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Culex mosquitoes are experimentally unable to transmit Zika virus

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We report that two laboratory colonies of *Culex quinquefasciatus* and *Culex pipiens* mosquitoes were experimentally unable to transmit ZIKV either up to 21 days post an infectious blood meal or up to 14 days post intrathoracic inoculation. Infectious viral particles were detected in bodies, heads or saliva by a plaque forming unit assay on Vero cells. We therefore consider it unlikely that *Culex* mosquitoes are involved in the rapid spread of ZIKV.

Outbreaks due to Zika virus (ZIKV) are expanding and affecting most tropical regions [1]. The rapid spread may be related to the efficiency of human-biting *Aedes aegypti* and *Aedes albopictus* mosquitoes, which are ZIKV vectors. However, both mosquito species were unexpectedly poorly competent vectors for ZIKV as shown by our laboratory in a previous study [2]. Other factors have been suggested to explain the rapid spread of ZIKV across the Americas [2]: a human population immunologically naive for the newly introduced virus, higher densities of *Ae. aegypti* or the involvement of other anthropophilic vectors such as *Culex* mosquitoes. In light of this, we experimentally infected two laboratory colonies of *Culex* species, *Cx. quinquefasciatus* and *Cx. pipiens*, with an Asian genotype of ZIKV and showed an absence of transmission up to 21 days post infection.

Mosquito experimental infections

In May and June 2016, we performed mosquito experimental infections on two laboratory mosquito colonies used in this study: *Cx. pipiens* collected in Tabarka, Tunisia, in 2010 [3] and *Cx. quinquefasciatus* collected in San Joaquin Valley in California, United States, in 1950 [4]. The latter is a colony of reference in studies on this mosquito [5]. Testing these colonies experimentally should allow us to determine whether the two species are genetically capable of transmitting ZIKV.

About 200 female mosquitoes of each species were successfully fed, with a total of 188 *Cx. pipiens* mosquitoes and 170 *Cx. quinquefasciatus* examined for vector competence. Mosquitoes were orally infected with an Asian genotype ZIKV (strain NC-2014–5132), originally isolated from a patient in New Caledonia in April 2014. The ZIKV strain is phylogenetically closely related to those currently circulating in Brazil [6]. One week-old female mosquitoes were provided with a blood meal containing a suspension of ZIKV [2] at a titre of $10^{7.2}$ plaque-forming units (PFU)/mL. Engorged females were kept in cardboard containers and maintained at 28 °C with 10% sucrose solution as food. We analysed 40–48 mosquitoes each time at 3, 7, 14 and 21 days post-infection (dpi), to estimate three parameters describing vector competence: (i) infection rate, which measures the proportion of mosquitoes with an infected body (including the midgut) among the number of analysed mosquitoes; this parameter indicates if the mosquito is able to be infected after the infectious blood meal; (ii) dissemination efficiency, which corresponds to the percentage of mosquitoes with an infected head among the number of analysed mosquitoes; it measures the ability of the virus to cross the midgut barrier, penetrate the mosquito haemocoel and infect internal organs; and (iii) transmission efficiency, which estimates the overall proportion of mosquitoes presenting virus in saliva among the number of tested mosquitoes. Head/body homogenates and saliva were titrated by PFU assay on Vero E6 cell monolayers as previously described [7].

Vector competence analysis

To confirm that the mosquitoes had ingested the virus, two engorged mosquitoes from each species were homogenised and the virus was titrated just after blood feeding: the two *Cx. pipiens* mosquitoes had ingested 6.4×10^4 viral particles and *Cx. quinquefasciatus*, 9×10^4.

Viral infection rate

Viral infection rates were similar for both *Culex* populations at 3, 7 and 21 dpi (Fisher's exact test: $p > 0.05$);

they were respectively 0/42, 1/47 and 5/40 for *Cx. quinquefasciatus* and 1/48, 3/47 and 6/46 for *Cx. pipiens*. However, at 14 dpi, 7/41 of the *Cx. quinquefasciatus* mosquitoes were infected, whereas none of the 47 *Cx. pipiens* mosquitoes were (Fisher's exact test, $p = 0.003$). When estimating the number of viral particles in the mosquito body, no difference was detected between the two mosquito species at each time point (Kruskal–Wallis test, $p > 0.05$) with higher viral loads detected in both species at 21 dpi: mean of 44 (standard deviation (SD): 60) for *Cx. quinquefasciatus* and 56 (SD: 90) for *Cx. pipiens*. Viral loads ranged from 10 to 36 particles for other time points.

Viral dissemination efficiency

Only a few *Cx. quinquefasciatus* mosquitoes were able to disseminate the virus at 14 dpi (1/41 mosquitoes analysed) and at 21 dpi (3/40). Upon examination of these mosquitoes, no more than 15 viral particles were detected in mosquito heads. For *Cx. pipiens*, no mosquitoes were detected with virus in the heads.

Viral transmission efficiency

No mosquitoes were found with ZIKV in saliva. Therefore, the tested *Cx. quinquefasciatus* and *Cx. pipiens* were able to be infected, *Cx. quinquefasciatus* only was able to disseminate virus at a low level, and both species were unable to transmit ZIKV up to 21 dpi.

Intrathoracic inoculation of mosquitoes

One batch of 100 one-week-old females of each mosquito species, *Cx. quinquefasciatus* and *Cx. pipiens* were inoculated intrathoracically with ca 2,530 PFU of the same ZIKV strain (NC-2014–5132). This dose corresponds to 10 times the maximum number of viral particles detected in mosquitoes analysed for vector competence. Viral dissemination was analysed by estimating viral load in mosquito heads at 3, 7 and 14 dpi. Viral dissemination was observed at 3 dpi (1/23) for *Cx. quinquefasciatus*, and at 7 dpi (3/21) and 14 dpi (1/24) for *Cx. pipiens*. No viral transmission (ZIKV in saliva) was detected in either species up to 14 dpi. Thus bypassing the midgut barrier by inoculating a high dose of ZIKV suspension in mosquitoes favoured neither viral dissemination nor transmission.

Background

First discovered in 1947 in Uganda, ZIKV became a major public health concern after its emergence in Yap Island, Micronesia, in 2007 [8] and French Polynesia in 2013–14 [9]. Its arrival in Latin America in 2015 led to a rapid regional spread of outbreaks of ZIKV infection associated with unusually severe effects, Guillain–Barré syndrome [10] and microcephaly in newborns [11]. Up to the first six months of 2016, more than two million people have been infected, in at least 45 countries in Latin America and the Caribbean [12].

The virus (genus *Flavivirus*, family *Flaviviridae*) circulated originally in an enzootic cycle between arboreal canopy-dwelling *Aedes* mosquitoes and non-human primates [13]. In addition to forested habitats, ZIKV has also been isolated in urban settings, with *Ae. aegypti* being the main vector [14]. *Ae. aegypti* mainly colonises tropical areas and can share the same regions with *Ae. albopictus*, which has also succeeded in invading some temperate countries [15].

The aim of our study was to assess the putative role of two mosquito species from the *Culex pipiens* complex, namely *Cx. pipiens* and *Cx. quinquefasciatus*, in ZIKV transmission. Because they are commonly found in temperate and tropical regions [16], respectively, they could strongly increase the risk of urban ZIKV outbreaks occurring.

Discussion

Members of the *Cx. pipiens* species complex are among the most widely distributed mosquitoes in the world and can act as disease vectors [17]. The species complex comprises several members including *Cx. pipiens* and *Cx. quinquefasciatus*, which are the most abundant *Culicinae* mosquitoes in temperate and tropical regions, respectively [16]. *Cx. pipiens* is the most ubiquitous mosquito species in temperate regions, occurring in rural and domestic environments [16] and can be found in nature in two biological forms, *pipiens* and *molestus*, which are morphologically indistinguishable [18]. The Tabarka strain, used in this study, is a mix of both forms [3] and has been shown to be a primary vector of West Nile virus (WNV) in the Mediterranean basin [19]. *Cx. quinquefasciatus* is mainly associated with human habitats and can experimentally transmit WNV, making it an ideal vector for domestic/urban transmission of WNV in tropical regions [20]. Our results show that laboratory colonies of *Cx. quinquefasciatus* and *Cx. pipiens* were unable to transmit an Asian genotype of ZIKV. Using mosquito colonies for vector competence studies can be considered as a proxy for measuring the genetic ability of one species to transmit a given pathogen [21]. In addition, the experimental ability to transmit a pathogen – vector competence – can vary according to specific combinations of virus and mosquito genotypes, which can be affected by environmental factors such as temperature [22]. The mosquito midgut barrier is the site where the initial steps such as viral attachment, penetration and replication take place before the release of newly produced virions into the mosquito haemocoel. We have shown that bypassing this midgut barrier, by inoculating viral particles into the haemocoel, did not favour viral dissemination nor transmission. Thus, our results strongly suggest that the *Cx. quinquefasciatus* and *Cx. pipiens* colonies were unable to transmit ZIKV, as has already been suggested for natural populations of *Cx. quinquefasciatus* collected during an outbreak of ZIKV infection in Mexico [23] and demonstrated for laboratory colonies of *Culex* mosquitoes [24,25].

Both mosquito species can tolerate environments highly charged with organic matter and high levels of chemical pollutants including insecticides [26].

Repeatedly confronted with insecticidal molecules, mosquito populations have developed resistance to insecticides, making vector control more difficult [27]. As *Aedes* and *Culex* mosquitoes do not share the same breeding sites, control measures targeting each of them are basically different. On the basis of our results, we consider that vector control should continue to focus on larval and adult habitats specific to *Aedes* mosquitoes, in order to efficiently control ZIKV vectors. While a vaccine is pending, surveillance and vector control should be reinforced against *Ae. aegypti* and *Ae. albopictus*, species that are able to transmit dengue virus, chikungunya virus and ZIKV.

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Conflict of interest

None declared.

Authors' contributions

FA and CAN designed and performed the research. AVR and RLO participated in producing reagents for mosquito experiments. MV produced viral stocks and performed mosquito inoculations. ABF designed the research, analysed the data and wrote the paper. All authors reviewed the paper.

References

1. Fauci AS , Morens DM. 2016. Zika Virus in the Americas--Yet Another Arbovirus Threat. N Engl J Med. 2016 Feb 18;374(7):601-4.
2. Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, et al. Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika Virus. PLoS Negl Trop Dis. 2016;10(3):e0004543. DOI: 10.1371/journal.pntd.0004543 PMID: 26938868
3. Krida G, Rhim A, Daaboub J, Failloux AB, Bouattour A. New evidence for the potential role of Culex pipiens mosquitoes in the transmission cycle of West Nile virus in Tunisia.Med Vet Entomol. 2015;29(2):124-8. DOI: 10.1111/mve.12107 PMID: 25586151
4. Georghiou GP. Research on mosquito resistance to insecticides at the University of California, Riverside.Proc Pap Annu Conf Calif Mosq Control Assoc. 1966;34:71-3.PMID: 5964233
5. Pocquet N, Milesi P, Makoundou P, Unal S, Zumbo B, Atyame C, et al. Multiple insecticide resistances in the disease vector Culex p. quinquefasciatus from Western Indian Ocean. PLoS One. 2013;8(10):e77855. DOI: 10.1371/journal.pone.0077855 PMID: 24204997
6. Zanluca C, Melo VC, Mosimann AL, Santos GI, Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil.Mem Inst Oswaldo Cruz. 2015;110(4):569-72. DOI: 10.1590/0074-02760150192 PMID: 26061233
7. Jupille H, Seixas G, Mousson L, Sousa CA, Failloux AB. Zika Virus, a New Threat for Europe?PLoS Negl Trop Dis. 2016;10(8):e0004901. DOI: 10.1371/journal.pntd.0004901 PMID: 27505002
8. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360(24):2536-43. DOI: 10.1056/NEJMoa0805715 PMID: 19516034
9. Cao-Lormeau VM, Roche C, Teissier A, Robin E, Berry AL, Mallet HP, et al. Zika virus, French polynesia, South pacific, 2013. Emerg Infect Dis. 2014;20(6):1085-6. DOI: 10.3201/eid2006.140138 PMID: 24856001
10. Brasil P, Sequeira PC, Freitas AD, Zogbi HE, Calvet GA, de Souza RV, et al. Guillain-Barré syndrome associated with Zika virus infection. Lancet. 2016;387(10026):1482. DOI: 10.1016/S0140-6736(16)30058-7 PMID: 27115821
11. Duarte Dos Santos CN, Goldenberg S. Zika Virus and Microcephaly: Challenges for a Long-Term Agenda.Trends Parasitol. 2016;32(7):508-11. DOI: 10.1016/j.pt.2016.03.008 PMID: 27105931
12. United States Centers for Disease Control and Prevention (CDC). All countries & territories with active Zika virus transmission. Atlanta, GA: CDC. [Accessed 16 Aug 2016]. Available from: http://www.cdc.gov/zika/geo/active-countries.html
13. Diallo D, Sall AA, Diagne CT, Faye O, Faye O, Ba Y, et al. Zika virus emergence in mosquitoes in southeastern Senegal, 2011. PLoS One. 2014;9(10):e109442. DOI: 10.1371/journal.pone.0109442 PMID: 25310102
14. Marchette NJ, Garcia R, Rudnick A. Isolation of Zika virus from Aedes aegypti mosquitoes in Malaysia.Am J Trop Med Hyg. 1969;18(3):411-5.PMID: 4976739
15. Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, Zeller H, et al. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector Borne Zoonotic Dis. 2012;12(6):435-47. DOI: 10.1089/vbz.2011.0814 PMID: 22448724
16. Farajollahi A, Fonseca DM, Kramer LD, Marm Kilpatrick A. "Bird biting" mosquitoes and human disease: a review of the role of Culex pipiens complex mosquitoes in epidemiology. Infect Genet Evol. 2011;11(7):1577-85. DOI: 10.1016/j.meegid.2011.08.013 PMID: 21875691
17. Ciota AT, Kramer LD. Vector-virus interactions and transmission dynamics of West Nile virus.Viruses. 2013;5(12):3021-47. DOI: 10.3390/v5123021 PMID: 24351794
18. Tempelis CH. Host-feeding patterns of mosquitoes, with a review of advances in analysis of blood meals by serology.J Med Entomol. 1975;11(6):635-53. DOI: 10.1093/jmedent/11.6.635 PMID: 235647
19. Amraoui F, Krida G, Bouattour A, Rhim A, Daaboub J, Harrat Z, et al. Culex pipiens, an experimental efficient vector of West Nile and Rift Valley fever viruses in the Maghreb region. PLoS One. 2012;7(5):e36757. DOI: 10.1371/journal.pone.0036757 PMID: 22693557
20. Fall G, Diallo M, Loucoubar C, Faye O, Sall AA. Vector competence of Culex neavei and Culex quinquefasciatus (Diptera: Culicidae) from Senegal for lineages 1, 2, Koutango and a putative new lineage of West Nile virus.Am J Trop Med Hyg. 2014;90(4):747-54. DOI: 10.4269/ajtmh.13-0405 PMID: 24567319
21. Vazeille-Falcoz M, Mousson L, Rodhain F, Chungue E, Failloux AB. Variation in oral susceptibility to dengue type 2 virus of populations of Aedes aegypti from the islands of Tahiti and Moorea, French Polynesia.Am J Trop Med Hyg. 1999;60(2):292-9. PMID: 10072154
22. Zouache K, et al. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. Proc Biol Sci. 1792;2014:281.
23. Guerbois M, Fernandez-Salas I, Azar SR, Danis-Lozano R, Alpuche-Aranda CM, Leal G, et al. Outbreak of Zika virus infection, Chiapas State, Mexico, 2015, and first confirmed transmission by Aedes aegypti mosquitoes in the Americas. J Infect Dis. 2016; pii:jiw302. DOI: 10.1093/infdis/jiw302 PMID: 27436433
24. Aliota MT, Peinado SA, Osorio JE, Bartholomay LC. Culex pipiens and Aedes triseriatus Mosquito Susceptibility to Zika Virus.Emerg Infect Dis. 2016;22(10). DOI: 10.3201/eid2210.161082 PMID: 27434194
25. Huang YS, Ayers VB, Lyons AC, Unlu I, Alto BW, Cohnstaedt LW, et al. Culex Species Mosquitoes and Zika Virus. Vector Borne Zoonotic Dis. 2016 Aug 24.
26. Krida G, Rhaiem A, Bouattour A. Effet de la qualité des eaux sur l'expression du potentiel biotique du moustique Culex pipiens L. dans la région de Ben Arous (Sud de Tunis). [Effect of water quality on the expression of biotic potential of the mosquito Culex pipiens L. in the region of Ben Arous (South of Tunis)].Bull Soc Entomol Fr. 1997;102:143-50.French.

