 (0.28–2.89)
3	45/179	1.01 (0.64–1.59)
4	31/52	2.40 (1.39–4.13)
5	67/99	2.72 (1.75–4.26)

CI: confidence interval; NA: not applicable.

Figure 3

Map of the land cover and climate classification of the 500 m-radius buffers in the nine French départements most affected by alveolar echinococcosis, 1982–2007



Hierarchical clustering on principle components analysis determined five categories of habitats. Significant disparities were observed between the residence location of cases and controls even at this fine scale. Category 1 is the reference class (habitats where residents were least at risk of developing alveolar echinococcosis (AE).

Persons living in Category 4- and 5-type habitats were at higher risk of contracting AE compared with individuals residing in the rest of the area (see Tables 2 and 3). For ethical reasons, a Voronoï polygon map is shown instead of buffers.

At a local scale in China, Pleydell et al. observed marked variations in AE prevalence between villages located less than 10 km apart in Zhang County, Ningxia, China, in an area spanning about 4,000 km² [16]. Moreover, human AE cases in France are irregularly dispersed, as hotspots of higher endemicity were observed at a finer scale within hyperendemic clusters [37]. Our study shows that the heterogeneous pattern of human AE incidence correlates with climate and land cover distribution at a local scale. AE incidence is also closely associated with human life habits, which change over time. Depending on the country, the most frequent individual AE risk factors include a person's sex, region of residence, agricultural or pastoral occupation, dog ownership and gardening practices [3,4,6-8]. In neighbouring Germany, the highest-risk behaviour associated with AE was farming, and gardeners were only at risk if they cultivated leaf or root vegetables [4]. These behavioural factors may partially explain the heterogeneous pattern of human AE distribution.

Our study of human AE cases complements investigations focusing on the sylvatic life cycle of the parasite [12,36-38]. From 2001 to 2005, in an area of only 900 km² of the French Ardennes, Guislain et al. observed a north/south gradient of infestation among foxes ranging from 20% to 80%, respectively [38]. In four zones in the canton of Zurich in Switzerland, approximately 1 km in radius separated by less than 15 km, the prevalence of *E. multilocularis* in foxes varied from 11.2% (95% CI: 12.7–27.2) to 60.7% (95% CI: 40.6–78.5) [39]. The populations of intermediate hosts, such as voles, fluctuate partially due to human behaviour [40] and thereby act as a metapopulation, as observed in China by Pleydell et al. [16]. This observation therefore explains the observed variability in voles' contribution to fox alimentation and subsequent variability in fox infestation [12].

We acknowledge that this study has several limitations. First, our approach addressed the risk of contracting AE among humans over an extended time

period in a relatively expansive area. Due to the vast area and extended period, the human data are probably not 100% exhaustive. The process of data collection [17] was based on repeated inquiries with all health professionals potentially involved in case management, ranging from those diagnosing the disease to those delivering specific treatments. As AE incidence is low in most regions of France, the disease is irregularly recognised by physicians, which may lead to under-diagnosis. However, diagnostic imaging and serological testing has greatly improved over the last few decades [41] and persistent misdiagnosis of such a chronic and severe disease is becoming less common. Additionally, if under-diagnosed cases minimised AE incidence in some areas, it is unlikely that the subsequent bias would yield such a perfect fit between AE cases and climatic conditions.

Second, as AE exhibits a long incubation period, i.e. between five and 15 years [1], the environment of cases may have changed between the dates of infection and diagnosis. However, previous analysis revealed that way of life and lifelong residence location of the same group of cases was markedly stable [3]. In France, climate and rural landscape change gradually over the course of several human generations. Therefore, the area associated with an increased risk of disease transmission to humans likely remained rather stable during the study period and will not radically change in the near future. It should, however, be emphasised that omitted parameters, such as fox population and the behaviour of humans and foxes (e.g. increase in urban fox populations), may also play a role in disease transmission. Combes et al. [42] have reported *E. multilocularis* infection in foxes far west of documented endemic areas in France (eastern and central France), even if parasitic loads observed in foxes in western areas were low compared with that in endemic areas of eastern France [43]. Therefore, as AE epidemiology is still evolving in France and Europe, it is important to continue diligent surveillance of human and fox infestation.

The FrancEchino Network

Annecy: VITRAT Virginie. Besançon: BARDONNET Karine; BARTHOLOMOT Brigitte; BEURTON-CHATAIGNER Isabelle; BLAGOSKLONOV Oleg; BRESSON-HADNI Solange; BRIENTINI Marie Pascale; CAPPELLE Sylvie; DELABROUSSE Eric; DI MARTINO Vincent; EVRARD Philippe; FELIX Sophie; GIRAUDOUX Patrick; GRENOUILLET Frédéric; HEYD Bruno; KANTELIP Bernadette; KNAPP Jenny; KOCH Stéphane; MANTION Georges; MILLON Laurence; RAOUL Francis; RICHOU Carine; VANLEMMENS Claire; VUITTON Lucine; VUITTON Dominique Angèle. Bourg en Bresse: PROST Patricia. Charleville Mezière: GODET Claire. Clermont Ferrand: ABERGEL Armand; BEYTOUT Jean; CAMBON Monique. Dijon: BESANCENOT Jean François; CUSENIER Bernadette; HILLON Patrick; MINELLO Anne. Grenoble: FAURE Odile; LETOUBLON Christian. Lyon: CHYDERIOTYS Georges; DUMORTIER Jérôme; GUILLAUD Olivier; PARTENSKY Christian; RABODONIRINA Meja; WALLON Martine. Marseille: PIARROUX Martine; PIARROUX Renaud. Metz: CHATELAIN Eric; JOHANN Marc; RAABE Jean-Jacques. Mulhouse: SONDAG Daniel. Nancy: GERARD Alain; LETRANCHANT Lorraine; MACHOUART Marie; WATELET Jérôme. Paris: FARGES Olivier; SAMUEL Didier. Reims: CHEMLA Cathy; DELATTRE Jean Francois. Rodez: GUERIN Bruno. Saint Ouen l'Aumône: DEBRUYNE Monique. Strasbourg: ABOU-BACAR Ahmed; AUDET Maxime; HANSMANN Yves; LEFEBVRE Nicolas. Thonon: LI Véronique. Vesoul: ALBY-LEPRESLE Blandine.

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Conflict of interest

None declared.

Authors' contributions

MP, JG and RP conceived and designed the surveys used in this article, MP and JG conducted statistical analyses, MP, JG, BF, DAV and RP contributed to the interpretation of data and wrote the first draft, and all others authors contributed to collecting data, revised the article critically and approved the final version.

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Clindamycin resistant *emm33 Streptococcus pyogenes* emerged among invasive infections in Helsinki metropolitan area, Finland, 2012 to 2013

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In 2012, blood, skin and soft tissue infections caused by clindamycin resistant *Streptococcus pyogenes* (group A streptococcus; GAS) appeared to be increasing in the Helsinki metropolitan area. We compared monthly percentages of clindamycin resistant isolates in the area between 2012 and 2013, with those in 2010 and 2011. Resistance frequency in terms of patient age was also studied. We reviewed the medical records of bacteraemic cases in 2012 and 2013 and linked the data to *emm* types. To inform on the *emm* distribution among GAS isolated from skin and soft tissue infections during the epidemic, GAS isolates of one month (March 2013) were *emm* typed. For GAS blood, skin, and soft tissue isolates taken together, the proportions of clindamycin resistant isolates were significantly higher in 2012 and 2013 (23% and 17%, respectively) compared with the two previous years (3%, p<0,001). The erythromycin resistance percentages were almost equal to clindamycin (22% and 17%) in 2012 and 2013, respectively. Clindamycin resistance was most frequent in GAS isolates of 40 to 60 year-old patients (148/417; 36%). Among clindamycin resistant isolates, 12 of 14 blood isolates from 2012 to 2013, and 11 of 13 skin and soft tissue isolates from March 2013, were *emm33*. *Emm33* GAS bacteraemia was associated with clindamycin and erythromycin resistance (odds ratio (OR): 7.0; 95% confidence interval (CI): 1.9–25.3). Infection focus was mainly the skin; either cellulitis (7/12) or necrotising fasciitis (3/12). All *emm33* GAS isolates harboured the *ermTR* resistance gene with constitutive macrolides, lincosamides and streptogramines B (MLS_B) phenotype. *Emm33* GAS was responsible for the higher proportion of clindamycin resistance in skin, soft tissue, and blood isolates locally in 2012 and 2013.

Introduction

Streptococcus pyogenes (group A streptococcus; GAS) causes pharyngitis, skin and soft tissue infections, and invasive septic diseases [1]. Certain GAS *emm* types have been associated with tissue-specific infections [2], antibiotic resistance [3], and local epidemics [3]. Erythromycin resistance has been linked to various *emm* types, such as 4, 6, 12, 75, and 77 [4-7]. Most of these *emm* types have been identified in throat isolates.

Depending on the erythromycin resistance mechanism, isolates may also be resistant to clindamycin, although relatively rarely [8].

There is limited information concerning clindamycin resistance in GAS isolates causing skin and soft tissue infections. In Finland, the annual percentages of erythromycin and clindamycin resistance was only 2 to 3% in 2012 when all GAS isolates (including throat isolates) were analysed together [9]. The figures for all GAS isolates have been the same also in Helsinki metropolitan area [10]. In February 2013, while making the annual local antibiotic resistance statistics of 2012, a high proportion of clindamycin resistance was noticed among blood, skin and soft tissue GAS isolates.

In this study, we investigated whether a specific *emm* type was behind this phenomenon, by examining the laboratory data of GAS isolates in 2012 and 2013 in the Helsinki metropolitan area. We used baseline data of years 2010 and 2011 for comparison. The invasive GAS cases of 2012 and 2013 were analysed in detail and linked to *emm* types (blood isolates) to characterise common denominators behind the increase in clindamycin resistance. To obtain more information on the *emm* distribution in GAS isolates from skin and soft tissue infections, *emm* typing was performed on a set of such GAS isolates obtained during March 2013 in the Helsinki metropolitan area.

Methods

Setting

In Finland, the Division of Clinical Microbiology at HUSLAB is a clinical diagnostic laboratory that serves the Helsinki metropolitan area of ca 1.5 million population. It receives from the local laboratories all blood cultures flagged positive for bacteria by the BacT/ALERT3D system (bioMerieux, Marcy l'Etoile, France) for bacterial identification and resistance analyses. The GAS blood isolates are routinely stored and sent to the national reference laboratory at the National Institute for Health and Welfare for *emm* typing. HUSLAB does the final identification and resistance analyses also for all other GAS isolates e.g. throat, skin, and soft tissue isolates of the Helsinki metropolitan area and keeps records of the resistance data for statistical analyses, however the bacterial isolates are not routinely stored. For this study, GAS isolates of skin and soft tissue infections of March 2013 were collected specifically, and stored at -70 °C. This was done to characterise the *emm* type distribution of both clindamycin susceptible and resistant GAS isolates from skin and soft tissue infections (while the epidemic was still going on), and to verify if the distribution was the same in these isolates compared with the routinely stored blood isolates. We were able to gather 78% (45/58) of the total skin and soft tissue GAS isolates of the month.

Microbiological methods

At HUSLAB GAS isolates are routinely identified by colony morphology with betahaemolysis on sheep blood agar and Lancefield grouping with latex agglutination (Latex Reagent A, Oxoid Ltd, Basingstoke, Hants, England). The resistance for erythromycin and clindamycin is routinely determined using the double-disc diffusion method on Mueller-Hinton agar with 5% defibrinated horse blood with 20 mg/L beta-NAD (MH-F broth). In this study, additional minimum inhibitory concentrations (MICs) were determined for the clindamycin resistant skin and soft tissue GAS isolates of March 2013 and blood isolates of 2012 to 2013 after twice sub-culturing on horse blood agar. MICs were determined by Etests (bioMerieux SA, Marcy l'Etoile, France) for azithromycin, clindamycin, doxycycline, erythromycin, levofloxacin, moxifloxacin, tetracycline, and vancomycin, on Mueller-Hinton (MH)-F broth using 0.5 McFarland inoculum and incubated for 18 ± 2h with 5% CO2 at 35 ± 1 °C. Telithromycin susceptibility was tested by disc-diffusion method in similar conditions as in MIC determinations. European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2013 breakpoints were used to determine whether the zone inhibitions or MICs were considered susceptible (S), intermediate (I) or resistant (R).

Emm typing and resistance gene analysis of the collected group A streptococcus isolates

At the National Institute for Health and Welfare, the 45 GAS isolates of March 2013 and 109 blood GAS isolates from 2012 to 2013 were *emm* typed according to the guidelines provided by Centres for Disease Control and Prevention (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp) as previously described [11].

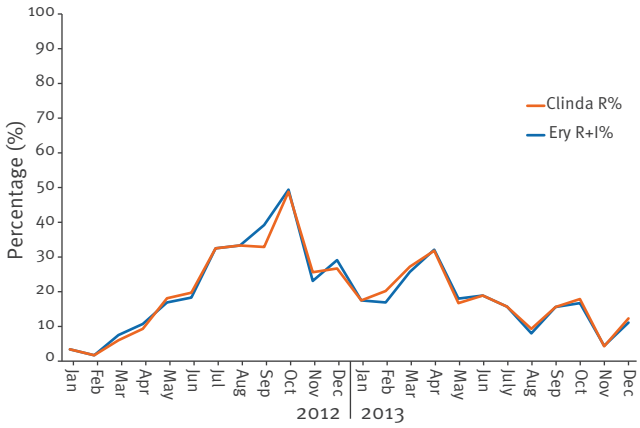
DNA of the erythromycin and clindamycin resistant GAS isolates (blood isolates of 2012–2013; n = 14 and isolates of March 2013; n = 13) was extracted by suspending the colonies in 100 µl of TE buffer and boiling for 15 min, followed by centrifugation at 13,000 rpm for 2 min. The presence of *erm*, including *ermB* and *ermTR*, and *mefA* genes was detected by multiplex-polymerase chain reaction (PCR) including primers for amplification of *mefA*, *ermB* and *ermTR* genes, as described previously [12]. Positive controls were *S. pyogenes* A569 for *mefA*, *Escherichia coli* with plasmid pJIR229 for *ermB*, and *S. pyogenes* A200 for *ermTR* [13].

Susceptibility data analysis

The susceptibility analyses of clinical isolates were made using WHONET 5.6 software. We analysed the resistance figures of GAS isolates of the Helsinki metropolitan area between January 2012 and December 2013, and compared the data to the baseline years, namely 2010 and 2011. Blood, skin, and soft tissue isolates were analysed separately from throat isolates. One isolate per patient, the most resistant one, was included in the analysis (WHONET definition). Data were expressed as percentage of isolates resistant or intermediate for erythromycin together and as percentage of isolates resistant for clindamycin, according to the EUCAST 2013 standard (http://www.eucast.org). The reason for this was that the standard did not include a zone diameter breakpoint for intermediate

Figure 1

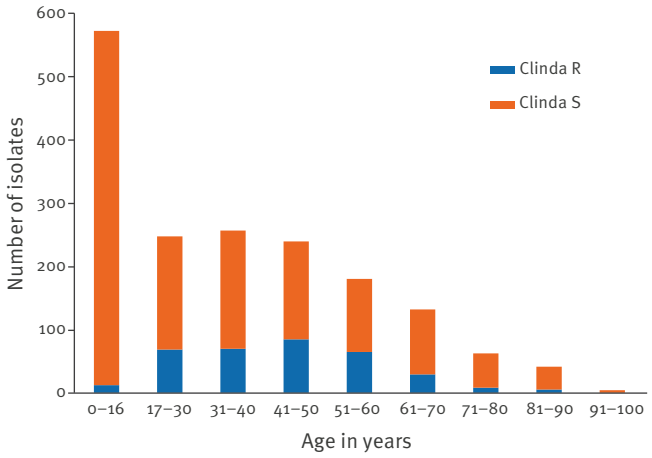
Percentage of skin, soft tissue, and blood group A streptococcus isolates showing reduced susceptibility to clindamycin and erythromycin in Helsinki metropolitan area, Finland, 2012–2013 (n = 1,765)



Clinda R%: percentage of isolates considered resistant to clindamycin according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2013 breakpoints; Ery R+I%: percentage of isolates considered intermediate-resistant and resistant to erythromycin, according to EUCAST 2013 breakpoints.

Figure 2

Clindamycin susceptibility of skin, soft tissue, and blood group A streptococcus isolates by age group, Helsinki metropolitan area, Finland, 2012–2013 (n = 1,765)



Clinda R: resistant to clindamycin; Clinda S: susceptible to clindamycin.

for clindamycin, but for erythromycin it did. Inducible clindamycin resistance was detected by antagonism of clindamycin activity by erythromycin (the D phenomenon) and if not present the isolate was reported susceptible. In HUSLAB the detected antagonism was reported resistant for clindamycin.

Analysis of clinical data and statistics

Electronic medical records of patients with a GAS positive blood culture between January 2012 and December 2013 in Helsinki metropolitan area were reviewed to identify underlying conditions and any common exposure between the patients. Age, sex, C-reactive protein (CRP) value, and leucocyte count at the time of diagnosis were registered. Diagnosed diabetes was recorded. Alcohol abuse was defined as a known social or medical problem caused by alcohol noted in the medical records. Intravenous drug abuse was registered similarly when mentioned in the records. Suspected focus of infection was registered. Presence of a cutaneous infection was described as either cellulitis (infections of the skin and underlying tissues such as erysipelas and deeper non-necrotising soft-tissue infection) or necrotising fasciitis (progressive, destructive, subcutaneous streptococcal infection with necrosis observed either directly or under surgery). Need for surgical procedures, complications, and stay at an intensive care unit due to GAS bacteraemia was recorded. Mortality within seven days after GAS positive blood culture was recorded. Data were analysed and compared using Fisher's exact or Pearson chi-squared tests, or t-test, Mann–Whitney U-test or analysis of variance (ANOVA), when appropriate, using SPSS for Windows statistical package (SPSS Inc., Chicago, IL). Logistic regression analysis was used to identify the risk factors. A p-value < 0.05 was considered statistically significant.

Results

Resistance data of clinical group A streptococcus isolates

When the resistance data were analysed without throat isolates, the proportions of skin, soft tissue, and blood GAS isolates obtained from Helsinki metropolitan area that were clindamycin resistant in 2012 and 2013 were respectively 23% (199/866) and 17% (153/899). The baseline figures were 3% (22/745) in 2010 and 3% (24/734) in 2011 (p < 0,001; 2012 and 2013 figures compared with 2010 and 2011 figures). The proportions of erythromycin intermediate-resistant and resistant isolates were almost equal to those for clindamycin, namely 22% (191/866) and 17% (152/899) in 2012 and 2013, respectively. Baseline proportions of erythromycin intermediate-resistant and resistant isolates were 4% (26/745) and 5% (33/734) in 2010 and 2011 (p < 0,001; 2012 and 2013 figures compared with 2010 and 2011 figures), respectively.

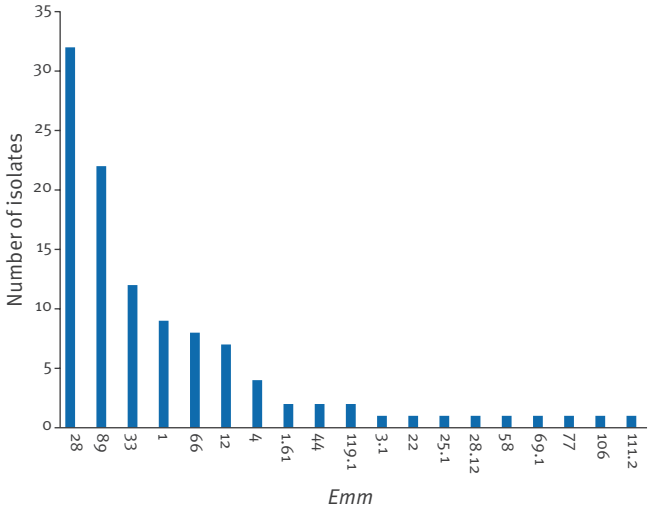
The increase in proportion of isolates with clindamycin resistance began in the spring 2012 and was the highest at 49% (40/82) in October 2012 (Figure 1).

At the end of the study period, in December 2013, still 12% (10/81) of skin, soft tissue, and blood GAS isolates were clindamycin resistant. The proportion of clindamycin resistant isolates varied between age groups, and was highest in the 41 to 50 (35%; 84/238) and 51 to 60 year-olds (36%; 64/179), and lowest among those < 16 years of age (2%; n = 12/569; Figure 2).

The proportion of clindamycin resistant throat isolates remained at the baseline level being 3% (233/8,953), and 4% (354/9,083) in 2012 and 2013, respectively. The proportion of throat isolates which were intermediate-resistant or resistant to erythromycin were also

Figure 3

Emm type distribution of invasive group A streptococcus isolates, Helsinki metropolitan area, Finland, 2012–2013 (n = 109)



3% (278/8,953) and 4% (391/9,083) in 2012 and 2013, respectively.

Emm types, clindamycin resistance, and clinical data of invasive group A streptococcus cases in 2012 and 2013

A total of 109 GAS positive blood isolates were identified in the Helsinki metropolitan area between January 2012 and December 2013. Figure 3 shows the *emm* type distribution of these invasive isolates.

Of the 109 GAS positive blood isolates, 14 were clindamycin resistant and these included 12 *emm33*, one *emm28*, and one *emm89*. Figure 4 shows the time distribution of the invasive GAS isolates resistant or sensitive for clindamycin with respective resistance genes. None of the *emm33* isolates were susceptible for clindamycin. During the baseline years 2010 and 2011, when clindamycin resistance among isolates was at a low level, no invasive *emm33* GAS were isolated in the Helsinki metropolitan area.

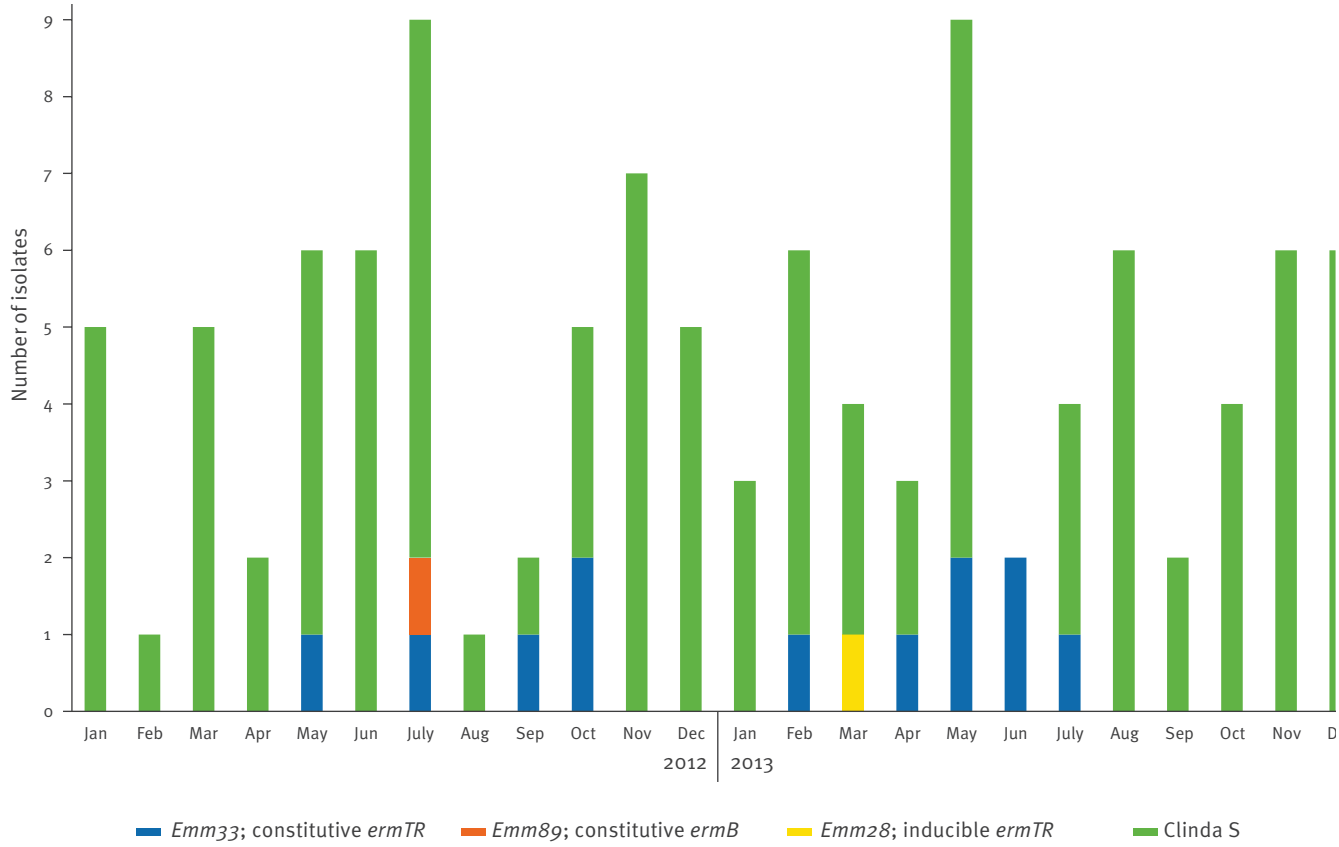
Table 1 compares the clinical data of *emm33* cases to the cases with another *emm* type. In logistic regression analysis clindamycin and erythromycin resistance, alcoholism, and intravenous drug abuse (Table 1) associated with *emm33* GAS bacteraemia. Of the 12 *emm33* cases, 10 had infection focus on the skin or soft tissue. Three *emm33* cases underwent a surgical procedure due to complications of GAS infection. There were no re-infections or need for intensive care in *emm33* cases.

Emm types and laboratory referral data of skin and soft tissue group A streptococcus isolates of March 2013

A total of 45 GAS isolates from skin and soft tissue infections were gathered in March 2013 and *emm* typed. *Emm* typing revealed two isolates being *S. dysgalactiae* subsp. *equisimilis* and these were discarded from the analysis. The remaining 43 isolates represented *emm* types shown in Figure 5. Of these, 13 showed clindamycin resistance and these included 11, which were *emm33*. None of the *emm33* were susceptible for clindamycin. Ten *emm33* isolates were from a skin lesion or abscess as shown by the laboratory referral data in Table 2.

Figure 4

Time distribution of invasive group A streptococcus isolates, Helsinki metropolitan area, 2012–2013 (n = 109)



Clinda S: isolates susceptible to clindamycin.
Clindamycin resistant isolates with respective resistance genes are shown separately (all *emm33* (n =12), one *emm89*, and one *emm28*).

Table 1

Invasive *emm33* cases compared with invasive non-*emm33* cases, Helsinki metropolitan area, Finland, 2012–2013 (n = 109)

	Emm33	Non-*emm33*	P	OR (95%CI)
Number of cases (male/female)	12 (7/5)	97 (47/50)	NS	–
Age in years: mean (range)	54 (22–80)	51 (0–89)	NS	–
C-reactive protein in mg/L: mean (range)	164 (4–393)	189 (4–573)	NS	–
Leucocyte count: mean (range)	13 (4–26)	14 (1–39)	NS	–
Cases with alcohol abuse: n/N	8/12	17/97	<0.001	11.9 (2.9–49.7)
Cases with intravenous drug abuse: n/N	4/12	5/97	<0.01	9.1 (2.0–40.8)
Cases with erythromycin resistant isolates[a]: n/N	12/12	2/97	<0.001	7.0 (1.9–25.3)
Cases with clindamycin resistance: n/N	12/12	2/97	<0.001	7.0 (1.9–25.3)
Cases with cellulitis: n/N	7/12	41/97	NS	–
Cases with necrotising fasciitis: n/N	3/12	4/97	NS	–
Cases with diabetes: n/N	3/12	18/97	NS	–
7-day mortality: n/N	0/12	4/97	NS	–

CI: confidence interval; OR: odds ratio; NS: not significant, p>0.05.

[a] Including intermediate-resistant and resistant isolates, according to EUCAST 2013 breakpoints.

Susceptibility and resistance genes of the collected *emm33* group A streptococcus isolates

All studied *emm33* GAS (skin and soft tissue isolates of March 2013 and blood isolates of 2012–2013, totally n=23) showed the constitutive macrolides, lincosamides and streptogramines B (MLS_B) phenotype with similar antibiotic resistance profiles and harboured the *ermTR* resistance gene. The isolates were non-susceptible for azithromycin (MIC range: 12–>256 mg/L), clindamycin (all MICs>256 mg/L), and erythromycin (MIC range: 2–8 mg/L). They showed susceptibility for doxycycline (MIC range: 0.125–0.38 mg/L), levofloxacin (MIC range: 0.25–0.75 mg/L), moxifloxacin (MIC range: 0.064–0.19 mg/L), tetracycline (MIC range: 0.25–1.0 mg/L), and vancomycin (MIC range: 0.38–0.75 mg/L). All isolates were susceptible for telithromycin (disc-diameter range: 27–38mm), which was tested by disc-diffusion method.

Discussion

During 2012 and 2013 *emm33* GAS caused a local epidemic of skin and soft tissue infections in the adult population in Helsinki metropolitan area, Finland. The outbreak was detected as a marked increase in the proportion of isolates resistant to erythromycin and clindamycin. In most cases the primary infection focus was the skin, but a few GAS *emm33* infections were invasive and caused necrotising fasciitis. All *emm33* isolates were resistant to both erythromycin and clindamycin.

There was an association of *emm33* with alcohol and intravenous drug abuse, however the number of patients was very low so these results have to be interpreted with caution. Alcohol abuse was marked positive if mentioned in the patient records. Since alcoholism is not always evident and not even always actively asked about by the doctor in the hospital, this information is most probably partly lacking from our data. The same counts for intravenous drug abuse. An association with alcohol abuse has nevertheless been reported for certain other *emm* types, such as *emm59* and *emm1* [14,15].

Emm33 belongs to the *emm* superfamily group D, which includes *emm* types causing skin infections, such as impetigo [2,16]. *Emm33* has further been characterised as a member of the D4 *emm*-cluster, which is able to bind plasminogen [17]. Plasminogen binding may contribute to the skin tissue tropism by possible break down of tissue barriers facilitating dissemination and prolonged bacterial persistence in the skin [18]. There is limited data concerning infections caused by this *emm* type. It caused some of the severe GAS infections of intravenous drug users reported in a study in the United Kingdom (UK) in 2003 and 2004, but was

Figure 5

Emm type distribution of skin and soft tissue group A streptococcus isolates, Helsinki metropolitan area, Finland, March 2013 (n = 43)

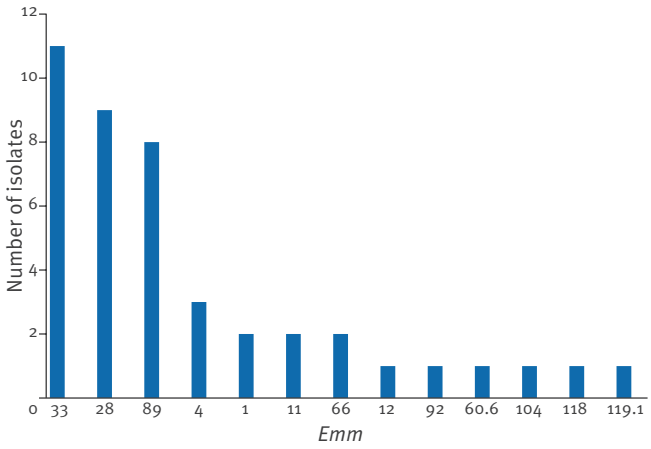


Table 2

Laboratory referral data of the group A streptococcus isolates from skin and soft tissue that were clindamycin resistant, Helsinki metropolitan area, Finland, March 2013 (n = 13)

Sex	Age groups in years	*Emm* type	Resistance gene	Infection focus
Female	81–90	*33*	*ermTR*[a]	Decubital wound, sacrum
Male	31–40	*33*	*ermTR*[a]	Auricular canal
Male	41–50	*33*	*ermTR*[a]	Suppurative wounds, foot and elbow
Female	31–40	*33*	*ermTR*[a]	Postoperative wound infection, gynaecological
Female	51–60	*33*	*ermTR*[a]	Postoperative wound infection, ankle
Male	41–50	*33*	*ermTR*[a]	Abscess, finger
Male	17–30	*33*	*ermTR*[a]	Impetigo, perioral
Male	41–50	*33*	*ermTR*[a]	Postoperative wound infection, finger
Female	17–30	*33*	*ermTR*[a]	Abscess, leg
Female	31–40	*33*	*ermTR*[a]	Nasal discharge
Male	17–30	*33*	*ermTR*[a]	Impetigo, foot
Male	17–30	*92*	*ermB*[b]	Suppurative wound, heel
Male	61–70	*11*	*ermB*[c]	Penile sores

[a] Constitutive *ermTR*.
[b] Inducible *ermB*.
[c] Constitutive *ermB*.

not the most common *emm* type in that study [19]. Intravenous drug abuse has been shown to be a risk factor for severe disease caused by GAS [20].

GAS is able to cause very local and timely limited epidemics, as shown in intravenous drug users in the UK [19]. Interestingly, in 2012 in our neighbouring country, Sweden, there was an increase of invasive *emm1* GAS diseases occurring mostly in patients over 80 years of age [21]. The invasive *emm1* numbers have remained stable in Helsinki metropolitan area between 2010 and 2013 indicating that the Swedish epidemic is local or has not reached Finland yet.

The Finnish National Institute for Health and Welfare receives all invasive GAS isolates from Finland for genotyping and strain collection. Since 2007, the main genotyping method has been *emm* typing. In Finland, the first *emm33* invasive GAS isolates were found only in 2012 and they all originated from the Helsinki metropolitan area. From June 2013 onwards sporadic *emm33* cases have been found also in other hospital districts, however *emm33* still remains an uncommon genotype in Finland (Pieter Smit, personal communication, April 2014).

Several mechanisms underlie the macrolide and lincosamide resistance. M phenotype isolates carry the *mefA* gene, which causes efflux of the antibiotic and confers resistance to many macrolides with preserved susceptibility to clindamycin and streptogramin B [22]. *Emm4* GAS with *mefA* has previously caused high erythromycin resistance locally in Finland [23]. Interestingly, in our study none of the tested isolates carried the *mefA* resistance gene. The resistance data of the Helsinki metropolitan area showed also that most of the isolates with decreased susceptibility to erythromycin were also clindamycin resistant (either with inducible or constitutive phenotype) suggesting that the M phenotype was not generally present in the GAS isolates during the years 2012 to 2013.

Ribosomal methylation of the target of the antibiotics (*ermA*, *ermB* or *ermTR*) prevents binding of the antibiotics by causing a conformational change in the 23S ribosome [24]. The *ermB* isolates usually show constitutive MLS_B ($cMLS_B$) resistance to macrolides, clindamycin, and streptogramin B, while the *ermA* and *ermTR* isolates may show macrolide-induced resistance to clindamycin [13]. In our study all *emm33* isolates shared the *ermTR* macrolide resistance gene with a $cMLS_B$ resistance phenotype. This resistance phenotype is more common among *ermB* but has in rare occasions also been shown by isolates carrying the *ermTR* gene [25].

All the *emm33* isolates were also susceptible to telithromycin. Usually isolates with the $cMLS_B$ phenotype are resistant or intermediate to telithromycin, however, such phenotypes harbour typically the *ermB* gene [26]. Accordingly, two isolates in our study (a blood *emm89* and a skin isolate of March 2013 *emm11*) with $cMLS_B$ phenotype with *ermB* gene showed intermediate susceptibility to telithromycin (data not shown). In contrast, an isolate with inducible MLS_B phenotype with *ermB* (skin isolate of March 2013 *emm92*) was susceptible for telithromycin. The only isolate with inducible MLS_B phenotype with *ermTR* gene (a blood isolate *emm28*) was susceptible for telithromycin, as were all the *emm33* isolates. Similar results have shown Giovanetti et al. regarding inducible *ermTR* GAS isolates [27]. All the *emm33* isolates in our study showed large disc-diameters growth suppression by telithromycin. Unfortunately at the time of investigation, telithromycin Etests were not available in our laboratory, so we were not able to determine MICs, which would have represented more precise data.

The *emm33* isolates in our study were also tetracycline susceptible, while Kataja et al. showed that inducible *ermTR* GAS isolates in Finland in 1994 and 1995 were mostly tetracycline resistant [25]. To fully understand the resistance mechanism underlying *emm33*, the isolates should be examined in more detail. The fact that

emm33 isolates had the same resistance gene and similar antibiotic resistance patterns supports the idea that they belong to the same clone (Pieter Smit, personal communication, April 2014).

Clindamycin is an important drug in the primary healthcare, especially, as it is used for treating GAS infections in penicillin allergic patients. The skin and soft tissue infections of intravenous drug abusers and of diabetic patients are often polymicrobial with anaerobic bacteria and staphylococci present making clindamycin the drug of choice for empirical treatment. In invasive, septic GAS infections clindamycin is used in combination with penicillin for better outcome possibly diminishing the bacterial toxin production [28]. Spreading of a skin-tropic *emm* type with clindamycin resistance is of concern considering the empirical antibiotic treatment of the abovementioned patient groups. An announcement, aimed at the primary care and hospital doctors of the city of Helsinki, was released in spring 2013 by HUSLAB together with the infectious disease specialists of the Helsinki city hospitals concerning the proportion of clindamycin resistant GAS figures. It guided the empirical therapy of adult skin infections recommending that clindamycin should not have been used as monotherapy. The infectious disease specialists of the whole Helsinki metropolitan area were also informed, and additional antimicrobial susceptibility testing was conducted for the clindamycin resistant GAS isolates to find alternative drugs for penicillin-allergic patients. Surveillance of the situation is important because *emm33* GAS may spread to children since it is a potential impetigo-causing *emm* type [2].

The study shows that for resistance statistics it is important to analyse skin and soft tissue GAS separately from the numerous throat GAS. Different *emm* types are typical for distinct anatomical locations and important resistance phenomena may be masked if isolates are analysed only together. Here we documented a single, local, epidemic of a previously rare *emm33* GAS causing skin and soft tissue infections with also invasive cases. This *emm* type caused rapid changes in macrolide and clindamycin resistance locally in the adult population. These findings had an impact on the empirical treatment of skin and soft tissue infections of the area.

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Conflict of interest

None declared.

Authors' contributions

Anne-Katrine Pesola investigated the medical records of the bacteraemic patients of years 2012-2013 and was mainly responsible for the statistical analyses. Reetta Sihvonen made the antimicrobial susceptibility testing (MIC determinations) for the resistant skin, soft tissue, and blood GAS isolates and determined the *erm* genes of the resistant isolates. Laura Lindholm was responsible for the *emm* typing of the GAS isolates. Anu Pätäri-Sampo made the original finding of the high resistance figures of GAS isolates in the Helsinki metropolitan area. Pätäri-Sampo designed the study, applied for the permission to run the study in HUSLAB, and was primarily responsible for the writing of the manuscript. All authors contributed substantially to the manuscript, and have seen and approved the final version.

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Haemagglutinin mutations and glycosylation changes shaped the 2012/13 influenza A(H3N2) epidemic, Houston, Texas

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While the early start and higher intensity of the 2012/13 influenza A virus (IAV) epidemic was not unprecedented, it was the first IAV epidemic season since the 2009 H1N1 influenza pandemic where the H3N2 subtype predominated. We directly sequenced the genomes of 154 H3N2 clinical specimens collected throughout the epidemic to better understand the evolution of H3N2 strains and to inform the H3N2 vaccine selection process. Phylogenetic analyses indicated that multiple co-circulating clades and continual antigenic drift in the haemagglutinin (HA) of clades 5, 3A, and 3C, with the evolution of a new 3C subgroup (3C-2012/13), were the driving causes of the epidemic. Drift variants contained HA substitutions and alterations in the potential N-linked glycosylation sites of HA. Antigenic analysis demonstrated that viruses in the emerging subclade 3C.3 and subgroup 3C-2012/13 were not well inhibited by antisera generated against the 3C.1 vaccine strains used for the 2012/13 (A/Victoria/361/2011) or 2013/14 (A/Texas/50/2012) seasons. Our data support updating the H3N2 vaccine strain to a clade 3C.2 or 3C.3-like strain or a subclade that has drifted further. They also underscore the challenges in vaccine strain selection, particularly regarding HA and neuraminidase substitutions derived during laboratory passage that may alter antigenic testing accuracy.

Introduction

Influenza viruses cause significant annual morbidity and mortality in the global human population [1]. Epidemics occur during the winter months, cycling roughly every six months between the northern and southern hemispheres. Two influenza A virus (IAV) subtypes, H3N2 and H1N1pdm09, and two influenza B virus (IBV) lineages, B/Yamagata and B/Victoria, have been circulating among humans since 2009. Epidemics caused by each of these subtypes/lineages vary from season to season; this is due, in part, to the selective advantage acquired by one subtype/lineage in a given season. Although the error-prone IAV RNA polymerase frequently generates nucleotide substitutions that can lead to a selective advantage in all eight genomic viral RNA segments (vRNAs), new epidemic variants are most frequently due to accumulated substitutions in the two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Substitutions in HA/NA can give rise to variants that escape host immunity from previous exposures or vaccinations and are selected in the non-naïve host population through a process called antigenic drift. Antigenic drift primarily occurs in epitopes recognised by antibodies that neutralise viral infectivity by blocking the interaction of HA with sialic acid receptors on host cell glycoproteins [2,3]. Antigenic drift necessitates frequent updating of the strains used in the influenza vaccine [3,4]. This requires global surveillance of the antigenic profile of circulating strains to inform the decisions made at biannual World Health Organization (WHO) meetings for the selection of influenza vaccine strains [4-6]. The acquisition of as few as one to five mutations in HA can necessitate an updated vaccine strain to optimally protect the public [7,8].