27. Raymond M, Callaghan A, Fort P, Pasteur N. Worldwide migration of amplified insecticide resistance genes in mosquitoes.Nature. 1991;350(6314):151-3. DOI: 10.1038/350151a0 PMID: 2005964

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Experimental investigation of the susceptibility of Italian *Culex pipiens* mosquitoes to Zika virus infection

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We investigated the susceptibility of an Italian population of *Culex pipiens* mosquitoes to Zika virus (ZIKV) infection, tested in parallel with *Aedes aegypti*, as a positive control. We analysed mosquitoes at 0, 3, 7, 10, 14, 20 and 24 days after an infectious blood meal. Viral RNA was detected in the body of *Cx. pipiens* up to three days post-infection, but not at later time points. Our results indicate that *Cx. pipiens* is not susceptible to ZIKV infection.

Since its emergence in South and Central America in 2014, Zika virus (ZIKV) has spread rapidly, resulting in an unprecedentedly large number of infections [1-4]. It is well accepted that *Aedes* species are the main vectors of ZIKV [5-7]. However, in order to assess the risk of spread of this infection to new areas, it is pivotal to investigate the possibility that mosquito species belonging to other genera could contribute to sustaining virus transmission. *Culex pipiens* is widespread in Mediterranean countries [8], and little is known at present about its potential role as ZIKV vector. We report here our findings on experimental infection of an Italian population of *Culex pipiens* mosquitoes with ZIKV, using *Ae. aegypti* mosquitoes as a positive control. Using quantitative reverse transcription PCR (qRT-PCR) to detect viral RNA, our findings indicate that *Cx. pipiens* is not susceptible to ZIKV infection.

Experimental infection of mosquitoes

Experimental infection of the mosquitoes, starting in April 2016, was performed using the ZIKV H/PF/2013 strain, of the Asian genotype (kindly provided by Dr Isabelle Leparc-Goffart of the French National Reference Centre for Arboviruses in Marseille) isolated from a patient returning from French Polynesia in 2013 [9]. We exposed 10 day-old female mosquitoes from an Italian *Cx. pipiens* population (collected in Rome, Latium Region, in the summer of 2015) and from a long-established colony of *Ae. aegypti* (collected in Reynosa, Mexico, in 1998) to an infectious blood meal for one hour, through a membrane feeding apparatus.

The virus was diluted in rabbit blood (final virus concentration: 6.46 $\log_{10}$ plaque-forming units (PFU)/mL) and maintained at 37°C by a warm-water circulation system. After the blood meal, fully engorged females were transferred to other cages and maintained on a 10% sucrose solution in a climatic chamber (26 ± 1°C; 70% relative humidity; 14 hour light:10 hour dark cycle) for 24 days. A total of 8–10 mosquitoes from both species were processed individually at 0, 3, 7, 10, 14, 20 and 24 dpi.

To evaluate viral infection, dissemination and transmission, body (head, thorax and abdomen), legs plus wings, and saliva were analysed, as previously described [10]. The viral titre was evaluated by qRT-PCR. Specific primers ZIKV 1086 and ZIKV 1162c were used, with 5-FAM as the reporter dye for the probe (ZIKV 1107-FAM) [11]. Crossing point values were compared with a standard curve obtained from 10-fold serial dilutions of virus stock of known concentration [7].

Mosquito bodies were analysed in order to evaluate the infection rate, calculated as the number of ZIKV-positive mosquito bodies out of the total number of fed females. Legs plus wings were tested to assess the dissemination rate, calculated as the number of the specimens with ZIKV-positive legs plus wings among the tested mosquitoes. The saliva of the potentially infected females was processed to assess the transmission rate, defined as the number of mosquitoes with ZIKV-positive saliva among the number of tested mosquitoes [7,10].

Vector competence analysis

All the *Cx. pipiens* (n=10) and *Ae. aegypti* (n=8) bodies analysed at day 0 (i.e. immediately after the infectious blood meal) showed positive results, with mean viral titres of 4.23 (standard deviation (SD): 0.07) $\log_{10}$ PFU/mL and 3.7 (SD: 0.18) $\log_{10}$ PFU/mL, respectively, confirming the ingestion of viral particles.

At 3 dpi, only one of 10 *Cx. pipiens* mosquitoes analysed was infected. In the *Cx. pipiens* body, viral RNA was detected at a low concentration (0.17 $\log_{10}$ PFU/mL), whereas no viral RNA was detected at the later collection times. Viral RNA was never detected in legs plus wings and in the saliva of the *Cx. pipiens* (Table).

These findings differed greatly with those obtained with *Ae. aegypti*. As expected, in *Ae. aegypti*, the viral titres detected in the mosquito bodies increased gradually, reaching a mean value of 5.12 (SD: 0.06) $\log_{10}$ PFU/mL at 14 dpi, as well as in legs plus wings and in the saliva, showing an extrinsic incubation period similar to that previously described [7]. The infection rate at 7 dpi was 6/12 as was found for the dissemination rate. At the same collection time, ZIKV was detected also in the saliva with a transmission rate of 2/12 and a mean viral titre of 1.80 (SD: 0.14) $\log_{10}$ PFU/mL. In the later collection points, ZIKV was detected in body, legs plus wings and saliva confirming the expected vector competence of this mosquito species (Table).

Discussion

In countries where ZIKV has recently spread, *Ae. aegypti* and *Ae. albopictus* have been recognised as the most efficient vectors [5-7]. There is limited evidence that ZIKV can infect other mosquito species naturally: the presence of the virus has been reported in species of the *Culex* genus in Senegal and in Brazil [12,13]. Following our study on ZIKV competence of an Italian *Ae. albopictus* population [7], we investigated the susceptibility of an Italian population of the widespread indigenous species *Cx. pipiens* [8] to ZIKV infection under laboratory conditions. Increasing concern about the spread of ZIKV and its epidemic potential [1-4] makes it particularly important to fill gaps in knowledge about the role that mosquitoes other than *Ae. albopictus* and *Ae. aegypti* may have in the circulation and transmission of this virus in the Mediterranean area.

We focused our attention on *Cx. pipiens* mosquitoes as a potential ZIKV vector, since these mosquitoes are ubiquitous in temperate and tropical areas, where they are involved in the transmission of a range of human and zoonotic pathogens, such as West Nile virus, St Louis encephalitis virus, Rift Valley Fever virus, filarial worms and avian malaria [14,15]. The important vector role of *Cx. pipiens* arises from its opportunistic host feeding behaviour and on the high abundance it can reach in rural as well as in urban settings [14,15].

Our results show that the Italian *Cx. pipiens* population tested was not susceptible to ZIKV; the short persistence of the virus in the mosquito's body does not allow viral replication and, consequently, viral dissemination in the salivary glands. Conversely, our results showed *Ae. aegypti* to be competent for ZIKV transmission, as previously reported [7].

Table

Competence for Zika virus (infection, dissemination and transmission rates)[a] and Zika virus titres in body, legs plus wings and saliva of *Culex pipiens* and *Aedes aegypti* colonies fed orally[b,c]

Days post-infection	*Cx. pipiens*						*Ae. aegypti*					
	Infection rate	Mean viral titre[d] (SD) in body	Dissemination rate	Mean viral titre[d] in legs + wings	Transmission rate	Mean viral titre[d] in saliva	Infection rate	Mean viral titre[d] (SD) in body	Dissemination rate	Mean viral titre[d] (SD) in legs + wings	Transmission rate	Mean viral titre[d] (SD) in saliva
0[e]	10/10	4.23 (0.07)	0/10	0	0/10	0	8/8	3.73 (0.18)	0/8	0	0/8	0
3	1/10	0.17	0/10	0	0/10	0	ND	ND	ND	ND	ND	ND
7	0/10	0	0/10	0	0/10	0	6/12	3.76 (1.25)	6/12	2.57 (0.32)	2/12	1.80 (0.14)
10	0/10	0	0/10	0	0/10	0	ND	ND	ND	ND	ND	ND
14	0/10	0	0/10	0	0/10	0	4/8	5.12 (0.06)	4/8	3.11 (0.36)	3/8	2.05 (0.97)
20	0/10	0	0/10	0	0/10	0	4/10	4.60 (0.21)	3/10	3.08 (0.28)	3/10	2.10 (0.39)
24	0/10	0	0/10	0	0/10	0	ND	ND	ND	ND	ND	ND

ND: not detected; SD: standard deviation.

[a] Infection rate: number of virus-positive bodies/number of tested females; dissemination rate: number of virus-positive legs plus wings/number of tested females; transmission rate: number of virus-positive saliva samples/number of tested females.

[b] The mosquitoes were kept at 26 °C and collected at various days post-infection.

[c] The viral titre was evaluated by quantitative reverse transcription PCR (qRT-PCR). Crossing point values were compared with a standard curve obtained from 10-fold serial dilutions of virus stock of known concentration [7].

[d] Expressed as log10 plaque-forming units/mL.

[e] Immediately after the infectious blood meal.

Similar results were reported in a recent study on ZIKV susceptibility of a *Cx. pipiens* population from the United States [16], showing that this species is not a competent vector for ZIKV. However, in Brazil, current studies have reported ZIKV detection in the salivary glands of *Cx. quinquefasciatus* that were artificially fed with ZIKV-infected blood, and tested 7 and 15 days post-feeding [13,17].

We did not carry out viral titration by plaque formation as we observed in a previous study a high correlation between titration by this method and viral RNA detection [10]: this may constitute a limitation of this study.

In conclusion, the findings of the studies conducted on Italian and United States populations of *Cx. pipiens* mosquitoes have important public health implications, and help to optimise the vector control activities in Italy, should autochthonous ZIKV transmission occur. *Cx. pipiens* mosquito populations in Italy are unlikely to be competent vectors for ZIKV. Thus, to date, *Ae. albopictus* is the only mosquito established in Italy for which vector competence for ZIKV has been demonstrated [7]. However, even if a low epidemic potential risk of ZIKV in Italy was estimated [18], it should be considered that arboviruses have the potential to rapidly change their vector–host associations [19]. Therefore further vector competence studies should be undertaken in order to plan evidence-based interventions.

Acknowledgements

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Conflict of interest

None declared.

Authors' contributions

BD, TL, DLM, SF, RME, SM, VG and FC performed the experiments; BD, TL, DLM, SF, VG and FC analysed the data; BD, TL, DLM, SF, VG, RR, VG, RG and FC wrote the manuscript.

References

1. Hennessey M, Fischer M, Staples JE. Zika virus spreads to new areas — region of the Americas, May 2015–January 2016. MMWR Morb Mortal Wkly Rep. 2016;65(3):55-8. DOI: 10.15585/mmwr.mm6503e1 PMID: 26820163
2. United States Centers for Disease Control and Prevention (CDC). Countries and Territories with Active Local Zika Virus Transmission. Atlanta, GA: CDC. [Accessed 31 Aug 2016]. Available from: http://www.cdc.gov/zika/geo/active-countries.html
3. Campos GS, Bandeira AC, Sardi SI. Zika virus outbreak, Bahia, Brazil.Emerg Infect Dis. 2015;21(10):1885-6. DOI: 10.3201/eid2110.150847 PMID: 26401719
4. Zika virus outbreaks in the Americas. Wkly Epidemiol Rec. 2015;90(45):609-10.PMID: 26552108
5. Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. eLife. 2015;4:e08347. DOI: 10.7554/eLife.08347 PMID: 26126267
6. Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D. Differential susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika virus. PLoS Negl Trop Dis. 2016;10(3):e0004543. eCollection 2016. DOI: DOI: 10.1371/journal.pntd.0004543
7. Di Luca M, Severini F, Toma L, Boccolini D, Romi R, Remoli ME, et al. Experimental studies of susceptibility of Italian Aedes albopictus to Zika virus. Euro Surveill. 2016;21(18):30223. DOI: 10.2807/1560-7917.ES.2016.21.18.30223 PMID: 27171034
8. Di Luca M, Toma L, Boccolini D, Severini F, La Rosa G, Minelli G, et al. Ecological Distribution and CQ11 Genetic Structure of Culex pipiens Complex (Diptera: Culicidae) in Italy. PLoS One. 2016;11(1):e0146476. DOI: DOI: 10.1371/journal.pone.0146476 . e Collection 2016.
9. Baronti C, Piorkowski G, Charrel RN, Boubis L, Leparc-Goffart I, de Lamballerie X. Complete coding sequence of zika virus from a French polynesia outbreak in 2013.Genome Announc. 2014;2(3):e00500-14. DOI: 10.1128/genomeA.00500-14 PMID: 24903869
10. Fortuna C, Remoli ME, Di Luca M, Severini F, Toma L, Benedetti E, et al. Experimental studies on comparison of the vector competence of four Italian Culex pipiens populations for West Nile virus. Parasit Vectors. 2015;8(1):463. DOI: 10.1186/s13071-015-1067-z PMID: 26383834
11. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14(8):1232-9. DOI: 10.3201/eid1408.080287 PMID: 18680646
12. Diallo D, Sall AA, Diagne CT, Faye O, Faye O, Ba Y, et al. Zika virus emergence in mosquitoes in southeastern Senegal. 2011. PLoS One. 2014;9(10):e109442. doi: DOI: 10.1371/journal.pone.0109442 . eCollection 2014.
13. Fundação Oswaldo Cruz (Fiocruz). Fiocruz identifica Culex no Recife com potencial para transmitir o vírus zika). [Fiocruz identifies in Recife Culex with the potential to transmit Zika virus].21 July 2016. Rio de Janeiro: Fiocruz; 2016. Brazilian. Available from: https://agencia.fiocruz.br/fiocruz-identifica-culex-no-recife-com-potencial-para-transmitir-o-virus-zika
14. Turell MJ. Members of the Culex pipiens complex as vectors of viruses.J Am Mosq Control Assoc. 2012;28(4) Suppl;123-6. DOI: 10.2987/8756-971X-28.4.123 PMID: 23401952
15. Farajollahi A, Fonseca DM, Kramer LD, Marm Kilpatrick A. "Bird biting" mosquitoes and human disease: a review of the role of Culex pipiens complex mosquitoes in epidemiology. Infect Genet Evol. 2011;11(7):1577-85. DOI: 10.1016/j.meegid.2011.08.013 PMID: 21875691
16. Aliota MT, Peinado SA, Osorio JE, Bartholomay LC. Culex pipiens and Aedes triseriatus Mosquito Susceptibility to Zika virus.Emerg Infect Dis. 2016;22(10). DOI: 10.3201/eid2210.161082 PMID: 27434194
17. Franca RFO, Neves MHL, Ayres CFJ, Melo-Neto OP, Brandão Filho SP. First International Workshop on Zika Virus Held by Oswaldo Cruz Foundation FIOCRUZ in Northeast Brazil March 2016 – A Meeting Report. PLoS Negl Trop Dis. 2016;10(6):e0004760. doi: DOI: 10.1371/journal.pntd.0004760 . eCollection 2016.
18. Guzzetta G, Poletti P, Montarsi F, Baldacchino F, Capelli G, Rizzoli A, et al. Assessing the potential risk of Zika virus epidemics in temperate areas with established Aedes albopictus populations. Euro Surveill. 2016;21(15):30199. DOI: 10.2807/1560-7917.ES.2016.21.15.30199 PMID: 27104366
19. Coffey LL, Failloux AB, Weaver SC. Chikungunya virus-vector interactions.Viruses. 2014;6(11):4628-63. DOI: 10.3390/v6114628 PMID: 25421891