The H3N2 subtype caused a severe epidemic during the 2012/13 influenza season in North America and contributed to a longer than normal season, with increased morbidity also in Europe [9,10]. Human H3N2 viruses represent a very successful lineage that has

circulated since the 'Hong Kong Flu' pandemic of 1968 [11,12]. New H3N2 strains evolve continually, resulting in annual epidemics that are periodically severe, such as the 2003/04 season (A/Fujian/411/2002-like viruses) [3,7]. The 2012/13 season in the United States (US) was notable in both greater epidemic severity and limited vaccine effectiveness, especially in the elderly [13-18]. Our incomplete understanding of the factors shaping the emergence of epidemic IAV/IBV variants impairs our ability to accurately predict strain fitness and select appropriate vaccine strains.

We hypothesised that the 2012/13 epidemic was caused by the emergence of a new HA variant that rapidly displaced previously circulating strains. To gain a better understanding of the genetic and molecular mechanisms central to the intensity and severity of the 2012/13 epidemic, we sequenced 154 coding-complete genomes from H3N2-positive nasopharyngeal swabs collected throughout the 2012/13 season from a large hospital network in Houston, Texas, US. This demographically diverse system of five hospitals provides a reasonable representation of the viruses circulating in the US during a non-pandemic seasonal influenza epidemic [19], which made it a suitable site for studying IAV genetic variation. Direct sequencing of primary swab specimens is critical because H3N2 viruses are known to rapidly acquire adaptive mutations that change their antigenicity when passaged in cell cultures or eggs [5,20]. It allows accurate analysis of HA and NA, the identification of substitutions, and the detection of reassortment among vRNAs encoding internal proteins that contribute to viral fitness.

Methods

Hospital system and coding-complete viral genome sequencing

The Methodist Hospital System in Houston, Texas consists of five hospitals that serve a large population of ca 4 million people from the most ethnically diverse population in the United States (ca 32% Caucasian, 41% Hispanic/Latino, 20% African-American and 7% Asian) [21,22]. The IAV-positive human nasopharyngeal swab specimens used in this study were collected between 3 November 2012 and 8 February 2013. During this period, ca 20% of the specimens were from inpatients and 80% were from outpatients. The majority of inpatient isolates were obtained from patients admitted with influenza-like illness through the emergency department, who therefore did not have nosocomially acquired infections.

These viral samples were sequenced using the J. Craig Venter Institute's (JCVI's) high-throughput next-generation sequencing pipeline. Briefly, IAV vRNAs were isolated directly from the swab specimens, and the entire genome was amplified from 3 µl of RNA template using a multi-segment RT-PCR strategy (M-RTPCR) [23,24]. The amplicons were sequenced using the Ion Torrent PGM (Thermo Fisher Scientific, Waltham, Massachusetts, US) and/or the Illumina MiSeq v2 (Illumina, Inc., San Diego, California, US) instruments. When sequencing data from both platforms was available, the data were merged and assembled together; the resulting consensus sequences were supported by reads from both technologies.

Sequence curation

Reference sequences for IAV HA and NA nucleotide sequences were obtained from GenBank (National Center for Biotechnology Information) and the EpiFlu database (Global Initiative on Sharing All Influenza Data; GISAID). For phylogenetic analyses, three H3N2 reference sets were used: (i) all available strains presented by the Centers for Disease Control and Prevention (CDC) at the 2012/13 US vaccine composition meeting (available at [25]), (ii) all available strains derived from a phylogenetic tree in the 2013 southern hemisphere vaccine composition report [26] and (iii) a random selection of 50 isolates from public databases collected globally between 1 September 2012 and 8 February 2013. For the genome constellation analysis, all available coding-complete human H3N2 genomes in the EpiFlu database collected between 1 October 2011 and 20 February 2013 were downloaded on 6 June 2013.

Phylogenetic analyses

HA and NA nucleotide sequences and HA amino sequences were each aligned using MAFFT v7.158b with default settings [27-29] and trimmed using Qiagen's CLC Genomics Workbench v6.0. Four independent maximum likelihood (ML) dendrograms were inferred using GARLI [30,31], and the trees with the best log likelihood scores were analysed further with 500 bootstrap replicates. The ML tree and bootstrap values were combined using SumTrees in DendroPy [32]. For nucleotide sequences, the GTR G+I nucleotide substitution model with five rate categories was used, as determined by jModelTest v2.1.4 [33,34]. For protein sequences, the JTT G+I substitution model with four rate categories and empirically derived residue frequencies was used, as determined by ProtTest v2.4 [35].

Genome constellation analyses

Study and reference H3N2 genomes were parsed into gene segment-specific multi-FASTA files. MAFFT alignments for each segment were converted to a distance matrix using ANDES [36]. Dendrograms were generated using the complete linkage (farthest neighbour) hierarchical clustering technique in ANDES. Dendrograms were cut at a specified per cent identity and vRNA segments were assigned to groups according to their cluster at that cut-off. The segment group assignments were combined to obtain a genome constellation that was visualised using OrionPlot [37].

Viral isolation and propagation

A subset of viruses was selected to represent each co-circulating clade. Viruses were isolated from the primary swab specimen for subsequent propagation and antigenic analysis. Selected strains were passaged

Table 1

Antigenic properties of study samples measured by HI assays using ferret or sheep antisera raised against contemporary influenza A(H3N2) strains

	HA GenBank or EpiFlu[a] accession number	HA clade	Antigens used to generate ferret sera				Antigens used to generate sheep sera	
			rA/Perth/16/2009	rA/Victoria/361/2011-like[b] (cell-grown)	rA/Victoria/361/2011 (egg-grown)	rA/Indiana/8/2011 (H3N2v)	A/Victoria/361/2011	A/Texas/50/2012
Reference antigens[c]								
rA/Perth/16/2009 (cell-grown)	GQ293081.1	1	320	320	113	<20	453	1,280
rA/Victoria/361/2011 (cell-grown)	EPI349103	3C.1	640	453	538	20	905	>2,560
rA/Victoria/361/2011 (egg-grown)	EPI353906	3C.1	226	320	905	40	1,280	>2,560
rA/Indiana/8/2011 (H3N2v)	JN638733.1	N/A	<20	<20	<20	320	<80	<80
Test antigens								
A/Texas/JMM_3/2012	CY134748.1	5	226	453	113	<20	320	1,810
A/Texas/JMM_48/2012	CY135076.1	3A	226	57	160	<20	<80	905
A/Texas/JMM_20/2012	CY134868.1	3C.2	320	226	320	<20	320	1,810
A/Texas/JMM_37/2012	CY134996.1	3C.3	80	<20	28	<20	<80	<80
A/Texas/JMM_4/2012	CY134756.1	3C-2012/13	80	<20	40	<20	<80	<80

HA: haemagglutinin; NA: neuraminidase; N/A: not applicable.

[a] The authors gratefully acknowledge the originating (WHO Collaborating Centre for Reference and Research on Influenza in North Melbourne, Victoria, Australia) and submitting (Centers for Disease Control and Prevention, Atlanta, Georgia, US) laboratories that contributed the cell- and egg-grown A/Victoria/361/2011 sequences to GISAID's EpiFlu database.

[b] rA/South_Australia/3/2011, which has the same amino acid sequence as A/Victoria/361/2011 (cell-grown) except for one change in the signal peptide.

[c] These reassortant viruses are named for the strain from which the HA and NA were obtained; the six internal genes match A/Puerto Rico/8/1934(H1N1), A/New York/238/2005(H3N2) or A/New York/1682/2009(H1N1pdm09) sequences.

Highlighted cells indicate two-way HI results, where the specified serum is tested using the same (or a very similar) antigen to that used to generate the serum. An 'r' before the strain name indicates the virus was generated using gene synthesis and reverse genetics. Results from one experimental replicate are presented and are representative of the results obtained from at least two additional experimental replicates performed on a different day. All HA controls were between 3 and 8 HA units.

twice in Madin-Darby canine kidney (MDCK) cells to avoid selecting for mutations that frequently arise when IAV is isolated in embryonated chicken eggs. Supernatants from P2 viral stocks were clarified by centrifugation at 1,800 × g for 10 min at 4 °C, aliquoted, stored at −80 °C and sequenced.

Influenza virus rescue using gene synthesis and reverse genetics

To avoid spurious substitutions and generate a uniform virus population, positive (A/Perth/16/2009(H3N2) and A/Victoria/361/2011(H3N2)) and negative (A/Indiana/8/2011(H3N2v)) control viruses for antigenic testing were generated using gene synthesis [38] and a modified reverse genetics system [23,24] and were confirmed by sequencing. Briefly, 6:2 reassortant viruses (designated with an 'r' before the strain name, e.g. rA/Perth/16/2009) were rescued from plasmids encoding the six internal protein vRNAs from the strains indicated in Table 1 (e.g. A/Puerto Rico/8/1934) and the linear synthesised HA and NA genes of the desired H3N2 viruses.

Antisera generation/acquisition and haemagglutination inhibition

Ferret antisera were generated by inoculating ferrets (two individuals per virus) intranasally with ca 1 × 10^6 $TCID_{50}$ of clarified virus supernatant in 1 ml, and antisera were collected 30 days after inoculation. These ferret experiments were conducted by an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited company, BIOQUAL, Inc. (Rockville, Maryland, US). Antisera from the two ferrets inoculated with the same strain were pooled 1:1 and treated with three times their volume of receptor-destroying enzyme (RDE; Hardy Diagnostics, Santa Maria, California, US) overnight at 37 °C. RDE was inactivated at 56 °C for 30 min, and the RDE-treated antisera were diluted with six times the original antisera volume using phosphate-buffered saline. Lyophilised sheep antisera raised against A/Victoria/361/2011 and A/Texas/50/2012 were provided by the Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), Department of Health and Human Services, US, and were also RDE-treated. Haemagglutination inhibition (HI) assays were performed [39] to determine the ability of various antisera to inhibit binding of the IAV isolates to guinea pig red blood cells.

Modelling H3 N-linked glycosylation

All potential HA N-linked glycosylation sites among contemporary H3N2 clades in this study were deduced from sequons and compared with the HA of A/Finland/486/2004(H3N2), for which the H3 structure has been solved [40]. Comparing the asparagine (N)

residues of predicted sequons with the three-dimensional H3 structure confirmed that they would all be on the protein surface. While crystal structures can often detect the core of N-linked glycans, the actual composition and structure of the high-mannose and complex sugars is poorly understood. Here, we use the high-mannose glycan deduced from small-angle X-ray scattering data for the N165-linked glycan of X31 (H3 structural numbering) [41] as a 'type glycan' modelled onto the A/Finland/483/2004 H3 structure (Protein Data Bank ID: 2YP3 [40]) using the AllosMod server [41] which implements an all-atom modelling approach allowing for limited protein conformational flexibility. In brief, AllosMod uses CHARMM [42,43] force field parameters to derive preliminary glycan geometries and then optimises combined protein–glycan structures with MODELLER [44]. The all-atom structural model was visualised in PyMOL v1.7.1.7 [45]. Scripts that implement this modelling and visualisation protocol are available at [46].

Results

Phylogenetic analyses to understand H3N2 evolution

The coding-complete genomes of 154 IAV H3N2 specimens were sequenced and their genome sequences were submitted to GenBank (Table 2).

Phylogenetic analyses of HA nucleotide sequences showed that the majority of circulating H3N2 strains arose from three major H3 clades: 5, 3A, and 3C (Figure 1A; clade nomenclature was adopted from US CDC [25]).

The backbone of the phylogeny had a ladder-like structure with relatively short terminal branches indicative of seasonal IAV antigenic drift arising from immune escape. While multiple older clade 5 strains were co-circulating in Houston, all circulating clade 3A strains were derived from a single monophyletic group. Importantly, a large number of the study specimens (66/154 = 42.9%) contained viruses that formed an emerging monophyletic group, which formed a new 3C subgroup that we designated 3C-2012/13. The majority of 3C viruses fell into subclades 3C.2 and 3C.3 or subgroup 3C-2012/13, while only two strains fell within subclade 3C.1, which was the subclade of the vaccine seed strains chosen for the 2012/13 and 2013/14 seasons (A/Victoria/361/2011 and A/Texas/50/2012, respectively). Analysis of H3N2 viruses worldwide from the onset of 2012/13 season in the northern hemisphere through June 2014 demonstrated that 3C.3 and 3C-2012/13 viruses also circulated in Europe and Asia (e.g. 3C.3-like A/Roma/11/2014 and 3C-2012/13-like A/Saint-Petersburg/170/2013; data not shown), suggesting that influenza surveillance in Houston, Texas, provided a reasonable sampling of circulating strains. However, it appears the 3C-2012/13 subgroup circulated through 2013 and then died out (data not shown). A larger phylogenetic analysis that included strains from recent seasons also placed our 2012/13 study strains in the context of the newest WHO-designated subclades for 3C.2 (i.e. 3C.2a) and 3C.3 (i.e. 3C.3a and 3C3.b) [47]. These new subclades are related to, but didn't arise directly from the 2012/13 viruses circulating in Texas; their placements based on this larger analysis are labelled on the left of the HA nucleotide phylogeny (Figure 1A).

Strains from all three major clades (5, 3A, and 3C) were co-circulating in Houston throughout the early, middle, and late 2012/13 epidemic (data not shown), indicating that strong temporal segregation of viral clades was not occurring within a single IAV season (Figure 1A). This shows that the emergence of a genetically novel virus early in the season did not rapidly displace other co-circulating strains.

Analysis of HA protein sequences for the same dataset showed that there were relatively few residue changes across all HA clades (Figure 2). This is particularly true for the HA proteins of the subclade 3C.3 and subgroup 3C-2012/13, which is illustrated by the interleaving of colours based on the nucleotide clade assignments (Figure 1B). This HA protein phylogeny emphasises how subclades 3C.2 and 3C.3, as well as subgroup 3C-2012/13, are more closely related to each other than to clade 3C.1 viruses.

The NA nucleotide tree also formed a backbone characteristic of IAV that shows evidence of limited genetic drift in NA (Figure 1C). The NA phylogeny also showed three main groupings for the study samples, which roughly corresponded to the HA clade assignments 5, 3A, and 3C. However, colouring the NA tree using the HA clade assignments demonstrated that intrasubtypic reassortment between H3 and N2 occurs readily, as indicated by the interleaving of HA clade colours (Figure 1C). This was primarily found among 3C subclades and between clades 5 and 6. The most pronounced intrasubtypic reassortment was seen in A/Texas/JMM_27/2012, which has an HA belonging to clade 3A and an NA that groups with 3C-2012/13 viruses.

Genome constellation analyses to identify reassortment

To better understand the extent of intrasubtypic reassortment occurring among seasonal H3N2 viruses, we performed genome constellation analyses using 198 genomes (154 from our study samples and 44 additional strains). A 98% nucleotide identity cut-off value provided a genome constellation resolution that matched that of the major HA clade designations (i.e. 1, 5, 6, 3A, 3B, and 3C) (Figure 3A).

This yielded 12 genome constellations and demonstrated that vRNAs coding for internal proteins are also involved in intrasubtypic reassortment. While all constellations shared highly similar PA and M segments, seven monophyletic HA 3C.2 samples had reassorted PB2 segments (constellation 12 in Figure 3A) that grouped with the PB2 of HA clade 3A (constellations 2

Table 2A

GenBank accession numbers of coding-complete influenza A(H3N2) genomes sequenced for this study, Texas, 3 November 2012–8 February 2013 (n = 154)

Virus name	PB2	PB1	PA	HA	NP	NA	M	NS
A/Texas/JMM_1/2012	CY134739.1	CY134738.1	CY134737.1	CY134732.1	CY134735.1	CY134734.1	CY134733.1	CY134736.1
A/Texas/JMM_2/2012	CY134747.1	CY134746.1	CY134745.1	CY134740.1	CY134743.1	CY134742.1	CY134741.1	CY134744.1
A/Texas/JMM_3/2012	CY134755.1	CY134754.1	CY134753.1	CY134748.1	CY134751.1	CY134750.1	CY134749.1	CY134752.1
A/Texas/JMM_4/2012	CY134763.1	CY134762.1	CY134761.1	CY134756.1	CY134759.1	CY134758.1	CY134757.1	CY134760.1
A/Texas/JMM_5/2012	CY134771.1	CY134770.1	CY134769.1	CY134764.1	CY134767.1	CY134766.1	CY134765.1	CY134768.1
A/Texas/JMM_8/2012	CY134779.1	CY134778.1	CY134777.1	CY134772.1	CY134775.1	CY134774.1	CY134773.1	CY134776.1
A/Texas/JMM_9/2012	CY134787.1	CY134786.1	CY134785.1	CY134780.1	CY134783.1	CY134782.1	CY134781.1	CY134784.1
A/Texas/JMM_10/2012	CY134795.1	CY134794.1	CY134793.1	CY134788.1	CY134791.1	CY134790.1	CY134789.1	CY134792.1
A/Texas/JMM_11/2012	CY134803.1	CY134802.1	CY134801.1	CY134796.1	CY134799.1	CY134798.1	CY134797.1	CY134800.1
A/Texas/JMM_12/2012	CY134811.1	CY134810.1	CY134809.1	CY134804.1	CY134807.1	CY134806.1	CY134805.1	CY134808.1
A/Texas/JMM_13/2012	CY134819.1	CY134818.1	CY134817.1	CY134812.1	CY134815.1	CY134814.1	CY134813.1	CY134816.1
A/Texas/JMM_14/2012	CY134827.1	CY134826.1	CY134825.1	CY134820.1	CY134823.1	CY134822.1	CY134821.1	CY134824.1
A/Texas/JMM_15/2012	CY134835.1	CY134834.1	CY134833.1	CY134828.1	CY134831.1	CY134830.1	CY134829.1	CY134832.1
A/Texas/JMM_16/2012	CY134843.1	CY134842.1	CY134841.1	CY134836.1	CY134839.1	CY134838.1	CY134837.1	CY134840.1
A/Texas/JMM_17/2012	CY134851.1	CY134850.1	CY134849.1	CY134844.1	CY134847.1	CY134846.1	CY134845.1	CY134848.1
A/Texas/JMM_18/2012	CY134859.1	CY134858.1	CY134857.1	CY134852.1	CY134855.1	CY134854.1	CY134853.1	CY134856.1
A/Texas/JMM_19/2012	CY134867.1	CY134866.1	CY134865.1	CY134860.1	CY134863.1	CY134862.1	CY134861.1	CY134864.1
A/Texas/JMM_20/2012	CY134875.1	CY134874.1	CY134873.1	CY134868.1	CY134871.1	CY134870.1	CY134869.1	CY134872.1
A/Texas/JMM_21/2012	CY134883.1	CY134882.1	CY134881.1	CY134876.1	CY134879.1	CY134878.1	CY134877.1	CY134880.1
A/Texas/JMM_22/2012	CY134891.1	CY134890.1	CY134889.1	CY134884.1	CY134887.1	CY134886.1	CY134885.1	CY134888.1
A/Texas/JMM_23/2012	CY134899.1	CY134898.1	CY134897.1	CY134892.1	CY134895.1	CY134894.1	CY134893.1	CY134896.1
A/Texas/JMM_24/2012	CY134907.1	CY134906.1	CY134905.1	CY134900.1	CY134903.1	CY134902.1	CY134901.1	CY134904.1
A/Texas/JMM_25/2012	CY134915.1	CY134914.1	CY134913.1	CY134908.1	CY134911.1	CY134910.1	CY134909.1	CY134912.1
A/Texas/JMM_26/2012	CY134923.1	CY134922.1	CY134921.1	CY134916.1	CY134919.1	CY134918.1	CY134917.1	CY134920.1
A/Texas/JMM_27/2012	CY134931.1	CY134930.1	CY134929.1	CY134924.1	CY134927.1	CY134926.1	CY134925.1	CY134928.1
A/Texas/JMM_29/2012	CY134939.1	CY134938.1	CY134937.1	CY134932.1	CY134935.1	CY134934.1	CY134933.1	CY134936.1
A/Texas/JMM_30/2012	CY134947.1	CY134946.1	CY134945.1	CY134940.1	CY134943.1	CY134942.1	CY134941.1	CY134944.1
A/Texas/JMM_31/2012	CY134955.1	CY134954.1	CY134953.1	CY134948.1	CY134951.1	CY134950.1	CY134949.1	CY134952.1
A/Texas/JMM_32/2012	CY134963.1	CY134962.1	CY134961.1	CY134956.1	CY134959.1	CY134958.1	CY134957.1	CY134960.1
A/Texas/JMM_33/2012	CY134971.1	CY134970.1	CY134969.1	CY134964.1	CY134967.1	CY134966.1	CY134965.1	CY134968.1
A/Texas/JMM_34/2012	CY134979.1	CY134978.1	CY134977.1	CY134972.1	CY134975.1	CY134974.1	CY134973.1	CY134976.1
A/Texas/JMM_35/2012	CY134987.1	CY134986.1	CY134985.1	CY134980.1	CY134983.1	CY134982.1	CY134981.1	CY134984.1
A/Texas/JMM_36/2012	CY134995.1	CY134994.1	CY134993.1	CY134988.1	CY134991.1	CY134990.1	CY134989.1	CY134992.1
A/Texas/JMM_37/2012	CY135003.1	CY135002.1	CY135001.1	CY134996.1	CY134999.1	CY134998.1	CY134997.1	CY135000.1
A/Texas/JMM_38/2012	CY135011.1	CY135010.1	CY135009.1	CY135004.1	CY135007.1	CY135006.1	CY135005.1	CY135008.1
A/Texas/JMM_39/2012	CY135019.1	CY135018.1	CY135017.1	CY135012.1	CY135015.1	CY135014.1	CY135013.1	CY135016.1
A/Texas/JMM_40/2012	CY135027.1	CY135026.1	CY135025.1	CY135020.1	CY135023.1	CY135022.1	CY135021.1	CY135024.1
A/Texas/JMM_41/2012	CY135035.1	CY135034.1	CY135033.1	CY135028.1	CY135031.1	CY135030.1	CY135029.1	CY135032.1
A/Texas/JMM_43/2012	CY135043.1	CY135042.1	CY135041.1	CY135036.1	CY135039.1	CY135038.1	CY135037.1	CY135040.1
A/Texas/JMM_44/2012	CY135051.1	CY135050.1	CY135049.1	CY135044.1	CY135047.1	CY135046.1	CY135045.1	CY135048.1
A/Texas/JMM_45/2012	CY135059.1	CY135058.1	CY135057.1	CY135052.1	CY135055.1	CY135054.1	CY135053.1	CY135056.1
A/Texas/JMM_46/2012	CY135067.1	CY135066.1	CY135065.1	CY135060.1	CY135063.1	CY135062.1	CY135061.1	CY135064.1
A/Texas/JMM_47/2012	CY135075.1	CY135074.1	CY135073.1	CY135068.1	CY135071.1	CY135070.1	CY135069.1	CY135072.1
A/Texas/JMM_48/2012	CY135083.1	CY135082.1	CY135081.1	CY135076.1	CY135079.1	CY135078.1	CY135077.1	CY135080.1
A/Texas/JMM_49/2012	CY135091.1	CY135090.1	CY135089.1	CY135084.1	CY135087.1	CY135086.1	CY135085.1	CY135088.1
A/Texas/JMM_50/2012	CY135099.1	CY135098.1	CY135097.1	CY135092.1	CY135095.1	CY135094.1	CY135093.1	CY135096.1
A/Texas/JMM_51/2012	CY135107.1	CY135106.1	CY135105.1	CY135100.1	CY135103.1	CY135102.1	CY135101.1	CY135104.1
A/Texas/JMM_54/2012	CY135131.1	CY135130.1	CY135129.1	CY135124.1	CY135127.1	CY135126.1	CY135125.1	CY135128.1
A/Texas/JMM_56/2012	CY135139.1	CY135138.1	CY135137.1	CY135132.1	CY135135.1	CY135134.1	CY135133.1	CY135136.1
A/Texas/JMM_57/2012	CY135147.1	CY135146.1	CY135145.1	CY135140.1	CY135143.1	CY135142.1	CY135141.1	CY135144.1
A/Texas/JMM_58/2012	CY135155.1	CY135154.1	CY135153.1	CY135148.1	CY135151.1	CY135150.1	CY135149.1	CY135152.1
A/Texas/JMM_59/2012	CY135163.1	CY135162.1	CY135161.1	CY135156.1	CY135159.1	CY135158.1	CY135157.1	CY135160.1
A/Texas/JMM_60/2012	CY135171.1	CY135170.1	CY135169.1	CY135164.1	CY135167.1	CY135166.1	CY135165.1	CY135168.1
A/Houston/JMM_61/2012	CY182696.1	CY182695.1	CY182694.1	CY182689.1	CY182692.1	CY182691.1	CY182690.1	CY182693.1
A/Houston/JMM_62/2012	CY182704.1	CY182703.1	CY182702.1	CY182697.1	CY182700.1	CY182699.1	CY182698.1	CY182701.1
A/Houston/JMM_63/2012	CY182712.1	CY182711.1	CY182710.1	CY182705.1	CY182708.1	CY182707.1	CY182706.1	CY182709.1
A/Houston/JMM_65/2012	CY182728.1	CY182727.1	CY182726.1	CY182721.1	CY182724.1	CY182723.1	CY182722.1	CY182725.1
A/Houston/JMM_66/2012	CY182736.1	CY182735.1	CY182734.1	CY182729.1	CY182732.1	CY182731.1	CY182730.1	CY182733.1
A/Houston/JMM_67/2012	CY182744.1	CY182743.1	CY182742.1	CY182737.1	CY182740.1	CY182739.1	CY182738.1	CY182741.1
A/Houston/JMM_68/2012	CY182752.1	CY182751.1	CY182750.1	CY182745.1	CY182748.1	CY182747.1	CY182746.1	CY182749.1

Table 2B

GenBank accession numbers of coding-complete influenza A(H3N2) genomes sequenced for this study, Texas, 3 November 2012–8 February 2013 (n = 154)

Virus name	PB2	PB1	PA	HA	NP	NA	M	NS
A/Houston/JMM_69/2012	CY182760.1	CY182759.1	CY182758.1	CY182753.1	CY182756.1	CY182755.1	CY182754.1	CY182757.1
A/Houston/JMM_70/2012	CY182768.1	CY182767.1	CY182766.1	CY182761.1	CY182764.1	CY182763.1	CY182762.1	CY182765.1
A/Houston/JMM_71/2012	CY182776.1	CY182775.1	CY182774.1	CY182769.1	CY182772.1	CY182771.1	CY182770.1	CY182773.1
A/Houston/JMM_72/2012	CY182784.1	CY182783.1	CY182782.1	CY182777.1	CY182780.1	CY182779.1	CY182778.1	CY182781.1
A/Houston/JMM_73/2012	CY182792.1	CY182791.1	CY182790.1	CY182785.1	CY182788.1	CY182787.1	CY182786.1	CY182789.1
A/Houston/JMM_74/2012	CY182800.1	CY182799.1	CY182798.1	CY182793.1	CY182796.1	CY182795.1	CY182794.1	CY182797.1
A/Houston/JMM_75/2012	CY182808.1	CY182807.1	CY182806.1	CY182801.1	CY182804.1	CY182803.1	CY182802.1	CY182805.1
A/Houston/JMM_76/2012	CY182816.1	CY182815.1	CY182814.1	CY182809.1	CY182812.1	CY182811.1	CY182810.1	CY182813.1
A/Houston/JMM_77/2012	CY182824.1	CY182823.1	CY182822.1	CY182817.1	CY182820.1	CY182819.1	CY182818.1	CY182821.1
A/Houston/JMM_78/2012	CY182832.1	CY182831.1	CY182830.1	CY182825.1	CY182828.1	CY182827.1	CY182826.1	CY182829.1
A/Houston/JMM_79/2012	CY182840.1	CY182839.1	CY182838.1	CY182833.1	CY182836.1	CY182835.1	CY182834.1	CY182837.1
A/Houston/JMM_80/2012	CY182848.1	CY182847.1	CY182846.1	CY182841.1	CY182844.1	CY182843.1	CY182842.1	CY182845.1
A/Houston/JMM_81/2012	CY182856.1	CY182855.1	CY182854.1	CY182849.1	CY182852.1	CY182851.1	CY182850.1	CY182853.1
A/Houston/JMM_82/2012	CY182864.1	CY182863.1	CY182862.1	CY182857.1	CY182860.1	CY182859.1	CY182858.1	CY182861.1
A/Houston/JMM_84/2012	CY182872.1	CY182871.1	CY182870.1	CY182865.1	CY182868.1	CY182867.1	CY182866.1	CY182869.1
A/Houston/JMM_85/2012	CY182880.1	CY182879.1	CY182878.1	CY182873.1	CY182876.1	CY182875.1	CY182874.1	CY182877.1
A/Houston/JMM_86/2012	CY182888.1	CY182887.1	CY182886.1	CY182881.1	CY182884.1	CY182883.1	CY182882.1	CY182885.1
A/Houston/JMM_87/2012	CY182896.1	CY182895.1	CY182894.1	CY182889.1	CY182892.1	CY182891.1	CY182890.1	CY182893.1
A/Houston/JMM_88/2012	CY182904.1	CY182903.1	CY182902.1	CY182897.1	CY182900.1	CY182899.1	CY182898.1	CY182901.1
A/Houston/JMM_90/2012	CY182912.1	CY182911.1	CY182910.1	CY182905.1	CY182908.1	CY182907.1	CY182906.1	CY182909.1
A/Houston/JMM_91/2012	CY182920.1	CY182919.1	CY182918.1	CY182913.1	CY182916.1	CY182915.1	CY182914.1	CY182917.1
A/Houston/JMM_92/2012	CY182928.1	CY182927.1	CY182926.1	CY182921.1	CY182924.1	CY182923.1	CY182922.1	CY182925.1
A/Houston/JMM_94/2012	CY182944.1	CY182943.1	CY182942.1	CY182937.1	CY182940.1	CY182939.1	CY182938.1	CY182941.1
A/Houston/JMM_95/2012	CY182952.1	CY182951.1	CY182950.1	CY182945.1	CY182948.1	CY182947.1	CY182946.1	CY182949.1
A/Houston/JMM_96/2012	CY182960.1	CY182959.1	CY182958.1	CY182953.1	CY182956.1	CY182955.1	CY182954.1	CY182957.1
A/Houston/JMM_97/2012	CY182968.1	CY182967.1	CY182966.1	CY182961.1	CY182964.1	CY182963.1	CY182962.1	CY182965.1
A/Houston/JMM_98/2012	CY182976.1	CY182975.1	CY182974.1	CY182969.1	CY182972.1	CY182971.1	CY182970.1	CY182973.1
A/Houston/JMM_99/2012	CY182984.1	CY182983.1	CY182982.1	CY182977.1	CY182980.1	CY182979.1	CY182978.1	CY182981.1
A/Houston/JMM_101/2013	CY182992.1	CY182991.1	CY182990.1	CY182985.1	CY182988.1	CY182987.1	CY182986.1	CY182989.1
A/Houston/JMM_102/2013	CY183000.1	CY182999.1	CY182998.1	CY182993.1	CY182996.1	CY182995.1	CY182994.1	CY182997.1
A/Houston/JMM_103/2013	CY183008.1	CY183007.1	CY183006.1	CY183001.1	CY183004.1	CY183003.1	CY183002.1	CY183005.1
A/Houston/JMM_104/2013	CY183016.1	CY183015.1	CY183014.1	CY183009.1	CY183012.1	CY183011.1	CY183010.1	CY183013.1
A/Houston/JMM_105/2013	CY183024.1	CY183023.1	CY183022.1	CY183017.1	CY183020.1	CY183019.1	CY183018.1	CY183021.1
A/Houston/JMM_106/2013	CY183032.1	CY183031.1	CY183030.1	CY183025.1	CY183028.1	CY183027.1	CY183026.1	CY183029.1
A/Houston/JMM_107/2013	CY183040.1	CY183039.1	CY183038.1	CY183033.1	CY183036.1	CY183035.1	CY183034.1	CY183037.1
A/Houston/JMM_108/2013	CY183048.1	CY183047.1	CY183046.1	CY183041.1	CY183044.1	CY183043.1	CY183042.1	CY183045.1
A/Houston/JMM_109/2013	CY183056.1	CY183055.1	CY183054.1	CY183049.1	CY183052.1	CY183051.1	CY183050.1	CY183053.1
A/Houston/JMM_110/2013	CY183064.1	CY183063.1	CY183062.1	CY183057.1	CY183060.1	CY183059.1	CY183058.1	CY183061.1
A/Houston/JMM_111/2013	CY183072.1	CY183071.1	CY183070.1	CY183065.1	CY183068.1	CY183067.1	CY183066.1	CY183069.1
A/Houston/JMM_112/2013	CY183080.1	CY183079.1	CY183078.1	CY183073.1	CY183076.1	CY183075.1	CY183074.1	CY183077.1
A/Houston/JMM_113/2013	CY183088.1	CY183087.1	CY183086.1	CY183081.1	CY183084.1	CY183083.1	CY183082.1	CY183085.1
A/Houston/JMM_115/2013	CY183096.1	CY183095.1	CY183094.1	CY183089.1	CY183092.1	CY183091.1	CY183090.1	CY183093.1
A/Houston/JMM_116/2013	CY183104.1	CY183103.1	CY183102.1	CY183097.1	CY183100.1	CY183099.1	CY183098.1	CY183101.1
A/Houston/JMM_117/2013	CY183112.1	CY183111.1	CY183110.1	CY183105.1	CY183108.1	CY183107.1	CY183106.1	CY183109.1
A/Houston/JMM_118/2013	CY183120.1	CY183119.1	CY183118.1	CY183113.1	CY183116.1	CY183115.1	CY183114.1	CY183117.1
A/Houston/JMM_119/2013	CY183128.1	CY183127.1	CY183126.1	CY183121.1	CY183124.1	CY183123.1	CY183122.1	CY183125.1
A/Houston/JMM_120/2013	CY183136.1	CY183135.1	CY183134.1	CY183129.1	CY183132.1	CY183131.1	CY183130.1	CY183133.1
A/Houston/JMM_121/2013	CY183144.1	CY183143.1	CY183142.1	CY183137.1	CY183140.1	CY183139.1	CY183138.1	CY183141.1
A/Houston/JMM_123/2013	CY183160.1	CY183159.1	CY183158.1	CY183153.1	CY183156.1	CY183155.1	CY183154.1	CY183157.1
A/Houston/JMM_124/2013	CY183168.1	CY183167.1	CY183166.1	CY183161.1	CY183164.1	CY183163.1	CY183162.1	CY183165.1
A/Houston/JMM_125/2013	CY183176.1	CY183175.1	CY183174.1	CY183169.1	CY183172.1	CY183171.1	CY183170.1	CY183173.1
A/Houston/JMM_126/2013	CY183184.1	CY183183.1	CY183182.1	CY183177.1	CY183180.1	CY183179.1	CY183178.1	CY183181.1
A/Houston/JMM_127/2013	CY183192.1	CY183191.1	CY183190.1	CY183185.1	CY183188.1	CY183187.1	CY183186.1	CY183189.1
A/Houston/JMM_128/2013	CY183200.1	CY183199.1	CY183198.1	CY183193.1	CY183196.1	CY183195.1	CY183194.1	CY183197.1
A/Houston/JMM_129/2013	CY183208.1	CY183207.1	CY183206.1	CY183201.1	CY183204.1	CY183203.1	CY183202.1	CY183205.1
A/Houston/JMM_130/2013	CY183216.1	CY183215.1	CY183214.1	CY183209.1	CY183212.1	CY183211.1	CY183210.1	CY183213.1
A/Houston/JMM_132/2013	CY183232.1	CY183231.1	CY183230.1	CY183225.1	CY183228.1	CY183227.1	CY183226.1	CY183229.1
A/Houston/JMM_134/2013	CY183240.1	CY183239.1	CY183238.1	CY183233.1	CY183236.1	CY183235.1	CY183234.1	CY183237.1
A/Houston/JMM_135/2013	CY183248.1	CY183247.1	CY183246.1	CY183241.1	CY183244.1	CY183243.1	CY183242.1	CY183245.1
A/Houston/JMM_136/2013	CY183256.1	CY183255.1	CY183254.1	CY183249.1	CY183252.1	CY183251.1	CY183250.1	CY183253.1

Table 2C

GenBank accession numbers of coding-complete influenza A(H3N2) genomes sequenced for this study, Texas, 3 November 2012–8 February 2013 (n = 154)

Virus name	PB2	PB1	PA	HA	NP	NA	M	NS
A/Houston/JMM_137/2013	CY183264.1	CY183263.1	CY183262.1	CY183257.1	CY183260.1	CY183259.1	CY183258.1	CY183261.1
A/Houston/JMM_139/2013	CY183272.1	CY183271.1	CY183270.1	CY183265.1	CY183268.1	CY183267.1	CY183266.1	CY183269.1
A/Houston/JMM_140/2013	CY183280.1	CY183279.1	CY183278.1	CY183273.1	CY183276.1	CY183275.1	CY183274.1	CY183277.1
A/Houston/JMM_141/2013	CY183288.1	CY183287.1	CY183286.1	CY183281.1	CY183284.1	CY183283.1	CY183282.1	CY183285.1
A/Houston/JMM_142/2013	CY183296.1	CY183295.1	CY183294.1	CY183289.1	CY183292.1	CY183291.1	CY183290.1	CY183293.1
A/Houston/JMM_144/2013	CY183304.1	CY183303.1	CY183302.1	CY183297.1	CY183300.1	CY183299.1	CY183298.1	CY183301.1
A/Houston/JMM_145/2013	CY183312.1	CY183311.1	CY183310.1	CY183305.1	CY183308.1	CY183307.1	CY183306.1	CY183309.1
A/Houston/JMM_146/2013	CY183320.1	CY183319.1	CY183318.1	CY183313.1	CY183316.1	CY183315.1	CY183314.1	CY183317.1
A/Houston/JMM_147/2013	CY183328.1	CY183327.1	CY183326.1	CY183321.1	CY183324.1	CY183323.1	CY183322.1	CY183325.1
A/Houston/JMM_149/2013	CY186026.1	CY186025.1	CY186024.1	CY186019.1	CY186022.1	CY186021.1	CY186020.1	CY186023.1
A/Houston/JMM_150/2013	CY186034.1	CY186033.1	CY186032.1	CY186027.1	CY186030.1	CY186029.1	CY186028.1	CY186031.1
A/Houston/JMM_151/2013	CY186042.1	CY186041.1	CY186040.1	CY186035.1	CY186038.1	CY186037.1	CY186036.1	CY186039.1
A/Houston/JMM_153/2013	CY186058.1	CY186057.1	CY186056.1	CY186051.1	CY186054.1	CY186053.1	CY186052.1	CY186055.1
A/Houston/JMM_154/2013	CY186066.1	CY186065.1	CY186064.1	CY186059.1	CY186062.1	CY186061.1	CY186060.1	CY186063.1
A/Houston/JMM_156/2013	CY186082.1	CY186081.1	CY186080.1	CY186075.1	CY186078.1	CY186077.1	CY186076.1	CY186079.1
A/Houston/JMM_157/2013	CY186090.1	CY186089.1	CY186088.1	CY186083.1	CY186086.1	CY186085.1	CY186084.1	CY186087.1
A/Houston/JMM_158/2013	CY186098.1	CY186097.1	CY186096.1	CY186091.1	CY186094.1	CY186093.1	CY186092.1	CY186095.1
A/Houston/JMM_160/2013	CY186114.1	CY186113.1	CY186112.1	CY186107.1	CY186110.1	CY186109.1	CY186108.1	CY186111.1
A/Houston/JMM_161/2013	CY186122.1	CY186121.1	CY186120.1	CY186115.1	CY186118.1	CY186117.1	CY186116.1	CY186119.1
A/Houston/JMM_162/2013	CY186130.1	CY186129.1	CY186128.1	CY186123.1	CY186126.1	CY186125.1	CY186124.1	CY186127.1
A/Houston/JMM_164/2013	CY186146.1	CY186145.1	CY186144.1	CY186139.1	CY186142.1	CY186141.1	CY186140.1	CY186143.1
A/Houston/JMM_166/2013	CY186154.1	CY186153.1	CY186152.1	CY186147.1	CY186150.1	CY186149.1	CY186148.1	CY186151.1
A/Houston/JMM_167/2013	CY186162.1	CY186161.1	CY186160.1	CY186155.1	CY186158.1	CY186157.1	CY186156.1	CY186159.1
A/Houston/JMM_168/2013	CY186170.1	CY186169.1	CY186168.1	CY186163.1	CY186166.1	CY186165.1	CY186164.1	CY186167.1
A/Houston/JMM_169/2013	CY186178.1	CY186177.1	CY186176.1	CY186171.1	CY186174.1	CY186173.1	CY186172.1	CY186175.1
A/Houston/JMM_170/2013	CY186186.1	CY186185.1	CY186184.1	CY186179.1	CY186182.1	CY186181.1	CY186180.1	CY186183.1
A/Houston/JMM_172/2013	CY186202.1	CY186201.1	CY186200.1	CY186195.1	CY186198.1	CY186197.1	CY186196.1	CY186199.1
A/Houston/JMM_174/2013	CY186210.1	CY186209.1	CY186208.1	CY186203.1	CY186206.1	CY186205.1	CY186204.1	CY186207.1
A/Houston/JMM_175/2013	CY186218.1	CY186217.1	CY186216.1	CY186211.1	CY186214.1	CY186213.1	CY186212.1	CY186215.1
A/Houston/JMM_176/2013	CY186226.1	CY186225.1	CY186224.1	CY186219.1	CY186222.1	CY186221.1	CY186220.1	CY186223.1
A/Houston/JMM_177/2013	CY186234.1	CY186233.1	CY186232.1	CY186227.1	CY186230.1	CY186229.1	CY186228.1	CY186231.1
A/Houston/JMM_178/2013	CY186242.1	CY186241.1	CY186240.1	CY186235.1	CY186238.1	CY186237.1	CY186236.1	CY186239.1
A/Houston/JMM_179/2013	CY186250.1	CY186249.1	CY186248.1	CY186243.1	CY186246.1	CY186245.1	CY186244.1	CY186247.1
A/Houston/JMM_180/2013	CY186258.1	CY186257.1	CY186256.1	CY186251.1	CY186254.1	CY186253.1	CY186252.1	CY186255.1