Descriptive epidemiology of *Escherichia coli* bacteraemia in England, April 2012 to March 2014

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We determined the incidence, risk factors and antimicrobial susceptibility associated with *Escherichia coli* bacteraemia in England over a 24 month period. Case data were obtained from the national mandatory surveillance database, with susceptibility data linked from LabBase2, a voluntary national microbiology database. Between April 2012 and March 2014, 66,512 *E. coli* bacteraemia cases were reported. Disease incidence increased by 6% from 60.4 per 100,000 population in 2012–13 to 63.5 per 100,000 population in 2013–14 ($p < 0.0001$). Rates of *E. coli* bacteraemia varied with patient age and sex, with 70.5% (46,883/66,512) of cases seen in patients aged ≥ 65 years and 52.4% (33,969/64,846) of cases in females. The most common underlying cause of bacteraemia was infection of the genital/urinary tract (41.1%; 27,328/66,512), of which 98.4% (26,891/27,328) were urinary tract infections (UTIs). The majority of cases (76.1%; 50,617/66,512) had positive blood cultures before or within two days of admission and were classified as community onset cases, however 15.7% (10,468/66,512) occurred in patients who had been hospitalised for over a week. Non-susceptibility to ciprofloxacin, third-generation cephalosporins, piperacillin–tazobactam, gentamicin and carbapenems were 18.4% (8,439/45,829), 10.4% (4,256/40,734), 10.2% (4,694/46,186), 9.7% (4,770/49,114) and 0.2% (91/42,986), respectively. Antibiotic non-susceptibility was higher in hospital-onset cases than for those presenting from the community (e.g. ciprofloxacin non-susceptibility was 22.1% (2,234/10,105) for hospital-onset vs 17.4% (5,920/34,069) for community-onset cases). Interventions to reduce the incidence of *E. coli* bacteraemia will have to target the community setting and UTIs if substantial reductions are to be realised.

Introduction

Data from voluntary laboratory-based surveillance in England, Wales and Northern Ireland has consistently shown *Escherichia coli* to be the most prevalent pathogen causing bacteraemia, with sustained annual increases [1]. In 2013 *E. coli* accounted for approximately 32% of all bacteraemia reports, an increase from 27% in 2009 [1]. Year-on-year increases in cases of bacteraemia due to *E. coli* have been observed across Europe [2]. This is reinforced by studies from Austria, China and the United States, which have implicated *E. coli* as the first and second most common cause of community-acquired and hospital-acquired bloodstream infection (BSI) respectively [3-5]. A further study from England estimated the all-cause mortality rate in *E. coli* bacteraemia patients to be 18.2% between July 2011 and June 2012 [6]. In addition to a high mortality burden, *E. coli* bacteraemia has been associated with increases in length of hospital stay and difficulties with antibiotic treatment due to infections caused by resistant strains [2,7]. All of these factors increase healthcare costs and have a substantial clinical and economic impact [8].

In June 2011 in England, centralised reporting of cases of *E. coli* bacteraemia by National Health Service (NHS) hospital Trust (groups of hospitals under the same management) was made mandatory with the aim of better elucidating the increases and patterns observed in the voluntary surveillance programme. The present study is an analysis of the first two years of mandatory surveillance data, providing a comprehensive review of the current situation across the entire English NHS.

Methods

Data collection

The study period comprised two years from 1 April 2012 to 31 March 2014, during which time all NHS acute Trusts (n = 167) in England reported all cases of bacteraemia due to *E. coli* to Public Health England (PHE, formerly the Health Protection Agency). Cases were reported via a web-based system originally developed for the mandatory surveillance of *Clostridium difficile* infection and bacteraemia caused by *Staphylococcus aureus*. Only the first blood culture positive for *E. coli* was reported, with further positive blood cultures

taken from the same patient within 14 days of the first sample regarded as the same episode of bacteraemia and not reported. Data items collected included the specimen date, patient demographics and care details at the time the blood culture was taken.

Patient identifiers from the mandatory *E. coli* dataset (i.e. patient name, date of birth, NHS number and hospital number) were used to link with antibiotic susceptibility data for the same bacteraemia case reported by Trust laboratories on a voluntary basis to a national database, LabBase2, maintained by PHE.

Data analyses

Data processing and analyses were performed using Stata12 (Stata Corporation, College Station, TX, US). *E. coli* population-level incidence rates were calculated using the Office for National Statistics (ONS) mid 2012 and 2013 resident population estimates, based on the results of the 2011 census [9]. National or regional rates of *E. coli* bacteraemia were presented per 100,000 population. Trust-level incidence rates were presented per 100,000 bed days, with the denominator being derived using 2013–14 KH03 data (organisational-level average daily number of occupied beds) [10]. Relevant KH03 information for each NHS acute Trust was multiplied by the number of days in the study period to provide the total bed day denominator. Incidence risk ratios (RR) were expressed as risks with 95% confidence intervals (CIs). Differences in categorical variables were assessed using a chi-squared test and considered statistically significant if two-tailed $p<0.05$. Subnational analyses mapped cases to the four regions of England, and the fifteen PHE Centres (PHECs).

To compare *E. coli* rates between similar types of hospitals, Trusts were grouped into five categories: small, medium or large acute Trusts, acute Specialist Trusts and acute Teaching Trusts. The groupings were based on a cross-tabulation of Estates Return Information Collection (ERIC) Trust categorisations and KH03 hospitals bed day capacity information [10,11]. Acute Specialist and acute Teaching Trusts were identified solely using the ERIC classifications. The remaining Trusts were divided into large, medium and small Trusts by ordering the ERIC categorisations and the total occupied hospital bed KH03 (2013–14) data, and applying a 75% inclusion of the Trusts which fell under the same classification in both datasets.

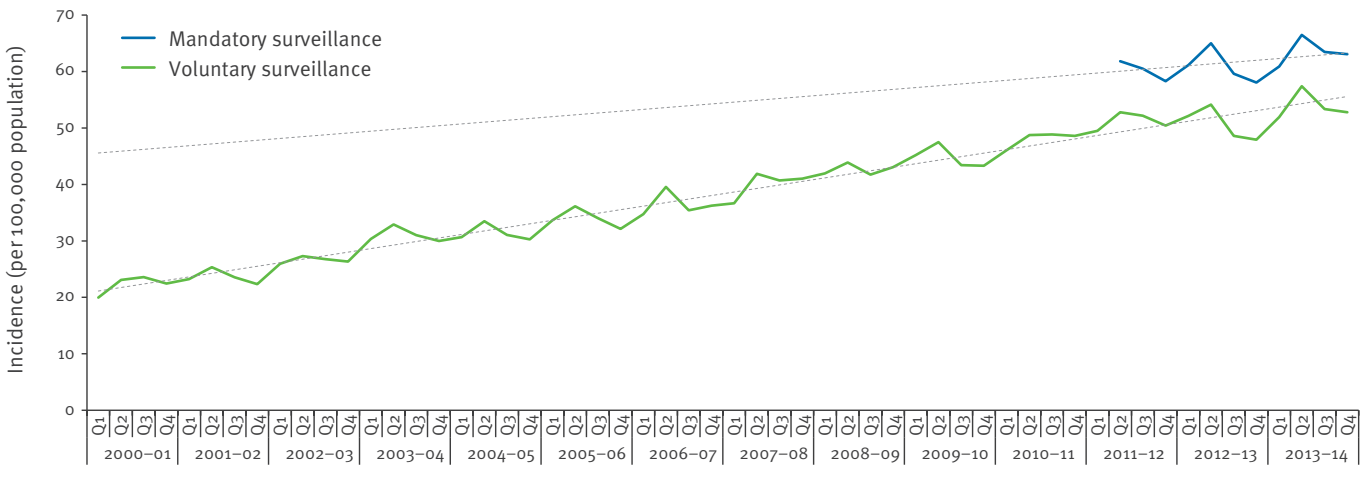
Cases were deemed to be hospital-onset (HO) cases if a patient's specimen date was on or after the third day of hospital admission (where the day of admission was day one). Patients who had a bacteraemia detected before or within 2 days of admission were classified as community-onset (CO) [12]. Cases were categorised as an unknown onset if admission date was not recorded.

Antibiotic susceptibility

Following the linkage of the mandatory surveillance to the LabBase2 datasets, the susceptibility of *E. coli* to key antibiotic groups was evaluated, namely the beta-lactam/beta-lactamase inhibitor combination piperacillin–tazobactam, third-generation cephalosporins (ceftazidime and cefotaxime), a fluoroquinolone (ciprofloxacin), carbapenems (imipenem, meropenem and ertapenem) and an aminoglycoside (gentamicin). LabBase2 collects routinely generated antimicrobial susceptibility test results from hospital laboratories, 95% use European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [13]. For the purposes of analysis, intermediate and resistant isolates were combined and classified as 'non-susceptible'. An isolate was considered non-susceptible to any of the groups above if at least one of the antibiotics within the group was found to be non-susceptible.

Figure 1

Temporal incidence of *Escherichia coli* bacteraemia based on the voluntary and mandatory surveillance schemes, England, April 2000–March 2014



Results

National and regional trends of *Escherichia coli* bacteraemia

A total of 66,512 cases of *E. coli* bacteraemia were reported between April 2012 and March 2014. There was a 6% increase in the annual incidence over the two consecutive years (32,309 cases in 2012–13, incidence 60.4/100,000 population, 95% CI: 59.7–61.1 vs 34,203 cases in 2013–14, incidence 63.5/100,000 population, 95% CI: 62.8–64.2; p<0.0001). A comparable increase in cases reported on a voluntary basis to LabBase2 was also noted. A slight seasonal peak during the second quarter (July–September) of each year was seen in both datasets (Figure 1).

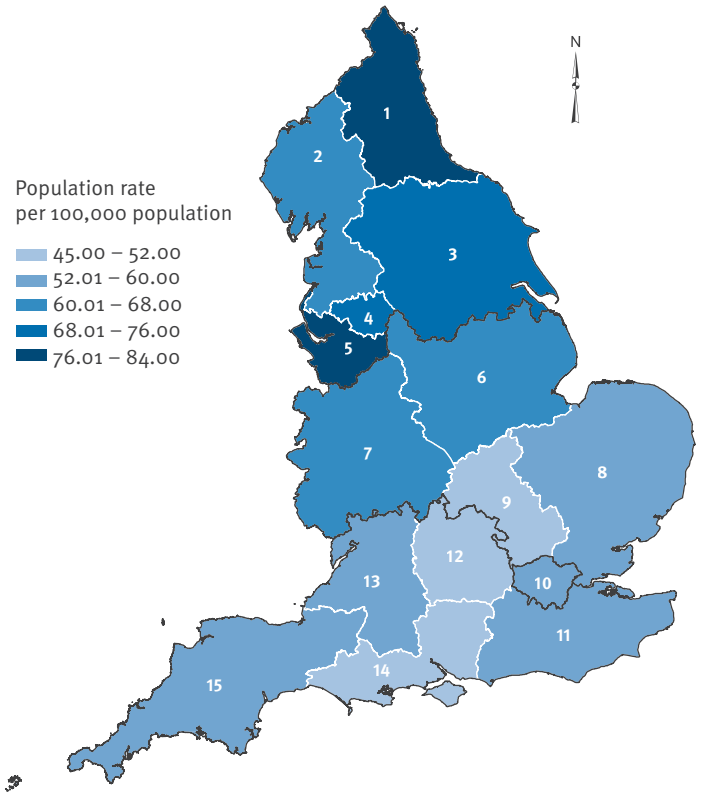
The variation in incidence among different geographical areas of England (based on PHECs and regions) is shown in Figure 2. There were statistically significant differences in regional rates (p<0.0001) between the highest rate in the North of England region (73.2/100,000 population/year) and the lowest in the South of England region (54.5/100,000 population/year), accompanied by a noticeable decreasing incidence gradient from the north to the south PHECs. When stratified by HO or CO of bacteraemia, both were highest in the North region (25.0 and 73.8/100,000 population/year, respectively), with 36% (5,604/15,393) of HO cases reported in the North region.

Disease incidence among different patient groups

The overall annual incidence of infection stratified by patient age and sex (sex data provided for 97.5%, 64,846 cases) is shown in Figure 3. The incidence of *E. coli* bacteraemia increased with patient age for both females and males, with the exception of children<1 year of age, where the incidence was higher than in patients aged 1 to 64 years (Figure 3). Approximately a quarter (25.8%; 283/1,096) of those aged<1 year were neonates aged≤7 days. The overall median age was 75 years (interquartile range (IQR): 61–83 years), with 70.5% (46,883/66,512) of cases occurring in patients≥65 years. Overall, 52.3% (33,969/64,846) of cases where sex was recorded were female (incidence 62.3 per 100,000 female population/year) and 47.6% (30,877/64,846) were male (incidence 58.4 per 100,000 male population/year), which translates to a 7% decreased RR in males compared with females (p<0.0001). Despite this, rates were higher among men across the majority of age groups. Rates were only higher among females in the following three age categories '1 to 14 years' (2.9 vs 2.0 per 100,000 population/year), '15 to 44 years' (19.6 vs 6.5 per 100,000

Figure 2

Region-specific average year rate of *Escherichia coli* bacteraemia in England, April 2012–March 2014 (n=66,324 patients)[a]



PHE region	PHE Centre	PHE Centre ID
North of England	North East	1
North of England	Cumbria and Lancashire	2
North of England	Yorkshire and Humber	3
North of England	Greater Manchester	4
North of England	Cheshire and Merseyside[a]	5
Midlands and East of England	East Midlands	6
Midlands and East of England	West Midlands	7
Midlands and East of England	Anglia and Essex	8
Midlands and East of England	South Midlands and Hertfordshire	9
London	London	10
South of England	Kent, Surrey and Sussex	11
South of England	Thames Valley	12
South of England	Avon, Gloucestershire and Wiltshire	13
South of England	Wessex	14
South of England	Devon, Cornwall and Somerset	15

NHS: National Health Service; PHE: Public Health England; PHEC: PHE Centres.