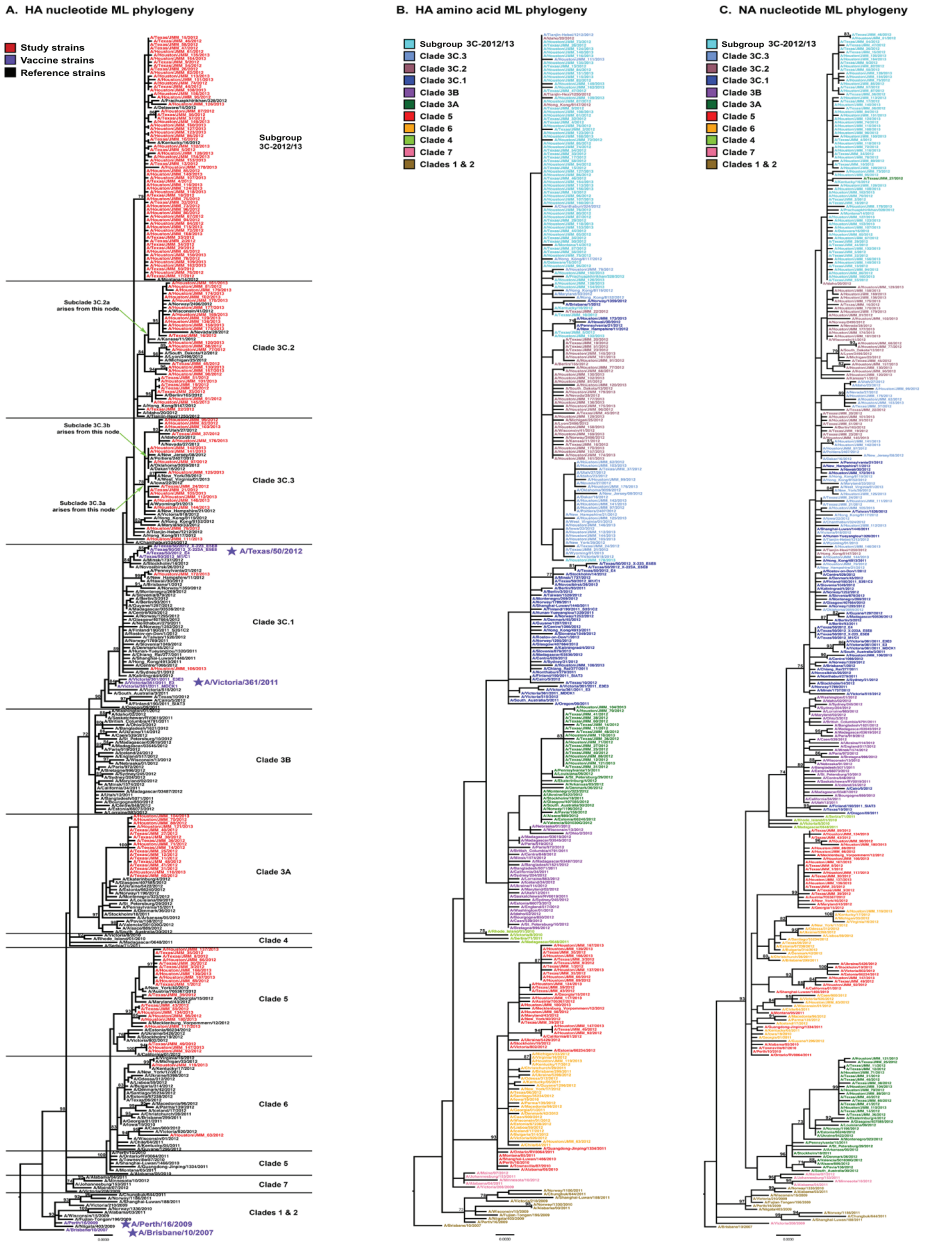
and 4 in Figure 3A). Similarly, while most HA clade 5 and 6 strains had unique PB2 and PB1 segments (blue boxes in the PB2 and PB1 columns of Figure 3A), three monophyletic HA clade 5 strains had retained the A/Perth/16/2009-like PB2 and PB1 segments (orange boxes for PB2 and PB1 in constellation 7 in Figure 3A). Monophyletic HA strains sharing common intrasubtypic reassortment genome constellations demonstrated that these reassortment events are likely to occur once, creating viral strains that transmit and spread to new individuals, rather than occurring as multiple independent reassortment events. NA diversity was also observed among clade 5 and 6 viruses, which can also be appreciated by the interleaving colours when HA clade designations were mapped onto the NA phylogeny (Figure 1C).

To stringently explore the diversity among clade 3C viruses, a genome constellation analysis containing only the clade 3C genomes was performed using a 99% nucleotide identity cut-off value (Figure 3B). For the most part, the four 3C subclades/subgroups each contained their own unique genome constellations (Figure 3B). We found that most 3C-2012/13 viruses contained a unique NS segment compared with other 3C viruses (constellation 9 in Figure 3B), and all 3C-2012/13 viruses had a unique NP segment (constellations 8 and 9 in Figure 3B). In addition, seven monophyletic HA 3C.2 specimens contained unique PB2, PA, HA, and M segments at the 99% cut-off (constellation 10 in Figure 3B), and five monophyletic HA 3C.3 samples had incorporated a unique NA (constellation 6 in Figure 3B).

Overall, genome constellation analysis revealed that intrasubtypic reassortment occurs frequently in human H3N2 viruses. In this collection alone, intrasubtypic reassortment resulted in the expansion of antigenic drift variants into new genome constellations, the generation of new HA/NA combinations, and the acquisition of a unique NS vRNA.

Figure 1

Phylogenetic relationships among the influenza A(H3N2) study samples, vaccine strains, and selected references inferred from maximum likelihood analyses of the HA nucleotide (A), HA protein (B), and NA nucleotide (C) sequences, Texas, 3 November 2012–8 February 2013 (n = 154)



CDC: Centers for Disease Control and Prevention; HA: haemagglutinin; ML: maximum likelihood; NA: neuraminidase; WHO: World Health Organization.

Bootstrap values for nodes with ≥70% support following 500 replicates are provided. Clades are classified following the CDC nomenclature [25] as closely as possible and are defined based on the HA nucleotide phylogeny (A). A new subgroup, designated 3C-2012/13, appears to have arisen during the 2012/13 influenza season. The nodes from which the new WHO-designated 3C.2a, 3C.3a, and 3C.3b subclades subsequently arise are marked on the phylogeny to place our 2012/13 study in the context of more recent seasons; placement was based on additional ML and neighbour-joining phylogenetic analyses using available H3 sequence data through 2014 (data not shown). For the HA protein (B) and NA nucleotide phylogenies (C), strain names are coloured by HA nucleotide clade definitions, showing that the HA protein and NA nucleotide clades largely match the nucleotide-based HA clade definitions. However, some interleaving of 3C subclades is observed in the HA protein tree, indicating that these subclades diverge largely through synonymous nucleotide mutations. The interleaving of colours in the NA nucleotide tree signifies intrasubtypic reassortment between the HA and NA gene segments. Scale bars indicate the average number of nucleotide changes per site.

The authors gratefully acknowledge the 87 originating and submitting laboratories who directly contributed sequences used in the phylogenetic analyses to GISAID: ADImmune Corporation, Taiwan; Alabama Department of Public Health, Bureau of Clinical Laboratories; Arkansas Children's Hospital; Austin Health, Australia; California Department of Health Services; Canterbury Health Services, New Zealand; US Centers for Disease Control and Prevention; CRR virus Influenza region Sud, France; Delaware Public Health Lab; Gart Naval General Hospital, United Kingdom; Georgia Public Health Laboratory; Government Virus Unit, Hong Kong; Health Protection Agency, England; Health Protection Inspectorate, Estonia; Hospital Clinic, Spain; Institute of Epidemiology and Infectious Diseases AMS of Ukraine; Institute of Epidemiology Disease Control and Research (IEDCR) & Bangladesh National Influenza Centre (NIC); Institute of Immunology and Virology Torlak, Serbia; Institute of Medical and Veterinary Science (IMVS), Australia; Institute of Public Health, Montenegro; Institute de Salud Publica de Chile; Institute Nacional de Saude, Portugal; Institut Pasteur de Dakar, Senegal; Institut Pasteur de Madagascar; Iowa State Hygienic Laboratory; Istituto Superiore di Sanit, Italy; Kansas Department of Health and Environment; Kentucky Division of Laboratory Services; Laboratory for Virology, National Institute of Public Health, Slovenia; Laboratory of Influenza and ILI, Belarus; Landspitali - University Hospital, Iceland; Louisiana Department of Health and Hospitals; Maine Health and Environmental Testing Laboratory; Maryland Department of Health and Mental Hygiene; Melbourne Pathology, Australia; Michigan Department of Community Health; Ministry of Health of Ukraine; Minnesota Department of Health; Monash Medical Centre, Australia; Montana Laboratory Services Bureau; Montana Public Health Laboratory; National Centre of Infectious and Parasitic Diseases, Bulgaria; National Institute for Communicable Disease, South Africa; National Institute for Health and Welfare, Finland; National Institute for Medical Research, United Kingdom; National Institute of Health, Korea; National Microbiology Laboratory, Health Canada; Nebraska Public Health Lab; Nevada State Health Laboratory; New Hampshire Public Health Laboratories; New Jersey Department of Health & Senior Services; New York City Department of Health; New York Medical College; New York State Department of Health; Norwegian Institute of Public Health; Oregon Public Health Laboratory; Pathwest QE II Medical Centre, Australia; Pennsylvania Department of Health; Prince of Wales Hospital, Australia; Queensland Health Scientific Services, Australia; Republic Institute for Health Protection, Macedonia; Rhode Island Department of Health; Robert Koch-Institute, Germany; San Antonio Metropolitan Health, Texas; South Dakota Public Health Lab; Southern Nevada Public Health Lab; Spokane Regional Health District, Washington; Statens Serum Institute, Denmark; State of Hawaii Department of Health; State of Idaho Bureau of Laboratories; Swedish Institute for Infectious Disease Control; Texas Department of State Health Services-Laboratory Services; University of Vienna, Austria; U.S. Air Force School of Aerospace Medicine; Utah Department of Health; VACSERA, Egypt; Victorian Infectious Diseases Reference Laboratory, Australia; Virginia Division of Consolidated Laboratories; Westmead Hospital, Australia; West Virginia Office of Laboratory Services; WHO Chinese National Influenza Center; WHO Collaborating Centre for Reference and Research on Influenza, Australia; WHO National Influenza Centre, Norway; WHO National Influenza Centre, National Institute of Medical Research (NIMR), United Kingdom; WHO National Influenza Centre Russian Federation; Wisconsin State Laboratory of Hygiene; and Wyoming Public Health Laboratory.

Figure 2

HA amino acid differences across among representative influenza A(H3N2) strains for various HA clades, Texas, 3 November 2012–8 February 2013 (n = 5)

		Residue changes in the HA1 domain of 2012/13 study samples and vaccine strains																																
H3 structural numbering:			2	8	33	45[a]	48	53	62	94	124[b]	128[c]	142	144[d]	145	156	186	194	198	209	212	214	219	221	223	226	230	261	278	280	292	312			
H3 numbering from Met:	9	18	24	49	61	64	69	78	110	140	144	158	160	161	172	202	210	214	225	228	230	235	237	239	242	246	277	294	296	308	328			
Antigenic site:							C	E	E	A		A	A	A	B	B		B			D	D			D			C						
Virus name and clade																																		
A/Perth/16/2009	1	Y	K	N	Q	S-	T	D	K	Y	S+	T+	R	K-	N	H	G	L	A	S	T	S	S	P	V	I	I	R	N	E	K	N	
Clade Consensus Sequence	3C.1	Y	K	N	R	N+	I	D	E	Y	S+	T+	R	N+	N	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	K	S	
A/Victoria/361/2011_WHO_MDCK	3C.1	H	K	N	Q	N+	I	D	E	Y	S+	T+	R	N+	N	H	G	L	S	S	A	I	S	P	I	I	I	R	N	E	K	S	
A/Victoria/361/2011_WHO_E3		H	K	N	Q	N+	I	D	E	Y	S+	T+	R	N+	N	R	V	L	S	S	A	I	S	P	I	I	I	R	N	E	K	S	
A/Victoria/361/2011_CDC_E3E3		H	K	N	Q	N+	I	D	E	Y	S+	T+	R	N+	N	Q	V	L	S	S	A	I	Y	P	I	I	I	R	N	E	K	S	
A/Texas/50/2012_M1C1	3C.1	Y	K	N	R	N+	I	D	E	Y	S+	N-	R	N+	N	H	G	L	P	S	A	I	S	P	I	I	I	R	K	E	K	S	
A/Texas/50/2012_E4		Y	K	N	R	N+	I	D	E	Y	S+	N-	R	N+	N	H	V	L	P	S	A	I	F	P	I	I	I	R	K	E	K	S	
A/Texas/50/2012_X223_E5E8		Y	K	N	R	N+	I	D	E	Y	S+	N-	R	N+	N	H	V	L	P	S	A	I	F	P	I	N	I	R	K	E	K	S	
A/Texas/50/2012_X223A_E5E8		Y	K	N	R	N+	I	D	E	Y	S+	N-	R	N+	N	H	V	L	P	S	A	I	F	P	I	N	I	R	K	E	K	S	
Clade Consensus Sequence	5	Y	E	D	Q	S-	T	N	E	H	S+	T+	R	N+	N	H	G	L	A	N	A	I	S	P	V	I	V	R	N	A	K	N	
A/Texas/JMM_3/2012 Po	5	Y	E	D	R	S-	T	N	E	H	S+	T+	R	N+	N	H	G	L	A	N	T	I	S	P	V	I	V	R	N	A	K	N	
A/Texas/JMM_3/2012 P2		Y	E	D	R	S-	T	N	E	H	S+	T+	R	N+	N	H	G	P	A	N	T	I	S	P	V	I	V	R	N	A	K	N	
Clade Consensus Sequence	3A	Y	K	N	Q	S-	T	D	E	Y	S+	T+	R	D-	S	H	G	L	A	S	S	I	S	P	I	I	I	Q	N	E	K	N	
A/Texas/JMM_48/2012 Po	3A	Y	K	N	Q	S-	T	D	E	Y	S+	T+	R	D-	S	H	G	L	A	S	S	L	S	P	I	I	I	Q	N	E	K	N	
A/Texas/JMM_48/2012 P2		Y	K	N	Q	S-	T	D	E	Y	S+	T+	R	D-	S	H	G	L	A	S	S	L	S	P	I	I	I	Q	N	E	K	N	
Clade Consensus Sequence	3C.2	Y	K	N	R	N+	I	D	E	Y	S+	T+	R	N+	S	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	K	S	
A/Texas/JMM_20/2012 Po	3C.2	Y	K	N	R	N+	I	D	E	Y	N-	T+	R	N+	S	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	R	S	
A/Texas/JMM_20/2012 P2		Y	K	N	R	N+	I	D	E	Y	N-	T+	R	N+	S	H	G	L	S	S	A	I	S	L	I	I	I	R	K	E	R	S	
Clade Consensus Sequence	3C.3	Y	K	N	R	N+	I	D	E	Y	S+	A-	G	N+	S	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	K	S	
A/Texas/JMM_37/2012 Po	3C.3	Y	K	N	Q	N+	M	D	E	Y	S+	A-	G	N+	S	H	G	L	S	S	A	I	S	P	I	V	I	R	K	E	K	S	
A/Texas/JMM_37/2012 P2		Y	K	N	Q	N+	M	D	E	Y	S+	A-	G	N+	S	H	G	L	S	S	A	I	S	P	I	V	I	R	K	E	K	S	
Clade Consensus Sequence	3C-2012/13	Y	K	N	R	N+	I	D	E	Y	S+	T+	R	N+	S	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	K	S	
A/Texas/JMM_4/2012 Po	3C-2012/13	Y	K	N	R	N+	I	D	E	Y	S+	T+	R	N+	S	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	K	S	
A/Texas/JMM_4/2012 P2		Y	K	N	R	N+	I	D	E	Y	S+	T+	R	N+	S	H	G	L	S	S	A	I	S	P	I	I	I	R	K	E	K	S	

HA: haemagglutinin; Met: methionine; +/- indicates whether the residue is associated with the gain (+) or loss (–) of a potential glycosylation site.

[a] S45N creates a potential glycosylation site (***S***SS to ***N***SS), with glycosylation occurring at N45.

[b] S124N removes a potential glycosylation site (NE***S*** to NE***N***), with glycosylation occurring at N122 (antigenic site A).

[c] T128N and T128A remove a potential glycosylation site (NW***T*** to NW***N*** or NW***A***), with glycosylation occurring at N126 (antigenic site A).

[d] N144D removes a potential glycosylation site (***N***SS to ***D***SS), with glycosylation occurring at N144 (antigenic site A).

All residue changes that exist in HA among the study samples (yellow), vaccine strains (purple), and clade consensus sequences (white) are provided. All sequences that match the A/Perth/16/2009 strain (clade 1) are coloured in pink, while residues that differ from that strain are shown in blue (first residue difference) or green (second residue difference). Residues associated with the gain (+) or loss (–) of a potential glycosylation site are indicated. Antigenic site assignments are derived from several sources [60-63]. Po indicates the original clinical swab specimen, while P2 indicates the second-passage viral stock grown in MDCK cells.

Figure 3

Genome constellations for coding-complete influenza A(H3N2) sequences from the 2012/13 influenza epidemic for all clades (A) and for clade 3 (B), Texas, 3 November 2012–8 February 2013 (n =154)

A.

	Genome constellation								HA clade	Count	
	PB2	PB1	PA	HA	NP	NA	M	NS		Study Samples	EpiFlu Samples
1									1	0	1
2									3A	17	3
3									3B	0	11
4									3A	1	0
5									6	1	2
6									6	0	6
7									5[a]	3	0
8									5	0	3
9									6	1	1
10									5	18	1
11									3C.1, 3C.2, 3C.3, 3C-2012/13	106	16
12									3C.2[b]	7	0

98% nucleotide identity clusters[e]:
- Cluster 1
- Cluster 2
- Cluster 3
- Cluster 4
- Cluster 5

B.

	Genome constellation								HA clade	Count	
	PB2	PB1	PA	HA	NP	NA	M	NS		Study Samples	EpiFlu Samples
1									3C.1	1	1
2									3C.1	1	2
3									3C.1	0	1
4									3C.3	1	2
5									3C.3, 3C.2[c]	12	3
6									3C.3[d]	5	1
7									3C.2	20	1
8									3C-2012/13	5	1
9									3C-2012/13	61	0
10									3C.2[b]	7	0

99% nucleotide identity clusters[e]:
- Cluster 1
- Cluster 2
- Cluster 3
- Cluster 4
- Cluster 5
- Cluster 6

[a] These study samples form their own HA monophyly within clade 5.

[b] These study samples form their own HA monophyly within clade 3C.2.

[c] Of the 13 strains in this constellation that are also in the HA phylogeny, only one strain falls within clade 3C.2.

[d] These study samples form their own HA monophyly within clade 3C.3.

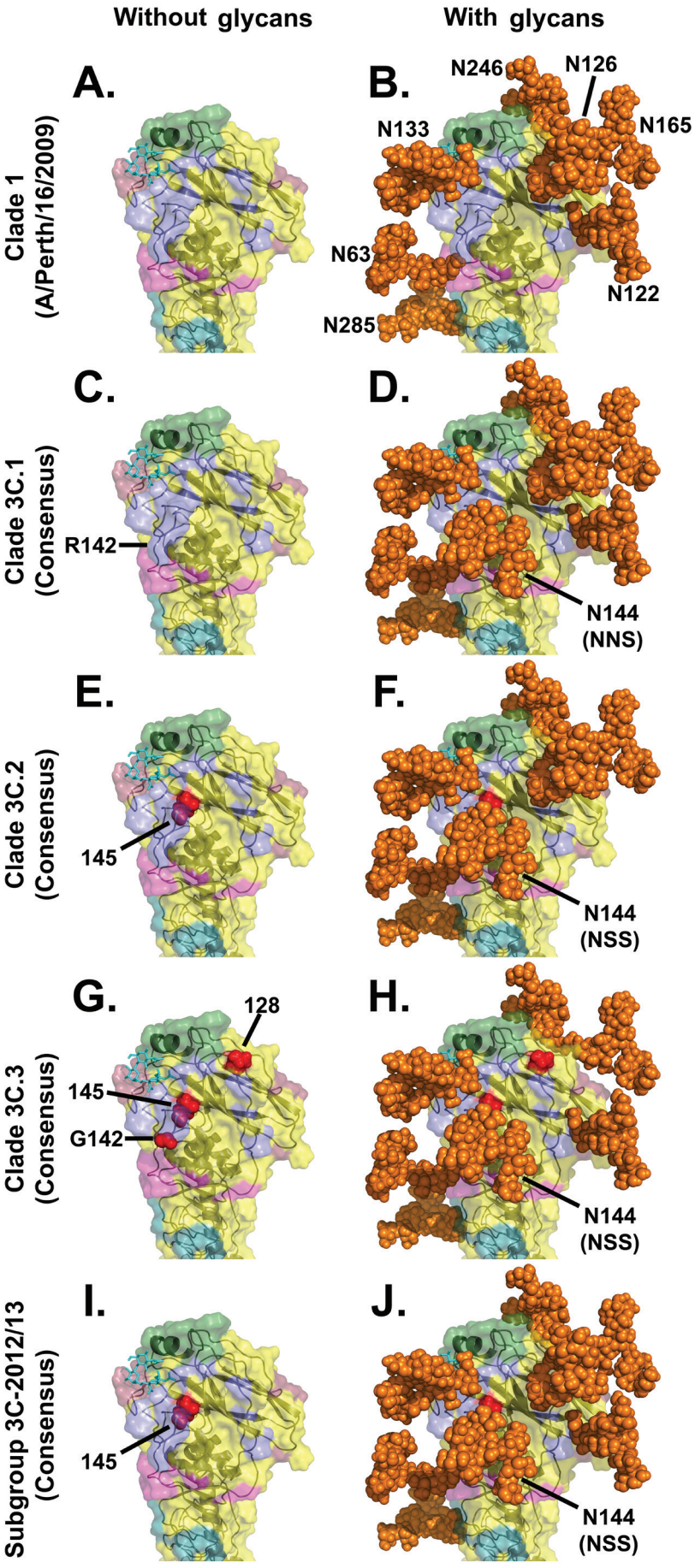
[e] Please note that each column is evaluated independently and cluster numbering is arbitrary.

The number of samples observed for each genome constellation is provided, along with an HA clade assignment, for all constellations for which at least one sequence was also included in the HA phylogeny (Figure 1). Results are sorted first by HA, followed by NA, PB2, PB1, PA, NP, M, and NS.
(A) Genome constellation 1 (orange) segments were defined as sharing 98% nucleotide identity with the A/Perth/16/2009 vaccine strain. Twelve unique genome constellations were identified at the 98% nucleotide identity cut-off, eight of which were observed in the study samples.
(B) Clade 3 genome constellation 1 (purple) segments were defined as sharing 99% nucleotide identity with the A/Victoria/361/2011 vaccine strain. 10 unique genome constellations were identified at the 99% nucleotide identity cut-off, nine of which were observed in the study samples.

The authors gratefully acknowledge the 32 originating and submitting laboratories who directly contributed sequences used in the constellation analysis to GISAID: Alaska State Virology Lab; Arizona Department of Health Services; Austin Health, Australia; California Department of Health Services; Canterbury Health Services, New Zealand; US Centers for Disease Control and Prevention; Institute of Medical and Veterinary Science (IMVS), Australia; Institut Pasteur New Caledonia; Iowa State Hygienic Laboratory; John Hunter Hospital, Virology Unit, Clinical Microbiology, Australia; Kentucky Division of Laboratory Services; Melbourne Pathology, Australia; Michigan Department of Community Health; New Mexico Department of Health; New York State Department of Health; Papua New Guinea Institute of Medical Research; Pathwest QE II Medical Centre, Australia; Pennsylvania Department of Health; Puerto Rico Department of Health; Queensland Health Scientific Services, Australia; Research Institute of Tropical Medicine, Philippines; Rhode Island Department of Health; Royal Hobart Hospital, Australia; Southern Nevada Public Health Lab; Spokane Regional Health District, Washington; State of Hawaii Department of Health; Texas Department of State Health Services-Laboratory Services; USAMC-AFRIMS Department of Virology, Cambodia; Utah Department of Health; Victorian Infectious Diseases Reference Laboratory, Australia; WHO Collaborating Centre for Reference and Research on Influenza, Australia; and WHO National Influenza Centre, National Institute of Medical Research (NIMR), United Kingdom.

Figure 4

Location of antigenic sites on the H3 monomer, along with key clade 3C substitutions and glycosylation sites



In all panels, the peptide backbone of the HA globular head is represented as a ribbon with a translucent solid surface using the A/Finland/486/2004 HA crystal structure, 2YP3, bound to a synthetic 2,6-sialic acid ligand (cyan stick structure) [40]. In panels on the right, all potential N-linked glycans in the H3 globular head were modelled using the AllosMod server [41] and rendered by PyMOL [45] as solid orange spheres.

Panels A and B: A representation of A/Perth/16/2009 (clade 1) illustrating previously defined H3 antigenic sites [60-63]: lavender (antigenic site A), green (antigenic site B), deep teal (antigenic site C), raspberry (antigenic site D), and light magenta (antigenic site E). A/Perth/16/2009 lacks the N144 glycosylation site that all subclade/subgroup 3C viruses have.

Panels C and D: A representation of 3C.1 viruses illustrating the H3 antigenic sites and the presence of the NNS sequon that may allow for glycosylation at N144, possibly at a reduced efficiency [55].

Panels E–J: Critical amino acid differences between the 3C.1 consensus and the indicated 3C subclade/subgroup consensuses are shown in red and labelled with H3 structural numbering.

Panel G: 3C.3 viruses have an R142G substitution, which removes most of the bulk of that protruding side chain; in this panel, the structure was modified to remove the bulky arginine (R) side chain (cf. the small red spheres for residue 142 with the larger lavender protrusion that extends downward in the other panels, as labelled in C).

Panel F: Some 3C.2 viruses have lost the N122 glycan.

Panel H: 3C.3 viruses lack the N126 glycan.

Panel J: 3C-2012/13 viruses are among the most heavily glycosylated.

Assessment of antigenic evolution in a single epidemic season

HI assays were performed [39] to determine the extent of antigenic drift among the H3 proteins during the 2012/13 epidemic. Two-way HI measurements, which paired an antigen with the antiserum raised against that antigen, provided HI titres that were typical for anti-H3N2 ferret and sheep antisera (Table 1). The specificity of the assay was shown by the negative control serum raised against a zoonotic H3N2 variant virus, (H3N2)v, which is antigenically similar to viruses that circulated in humans in the early to mid-1990s. Analysis of isolates propagated from specimens selected from the major clades showed that ferret and sheep polyclonal antisera against the recommended vaccine strains (A/Victoria/361/2011 and A/Texas/50/2012) prevented the binding of clade 5, 3C.1, and 3C.2 isolates to the sialic acid receptors on guinea pig red blood cells (Table 1). However, the emerging 3C.3 and 3C-2012/13 viruses escaped inhibition by these vaccine sera (›16-fold reduction) (Table 1). This illustrates that the few amino acid differences in the H3 proteins (e.g. residues 128, 142, and 145 using H3 structural numbering) between the emergent 3C.3 and 3C-2012/13 viruses vs the 3C.1 viruses have enabled escape from polyclonal antibody responses to natural infections or vaccinations.

The virus isolates tested in HI were sequenced and had the same HA amino acid sequences as the original swab specimens in all but two cases (Figure 2), while the NA vRNAs of 3C.3 and 3C-2012/13 viruses acquired a mutation (i.e. D151N/G) that has been shown to reduce NA activity [48], illustrating the difficulty in accurately analysing the true antigenicity of viruses circulating in humans using in vitro assays and the importance of directly sequencing the viral population in swab specimens rather than viruses isolated from eggs or tissue culture. Furthermore, the HA of many contemporary H3N2 viruses bind inefficiently to guinea pig RBCs and the cognate NA aids in binding to receptors on the RBCs. Addition of oseltamivir in the HA assay to reduce NA binding reduced the HA titre so much for these viruses that HI assays could not be performed in the presence of oseltamivir.

Examination of the HA and NA residue changes (Figure 2, Figure 4) demonstrated that a few simultaneously evolving amino acid substitutions, some causing the gain or loss of sequons for N-linked glycans, differentiated the HA molecules among strains in the 2012/2013 epidemic.

Discussion

The H3N2 subtype predominated in Houston during the 2012/13 season, as was the case throughout the US for this epidemic [10]. While viruses from several known clades were co-circulating throughout the season, the majority of IAV H3s belonged to the 3C subclades, 3C.2 and 3C.3, as well as a new subgroup designated here as 3C-2012/13. Subgroup 3C-2012/13 represented an emerging group of viruses that differed primarily in nucleotide, but not amino acid, sequence from subclades 3C.2 and 3C.3, suggesting the possibility of convergent evolution of these drift variants in the human population.

Our data demonstrate that intrasubtypic reassortment created some of the co-circulating viruses in the 2012/13 epidemic. This finding is consistent with a recent study [12] and demonstrates that intrasubtypic reassortment is another evolutionary mechanism that antigenic drift variants can employ to gain fitness advantages and spread among the viral population. In some cases, intrasubtypic reassortment events among H3N2 segments can become fixed in the population as part of periodic selective sweeps and incorporated into the trunk of the phylogeny [49,50]. Intrasubtypic reassortment may also serve as a mechanism whereby drift variants can recover from a putative fitness loss (e.g. reduced receptor binding by HA) by acquiring epistatic changes through reassortment (e.g. in NA or NS) and/or the subsequent increase in amino acid replacement rates that reassortment triggers [49,50]. The NS gene segment encodes the NS1 protein, which is important in the evasion of the innate host immune response [51,52]; therefore, it is reasonable to hypothesize that the acquisition of a unique NS gene segment in most 3C-2012/13 viruses may offer a fitness advantage in the host when paired with the 3C-2012/13 HA segment. Experimental testing of these types of reassortants (e.g. 3C-2012/13 constellations 9 and 10 in Figure 3B) is required to test this hypothesis and to better understand how various segments interact with drifted HA sequences to create successful H3N2 strains.

Importantly, our data show that the emerging 3C.3 and 3C-2012/13 subclades/subgroups represented the majority of the virus strains sequenced in this study, and the representative samples from these clades that were tested in HI assays reacted poorly with antisera raised against the vaccine strains used for the 2012/13 season (egg- and cell-grown A/Victoria/361/2011) and the 2013/14 season (A/Texas/50/2012) which are both in the 3C.1 subclade. While use of oseltamivir in the HA assay reduced the HA titre enough that HI assays could not be performed in the presence of oseltamivir, more recent data on vaccine effectiveness and vaccine strain updates have corroborated our findings that the 2012/13 H3N2 vaccine strain was generally poorly matched for the majority of circulating 2012/13 H3N2 strains [26,47,53].

Overall, our data demonstrate that a few simultaneously evolving amino acid substitutions played a central role in immune escape by the 2012/13 epidemic viruses. Our data indicate that the H3N2 viruses escape the human immune response through a combination of specific residue changes (e.g. N145S, H3 structural numbering) and the impact these changes have on N-linked glycosylation. Residue 145 is located on a loop of the H3 that is partly involved in receptor binding (Figure 4),

and it is one of seven residues shown to play a central role in the antigenic drift of H3 viruses in humans [54]. Viruses in 3C.2, 3C.3, and 3C-2012/13 have the S145 substitution, while the clade 3C.1 viruses have N145. This residue is part of an N-linked glycosylation sequon, and the N145S change is likely to increase glycosylation of N144 [55].

Some 3C.2 viruses acquired an S124N substitution, which removes the N122 glycosylation site; they are antigenically similar to previously circulating viruses in clades 1, 5, and 3C.1 and are therefore likely to die out. However, the new 3C.2a subclade that emerged from 3C.2 (Figure 1A) and expanded in the 2014/15 season [47] has lost glycosylation at 144 (N144S) and added a new putative glycosylation site at N158.

The 3C.3 viruses acquired accessory changes in or near antigenic site A, including T128A, which removes the N126 glycosylation site, and R142G; these substitutions are likely to be responsible for these viruses' antigenic difference from 3C.1 and 3C.2 viruses. The alterations of N-linked glycosylation sequons among the various clades (Figure 2, Figure 4) are likely to contribute to more complex structural differences in the HA due to the gain or loss of glycan shields [56-58]. In particular, it appears that glycans at HA residues N126 and N144 are the major differences among the 3C subclades/subgroups (Figure 2).

Furthermore, because 3C.3-like viruses and their descendants currently circulate globally (data not shown), vaccines based on A/Texas/50/2012 are likely to offer suboptimal protection. This hypothesis is supported by the moderate vaccine effectiveness for the 2012/13 season in the US (47% for IAV), where the H3N2 subtype predominated [13]. The data presented here indicate that an ideal vaccine candidate would probably be derived from subclade 3C.3a or 3C2a. The WHO influenza vaccine selection committee recently recommended an A/Switzerland/9715293/2013-like strain, which belongs to the 3C.3a subgroup of the 3C.3 viruses (Figure 1A), for the 2015 southern hemisphere season [59]. In addition to N145S, T128A and R142G substitutions, the 3C.3a viruses have acquired additional changes (A138S, F159S) that are likely to be antigenically important.

Conclusion

This study provides insights into the dynamics of the 2012/13 influenza epidemic by demonstrating that multiple H3N2 strains co-circulated during the epidemic, that different antigenic drift variants evolved concurrently and that intrasubtypic reassortment occurred frequently, suggesting that reassortment plays a role in the evolution of seasonal influenza viruses. The emergent 3C.3 and 3C-2012/13 viruses, which had substitutions impacting N-linked glycosylation at major antigenic sites, predominated, and we predict that they will come to define the new trunk of the phylogeny. Finally, our data show that the accumulation of relatively few HA mutations can convey large selective advantages, sometimes through epistatic interactions, and support the new 2015 southern hemisphere vaccine strain recommendation.

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Conflict of interest

None declared.

Authors' contributions

KMS, SAS, RJO, SRD, DEW, and JMM conceived and designed the study. RJO and JMM conducted clinical diagnostics. XL and RAH performed the sequencing, TBS performed the sequencing data analysis and computational genome assemblies, and NF performed the finishing analysis to assess the final sequence quality. KMS and SAS performed the phylogenetic and genome constellation analyses. KMS and HLH performed the laboratory experiments. AT modelled the H3 N-linked glycosylation, and AT and KMS created the final structural figures. KMS, SAS, SRD, DEW, and JMM wrote the manuscript, and all authors reviewed and approved the final version. Funding for the studies was obtained by DEW and JMM.

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Public health response to two incidents of confirmed MERS-CoV cases travelling on flights through London Heathrow Airport in 2014 – lessons learnt

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In May 2014, Public Health England was alerted to two separate laboratory-confirmed cases of Middle East respiratory syndrome coronavirus (MERS-CoV) infection who transited through London Heathrow Airport while symptomatic on flights from Saudi Arabia to the United States of America. We present the rationale for the public health response to both incidents, and report results of contact tracing. Following a risk assessment, passengers seated two seats around the cases were prioritised for contact tracing and a proactive media approach was used to alert all passengers on the planes of their possible exposure in both incidents. In total, 64 United Kingdom (UK) residents were successfully contacted, 14 of whom were sat in the priority area two seats all around the case(s). Five passengers reported respiratory symptoms within 14 days of the flight, but all tested were negative for MERS-CoV. Details of non-UK residents were passed on to relevant World Health Organization International Health Regulation focal points for follow-up, and no further cases were reported back. Different approaches were used to manage contact tracing for each flight due to variations in the quality and timeliness of the passenger contact information provided by the airlines involved. No evidence of symptomatic onward transmission was found.

Introduction

On 2 May 2014, Public Health England (PHE) was alerted by the public health authorities in the United States (US) of a laboratory-confirmed case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection who had transited through London Heathrow Airport, United Kingdom (UK) on a flight from Saudi Arabia to the US [1]. Nine days later, the US authorities notified PHE of a second, unlinked, laboratory-confirmed MERS-CoV case that had also travelled from Saudi Arabia to the US and transited through Heathrow Airport [1].

This article describes the contact tracing approaches undertaken for both in-bound flights, compares the issues and outcomes and provides recommendations on future approaches. The results of contact tracing of UK nationals on the same onward flights from London to the US as the cases are also reported.

MERS-CoV is a novel coronavirus, first detected in a patient in 2012 [2]. As of 16 April 2015, the World Health Organization (WHO) have been notified of 1,106 cases of MERS-CoV, with 421 deaths [3]. The vast majority of cases have occurred in Saudi Arabia and the United Arab Emirates. However, exported MERS-CoV cases have occurred in several countries outside the Middle East the majority travelling by air [4]. The public health response to many of these cases involved follow up of close contacts on planes to detect any evidence of onward transmission. Only limited secondary transmission has been demonstrated to date around such exported cases [5].

Two earlier imported laboratory-confirmed MERS-CoV cases from the Middle East have been documented in the UK. Of these, the first was transported to the UK via private air ambulance and the second travelled on a commercial flight. Both were symptomatic during their flights. No secondary cases were identified among the contacts on either of these flights, however two family contacts of the second case were later identified as confirmed cases following transmission in the UK in household and hospital settings [6,7].

Outside of these incidents, we are aware of four further published reports of confirmed MERS-CoV cases travelling on commercial flights, including two cases who were reported to have travelled while symptomatic to Greece and Italy, respectively [8,9]. Another was an asymptomatic case who travelled to Malaysia and later developed symptoms upon arrival [10]. In May 2014, two laboratory-confirmed cases travelled on the

same flight from Saudi Arabia to the Netherlands while symptomatic [11]. Variable approaches were used for the contact tracing of flight contacts in each of these incidents, however, all failed to identify any evidence of onward transmission to other passengers.

London Heathrow Airport is a key air transport hub, and more passengers from the Middle East travel to, or transit through the UK than any other country in Europe [12]. This paper describes the public health response to two further laboratory-confirmed MERS-CoV cases on commercial flights, provides further evidence on the potential transmissibility of MERS-CoV during air travel, and informs the public health response to possible future incidents. The paper also explores alternative options to identify possible cases by using the media and public information messages to raise awareness among those travelling to and returning from the Middle East, particularly in circumstances when passenger lists may not be available.

Incident 1 – Flight Riyadh to London Heathrow, 24 April 2014 (with onward travel to Chicago)

On Friday 2 May 2014, PHE was alerted by the US Centers for Disease Control and Prevention (CDC) of a reported probable case of MERS-CoV infection, positive on screening by PCR but awaiting confirmation by the national reference centre. The case had travelled eight days earlier from Riyadh, Saudi Arabia to Chicago, US, on 24 April, transiting through London Heathrow Airport. Confirmation was received from CDC at 8p.m. GMT on 2 May 2014 [1]. The case was a healthcare worker who had been working in a Saudi Arabian healthcare institution that treated MERS-CoV patients [1]. The case first felt unwell on 18 April. Six days later, they were symptomatic with fever and myalgia, but without respiratory symptoms, during the seven hour flight from Riyadh to London. They spent approximately two hours at London Heathrow before boarding a flight to Chicago. Following the initial notification on 2 May, PHE immediately convened an incident management team.

Considerations for contact tracing

Although the risk of onward transmission to passengers and crew on board the flight was considered to be very low, given the recent emergence of MERS-CoV it was agreed with the CDC to adopt a precautionary approach and undertake contact tracing on the basis of protecting public health, as well as to add to the existing evidence base for risk of transmission on aircraft and inform future responses.