[a] Wirral University Teaching Hospital NHS Foundation Trust did not report E. coli bacteraemia cases for the entire duration between April 2012–March 2013. Hence, associated Cheshire and Merseyside PHEC average rate across the two years will not include April 2012–March 2013 data for this acute Trust and may have caused an underestimation of the rate of infection for this PHEC.

population/year) and '45 to 54 years' (31.6 vs 27.7 per 100,000 population/year). All three age category rates by sex were statistically significantly different ($p < 0.005$). Notably the female rate in the '15 to 44 years' category was threefold that of the males and presented the highest RR in comparison to the other age categories (RR: 3.0; 95% CI: 2.8–3.3). The highest age and sex specific rate was among men aged ≥ 85 years, with an increased RR of 36% in males vs females (males: 749.2 per 100,000 population/year vs females: 486.7 per 100,000 population/year; RR: 0.6; 95% CI: 0.6–0.7; $p < 0.0001$).

Presentation of *Escherichia coli* bacteraemia

Seventy-four per cent (48,953/66,512) of *E. coli* bacteraemia cases were classified as CO, compared with 23.1% (15,393/66,512) HO; 3.3%, (2,166/66,512) of cases were with unknown onset. Approximately 15.7% (10,468/66,512) were classed as late HO, i.e. occurred seven or more days following hospital admission.

Ninety per cent (60,135/66,512) of *E. coli* bacteraemia reports included information on patient provenance. Approximately three quarters of reports indicated that the patient was admitted from home (50,610/66,512) (Table 1), 46.5% (23,517/50,610) of whom were patients aged ≥ 75 years.

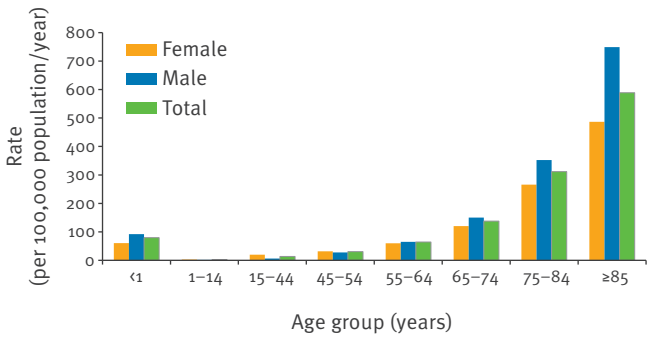
The median incidence of bacteraemia classified as HO was 20.5 per 100,000 bed days. The incidence of HO *E. coli* bacteraemia increased with Trust size, with annual median rates of 17.5, 19.7 and 22.6/100,000 bed days for the small, medium and large acute Trusts, respectively. These rates were not significantly different (Figure 4). The highest median HO rate of infection was seen in acute Teaching Trusts (24.6/100,000 bed days). The lowest median incidence (16/100,000 bed days) was in acute Specialist Trusts. The distribution of acute Specialist Trusts HO rates was wide, with the IQR for this Trust type entirely overlapping that of the small acute Trusts. There were a total of six outliers, the most extreme were related to 'Specialist' cancer centres (44.0 and 94.6 /100,000 bed days).

The boxes represent the 25th and 75th percentiles; the median line is present within the box. The lower and upper whiskers represent the 5th and 95th percentiles. Outliers are represented by dots.

The largest proportion (41.0%; 27,254/66,512) of reported *E. coli* bacteraemia cases occurred under the specialty of 'general medicine' (Table 1). 'Surgery' accounted for the second highest proportion of cases (12.8%; 8,506/66,512) followed by 'care of the elderly' at 8.7% (5,760/66,512).

Figure 3

Escherichia coli bacteraemia age and sex specific average year rates, England, April 2012–March 2014 (n=64,846 patients)[a]



[a] Of 66,512 cases of bacteraemia included in the study, information on sex was available for 64,846. Rates in the figure are based on the total of 64,846 patients

Table 1

Patient provenance, speciality and primary focus of *Escherichia coli* bacteraemia, England, April 2012–March 2014 (n = 66,512 patients)[a]

Patient provenance n (%)		Specialty n (%)		Primary focus of infection n (%)	
Home	50,610 (76.1)	General medicine	27,254 (41.0)	Genital/urinary tract	27,328 (41.1)
Nursing/residential home	5,352 (8.0)	Other[b]	9,525 (14.3)	Unknown	11,971 (18.0)
Not known	2,051 (3.1)	Surgery	8,506 (12.8)	Hepatobiliary	7,611 (11.4)
Hospital (UK or abroad, incl. private)	1,380 (2.1)	Care of the elderly	5,760 (8.7)	Gastrointestinal (not hepatobiliary)	3,493 (5.3)
Other[c]	469 (0.7)	A and E	5,381 (8.1)	Respiratory tract	2,065 (3.1)
PCT Hospital	156 (0.2)	Urology related	2,005 (3.0)	Other[d]	1,932 (2.9)
Non-UK resident	117 (0.2)	Oncology	1,644 (2.5)	Indwelling intravascular device	828 (1.2)
Blank	6,377 (9.6)	Paediatrics	1,351 (2.0)	Skin/soft tissue	610 (0.9)
		Not known	424 (0.6)	Blank	10,674 (16.0)
		Blank	4,662 (7.0)		

A and E: Accident and Emergency; Incl.: including; PCT: Primary Care Trust; UK: United Kingdom.

[a] Of 66,512 cases of bacteraemia included in the study, information on patient provenance was available for 60,135 cases, on speciality for 61,850 and on the underlying primary focus for 55,838. Percentages in the table are based on the total of 66,512 patients.

[b] Specialities which were not commonly reported were grouped as 'other'.

[c] Including temporary accommodation and penal establishment.

[d] Including: no clinical signs, bone and joint, central nervous system.

The underlying primary focus of the bacteraemia was reported in 84.0% (55,838/66,512) of cases. 'Genital/urinary tract' was thought to be the source for just under half of case (41.1%; 27,328/66,512); 98.4% (26,891/27,328) of these were urinary tract infections (UTIs). For 18.0% (11,971/66,512) of cases the primary focus was unknown. Genital/urinary tract source of infections were associated with 44.0% (21,526/48,953) of patients with a CO *E. coli* bacteraemia and 34.1% (5,247/15,393) of patients with HO.

E. coli bacteraemia with a urinary primary focus of infection were associated with a higher proportion of females than males (56.0% 15,058/26,891 vs 42.0% 11,274/26,891 respectively). Notably, the disparities according to sex were most evident between the three age groups which spanned 1 to 54 years of age (1–14 years: females 67.4% (62/92) vs males 29.3% (27/92), $p<0.0001$; 15–44 years: females 82.8% (2,094/2,528) vs males 14.7% (371/2,528), $p<0.006$; 45–54 years: females 62.8% (1,050/1,672) vs males 34.8% (582/1,672), $p<0.0009$).

Where the primary focus of infection was given as UTI, 69.5% (5,255/7,559) of the records with a response indicated the presence of a urinary catheter. However, this field was poorly completed and not representative, with 72% of cases missing this information.

Antibiotic susceptibility

Eighty-two per cent (54,301/66,512) of *E. coli* bacteraemia records from the mandatory surveillance were successfully linked to antibiotic susceptibility data. Non-susceptibility was highest for ciprofloxacin (18.4%; 8,439/45,829) and lowest for the carbapenems (0.2%; 91/42,986) (Table 2).

Although the proportions of isolates non-susceptible to the various antibiotics were similar between the two successive years, there was an increase in the number of isolates non-susceptible to these antibiotics. In particular, piperacillin–tazobactam non-susceptible cases increased by 10.9% (2,226 cases in 2012–13; 2,468 cases in 2013–14).

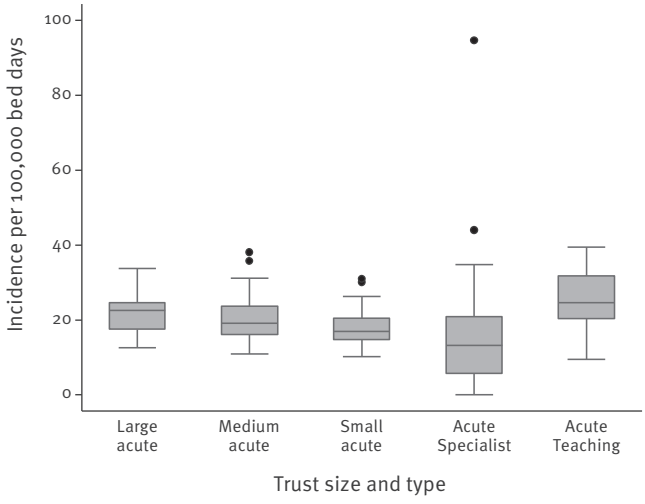
Similar levels of non-susceptibility were observed at the regional level compared with nationally, i.e. non-susceptibility to ciprofloxacin was the highest and carbapenem non-susceptibility was the lowest across all the PHECs. Although the ranking was similar there were nonetheless regional variations in the proportions of *E. coli* that were non-susceptible to antibiotics. Unlike the North–South variation seen with the incidence of *E. coli* bacteraemia, non-susceptibility was generally highest in the London region. The London PHEC had the highest proportion of non-susceptibility to ciprofloxacin (25.4%; 1,742/6,868), piperacillin–tazobactam (12.8%; 893/6,977), gentamicin (15.2%; 1,098/7,216), and one of the highest to third-generation cephalosporins (14.9%; 951/6,400). These were significantly different ($p<0.0001$) to the lowest levels of non-susceptibility seen in the North East PHEC (ciprofloxacin 13.4% 434/3,237; third-generation cephalosporins 6.3% 201/3,187; gentamicin 5.5% 183/3,342). Yorkshire and Humber, and Thames Valley PHECs were excluded from the analysis as only 61% and 56% of cases were successfully linked.

When stratified by onset, non-susceptibility to all study antibiotics was higher in HO cases (Table 2). Non-susceptibility in HO cases have marginally decreased over the two study years, particularly for ciprofloxacin and third-generation cephalosporins (10% and 11% decrease), whereas the CO have increased; 10% and 9% rise in non-susceptibility to third-generation cephalosporins and piperacillin–tazobactam, respectively ($p<0.05$). Piperacillin–tazobactam presented the largest disparity between HO and CO, with nearly twofold difference in the proportion of isolates showing non-susceptibility in HO cases (15.1%, 1,562/10,363) compared with the CO (8.7%, 2,986/34,175).

There were statistically significant differences in the proportion of antibiotic non-susceptibility in males compared with females for all antibiotics apart from the carbapenems, particularly for ciprofloxacin (males: 20.9% 4,433/21,236; female: 16.2%, 3,783/23,320; $p<0.0001$). Within the 15 to 44 year age group, the proportion of males with *E. coli* not susceptible to ciprofloxacin was significantly higher than the proportion for females (males: 20% 183/899; females: 11% 298/2,812; $p<0.0001$). Non-susceptibility for each antibiotic class, apart from carbapenems, increased with age, with the highest non-susceptibilities seen in infections in patients aged ≥ 65 years.

Figure 4

Box-and-whisker plots showing hospital-onset *Escherichia coli* bacteraemia annual rates, by Trust size and type, England, April 2012–March 2014



The boxes represent the 25th and 75th percentiles; the median line is present within the box. The lower and upper whiskers represent the 5th and 95th percentiles. Outliers are represented by dots.

Discussion

The linkage of *E. coli* bacteraemia cases, reported by Trusts as part of a mandatory surveillance scheme to susceptibility data reported by laboratories on a voluntary basis, has enabled a comprehensive analysis that gives insight into the national epidemiology and burden of *E. coli* bacteraemia across England. Mandatory surveillance of *E. coli* bacteraemia was implemented in June 2011 hence long-term trends over time have not been fully established; however the rise in incidence across the two years has mirrored the year-on-year increase in incidence seen in the voluntary surveillance dataset. The results presented here, along with an emerging body of evidence, suggest that there is seasonal variation in *E. coli* bacteraemia rates, with a peak during the summer [14,15].

Analysis of geographical variation in infection rates showed a North–South divide, with the South of England having a lower and the North a higher *E. coli* bacteraemia rate than the average for England. Various other regional data resonate with this division, with the North having higher health inequalities and poorer health outcomes [16]. There were differences in the proportion of HO rates, suggesting that the geographical heterogeneity may be associated with provision of healthcare.

E. coli bacteraemia incidence rates generally increased with age, across both sexes, with a high proportion of *E. coli* bacteraemia occurring in patients aged ≥ 65 years (85.5%). We identified a larger incidence among patients aged ≤ 1 year compared with those a few years

Table 2

Number and percentage of non-susceptible *Escherichia coli* bacteraemia strains to selected antibiotics, England, April 2012–March 2014

Criteria		Ciprofloxacin	Third generation cephalosporins[a]	Piperacillin–Tazobactam	Gentamicin	Carbapenems[b]
2012–14	Number tested	45,829	40,734	46,186	49,114	42,986
	Number of non-susceptible	8,439	4,256	4,694	4,770	91
	Non-susceptible (%)	18.4	10.4	10.2	9.7	0.21
Age group in years n/N[c] (%)	<1	56/673 (8.3)	34/661 (5.1)	32/671 (4.8)	55/749 (7.3)	0/615 (0.00)
	1–14	53/250 (21.2)	40/229 (17.5)	35/250 (14.0)	37/263 (14.1)	5/202 (2.48)
	15–44	493/3,821 (12.9)	268/3,380 (7.9)	311/3,842 (8.1)	333/4,105 (8.1)	10/3,521 (0.28)
	45–54	546/3,130 (17.4)	264/2,772 (9.5)	284/3,162 (9.0)	336/3,309 (10.2)	11/2,940 (0.37)
	55–64	1,027/5,376 (19.1)	501/4,779 (10.5)	554/5,391 (10.3)	551/5,742 (9.6)	12/5,024 (0.24)
	65–74	1,844/9,362 (19.7)	887/8,302 (10.7)	941/9,439 (10.0)	1,006/9,997 (10.1)	13/8,816 (0.15)
	75–84	2,379/13,003 (18.3)	1,238/11,511 (10.8)	1,404/13,101 (10.7)	1,358/13,964 (9.7)	23/12,215 (0.19)
	>84	2,041/10,214 (20.0)	1,024/9,100 (11.3)	1,133/10,330 (11.0)	1,094/10,985 (10.0)	17/9,653 (0.18)
Sex n/N[c] (%)	Female	3,783/23,320 (16.2)	2,013/20,685 (9.7)	2,235/23,462 (9.5)	2,252/25,025 (9.0)	40/21,817 (0.18)
	Male	4,433/21,236 (20.9)	2,102/18,845 (11.2)	2,322/21,443 (10.8)	2,403/22,749 (10.6)	51/19,948 (0.26)
	Unknown	223/1,273 (17.5)	141/1,204 (11.7)	137/1,281 (10.7)	115/1,340 (8.6)	0/1,221 (0.00)
Focus n/N[c] (%)	Genital/urinary tract	3,915/19,543 (20.0)	1,952/16,595 (11.8)	1,991/19,161 (10.4)	2,387/20,566(11.6)	34/18,086 (0.19)
	Indwelling intravascular device	122/529 (23.1)	57/471 (12.1)	73/534 (13.7)	78/553 (14.1)	2/477 (0.42)
	Skin/soft tissue	86/430 (20.0)	43/361 (11.9)	50/436 (11.5)	44/450 (9.8)	2/394 (0.51)
Onset setting n/N[c] (%)	Hospital	2,234/10,105 (22.1)	1,306/9,099 (14.4)	1,562/10,363 (15.1)	1,469/10,901 (13.5)	33/9,585 (0.34)
	Community	5,920/34,069 (17.4)	2,802/30,072 (9.3)	2,986/34,175 (8.7)	3,154/36,497(8.6)	56/31,816 (0.18)
Trust category n/N[c] (%)	Large acute	584/2,877 (20.3)	313/2,660 (11.8)	485/3,216 (15.1)	402/3,394 (11.8)	6/2,959 (0.20)
	Medium acute	489/2,417 (20.2)	280/2,040 (13.7)	307/2,352 (13.1)	320/2,563 (12.5)	7/2,331 (0.30)
	Small acute	270/1,179 (22.9)	184/1,051 (17.5)	176/1,163 (15.1)	184/1,314 (14.0)	4/1,094 (0.37)
	Acute Teaching	808/3,261 (24.8)	472/3,003 (15.7)	548/3,258 (16.8)	503/3,236 (15.5)	15/2,867 (0.52)
	Acute Specialist	NA[d]	NA[d]	NA[d]	NA[d]	NA[d]

NA: not applicable.

[a] Third-generation cephalosporins were represented by ceftazidime and cefotaxime. Isolates non-susceptible to any of these two antibiotics were considered as non-susceptible to third generation cephalosporins.

[b] Carbapenems were represented by imipenem, meropenem and ertapenem. Isolates non-susceptible to any of these three antibiotics were considered as non-susceptible to carbapenems. The proportions of isolates that are non-susceptibile to carbapenems in England is currently very low. To visualise differences between the groups (age/sex/focus/onset setting/trust type), the proportions of isolates that are non-susceptible to this particular antibiotic group are presented with a two decimal point precision.

[c] The numbers supporting the percentages presented are provided, whereby the denominators represent the total number of isolates tested per category within each group considered (age/sex/focus/onset setting/trust type).

[d] Only 52% of cases occurring in Specialist Trust were successfully linked to antibiotic susceptibility data; as a result of this further analysis was not performed on this Trust group.

older. These findings are in agreement with previous studies [5,16-20], and are related to the vulnerability of these groups to infection, with the very young being immunologically naive and older age patients having progressively deteriorating immune systems, increasing comorbidities and invasive healthcare procedures [21,22]. There was increasing infection with decreasing neonatal age, particularly in neonates aged less than a week, indicative of vertical transmission events. *E. coli* infections in preterm neonates, along with group B streptococcal infection, contribute a substantial burden of disease in this patient group [23].

Evident sex differences in the distribution of *E. coli* bacteraemia by age were present; rates were higher in females between 1 and 54 years of age and greater in males in the older age groups (›54), this is consistent with previous findings [17,18]. *E. coli* bacteraemia frequently occurs as a complication following a UTI. Indeed, the greater bacteraemia rate among females were likely due to a higher proportion of UTIs occurring among females aged between 1 and 54 years. Females have a higher predisposition for UTIs compared with males due to their urethras being shorter and in closer proximity to the rectum [24].

The most common source of infection leading to *E. coli* bacteraemia was the genital/urinary tract. This was associated with increasing age, with older patients becoming more susceptible to UTIs perhaps due to increasing urological co-morbidities and the increased use of catheters. Older males are more prone to prostate problems which can lead to urinary retention and UTIs; the performance of prostate biopsies is an additional risk factor for bacteraemia in males aged over 54 years [17].

A large percentage of cases were reported with an 'unknown' focus of infection (18.0%). Treatment of such infections may prove problematic, as without identification of the source it is difficult to target interventions that will remove or nullify it. An unresolved infection source risks the repeated seeding of the bacteria into the blood, leading to repeated episodes and prolonged patient exposure to antibiotics, increasing the risk of selecting for antibiotic resistant strains.

The study indicates that approximately three-quarters of *E. coli* bacteraemias were of CO. Other studies have also found higher rates in community-acquired bacteraemia [20,25]. Approximately 16% were late-HO patients and had been under the care of the Trust for a week or more before their bacteraemia establishing, thus they represent cases likely to be hospital acquired and therefore the most amenable to prevention via hospital based infection control measures.

A limitation which warrants further investigation is the need to differentiate CO infections that are community-acquired versus those that have an association with prior healthcare i.e. healthcare-associated infections. The simplistic categorisation of 'pre-day 2 of hospital admission' cases as 'community' fails to account for infections acquired as a result of outpatient care, or those occurring immediately after discharge [12,18,25,26]. A proportion of the 'community' cases observed in the study may, in part, be the result of this lack of precision. There was a high proportion of cases reported to have been admitted from home. These findings reflect the complexity of procedures which are now being delivered in the community or where the patient has been discharged from hospital to continue convalescing at home.

Larger acute Trusts were associated with a higher rate of infection. A larger Trust has a corresponding larger pool of susceptible individuals, immunocompromised patients, higher patient per nurse ratio, wider use of antimicrobials which could lead to selection pressures, and greater challenges in maintaining infection control measures [27,28]. The highest median HO rate of infection was seen in acute Teaching Trusts, these Trusts generally have more complex, tertiary care patients than general acute Trusts [29]. The acute Specialist Trusts had the highest variance, the outliers seen in this group were in two specialist cancer Trusts. Most cancer treatments affect a patient's susceptibility to infection [30]. The use of invasive devices (e.g. intravenous lines), prior exposure to antimicrobial therapy and multiple hospitalisations would increase a patient's risk of acquiring a bacteraemia [31]. The heterogeneity of case mix within and across Trust types, particularly Specialist Trusts, and the lack of statistical differences limits our ability to determine whether the findings were genuine or due to artefact.

The proportions of isolates non-susceptible to the antibiotics tested did not vary greatly between the two years. However, while the stability of resistance in *E. coli* over recent years has been highlighted in the literature [20,32], the increased incidence of bacteraemia caused by *E. coli*, means that the burden of resistant infections has nonetheless continued to rise [33].

Non-susceptibility to carbapenems remains low in England. The isolates non-susceptible to carbapenems were more closely associated with HO cases, than for any of the other antibiotic classes. Carbapenems are often considered as 'last-line' antibiotics for Enterobacteriaceae, as carbapenem-resistant isolates often exhibit resistance to multiple antibiotic classes, severely limiting the number of effective therapies available. Although carbapenems account for a minority of total antibiotic consumption, we have seen the consumption of carbapenems increase by 31.3% in England between 2010 and 2013 [33].

Non-susceptibility to ciprofloxacin was higher than for any of the other antimicrobials (18.4%). Resistance to a fluoroquinolone is often associated with resistance to other antibiotics frequently indicated for UTIs (e.g. trimethoprim), with ciprofloxacin itself currently

stated as the first line treatment for complicated UTIs [13]. English prescribing guidance over the past decade has reduced the recommended duration of trimethoprim treatment for uncomplicated cystitis and shifted to nitrofurantoin as the first-line option [34]. Previous suboptimal antibiotic consumption could have impacted on an increase in recurrent UTIs, propensity of bacteraemia and non-susceptibility [34].

During the last decade in England, there has been a prescribing shift away from fluoroquinolones and third-generation cephalosporins, towards higher use of beta-lactamase/inhibitor combinations and carbapenems; this may in part explain the rise in piperacillin–tazobactam non-susceptibility, however laboratories changing over from Clinical and Laboratory Standards Institute (CLSI) to EUCAST breakpoint and methods, could also explain the increases [13,33].

Unlike the geographical variation seen with *E. coli* incidence, non-susceptibility was generally highest in the London region. As the susceptibility data are collected by voluntary reporting, it could be that variations reflect differences in reporting. However this finding is in accordance with the higher prevalence of antimicrobial consumption reported in London [33].

Antibiotic non-susceptibility was generally higher in the HO cases, notably piperacillin–tazobactam. This is most probably due to greater selection pressures in the hospital environment. Piperacillin–tazobactam, is also predominantly used in the hospital setting [33]. Across the two years there has been a marginal increase in antibiotic non-susceptibility in CO cases. Recent studies show a rise in community prescribing, particularly in general practices [33].

E. coli bacteraemia in males were more likely to be non-susceptible than in females, agreeing with observations from other studies [32,35]. Since the proportion of non-susceptible *E. coli* is higher with older age (≥ 65 years) and older age categories are known to have higher rates of bacteraemia in males compared with females, it is likely that a higher proportion of males have more complicated infections, frequently with hospital strains, are exposed to more antimicrobial therapy, which increases selection pressure and results in higher proportions of non-susceptibility.

Increases in rates of *E. coli* bacteraemia are multifactorial and may in part be explained by an ageing population, increased international travel and consumption of antibiotics. The present study suggests that interventions targeting the source of infection, particularly UTIs, may be effective in reducing rates. Reduction in prescribing of broad-spectrum antibiotics also has the potential to decrease the rates of bacteraemia due to resistant bacterial strains. Further investigation into the true onset of bacteraemia would be beneficial. Similarly, research into the geographical variations observed would be advantageous. Ongoing surveillance will assist with the majority of the above and will help identify and assess potential interventions to ultimately reduce the emerging threat of antimicrobial resistance.

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Conflict of interest

None declared.

Authors' contributions

Sabine Bou-Antoun led the data collection, analysis, interpretation, writing and corrections of the manuscript. John Davies contributed to the data analysis, interpretation of the results and review of the manuscript. Rebecca Guy contributed to the data collection and review of the manuscript. Alan P. Johnson (Department Head), Elizabeth A. Sheridan and Russell J. Hope (Head of E. coli bacteraemia surveillance) contributed to the study conception and review of the manuscript. All authors had full access to all the data in the study, read and approved the final manuscript that was submitted.

References

1. Public Health England (PHE). Health Protection Report: Voluntary surveillance of Escherichia coli bacteraemia in England, Wales and Northern Ireland: 2009-2013. London: PHE; 16 May 2014. [Accessed January 2015]. Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/339992/Ecoli_bacteraemia_in_Eng_Wales_and_NI_2009_to_2013.pdf
2. Gagliotti C, Balode A, Baquero F, Degener J, Grundmann H, Gür D, et al. . Escherichia coli and Staphylococcus aureus: bad news and good news from the European Antimicrobial Resistance Surveillance Network (EARS-Net, formerly EARSS), 2002 to 2009.Euro Surveill. 2011;16(11):19819.PMID: 21435327
3. Berger J, Diab-Elschahawi M, Blacky A, Pernicka E, Spertini V, Assadian O, et al. A matched prospective cohort study on Staphylococcus aureus and Escherichia coli bloodstream infections: extended perspectives beyond resistance. Am J Infect Control. 2010;38(10):839-45. DOI: 10.1016/j.ajic.2010.04.212 PMID: 20650546
4. Bo S-N, Bo J, Ning Y-Z, Zhao Y, Lu X-L, Yang J-Y, et al. Relationship between time to positivity of blood culture with clinical characteristics and hospital mortality in patients with Escherichia coli bacteremia. Chin Med J (Engl). 2011;124(3):330-4.PMID: 21362328
5. Uslan DZ, Crane SJ, Steckelberg JM, Cockerill FR, St Sauver JL, Wilson WR, et al. Age- and sex-associated trends in bloodstream infection: a population-based study in Olmsted County, Minnesota. Arch Intern Med. 2007;167(8):834-9. DOI: 10.1001/archinte.167.8.834 PMID: 17452548
6. Abernethy JK, Johnson AP, Guy R, Hinton N, Sheridan EA, Hope RJ. Thirty day all-cause mortality in patients with Escherichia coli bacteraemia in England.Clin Microbiol Infect. 2015;21(3):251.e1-8. DOI: 10.1016/j.cmi.2015.01.001 PMID: 25698659
7. Melzer M, Petersen I. Mortality following bacteraemic infection caused by extended spectrum beta-lactamase (ESBL) producing E. coli compared to non-ESBL producing E. coli.J Infect. 2007;55(3):254-9. DOI: 10.1016/j.jinf.2007.04.007 PMID: 17574678
8. Cosgrove SE. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs.Clin Infect Dis. 2006;42(Suppl 2):S82-9. DOI: 10.1086/499406 PMID: 16355321
9. Office for National Statistics (ONS). Population Estimates for UK, England and Wales, Scotland and Northern Ireland,

Mid-2011 and Mid-2012 2013. Updated 08/08/20132014. London: ONS. Available from: http://webarchive.nationalarchives.gov.uk/20160105160709/http://www.ons.gov.uk/ons/rel/pop-estimate/population-estimates-for-uk--england-and-wales--scotland-and-northern-ireland/mid-2011-and-mid-2012/index.html