In the UK it was agreed that passengers in the immediate vicinity of the case were a priority and should be contacted and followed up. At the time of the incident no specific international guidance on contact tracing on aircraft for MERS-CoV cases existed, therefore guidance on severe acute respiratory syndrome (SARS) was considered as the most analogous and suitable for our investigation. The current European Centre for Disease Prevention and Control (ECDC) Risk Assessment Guidelines for Infectious Diseases transmitted on Aircraft (RAGIDA) suggests that for SARS, contact tracing should be comprehensive, and may extend to passengers up to seven rows around the case, or even the entire plane, whereas WHO guidance suggests two rows in front and behind the case and the row they are sat in [13,14]. Given that there has been little evidence from previous incidents to suggest MERS-CoV is easily transmissible on aircrafts, it was decided to adopt a more pragmatic approach based on the WHO guidance.

Passengers seated in the two seats all around the case were deemed to be 'priority' contacts. The remaining passengers and crew were to be alerted to the situation and advised on the actions to take should they develop respiratory symptoms within the 14 days following the flight.

The flight manifest and contact details of the passengers were requested from the airline. Previous experiences of obtaining passenger details for contact tracing suggested this information could take some time to obtain. A proactive media approach was developed to rapidly initiate the contact tracing process, on the basis that the forthcoming weekend of 3 to 5 May included a public holiday, and that eight days of the 14-day incubation period had already passed. The press release was timed to coincide with the announcement of confirmatory test results from the US and was issued on Friday 2 May with details of the flight number, and advice to any passengers with respiratory symptoms on that flight to seek medical advice through NHS 111, (a UK telephone service for health advice provided by the National Health Service), due to limited primary care access over the weekend. An algorithm was developed to support call handlers to manage calls from members of the public who had been on the flight.

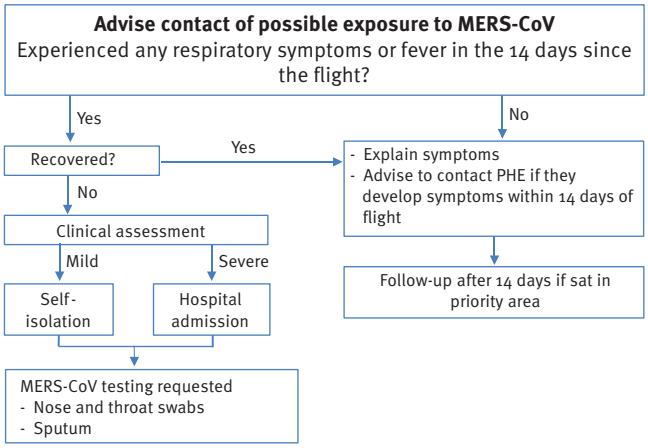
Follow-up and criteria for testing

The passenger contact details were provided by the airline within hours of request and contained names, nationalities and contact details for all passengers. Priority contacts who were identified as being resident in the UK from their contact details, were initially interviewed centrally by staff at the Centre for Infectious Disease Surveillance and Control (CIDSC) on the evening of Friday 2 May, and their details were passed to their local health protection team for follow-up. They were informed of a possible exposure to a confirmed MERS-CoV case and asked whether they had developed any respiratory symptoms since the flight.

The definition of a possible case, and criteria for testing, was anyone who was on the flight and who had experienced respiratory symptoms, within 14 days of the flight. This definition is synonymous to the PHE close contact definition for MERS-CoV [15]. Symptomatic contacts were assessed by a health professional over the phone, and were asked to self-isolate or attend

Figure 1

Algorithm for management of flight contacts in two incidents of confirmed MERS-CoV cases travelling on flights through London Heathrow Airport, United Kingdom, May 2014



MERS-CoV: Middle East respiratory syndrome coronavirus; PHE: Public Health England.

hospital if warranted. Lower respiratory samples and/or nose and throat swabs were arranged using appropriate infection control precautions [16]. Asymptomatic contacts were asked to report any respiratory symptoms or fever that developed within the remaining 14 day period from the flight. All priority contacts were followed-up by local health protection teams (Figure 1) for at least 14 days since their exposure – the maximum putative incubation period of MERS-CoV.

The details of all passengers that were non-UK residents were sent to relevant national focal points via the bilateral International Health Regulations (IHR) and European Early Warning Response System (EWRS) over the public holiday weekend, and feedback on the health status of these contacts was requested.

In addition to passengers on the Riyadh to London flight, details for those on the London to Chicago flight who had returned to the UK were sent to PHE by the CDC and were prioritised and followed up as above.

Following the weekend press release, all remaining passengers resident in the UK on the flight were actively contacted on Tuesday, 6 May, following the public holiday weekend. Where the address of passengers was known, their contact details were passed on to the relevant local health protection team to make contact. Otherwise passengers were informed centrally by CIDSC who gathered the relevant contact details. Passengers were asked to report whether they had experienced any respiratory symptoms within 14 days of the flight. If yes, they were managed in the same way as the priority contacts, but they were not followed-up after 14 days.

Incident 2 – Flight Jeddah to London Heathrow, 1 May 2014 (with onward travel to Boston)

On 11 May 2014, PHE was alerted to a second probable case of MERS-CoV infection (later confirmed) who had travelled from Jeddah, Saudi Arabia to Boston, US via London Heathrow Airport 10 days previously, on 1 May 2014 [1]. The case became symptomatic on the day of the flight and was symptomatic on board the six hour UK bound flight with symptoms of fever, chills, myalgia and a slight cough.

Contact tracing

A basic passenger manifest containing names and seat numbers of all passengers on the flight was available from the airline within 24 hours of the notification from the CDC. The passenger contact details were only provided on 14 May, at the end of the 14 day follow-up period.

In a similar fashion to incident 1, a pro-active press release was issued on 12 May to alert passengers, and the NHS 111 algorithm was adapted to include passengers on this flight. Given that the maximum incubation period of 14 days was exceeded by the time information was available, only the 17 passengers who sat in the immediate two adjacent seats to the case were identified for active contact tracing, however contact details were not available for seven of them. They were asked about any respiratory symptoms that might have developed within 14 days of the flight. If they had developed symptoms within 14 days of the flight, and were still symptomatic, they were clinically assessed, asked to self-isolate and sampling was undertaken as described above.

Where available, the details of non-UK residents sitting in the two seats all around the case were passed to the respective national focal point via bilateral IHR and EWRS mechanisms. The CDC provided details on passengers who were on the flight from London to Boston, but had returned to the UK, and they were contacted if they were seated within two seats all around the case.

Contact tracing results

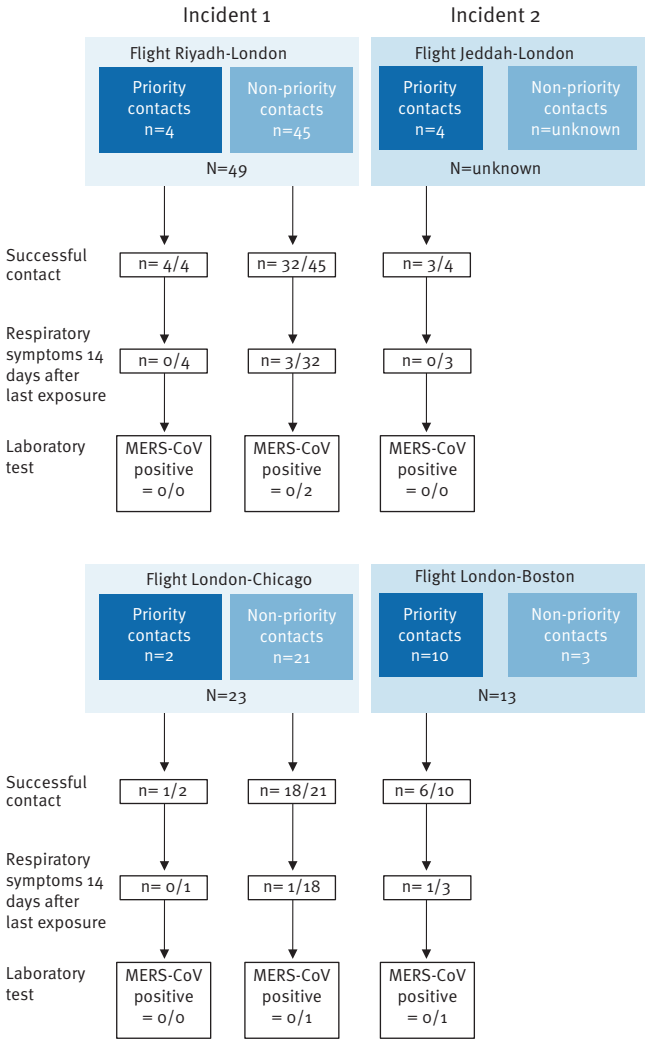
Results of the contact tracing are depicted in Figure 2.

Incident 1 – Flight Riyadh to London Heathrow

There were 178 passengers on the flight manifest on 24 April 2014, including the case. Of these, 49 passengers were UK residents of whom four were considered priority contacts as they were seated within two seats all around the case; the remaining 45 were seated elsewhere in the plane. PHE successfully contacted 36 of these passengers. None of the four priority passengers reported acute respiratory symptoms in the 14 days since the flight; however, three of the non-priority passengers reported mild respiratory symptoms during

Figure 2

Results of contact tracing for UK residents in two incidents of confirmed MERS-CoV cases travelling on flights through London Heathrow Airport, UK, May 2014



MERS-CoV: Middle East respiratory syndrome coronavirus; UK: United Kingdom.

Information on contact tracing of international contacts on the flights is not displayed in this figure.

this period and were still symptomatic. None required hospitalisation.

Testing of symptomatic contacts

The two symptomatic contacts were sampled and tested. Both were negative for MERS-CoV by PCR testing. One contact was positive for rhinovirus and the other one was positive for influenza B virus. The third symptomatic contact was not tested as they did not agree to be further contacted by the local health protection team.

Non-United Kingdom residents

Of the 128 passengers who were non-UK residents or nationals, 13 were seated within two seats all around the case, the remainder were seated elsewhere on the plane. Details of these passengers were passed to respective international counterparts for contact tracing. Of these 13 passengers that were non-UK residents and were seated in the priority area, seven were contacted and all were asymptomatic. There was no feedback received about the non-UK passengers seated elsewhere on the plane.

Incident 2 - Flight Jeddah to London Heathrow

The flight manifest identified 17 passengers on the flight on 1 May 2014 who were priority contacts. Four of these passengers were UK residents and six were international contacts. No contact information was available for seven of the passengers. Of the four UK contacts, three were successfully contacted, none of whom reported any acute respiratory symptoms within 14 days of the flight. No further information was received on the international passengers.

Onward flights from London Heathrow to the United States

The CDC informed PHE of contacts on the onward flights from London to the US.

Incident 1

The case travelled to Chicago on 24 April and 23 UK contacts were identified on this flight, two of these were considered priority contacts. Contact was made with one who did not report any respiratory symptoms within nine days from the flight. The other 21 UK residents on the flight were considered non-priority contacts, and 18 were successfully contacted, of whom one was symptomatic within the 14 days after the flight. Upper respiratory tract samples tested negative for MERS-CoV and for other respiratory viruses.

Incident 2

The case travelled to Boston on 1 May and 13 UK contacts were identified on this flight. Ten of these contacts were considered priority contacts, of which six were successfully contacted and one was found to be symptomatic. This contact was found to be positive for streptococcal A infection, but negative for MERS-CoV and a respiratory virus panel (adenovirus, respiratory syncytial virus, parainfluenza, rhinovirus, human metapneumovirus and influenza A and B virus).

Discussion and conclusions

Given the prominence of the UK, and particularly London Heathrow, as a key air transport hub connected with the Middle East and continued reporting of new MERS-CoV cases from that region, it is probable that similar incidents will occur in the future. This may happen with higher frequency during times of increased local transmission in the Middle East as was observed in April–May 2014. Although the incidence of MERS-CoV has remained low compared with this particular peak period, a smaller peak of cases occurred in Saudi Arabia during early spring 2015, suggesting there may

yet be an aspect of local seasonality to MERS-CoV transmission [17]. Contact tracing investigations, such as the one described, have significant resource implications for public health due to the effort required to identify and follow up passengers on a flight.

In total, there were 89 UK contacts on board these flights, of which 20 were priority contacts and 69 non-priority contacts. The contact tracing carried out for the two flights to the UK described in detail in this paper and among UK residents on the two flights to the US, did not identify any further symptomatic cases of MERS-CoV infection. This adds to the growing body of evidence of the lack of transmission to date from symptomatic MERS-CoV cases during air travel [8-11].

During the investigation we only took clinical samples from symptomatic contacts. This is a limitation, as MERS-CoV infection has been observed to cause mild disease and asymptomatic infections which we may not have identified by the approach outlined in this paper [6]. However, we used a low threshold for symptoms requiring testing to increase the likelihood of identifying mild cases. We also did not test contacts serologically unless there was an indication of secondary transmission from initial tests, as serology was not considered to be proportionate to the public health risk based on evidence from other investigations. We were unfortunately unable to contact all the people considered to be priority contacts, which is a further limitation of our investigation. However, no cases of MERS-CoV infection have been confirmed in the UK since the flights. One priority contact reported symptoms, however they did not accept further contact with the local health protection team, and would not attend for testing. They were provided with advice on the symptoms, and how to contact health services should their condition deteriorate.

The two incidents illustrate the possible challenges and unpredictability of public health investigations. In both, public health authorities only became aware of the incidents sometime after the exposure. In the first incident a full flight manifest with contact details for most of the passengers, was available within 12 hours of notification, enabling an effective, although resource intensive, response. In the second incident the contact details of passengers were not available until several days following the first notification. Moreover, the details, when available, were incomplete, and contact tracing did not take place until after the end of the maximum putative incubation period of 14 days, minimising the potential public health benefits of the investigation. We did not receive information on the health status of all the non-UK contacts that were passed to other national focal points. This is a further challenge in the investigation of these incidents, as the majority of flight contacts were non-UK nationals.

The difficulties of undertaking rapid and effective contact tracing on flights due to the variability of the information made available by the respective airlines, and the speed at which it is received, even if considerable effort and resources are committed is highlighted here. The public health responsibilities of conveyancers are outlined in the International Health Regulations [18]. There are specific regulations regarding the provision of passenger contact information when an incident of public health concern is identified during the flight. However, there is no public health legislation requiring conveyancers to retain information such as passenger manifests or contact details for contact tracing in the event that disease is identified after the flight. Although both airlines in these described incidents were very co-operative, it is recommended that the ability to rapidly access plane manifests with adequate information to enable public health investigation be further strengthened. Declaring MERS-CoV infection as a notifiable disease within the UK may contribute to this; however, administrative practices among airlines with regards to the length of time flight manifests and contact details are retained would also need to be addressed. New UK legislation (in development) is likely to mandate that best efforts are made to provide passenger contact information for such public health investigations, however, currently such airline co-operation is on a voluntary basis [19].

Due to past difficulties in obtaining passenger information, PHE sought to explore other mechanisms to alert passengers on these flights in a timely manner. In both incidents a pro-active media approach linked to a telephone help-line service was applied in an attempt to alert passengers in the UK prior to the passenger information becoming available. In practice no passenger contacts were identified through this mechanism that we are aware of. Although not formally measured, the incident response highlighted a lack of awareness of MERS-CoV infections among passengers travelling to and from the Middle East. Many of the passengers spoken to during the contact tracing expressed very limited knowledge of MERS-CoV symptoms or risks. Following the incidents, as a more general measure PHE adapted CDC information materials for display in airports in order to raise awareness of MERS-CoV among the general public and provide advice on the symptoms and how to access medical attention. These have also been circulated to airlines with direct flights to the Middle East; however, uptake and use has been limited due to airports expressing commercial concerns. Finally, the option of alerting all non-priority contacts via text message was considered, and although this was not done during the incidents, it could be a resource-effective approach as a phone number was the most consistently available contact information within the manifest.

The response to this incident required significant resources at both a national and local level, as well as international collaboration. Over the course of the weekend and the initial incident response, several incident meetings were held, with around 20 senior staff in attendance across epidemiology and surveillance,

port health, microbiology services, operational health protection staff, communications and the NHS. Within PHE four scientists, as well as seven specialist registrars worked full time on the contact tracing and incident response over several days. At a local level, 18 local health protection teams were involved in the contact tracing and follow-up. This sort of contact tracing requires significant person-time, and for this reason, any decision to deploy this level of resource should be taken judiciously, particularly in the emerging evidence of limited transmissibility in such settings.

Supranational organisations such as WHO and ECDC and the established information processes such as IHR national focal points, and the EWRS play a key role in incidents such as this. On both flights, the majority of persons were non-UK residents, and using these systems we were able to pass on details, and receive information on the health status of non-UK contacts with international counterparts. However, not all focal points responded, and so there were still gaps in our information. Given the international nature of air travel, an enhanced role of ECDC or WHO could be to encourage countries to respond to requests for information on identified contacts, providing more reliable information on transmission in the early stages of an emerging infection.

There are still some gaps in understanding of the risk of transmission of MERS-CoV from patients who are asymptomatic, or who do not have respiratory symptoms [20,21] There is evidence of transmission from cases with mild symptoms or who are asymptomatic [22]. However, documented episodes of human-to-human transmission have been concentrated in healthcare settings, or within households. This suggests that close and prolonged contact with the case is required [23]. The first case reported here had no respiratory symptoms during the flight and may have posed a very low transmission risk if MERS-CoV transmissibility is similar to other respiratory viruses. The second case had a slight cough, and so may have represented a slightly greater infection risk through the respiratory route. A better understanding of the risk of transmissibility will allow the scale of the public health response to be modified. However, until a better understanding is achieved, the public health response to such events needs to remain precautionary. In the meantime, the need to develop less resource-intensive methods of contact follow-up and investigation persists.

In light of the UK experience, and the limited evidence of in-flight transmission of MERS-CoV from other investigations, we would argue that contact tracing and follow-up of the entire plane, or up to the maximum range of seven rows either side of the case (as per the RAGIDA guidelines for SARS) is not a proportionate response for cases of MERS-CoV travelling on flights in general. We recommend that based on current evidence, contact tracing of two rows in front and behind, or as in our investigation, two seats all around the case, represents a more appropriate approach in most circumstances, although this needs to be evaluated on a case-by-case basis.

Members of the Incident Management Team

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Conflict of interest

None declared.

Authors' contributions

F Parry-Ford and N Boddington agreed the initial outline for the article and wrote the first draft. All authors made a substantive contribution to the writing of the final manuscript.

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The Italian registry of cystic echinococcosis (RIEC): the first prospective registry with a European future

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Cystic echinococcosis (CE), a worldwide zoonosis, is highly endemic in southern and eastern Europe. Its actual prevalence is unknown due to the lack of efficient reporting systems designed to take into account the particular features of the disease. Neglect of CE makes diagnosis and clinical management difficult outside referral centres, with inconsistencies in clinical practice and often unnecessary procedures carried out that have associated risks and costs. The Italian registry of CE (RIEC) is a prospective multicentre registry of CE patients seen from January 2012 in Italian health centres; data are voluntarily submitted to the registry. Its aims are to show the prevalence of CE in Italy, bring the importance of this infection to the attention of health authorities, encourage public health policies towards its control, and stimulate biological, epidemiological and clinical research on CE. From January 2012 to February 2014, a total 346 patients were enrolled in 11 centres, outnumbering national reports of many CE-endemic European countries. We discuss preliminary data and challenges of the RIEC, template for the European registry of CE, which has been implemented within the Seventh Framework Programme project HERACLES (Human cystic Echinococcosis ReseArch in CentraL and Eastern Societies) since September 2014.

Background

Cystic echinococcosis (CE) is a zoonotic disease affecting an estimated 1.2 million people worldwide, with 3 million disability-adjusted life years (DALYs) lost globally every year, although these figures are likely to be underestimates [1]. *Echinococcus granulosus*, the cestode causing the infection, completes its life cycle between dogs and other canids (definitive hosts) and livestock, especially sheep (intermediate hosts), with humans as accidental intermediate hosts. In humans, the parasite develops in its metacestode stage, forming cysts in organs and tissues, mainly in the liver. CE is mostly endemic in rural areas where sheep raising is practiced, such as central Asia and China, South America and Mediterranean countries [2]. In 2012 a joint FAO/World Health Organization (WHO) expert group classified E. granulosus second among the top eight ranked food-borne parasites of global public health importance [3]. Nevertheless, compared with other diseases of similar burden, CE has received much less attention and funding [4].

Human CE has been described as chronic, complex and neglected [5]. The lack of efficient reporting systems designed to take into account the particular features of this disease results in under-reporting and/or misreporting of CE. In a vicious circle, under-reporting leads to the perception that CE is not an important health problem, which in turn makes measurement of disease burden and impact on public health even more difficult. Neglect hampers the collection of good-quality data to inform control programmes and evidence-based diagnostic and therapeutic strategies. This ultimately leads to at least suboptimal clinical management of cases and allocation of public resources for treatment and control.