10. England National Health Service (NHS England). KH03: Bed Availability and Occupancy Data – Overnight. NHS Organisations in England for 2012-14. Leeds: NHS England; 2014. Available from: https://www.england.nhs.uk/statistics/statistical-work-areas/bed-availability-and-occupancy/bed-data-overnight/
11. Health and Social Care Information Centre (HSCIC). ERIC - Estates Return Information Collection: 2012-13 Data return. Leeds: HSCIC; 2014, [Accessed August 2014]. Available from: http://hefs.hscic.gov.uk/DataFiles.asp
12. Siegman-Igra Y, Fourer B, Orni-Wasserlauf R, Golan Y, Noy A, Schwartz D, et al. Reappraisal of community-acquired bacteremia: a proposal of a new classification for the spectrum of acquisition of bacteremia. Clin Infect Dis. 2002;34(11):1431-9. DOI: 10.1086/339809 PMID: 12015688
13. Livermore DM, Hope R, Reynolds R, Blackburn R, Johnson AP, Woodford N. Declining cephalosporin and fluoroquinolone non-susceptibility among bloodstream Enterobacteriaceae from the UK: links to prescribing change?J Antimicrob Chemother. 2013;68(11):2667-74. DOI: 10.1093/jac/dkt212 PMID: 23766490
14. Al-Hasan MN, Lahr BD, Eckel-Passow JE, Baddour LM. Seasonal variation in Escherichia coli bloodstream infection: a population-based study.Clin Microbiol Infect. 2009;15(10):947-50. DOI: 10.1111/j.1469-0691.2009.02877.x PMID: 19845704
15. Deeny SR, van Kleef E, Bou-Antoun S, Hope RJ, Robotham JV. Seasonal changes in the incidence of Escherichia coli bloodstream infection: variation with region and place of onset.Clin Microbiol Infect. 2015;21(10):924-9. DOI: 10.1016/j.cmi.2015.06.023 PMID: 26141255
16. Whitehead M, Doran T. The north-south health divide. BMJ. 2011;342(feb15 2):d584. DOI: 10.1136/bmj.d584 PMID: 21325006
17. Kennedy KJ, Roberts JL, Collignon PJ. Escherichia coli bacteraemia in Canberra: incidence and clinical features.Med J Aust. 2008;188(4):209-13.PMID: 18279126
18. Laupland KB, Gregson DB, Church DL, Ross T, Pitout JDD. Incidence, risk factors and outcomes of Escherichia coli bloodstream infections in a large Canadian region.Clin Microbiol Infect. 2008;14(11):1041-7. DOI: 10.1111/j.1469-0691.2008.02089.x PMID: 19040476
19. Laupland KB, Gregson DB, Flemons WW, Hawkins D, Ross T, Church DL. Burden of community-onset bloodstream infection: a population-based assessment.Epidemiol Infect. 2007;135(6):1037-42. DOI: 10.1017/S0950268806007631 PMID: 17156500
20. Williamson DA, Lim A, Wiles S, Roberts SA, Freeman JT. Population-based incidence and comparative demographics of community-associated and healthcare-associated Escherichia coli bloodstream infection in Auckland, New Zealand, 2005-2011.BMC Infect Dis. 2013;13(1):385. DOI: 10.1186/1471-2334-13-385 PMID: 23964864
21. Holt PG, Jones CA. The development of the immune system during pregnancy and early life.Allergy. 2000;55(8):688-97. DOI: 10.1034/j.1398-9995.2000.00118.x PMID: 10955693
22. Weng N-P. Aging of the immune system: how much can the adaptive immune system adapt?Immunity. 2006;24(5):495-9. DOI: 10.1016/j.immuni.2006.05.001 PMID: 16713964
23. Stoll BJ, Hansen NI, Sánchez PJ, Faix RG, Poindexter BB, Van Meurs KP, et al. . Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues.Pediatrics. 2011;127(5):817-26. DOI: 10.1542/peds.2010-2217 PMID: 21518717
24. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs.Dis Mon. 2003;49(2):53-70. DOI: 10.1067/mda.2003.7 PMID: 12601337
25. Nielsen SL, Pedersen C, Jensen TG, Gradel KO, Kolmos HJ, Lassen AT. Decreasing incidence rates of bacteremia: a 9-year population-based study.J Infect. 2014;69(1):51-9. DOI: 10.1016/j.jinf.2014.01.014 PMID: 24576825
26. Friedman ND, Kaye KS, Stout JE, McGarry SA, Trivette SL, Briggs JP, et al. Health care--associated bloodstream infections in adults: a reason to change the accepted definition of community-acquired infections. Ann Intern Med. 2002;137(10):791-7. DOI: 10.7326/0003-4819-137-10-200211190-00007 PMID: 12435215
27. Rafferty AM, Clarke SP, Coles J, Ball J, James P, McKee M, et al. Outcomes of variation in hospital nurse staffing in English hospitals: cross-sectional analysis of survey data and discharge records. Int J Nurs Stud. 2007;44(2):175-82. DOI: 10.1016/j.ijnurstu.2006.08.003 PMID: 17064706
28. Kanerva M, Ollgren J, Lyytikäinen O, Finnish Prevalence Survey Study Group. Interhospital differences and case-mix in a nationwide prevalence survey.J Hosp Infect. 2010;76(2):135-8. DOI: 10.1016/j.jhin.2010.05.017 PMID: 20663587
29. Factors Contributing to the Emergence of Resistance. In: Knobler SL, Lemon SM, Najafi M, et al., editors. The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary. Washington (DC): National Academies Press (US); 2003.
30. Bos MMEM, Smeets LS, Dumay I, de Jonge E. Bloodstream infections in patients with or without cancer in a large community hospital.Infection. 2013;41(5):949-58. DOI: 10.1007/s15010-013-0468-1 PMID: 23645474
31. Ha YE, Kang C-I, Cha MK, Park SY, Wi YM, Chung DR, et al. Epidemiology and clinical outcomes of bloodstream infections caused by extended-spectrum β-lactamase-producing Escherichia coli in patients with cancer. Int J Antimicrob Agents. 2013;42(5):403-9. DOI: 10.1016/j.ijantimicag.2013.07.018 PMID: 24071027
32. Courpon-Claudinon A, Lefort A, Panhard X, Clermont O, Dornic Q, Fantin B, et al. , COLIBAFI Group. Bacteraemia caused by third-generation cephalosporin-resistant Escherichia coli in France: prevalence, molecular epidemiology and clinical features.Clin Microbiol Infect. 2011;17(4):557-65. DOI: 10.1111/j.1469-0691.2010.03298.x PMID: 20649802
33. Public Health England (PHE). English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) 2014. London: PHE. Available from: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/362374/ESPAUR_Report_2014__3_.pdf
34. Steinke DT, Seaton RA, Phillips G, MacDonald TM, Davey PG. Prior trimethoprim use and trimethoprim-resistant urinary tract infection: a nested case-control study with multivariate analysis for other risk factors.J Antimicrob Chemother. 2001;47(6):781-7. DOI: 10.1093/jac/47.6.781 PMID: 11389110
35. Rodríguez-Baño J, Navarro MD, Romero L, Muniain MA, de Cueto M, Gálvez J, et al. Risk-factors for emerging bloodstream infections caused by extended-spectrum beta-lactamase-producing Escherichia coli. Clin Microbiol Infect. 2008;14(2):180-3. DOI: 10.1111/j.1469-0691.2007.01884.x PMID: 18005358

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Isolation of H5N6, H7N9 and H9N2 avian influenza A viruses from air sampled at live poultry markets in China, 2014 and 2015

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Zoonotic infections by avian influenza viruses occur at the human–poultry interface, but the modes of transmission have not been fully investigated. We assessed the potential for airborne and fomite transmission at live poultry markets in Guangzhou city and in Hong Kong Special Administrative Region (SAR), China, during 2014 and 2015. Viral genome and infectious avian influenza A viruses of H5N6, H7N9, and H9N2 subtypes were detected predominantly from particles larger or equal to 1 µm in diameter in the air sampled with cyclone-based bioaerosol samplers at the live poultry markets in Guangzhou. Influenza A(H9N2) viruses were ubiquitously isolated every month during the study period from air and environmental swabs, and different lineages of H9N2 virus were isolated from markets where chickens and minor land-based poultry were sold. The use of de-feathering devices increased the quantity of virus-laden airborne particles while market closure reduced the amount of such particles. The results highlight the possibility of airborne transmission of avian influenza viruses among poultry or from poultry to humans within such settings. This may explain epidemiological observations in which some patients with H7N9 infection reported being in markets but no direct contact with live poultry or poultry stalls.

Introduction

Influenza A viruses infect a wide range of animal species and are transmitted via virus-laden particles through multiple non-exclusive modes. Interplay between multiple viral, host and environmental factors determine influenza viral transmission efficiency [1-5]. Virus–host compatibility establishes viral tropism and the quantity of virus-laden particles that may be released from infected hosts [1,2]. Gravity limits the distance that virus-laden particles can travel; large droplets settle rapidly and contribute to fomite transmission while droplet nuclei less than 5 µm in diameter may remain suspended in the air and mediate airborne transmission [3,4]. Humidity and temperature may impact on particle size and viability of the virus [5].

Zoonotic infections by avian influenza viruses occur at the human–avian interface [6] and live poultry markets play a critical role in maintaining, amplifying and disseminating avian influenza viruses between poultry species and from poultry to humans [7]. Exposure to live poultry has been reported by many patients with illness due to H5N1 and H7N9 infection, but sometimes such exposure has been indirect, for example visiting a vegetable stall within a large market where live poultry were sold [8]. Thus the modes of transmission are not well defined. The importance of contact or fomite transmission is supported by the detection of avian influenza viruses from various environmental swabs (e.g. counter surfaces, cages, water) at live poultry markets [9,10]. In addition, virus-laden particles that may mediate droplet or airborne transmission could be released from infected birds or as a result of aerosol-generating procedures during poultry slaughtering at markets. Currently, however, there is no information on the quantity, particle size and viability of virus-laden particles at live poultry markets.

To systematically assess the potential modes of transmission of avian influenza viruses at the human–poultry interface, we conducted monthly air and environmental sampling during July 2014 and October 2015 at three types of live poultry markets in Guangzhou city, Guangdong Province, China, and at one wholesale market in Hong Kong Special Administrative Region (SAR), China. In Hong King SAR, a ban on keeping live poultry

overnight at retail live poultry markets has been implemented since 2008 [7].

Methods

Samples were obtained from three different market types in Guangzhou: one wholesale market (two sites), one mixed animal market (two sites) and one retail market (one site). In Hong Kong SAR, we sampled at one wholesale poultry market.

Sampling in the Guangzhou wholesale market and mixed animal market was carried out from July 2014 to October 2015. In the retail market, sampling was conducted from January to October 2015; in the Hong Kong SAR market, sampling was carried out in October and November in 2014 and March, April, July, August, September and October in 2015.

Bioaerosol and environmental sampling at live poultry markets

Two types of cyclone-based bioaerosol samplers were used. The NIOSH bioaerosol sampler (BC251) collects particles based on their aerodynamic diameters into > 4, 1–4, and < 1 µm fractions at a flow rate of 3.5 L per minute [11]. The NIOSH samplers were set 1.2 m above ground and 0.5 m distance from poultry housing; samplers without connection to a vacuum pump were similarly placed as negative controls. After 30 min, a total of 0.105 m^3 air was sampled; 1 mL of minimum essential media with 4% bovine serum albumin was added to each of the collection tubes and polytetrafluorethylene filters and transported on ice packs to the laboratories at Guangdong Provincial Center for Disease Control and Prevention or at the University of Hong Kong.

The Coriolis µ air sampler (referred hereafter to as Coriolis) (Bertin Technologies) collects air at 100–300 L per minute. After 10 min sampling using 300 L per minute, a total of 3.0 m^3 air was sampled into a conical vial containing 5 mL MEM, which was concentrated using the 100 kDa Amicon Ultra-15 (Millipore) to a final volume of 1.5 mL. The sampler was placed 1 m above the ground and 0.5 m distance from poultry housing.

In parallel, environmental swabs were also collected from drinking water, fresh faecal droppings, or surfaces (cages, de-feathering machine and waste bins) at the markets. Temperature and humidity were recorded using a hygro-thermometer (Extech).

Detection and quantification of influenza viral RNA genome

Viral RNA for testing by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR) was extracted from 400 µL of the specimen using the QIAGEN EZ Robot or the RNeasy Mini Kit (Qiagen) and eluted into 60 µL H_2O. Influenza viral RNA was detected using AgPath-ID One-Step RT-PCR Reagents (Life Technologies) with specific primers and probes [12], using 5 µL of the eluted RNA. The number of influenza A virus M gene copies per m^3 air was calculated, where V_w is the volume of medium added to the sampler, V_r is the volume of specimen used for RNA extraction, U is the airflow rate (m^3 per minute) and t is the sampling time.

Formula 1

M gene copies per cubic metre air =
copies per µL × 60 µL × VwVr ÷ (U × t)

The minimum linear range of quantification (LoQ) was two copies M gene per µL, and the LoQs were determined as 2,857 and 150 copies/m^3 air for the NIOSH and Coriolis samplers, respectively. Influenza A virus M gene-positive samples were subtyped using H5-, H7- or H9-specific primers and probes by qRRT-PCR [9].

Virus isolation in embryonated chicken eggs

All samples with threshold cycle (Ct) values ≤ 35 for influenza A virus M gene by qRRT-PCR were propagated in embryonated chicken eggs by injecting 0.2 mL of specimen into the allantoic cavity and incubated at 37 °C for 48–72 hours. Allantoic fluid that agglutinated chicken or turkey red blood cells were further characterised by qRRT-PCR; samples with increasing copy numbers for influenza viral H5, H7 or H9 gene (reduced Ct values relative to the original field samples) after egg propagation were considered positive by virus isolation.

Genome sequencing and phylogenetic analysis

Viral RNA from an isolated virus was extracted using the RNeasy Mini Kit (Qiagen), amplified by RT-PCR [13] and was subjected to dideoxynucleotide sequencing or next-generation sequencing using the Ion PGM System with PathAmp FluA Reagents (Life Technologies). The sequences were submitted to the Global Initiative on Sharing All Influenza Data (GISAID) [14] (EPI674320, EPI674374 to EPI674424, EPI676397 to EPI676400, EPI676490, EPI676491, EPI696727 and EPI696728). Phylogenetic analysis was performed with the H9 haemagglutinin (HA) coding sequence (1,093 nt, 115–1,207 nt from ATG) aligned with reference strains from GISAID (Table 1). Phylogenetic trees were constructed by maximum likelihood method with bootstrap analysis (n = 1,000) by MEGA (version 6.0).

Statistical analysis

Correlation analyses were done by determining Spearman's rank-correlation coefficients (r_s). Fisher's exact test was applied to assess if the subtypes detected were statistically significantly different. Statistical analyses were performed using Graphpad Prism 6.0.

Ethics statement

Permission from the vendors at the poultry markets was obtained before the bioaerosol and environmental sampling. All sampling was performed without

Table 1

Origin of the haemagglutinin sequences of influenza A(H9N2) isolates used for the phylogenetic analysis

Segment ID	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI573379	China	2014-Jul-04	A/chicken/Shanghai/015/2014	Shanghai Animal Disease Control Center	Ge,F., Mao,X. and Liu,J.
EPI339694	China	2011-Jul	A/chicken/Anhui/G29/2011	China Animal Health & Epidemiology Center	Chen,J., Liu,S., Jiang,W.M., Hou,G.Y., Li,J.P. and Chen,J.M.
EPI315926	China	2011-Feb	A/chicken/Anhui/ZTL/2011	South China Agricultural University	Liu,J.
EPI296439	China	2009-Jan	A/chicken/Baoshan/111/2009	Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory	Song,J., Ye,C. and Tian,J.
EPI572786	China	2013-Mar-09	A/chicken/Beijing/0309/2013	China Agricultural University	Pu,J., Wang,J., Zhang,G., Yin,Y., Lv,N., Zhu,B., et al.
EPI470965	China	1994	A/chicken/Beijing/1/1994	St. Jude Children's Research Hospital	Baranovich,T., Marathe,B.M., Bridges,O., Burnham,A., Carey,D., Cline,T.D., et al.
EPI597003	China	2014-Feb-21	A/chicken/Dongguan/1424/2014	The University of Hong Kong	Lam,T.T., Zhou,B., Wang,J., Chai,Y., Shen,Y., Chen,X., et al.
EPI315960	China	2011-Jan	A/chicken/Fujian/SL6/2011	South China Agricultural University	Liu,J.
EPI81684	China	2000	A/chicken/Guangdong/4/00	Harbin Veterinary Research Institute	Li,C., Yu,K., Tian,G., Yu,D., Liu,L., Jing,B., et al.
EPI315932	China	2011-Feb	A/chicken/Guangdong/FZH/2011	South China Agricultural University	Liu,J.
EPI621363	China	2015-Jan	A/chicken/Guangdong/KPL01/2015	South China Agricultural University	Su,X. and Xie,Q.
EPI239451	China	1994	A/Chicken/Guangdong/SS/94	Yangzhou University	Shi,H.Y., Liu,X.F., Chen,S.J., Sun,L. and Xin,C.H.A.
EPI81694	China	1999	A/chicken/Guangxi/10/99	Harbin Veterinary Research Institute	Li,C., Yu,K., Tian,G., Yu,D., Liu,L., Jing,B., et al.
EPI122456	China	2005	A/chicken/Guangxi/55/2005	Guangxi General Veterinary Prevention and Quarantine Services	Liu,Q., Xiong,Y., Qin,L., Liu,K., Zhu,W., Qin,F.Y., et al.
EPI122438	China	2000	A/chicken/Guangxi/6/2000	Guangxi General Veterinary Prevention and Quarantine Services	Liu,Q., Xiong,Y., Qin,L., Liu,K., Zhu,W., Qin,F.Y., et al.
EPI538912	China	2013-Nov-23	A/chicken/Guangxi/LS/2013	Guangxi Veterinary Research Institute	Li,H.M., Guo,J.G., Zhou,L.B., Chen,L., Long,J.M. and Pan,J.
EPI81698	China	2000	A/chicken/Hebei/31/00	Harbin Veterinary Research Institute	Li,C., Yu,K., Tian,G., Yu,D., Liu,L., Jing,B., et al.
EPI140897	China	2008-Aug-02	A/chicken/Hebei/B1/2001	Shandong Academy of Agricultural Science, Animal Husbandry and Veterinary Institute	Huang,Y., Hu,B., Wen,X., Cao,S., Gavrilov,B.K., Du,Q., et al.
EPI140900	China	2006	A/chicken/Hebei/L1/2006	Shandong Animal Husbandry and Veterinary Institute	Huang,Y., Hu,B., Wen,X., Cao,S., Xu,D., Zhang,X., et al.
EPI254331	China	2003	A/chicken/Heibei/8/2003	College of Life Science and Technology, Southwest University of Nationalities	Yue,H., Tang,C. and Li,M.Y.
EPI326397	China	1998	A/chicken/Henan/A3/1998	Henan Agriculture University	Wang,Z., Zhao,J., Cai,L., Zheng,L. and Wang,C.
EPI238802	China	1998	A/chicken/Henan/nd/1998	Beijing Genomics Institute, Chinese Academy of Sciences	Liao,X., Zhang,X., Wang,J., Yu,J. and Liu,J.
EPI470859	Hong Kong SAR	1997	A/chicken/Hong_Kong/G9/1997	St. Jude Children's Research Hospital	Baranovich,T., Marathe,B.M., Bridges,O., Burnham,A., Carey,D., Cline,T.D., et al.
EPI470915	Hong Kong SAR	2011	A/chicken/Hong_Kong/NT10/2011	St. Jude Children's Research Hospital	Baranovich,T., Marathe,B.M., Bridges,O., Burnham,A., Carey,D., Cline,T.D., et al.
EPI337045	China	1999-Feb-21	A/chicken/Hubei/01/1999	Huazhong Agricultural University	Zhang,Z., Hu,S., Li,Z., Liu,M., Li,S., Xiao,Y. et al.
EPI623618	China	2014-May-28	A/chicken/Hubei/2014	Wuhan Institute of Virology, Chinese Academy of Sciences	Wang,N., Liu,X.-J., Wang,B., Zhang,S.-H. and Ge,X.-Y.
EPI593620	China	2014-Jun-29	A/chicken/Jiangxi/19426/2014	Centre of Influenza Research, School of Public Health, The University of Hong Kong	Lam,T.T., Zhou,B., Wang,J., Chai,Y., Shen,Y., Chen,X., et al.
EPI559917	China	2012-Nov-10	A/chicken/Jilin/GYH1/2012	College of Veterinary Medicine, Jilin University	Cong,Y., Zhu,L. and Ran,W.

Segment ID	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI301458	Jordan	2004	A/chicken/Jordan/1436-1451/2004	Istituto Zooprofilattico Sperimentale Delle Venezie	Fusaro,A., Monne,I., Salviato,A., Valastro,V., Schivo, A., Amarin,N.M., et al.
EPI470818	South Korea	1996	A/chicken/Korea/25232-96006/1996	St. Jude Children's Research Hospital	Baranovich,T., Marathe,B.M., Bridges,O., Burnham,A., Carey,D., Cline,T.D., et al.
EPI5901	South Korea	1996	A/Chicken/Korea/38349-p96323/96	St. Jude Children's Research Hospital	Guan,Y., Shortridge,K.F., Senne,D., Krauss,S. and Webster,R.G.
EPI304611	South Korea	2007-Nov-24	A/chicken/Korea/GH2/2007	Biological Sciences, Inje University	Koo,Y.
EPI339649	China	2011-Jul	A/chicken/Ningxia/182/2011	Laboratory of Animal Epidemiological Surveillance, China Animal Health & Epidemiology Center	Chen,J., Liu,S., Jiang,W.M., Hou,G.Y., Li,J.P. and Chen,J.M.
EPI487143	China	2001-Jul-18	A/chicken/Shandong/241/2001	Avian Virus Disease Laboratory, LanZhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences	In,Z., Xu,C., Liu,B., Ji,Y., Fu,Y., Guo,J. and et al.
AF508570[a]	China	1996	A/Chicken/Shandong/6/96	Microbiology, The University of Hong Kong	Li,J.W., Yu,K.Z., Brown,I., Shortridge,K.F., Pieris,J.S.M. and Guan,Y.
EPI470818	China	2009-May	A/chicken/Shandong/H/2009	CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences	Lu,L., Bi,Y., Li,J., Sun,L. and Liu,W.
EPI272334	China	2008-Dec-01	A/chicken/Shandong/LY/2008	Department of Veterinary Prevention Medicine, College of Veterinary Medicine, China Agricultural University	Pu,J., Zhang,G. and Liu,J.
EPI239002	China	1998	A/Chicken/Shanghai/F/98	Animal Infectious Disease Laboratory, School of Veterinary Medicine, Yangzhou University	Lu,J.H., Liu,X.F., Shao,W.X., Liu,Y.L., Wei,D.P. and Liu,H.Q.
EPI470907	China	2005	A/chicken/Shantou/22116/2005	St. Jude Children's Research Hospital	Baranovich,T., Marathe,B.M., Bridges,O., Burnham,A., Carey,D., Cline,T.D., et al.
AF508572[a]	China	1997	A/chicken/Shenzhen/9/97	Microbiology, The University of Hong Kong	Li,J.W., Yu,K.Z., Brown,I., Shortridge,K.F., Pieris,J.S.M. and Guan,Y.
EPI573766	China	2014-Mar	A/chicken/Yangzhou/752/2014	Yang Zhou University	Yang,X.
EPI241325	China	2000-Sep-04	A/chicken/Zhejiang/HE6/2009	College of Animal Science, South China Agricultural University	Chen,C., Ji,J., Bai,S., Zuo,K. and Xie,Q.
EPI221855	China	2007	A/chicken/Zhejiang/HJ/2007	Avian Disease, Animal Husbandry and Veterinary Medicine of Fujian Academy of Agricultural Sciences	Wan,C. and Huang,Y.
EPI610285	China	2010-Oct-10	A/Chinese francolin/Guangxi/B7/2010	Guangxi Key Laboratory of Animal Vaccines and Diagnostics, Guangxi Veterinary Research Institute	Peng,Y., Xie,Z., Liu,J., Pang,Y., Xie,Z., Xie,L., et al.
EPI441826	Viet Nam	2006-Dec	A/Chinese Hwamei/Vietnam/38/2006	Kyoto Sangyo University, Faculty of Life Sciences	Takakuwa,H.
CY005632[a]	Hong Kong SAR	1979-Oct-09	A/duck/HK/784/1979	St. Jude Children's Research Hospital	Obenauer,J.C., Denson,J., Mehta,P.K., Su,X., Mukatira,S., Finkelstein,D.B., et al.
EPI10783	Hong Kong SAR	2003	A/Duck/Hong_Kong/289/78	Microbiology, The University of Hong Kong,	Li,K.S., Xu,K.M., Peiris,J.S., Poon,L.L., Yu,K.Z., Yuen,K.Y., et al.
EPI16562	Hong Kong SAR	1979	A/duck/Hong_Kong/552/79	Microbiology, The University of Hong Kong	Li,K.S., Xu,K.M., Peiris,J.S., Poon,L.L., Yu,K.Z., Yuen,K.Y., et al.
EPI5885	Hong Kong SAR	1997	A/Duck/Hong_Kong/Y280/97	St. Jude Children's Research Hospital	Guan,Y., Shortridge,K.F., Krauss,S. and Webster,R.G.
EPI5887	Hong Kong SAR	1997	A/duck/Hong_Kong/Y439/1997	St. Jude Children's Research Hospital	Baranovich,T., Marathe,B.M., Bridges,O., Burnham,A., Carey,D., Cline,T.D., et al.

Segment ID	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI497495	China	2013-Mar-13	A/environment/Chongqing/00516/2013	WHO Chinese National Influenza Center	Gao, R., Li, X., Zhang, Y., Zou, S., Zhao, X., Li, X., et al.
EPI457842	China		A/environment/Jiangxi/02895/2012	Harbin Veterinary Research Institute	Li,C., Yu,K., Tian,G., Yu,D., Liu,L., Jing,B., and et al.
EPI492254	China	2013-Mar-25	A/environment/Zhejiang/14/2013	Zhejiang Provincial Center for Disease Control and Prevention	Feng,Y., Mao,H., Xu,C., Jiang,J., Chen,Y., Yan,J., et al.
EPI232381	USA	2008	A/ferret/Maryland/P10-UMD/2008	University of Maryland	Sorrell,E.M., Wan,H., Araya,Y., Song,H. and Perez,D.R.
AJ404627[a]	Hong Kong SAR	1999	A/guinea_fowl/Hong_Kong/WF10/99	St. Jude Children's Research Hospital	Perez,D.R., Lim,W., Seiler,J.P., Yi,G., Peiris,M., Shortridge,K.F. et al.
EPI24300	Hong Kong SAR	2003	A/guineafowl/HongKong/NT184/03	St. Jude Children's Research Hospital	Choi,Y.K., Ozaki,H., Webby,R.J., Webster,R.G., Peiris,J.S., Poon,L., et al.
AJ404627[a]	Hong Kong SAR	1999	A/Hong_Kong/1074/99	Virology, National Institute for Medical Research, London	Lin,Y.P., Shaw,M., Gregory,V., Cameron,K., Lim,W., Klimov,A., et al.
EPI498037	Hong Kong SAR	2013-Dec-28	A/Hong_Kong/308/2014	Public Health Laboratory Services Branch, Centre for Health Protection	Mak,G.C., Cheng,P.K.C., Lo,J.Y.C.
CY055140[a]	Hong Kong SAR	2009-Oct-29	A/Hong_Kong/33982/2009	Public Health Lab Centre, Centre For Health Protection, Department of Health, Hong Kong	Cheng,P.K.C. and Lim,W.L.
EPI439653	China	2011-Feb	A/mallard/Jiangxi/42/2011	Institute of Molecular Ecology and Evolution, East China Normal University	Zhu,G., Wang,R., Xuan,F., Daszak,P., Anthony,S.J., Zhang,S., Zhang,L. and He,G.
EPI5889	Hong Kong SAR	1997	A/Quail/Hong_Kong/G1/97	St. Jude Children's Research Hospital	Guan,Y., Shortridge,K.F., Krauss,S. and Webster,R.G.
EPI573570	China	2013-Apr	A/quail/Jiangsu/WX3/2013	School of Veterinary Medicine, Yangzhou University	Zhu,Y., Yang,D., Ren,Q., Yang,Y., Liu,X., Xu,X., et al.
EPI113630	China	2005	A/quail/Shantou/19506/2005	Department of Microbiology, The University of Hong Kong,	Xu,K.M., Li,K.S., Smith,G.J.D., Li,J.W., Tai,H., Zhang,J.X., et al.
EPI113492	China	2000	A/quail/Shantou/782/2000	Department of Microbiology, The University of Hong Kong,	Xu,K.M., Li,K.S., Smith,G.J.D., Li,J.W., Tai,H., Zhang,J.X., et al.
EPI597411	China	2014-Feb-21	A/silkie_chicken/Dongguan/968/2014	Centre of Influenza Research, School of Public Health, The University of Hong Kong	Lam,T.T., Zhou,B., Wang,J., Chai,Y., Shen,Y., Chen,X., et al.
EPI5913	United States	1966	A/turkey/California/189/66	St. Jude Children's Research Hospital	Guan,Y., Shortridge,K.F., Krauss,S. and Webster,R.G.
EPI407924	United States	1966	A/turkey/Wisconsin/1/1966	St. Jude Children's Research Hospital	Wentworth,D.E., Dugan,V., Halpin,R., Lin,X., Wester,E., Bera,J., et al.

a Sequence downloaded from Influenza Research Database funded by the National Institute of Allergy and Infectious Diseases (NIAID) [36].

directly handling the poultry, thus animal ethics were not applicable for our study.

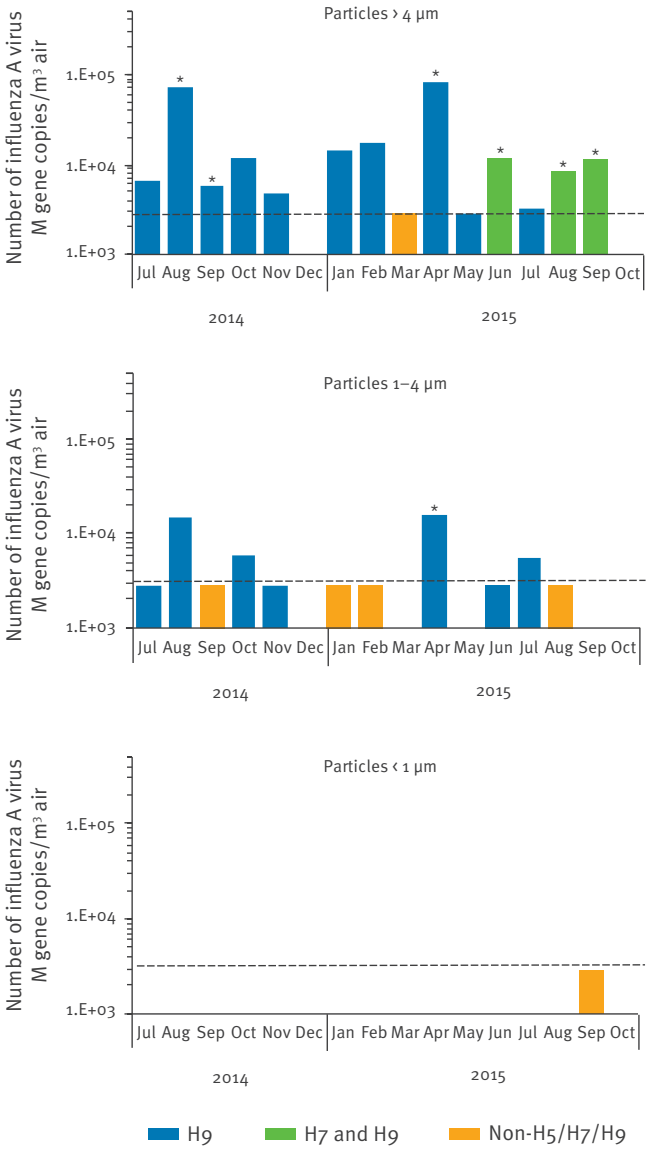
Results

Sampling at a wholesale market in Guangzhou

The wholesale market was organised into areas for holding live poultry, slaughtering and selling dressed poultry (poultry carcasses). Two sites were sampled. Site A1 was within the live poultry holding area of ca 5,500 m², where 10,000–20,000 poultry (predominantly chickens) were kept at any one time. Chickens were kept on a litter-bedded floor and were often sold to other retail markets within three days. Site A2 was a stall for chicken slaughtering with a de-feathering machine. There was one routinely scheduled market rest day per month; additional rest days may be scheduled in response to reports of human zoonotic infections.

Figure 1

Influenza A virus M gene copy number from particles in air sampled at a wholesale live poultry market in Guangzhou city, China, July 2014–October 2015



Site A1

Using the NIOSH sampler, influenza A virus M gene was detected by qRRT-PCR from particles›4 µm in 14/16 samples at 3,300–79,357 copies/m³ air, with 2/14 samples positive for the M gene but below the LoQ. In addition, the M gene was detected from particles 1–4 µm in 11/16 samples at 5,578–15,536 copies/m³ air (7/11 below LoQ) and from particles‹1 µm (1/16 sampling, 1/1 below LoQ) (Figure 1). In parallel, NIOSH samplers without a connection to a vacuum pump (as negative controls) were consistently negative for influenza A virus M gene by qRRT-PCR from particles›4, 1–4 or‹1 µm. H9 was the predominant HA subtype detected by qRRT-PCR, while mixed H7 and H9 or non-H5/H7/H9 RNA were also detected (Figure 1, Table 2).