Reasons for neglect

Neglect of CE is due to several factors [1]: firstly, the life cycle of E. granulosus is difficult to interrupt in the absence of sustained, expensive and well-coordinated programmes. Secondly, control of the infection in humans, as opposed to livestock, does not have an impact on the global spread of infection. CE is not perceived as an important animal health problem, which is therefore not tackled. Thirdly, the burden of CE is difficult to quantify because of its geographical dispersal in vast rural areas, absence of specific symptoms, and lack of an effective disease record system. Lastly, the disease affects mostly poor pastoral communities, with

Box

Data acquired in the Italian registry of cystic echinococcosis, January 2012–February 2014

Birth and residency data; contact details
Epidemiology
- residence in and visits to CE-endemic areas - field of employment at and before CE diagnosis - rural at-risk activities carried out before CE diagnosis
- risk factors: contact with at-risk dogs, or potentially contaminated soil, food or water - known relatives with CE
Clinical history
- patient classification (first diagnosis, follow-up, relapse) - year, place and imaging tool of first CE diagnosis
- past and current symptoms - past and current therapies/management strategy of CE (including length and complications)
Cysts characteristics at diagnosis and at each visit
- number
- organ and localisation within the organ (e.g. liver segment) - size (small<5 cm; medium 5–10 cm; large>10 cm) - stage (according to the WHO-IWGE classification [6])
- complications
Serology
- test (manufacturer, antigen, format) - result including titre where applicable

CE: cystic echinococcosis; WHO-IWGE: World Health Organization Informal Working Group on Echinococcosis.

Data are entered using fixed drop-down menus and multiple-choice options, organised in multiple data sheets (the title of each sheet is shown in bold). For all information, location, start date and end date of the event is indicated if possible. Fields for entering free text are also present where appropriate.

a low case fatality rate but with difficult and expensive diagnosis and treatment.

Implications of chronicity and complexity of cystic echinococcosis

In humans, echinococcal cysts grow slowly, passing through several stages (Figure 1), and often remain clinically silent for many years [6,7]. Thus, infected patients may be diagnosed long after infection and in a country different from where the infection was acquired. This compounds the difficulty in evaluating the distribution of CE. Diagnosis and clinical management of CE are complex and require a multidisciplinary approach, often available only in referral centres. Imaging, in particular ultrasound, is the mainstay of diagnosis and follow-up of abdominal CE [6,8] while serology has only an ancillary role [9]. In 2003, the WHO Informal Working Group on Echinococcosis (IWGE) implemented a consensus classification of cyst stages [6] (Figure 1). This followed the introduction of the first widely used ultrasound classification of hepatic CE proposed by Gharbi et al. [10]. The WHO-IWGE classification allows all morphological stages of cysts to be classified unequivocally, and groups them into clinical categories reflecting the current knowledge of the natural history of CE. Importantly, it provides a guide for the rational allocation of CE patients to different management options [11]. However, this consensus approach, and the use of CE classifications of any kind, are still widely underused [12], adding to the lack of prospective randomised trials evaluating the optimal stage-specific clinical management of CE, which hampers an evidence-based approach to this disease. Clinical management of CE still relies on expert opinion [11] and the management of the disease is often inappropriate, exposing patients (and health systems) to unnecessary treatments and costs.

Reporting systems and burden of cystic echinococcosis in Italy and Europe

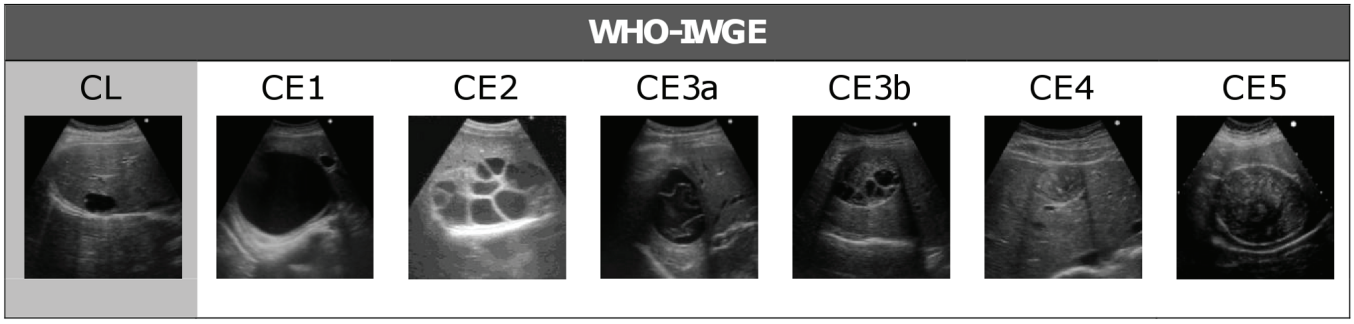
CE is endemic in Italy, although its burden is difficult to estimate. Despite being a notifiable infection in animals [13], being listed among reportable occupational diseases [14] and subject to surveillance according to European legislation [15], notification of human cases ceased de facto to be compulsory in 1991. Currently, health authorities are required to provide only a yearly summary of regional data [16]; it is up to the individual physician to notify cases. As a result, no official data are transmitted to the European Centre for Disease Prevention and Control (ECDC) [17-19]. At the national level, a mean of 1,379 (SD: 442.5) yearly hospital discharges with a diagnosis of CE were recorded in 2001–12, with annual incidence estimated to range from 0.18 to 6.78 per 100,000 population [20]. However, reporting based solely on hospital discharge records is inadequate, as the majority of CE cases are diagnosed and managed in an outpatient setting [21], besides the fact that asymptomatic cases can be only diagnosed during screening campaigns. For example, an ultrasound survey carried out by Caremani et al. [22] in 19 centres throughout Italy in 1988–90 found 424 cases diagnosed with CE by examining 333,144 patients, compared with 284 cases reported in the official data in the same period. Prevalence and incidence data in livestock are also incomplete [17,19].

Difficulties in reporting of CE do not apply to Italy alone. Very heterogeneous national surveillance systems for CE exist in Europe [17]. However, comparison of notified cases with hospital records indicates a clear discrepancy, with consistent underestimation of the burden of CE [17,18]. For example, Pardo et al. reported a two- to fourfold lower number of notified cases compared with hospital records in certain regions of Spain from 1996 to 2003 [23].

Besides inaccurate reporting of the number of cases, other limitations of current reporting systems include ambiguity in discrimination between autochthonous

Figure 1

World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) ultrasound classification of echinococcal cysts



CL (cystic lesion), CE1 and CE2 (active cysts), CE3a and CE3b (transitional cysts), and CE4 and CE5 (inactive cysts).
Source: [30].

and imported cases and between new and re-admitted cases, and lack of collection of important epidemiological data such as occupation and other risk factors. Given the chronicity and frequent relapses in CE [24,25], patients often access different health centres over time, which could lead to potential duplication of data, which would also contribute to inaccurate statistics. In addition, aspects such as cyst stage and therapy outcome, which have a profound impact on the clinical management of patients and in turn on public health resource allocation, are generally not collected in notification forms.

Setting up the Italian registry of cystic echinococcosis

To start tackling these long-standing problems, the WHO Collaborating Centre for the Clinical Management of Cystic Echinococcosis, (University of Pavia, San Matteo Hospital Foundation, in Pavia) and the Istituto Superiore di Sanità (the Italian National Health Institute, ISS, in Rome) set up the Italian registry of cystic echinococcosis (Registro Italiano Echinococcosi Cistica, RIEC). The aims of the registry are to show the prevalence of CE in Italy, bring the importance of this infection to the attention of health authorities, encourage public health policies towards its control, and stimulate biological, epidemiological and clinical research on CE.

Organisation and management of the Italian registry

The development of RIEC started in 2012 as part of a research project (IZS SA 07/10 RC) at the Sardinian Experimental Zooprophylactic Institute. The Project was managed by ISS and the Pavia WHO collaborating centre. The project group included a project manager (PR), project coordinators (FT, EB), and an information technology developer (FG). After approval from the ethics committee of the ISS, RIEC was launched in October 2012, and is accessible through the ISS website [26]. The RIEC website [27] includes sections freely accessible to the public providing educational and scientific material, and a restricted area, accessible only to users authorised to enter data. The project group performs regular monitoring and evaluation of the database. Ownership of data from individual centres belongs to the individual centres themselves; however, upon permission, the project group analyses and publishes a cumulative summary of data on a regular basis.

Data collection

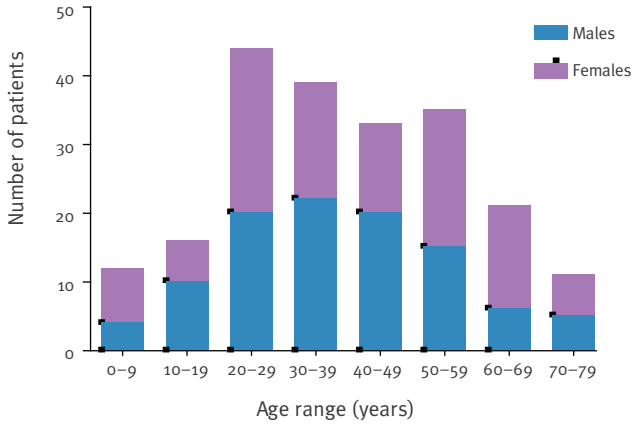
RIEC is a prospective, observational, multicentre registry of CE patients seen from January 2012 onward in Italian health centres that adhere voluntarily to the registration of their patients into RIEC. Clinicians of all disciplines in health centres potentially interested in joining RIEC are contacted by email and during scientific meetings. Clinicians are given a unique username and password to access the registry. Individual clinicians have access only to data of patients enrolled in their centres, and they can enrol and access data of CE patients previously recruited in another centre who subsequently moved and are then under their care.

The only inclusion criterion to enrol a new patient in the registry is that the patient has been diagnosed with probable or confirmed CE [11], whether hospitalised or in an outpatient setting and independent of country of birth and nationality. Upon first enrolment, a unique patient 'RIEC code' is generated automatically, avoiding duplication of records, should the patient present at another centre in the future, and facilitating data retrieval during follow-up. Following written informed consent concerning processing of sensitive data, other information can then be acquired (Box). These are entered using drop-down menus and multiple-choice options to facilitate data extraction and analysis, and can be updated following duplication and amendment.

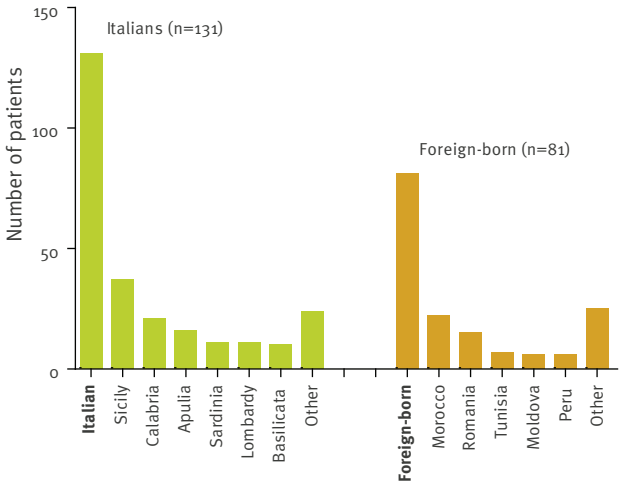
Figure 2

Preliminary data from patients with cystic echinococcosis enrolled in the Italian registry of cystic echinococcosis, Pavia, Italy (World Health Organization Collaborating Centre for Clinical Management of Cystic Echinococcosis), January 2012–February 2014 (n = 212)

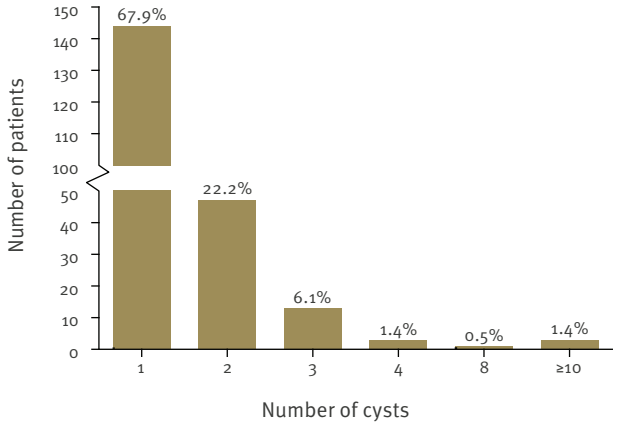
A Age at diagnosis and sex distribution



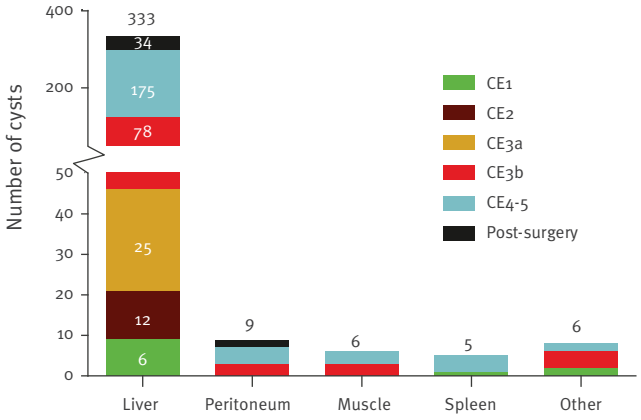
B Place of birth of patients



C Cysts per patient at first enrolment



D Organ localisation of cysts (n = 361) and cyst stages within organs at first enrolment



CE1 and CE2 (active cysts), CE3a and CE3b (transitional cysts), and CE4 and CE5 (inactive cysts).

Data safety and confidentiality

RIEC is established within the secured ISS information technology network, in compliance with national and international regulations on the protection and use of personal data. Data are stored in a three-stage cluster database server implemented in a redundant array of independent disks and made available by an SQL-relational database management system server. The connection between authorised user and web application is made via an HTTP protocol. All registered data and logs are backed up at short regular intervals, and copied on a tape library stored in different fireproof locations. Upon patient registration, sensitive data are automatically anonymised with the assignment of a unique code, which is uncoupled from sensitive data to make them encrypted.

Preliminary findings

By February 2014, 16 centres had adhered to the RIEC, with 346 patients enrolled in 11 centres. The remaining five centres had not yet had any enrolled patients. The majority of records (212 patients) were entered by the WHO collaborating centre in Pavia, showing a mean of 1.46 visits per patient (standard deviation (SD): 1.09; range: 1–4 visits/patient). Preliminary demonstrative results are derived from data from this centre.

A total of 110 male and 102 female CE patients were seen in this centre from January 2012 to February 2014 and were enrolled in the RIEC (Figure 2A). The mean age at diagnosis was 40 years (SD: 6.8; range: 5–77) (Figure 2A). Of the 212 patients, 131 (62%) were born in Italy, while 81 (38%) were foreign-born (Figure 2B).

Morocco and Romania were the countries of birth of the majority of foreign-born patients (n = 22 and n = 15, respectively), while the majority of Italian patients were born in the southern regions of Sicily (28%, n = 37) and Calabria (16%, n = 21) (Figure 2B). A total of 14 newly diagnosed cases were recorded in 2012 (13% of 108 visits in 2012) and 21 in 2013 (21% of 99 visits in 2013). During first enrolment, the majority (70%, n = 148) of patients had a single cyst (Figure 2C), and 333 of 361 cysts (92%) were hepatic (Figure 2D). Transitional (CE3a and CE3b) and inactive (CE4 and CE5) cysts stages were most commonly present at the time of enrolment (Figure 2D).

Discussion

The implementation of the RIEC responds to a long-standing need for a CE national registry with online data entry [11,21], similar to the European registry for alveolar echinococcosis [28]. Its design allows the collection of both basic epidemiology and detailed longitudinal clinical data in a simple and unequivocal way. This will provide valuable information on the prevalence and parasitic pressure (linked to the number of small, active cysts) in patients in endemic areas. Moreover, it will offer the opportunity to study prospectively and systematically the stage-specific effectiveness of clinical management options and clinical manifestations, the rate of adverse reactions, relapse rate and costs of CE infection.

During January 2012 to February 2014, the Pavia centre entered the majority of the data, while the other centres entered only minimal demographic data. This was probably due to the fact that the Pavia centre is a pilot centre for RIEC implementation and is a WHO collaborating centre. Downloadable tools that facilitate data collection and entry, e.g. online tutorials and paper-based report forms for data collection during routine practice, are provided. However, more effort is needed from the project group to encourage data entry and highlight the advantages of using the RIEC, even for individual clinicians. Indeed, the RIEC allows systematic recording of data and of the clinical management of patients, even those followed in more than one centre. It also allows clinicians to retrieve and analyse the centre's cumulative clinical data for internal use and publications. Additionally, data collection and entry is not very time consuming: in Pavia, human resources allocated to this task were calculated as less than 0.02 full-time equivalents for 2013.

The fact that data entry relies on the clinician's goodwill and time suggests that the current figures are far from representing the real national situation. Only compulsory notification of CE would provide more reliable data for surveillance of the disease in humans. A centralised registry eliminates the need to merge regional data, avoids data duplication, captures both inpatient and outpatient data, and makes both clinical and epidemiological data accessible to clinicians, epidemiologists and health authorities. This is also of particular importance in light of increasing migration. It is crucial to inform control measures for what is essentially a preventable disease, and to allow for better resource allocation. The preliminary data from the RIEC presented here show that CE is still present in Italy and the cases largely outnumber the total of national cases reported by most European endemic countries [17], further stressing the need for a better reporting system for CE at the European level.

HERACLES (Human cystic Echinococcosis ReseArch in CentraL and Eastern Societies) is a four-year collaborative project launched in October 2013, funded by the European Union, under the Seventh Framework Programme (grant agreement number 602051). Within HERACLES, the RIEC has been be the template for the creation of the European registry of cystic echinococcosis, with initial inclusion of data from Bulgaria, Italy, Romania and Turkey, while health centres from other European countries expressed an interest in joining [29]. The European registry was launched in September 2014, is available in English and national languages, and is subject to constant monitoring and evaluation and template optimisation. This tool provides an efficient and disease-tailored template to governments, the European Commission and related European agencies to harmonise data collection, monitoring and reporting of CE.

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Conflict of interest

None declared.

Authors' contributions

FT conceived, designed and pilot tested RIEC and ERCE, contributed to data entry and RIEC and ERCE monitoring and evaluation, analysed the data and wrote the manuscript. PR designed and implemented the RIEC and ERCE website, contributed to RIEC and ERCE design and monitoring and evaluation, data analysis and manuscript writing. FG designed, pilot tested, and implemented RIEC and ERCE and contributed to data analysis and manuscript writing. MM, GJN and FR contributed to data entry and analysis. AC conceived and coordinated the implementation of ERCE and contributed to manuscript writing. EP contributed to the design of RIEC. EB conceived RIEC and ERCE, contributed to their design, and

contributed to manuscript writing. All authors critically revised and approved the final version of the manuscript.

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ECDC adds new pages to online directory on infection prevention and control

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As part of its support to the 5 May **'SAVE LIVES: Clean Your Hands'** [1] campaign the European Centre for Disease Prevention and Control (ECDC) has updated its online directory [2] containing resources on infection prevention and control by adding two new pages: 'Guidance on hand hygiene in healthcare and 'Guidance on healthcare-associated pneumonia and ventilator-associated pneumonia'.

The **directory** lists guidance, available online, on how to prevent and control antimicrobial resistance and healthcare-associated infections. It consists of six parts:

- Carbapenem-resistant *Enterobacteriaceae* (CRE) Directory
- Meticillin-resistant *Staphylococcus aureus* (MRSA) Directory
- *Clostridium difficile* infection (CDI) Directory
- Organisation of infection prevention and control Directory
- Hand hygiene in healthcare Directory
- Healthcare-associated & ventilator-associated pneumonia Directory

The 'SAVE LIVES: Clean Your Hands' campaign is led by the World Health Organization (WHO). Arranged on 5 May every year, it is a part of a worldwide effort to support healthcare workers to improve hand hygiene in healthcare settings and by that to support the prevention of healthcare-associated infections. The campaign was launched in 2009 and is and extension to the WHO First Global Patient Safety Challenge: Clean Care is Safer Care.

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The Americas region declares that rubella has been eliminated

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At a Pan American Health Organization/World Health Organization (PAHO/WHO) meeting in April 2015, an international expert committee declared that the Americas region is free of the endemic transmission of rubella [1]. Rubella and congenital rubella syndrome are the third and fourth vaccine-preventable diseases to be eliminated in the Americas, after smallpox (1971) and polio (1994).

The eradication of rubella follows a 15-year initiative which involved widespread vaccination against measles, mumps and rubella (MMR) in the Western Hemisphere.

When contracted in early pregnancy, rubella can cause miscarriage or birth defects.

Read more **here**.

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