The quantity and subtypes of influenza virus-laden particles detected in the air using a NIOSH bioaerosol sampler, at particle sizes of›4, 1–4 and‹1 µm diameter are shown. The horizontal dotted lines indicate the linear range of quantification for the influenza A virus M gene by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR) assay. Samples in which virus was isolated after egg passage are indicated by an asterisk (*).

H9N2 viruses (five isolates) and mixture of H7N9/H9N2 viruses (one isolate) were further isolated from the air samples collected by the NIOSH sampler at the fraction of › 4 µm, with an isolation rate (number of isolates/number of PCR-positive samples) of 6/14 (Tables 2 and 3). From the fraction of 1–4 µm, one H9N2 virus was isolated from 11 influenza A virus M gene-positive samples after egg propagation (Table 2).

The HA and neuraminidase (NA) genes of the sample with mixed H7N9 and H9N2 (A/Environment-air/GZ/NIOSH-395/2015) from our study showed 99.3% and 99.6% homology to that of the A/Chicken/Guangdong/GZ068/15 (H7N9) virus (GISAID:EPI_ISL_176834), respectively.

The Coriolis air sampler showed comparable efficiency to the NIOSH sampler in detecting influenza A virus M gene in the air samples, with Spearman's $r_s = 0.68$ ($p = 0.01$). Influenza A virus M gene was detected from 12 of 14 samples at 310–21,413 copies/m³ air (Table 2). Four H9N2 viruses were isolated after one passage in embryonated eggs from 12 influenza A virus M gene-positive Coriolis samples, including one that was originally positive for both H9 and H7 RNA by qRRT-PCR (Tables 2 and 3).

Influenza A virus M gene was detected in 36 of 59 environmental swabs – with a total isolation rate of 15/36 – including drinking water, faecal droppings and surfaces (Table 2). Of samples that were influenza A virus M gene-positive, further subtyping demonstrated the

H9 subtype (19/36), mixed H7/H9 (14/36) and non-H5/H7/H9 specimens (3/36). A total of 12 H9N2 viruses and three mixtures of H7N9/H9N2 viruses were isolated (Table 2 and 3). The distribution of virus subtypes detected in the environmental swabs and the NIOSH air samplers were not significantly different (p = 0.51, Fisher's exact test).

We analysed if viral load or environmental conditions might be associated with virus isolation from the air samplers; however, the M gene copy numbers, temperature, and relative humidity were not significantly different between months in which virus was isolated and those in which it was not, using the NIOSH sampler (p = 0.17, 0.07 and 0.72, respectively, Mann–Whitney

Table 2

Influenza A viruses detected and isolated from air and environmental samples at live poultry markets, Guangzhou, China (3 markets), and Hong Kong SAR (1 market), July 2014–October 2015[a]

Market and sample type	Number of influenza A virus M gene-positive[b]/ total sampled	Number of isolates/ number of influenza A virus M gene-positive samples[c]	HA subtype of influenza A virus M gene-positive samples[d] (number of isolates)						
			H5	H7	H9	H5 and H9	H7 and H9	H5 and H7	Non-H5/H7/H9
Wholesale market, Guangzhou [e]									
Air (NIOSH sampler)									
Particles >4 µm	14/16	6/14	0	0	10 (3)	0	3 (3)	0	1
Particles 1–4 µm	11/16	1/11	0	0	7 (1)	0	0	0	4
Particles <1 µm	1/16	0/1	0	0	0	0	0	0	1
Air (Coriolis µ)	12/14	4/12	0	0	9 (3)	0	3 (1)	0	0
Drinking water	8/11	4/8	0	0	3 (1)	0	5 (3)	0	0
Faecal droppings and surfaces	28/48	11/28	0	0	16 (9)	0	9 (2)	0	3
Mixed animal market, Guangzhou[f]									
Air (NIOSH sampler, site B1)									
Particles >4 µm	15/16	5/15	0	0	11 (3)	2 (2)	0	0	2
Particles 1–4 µm	9/16	0/9	0	0	7	0	0	0	2
Particles <1 µm	1/16	0/1	0	0	0	0	0	0	1
Air (NIOSH sampler, site B2)									
Particles >4 µm	15/16	4/15	0	0	12 (2)	2 (2)	0	0	1
Particles 1–4 µm	11/16	0/11	0	0	7	0	0	0	4
Particles <1 µm	3/16	0/3	0	0	0	0	0	0	3
Air (Coriolis µ)	14/14	6/14	0	0	10 (4)	3 (2)	0	0	1
Drinking water	11/30	3/11	1 (1)	1	5 (2)	1	0	1	2
Faecal droppings and surfaces	54/79	15/54	4 (2)	1	32 (12)	3	1	2	11 (1)
Retail market, Guangzhou									
Air (NIOSH sampler)									
Particles >4 µm	10/10	1/10	0	0	5 (1)	2	1	0	2
Particles 1–4 µm	6/10	0/6	0	0	2	0	0	0	4
Particles <1 µm	1/10	0/1	0	0	0	0	0	0	1
Drinking water	4/13	0/4	0	1	1	0	1	0	1
Faecal droppings and surfaces	14/23	1/14	0	1	11 (1)	0	1	0	1
Wholesale market, Hong Kong SAR[g]									
Air (NIOSH sampler)	0/22	0/0	0	0	0	0	0	0	0
Air (Coriolis µ)	6/13	0/6	0	0	3	0	0	0	3
Faecal droppings and surfaces	0/39	0/0	0	0	0	0	0	0	0

HA: haemagglutinin; qRRT-PCR: quantitative real-time reverse transcription polymerase chain reaction.

[a] In the retail market in Guangzhou, sampling was conducted from January to October 2015; in the market in Hong Kong SAR, sampling was conducted in October and November in 2014 and in March, April, July, August, September and October in 2015.

[b] Influenza A virus M gene was detected using qRRT-PCR.

[c] The virus isolation rate was defined as the number of positive isolates after one passage in embryonic chicken eggs among influenza A virus M gene-positive samples.

[d] The M gene-positive samples were further subtyped by qRRT-PCR using primers and probes for H5, H7, H9 HA.

[e] The sampling site was located at the poultry holding area within the wholesale live poultry market (see site A1 in the text).

[f] Sites B1 and B2 were two separate vendors' stalls within the mixed animal market.

[g] No drinking water was provided in the wholesale market in Hong Kong SAR.

test) or the Coriolis sampler (p = 0.86, 0.49 and 0.32, respectively).

In December 2014 and October 2015, neither air sampler detected the influenza A virus M gene. In December 2014, sampling was coincidentally performed on the market rest day (when the market was closed); all chickens were removed from the market but the environment had not yet been disinfected. In October 2015, sampling was performed the day after market closure. These results suggest that market closure may effectively reduce the viral load at the markets for a short time period.

Site A2

We performed air sampling while the de-feathering machine at site A2 was in operation (five samples) or not in use (three samples). While the machine was in operation, influenza A virus M gene was detected by qRRT-PCR from particles › 4 µm in 5/5 samples at 4,157–28,929 copies/ m^3 air (2/5 below LoQ) and from particles 1–4 µm in 2/5 samples (2/2 below LoQ); no viral RNA was detected from particles ‹ 1 µm (0/5 samples). H9 RNA was detected in 4/5 samples and mixed H5/H9 RNA was detected in 1/5 samples from particles › 4 µm; one H9N2 virus was isolated from the air sample.

In contrast, influenza A virus M gene was not detected in air sampled while the de-feathering machine was not in use (0/3 samples). At the same time, environmental swabs collected from the de-feathering machine were consistently positive for the M gene by qRRT-PCR, regardless of whether the machine was in use or not. Overall, the results suggest that infectious influenza A virus-laden particles can be generated during the de-feathering process.

Sampling at a mixed animal market in Guangzhou

This mixed animal market sold live poultry, reptiles and mammals, although poultry were kept in a separate area. The predominant poultry species sold were aquatic birds (ducks and geese) and minor land-based poultries (pheasants, guinea fowls, chukar partridges, quails). Each vendor may have a few hundred birds of different species, which were kept in separate cages or pens of various sizes. There was no clear all-in/all-out policy or known routine market rest days.

NIOSH samplers were set up at two separate vendors' stalls (sites B1 and B2). At site B1, influenza A virus M gene was detected by qRRT-PCR from particles › 4 µm in 15/16 samples at 6,179–1,650,000 copies/m^3 air (2/15 below LoQ), from particles 1–4 µm in 9/16 samples at 3,450–210,714 copies/m^3 air (3/9 below LoQ) and from particles ‹ 1 µm in 1/16 samples (1/1 below LoQ) (Figure 2). At site B2, influenza A virus M gene was detected from particles › 4 µm in 15/16 samples at

Table 3

Influenza A virus isolation from samples with mixed H5, H7, H9 haemagglutinin subtypes from two live poultry markets in Guangzhou, China, July 2014–October 2015

Sample type	Sample ID	Date	HA subtype(s) detected	
			In market samples by qRRT-PCR	After egg passage[a]
Wholesale market, Guangzhou[b]				
NIOSH air sample	GZ331	Jun 2015	H7 and H9	H9
	GZ395	Aug 2015	H7 and H9	H7 and H9
	GZ437	Sep 2015	H7 and H9	H9
Coriolis µ air sample	GZ449	Sep 2015	H7 and H9	H9
Drinking water	GZ376	Aug 2015	H7 and H9	H7 and H9
	GZ378	Aug 2015	H7 and H9	H7 and H9
	GZ417	Sep 2015	H7 and H9	H9
Faecal droppings	GZ319	Jun 2015	H7 and H9	H7 and H9
	GZ420	Sep 2015	H7 and H9	H9
Mixed animal market, Guangzhou[c]				
NIOSH air sample (site B1)	GZ089	Oct 2014	H5 and H9	H5 and H9
	GZ184	Jan 2015	H5 and H9	H5
NIOSH air sample (site B2)	GZ124	Nov 2014	H5 and H9	H5 and H9
	GZ187	Jan 2015	H5 and H9	H5 and H9
Coriolis µ air sample (both sites B1 and B2)	GZ259	Mar 2015	H5 and H9	H9
	GZ289	Apr 2015	H5 and H9	H9

HA: haemagglutinin; qRRT-PCR: quantitative real-time reverse transcription polymerase chain reaction.

[a] A sample with copy numbers of influenza A virus H5, H7, or H9 genes (reduced threshold cycle (Ct) values by qRRT-PCR) higher than those of the original filed sample after egg propagation was considered positive by virus isolation.

[b] The sampling site was located at the poultry holding area within the wholesale live poultry market (see site A1 in the text).

[c] Sites B1 and B2 were two separate vendors' stalls within the mixed animal market.

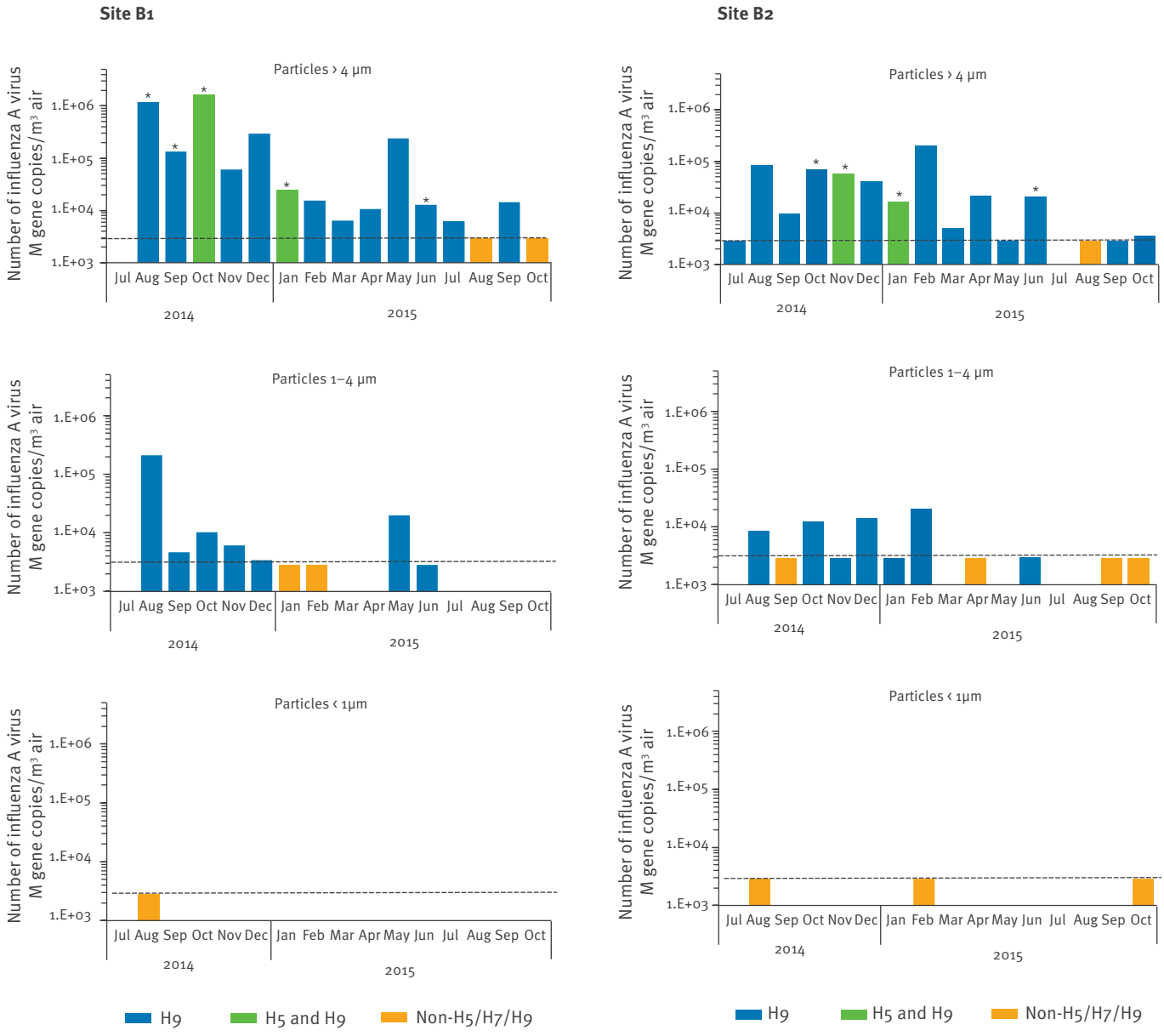
3,590–204,286 copies/m³ air (4/15 below LoQ), from particles 1–4 µm in 11/16 samples at 3,050–20,857 copies/m³ air (6/11 below LoQ) and from particles < 1 µm in 3/16 sampling (3/3 below LoQ) (Figure 2).

H9 and mixed H5/H9 RNA were detected from the M gene-positive samples by qRRT-PCR. H9N2 (n = 6) and mixed H9N2/H5N6 (n = 3) viruses were isolated from the fraction of particles > 4 µm, with isolation rates of 5/15 and 4/15 at sites B1 and B2, respectively (Tables 2 and 3). Higher M gene copy numbers (p = 0.01, Mann–Whitney test) and lower relative humidity (p = 0.04) were noted in the months when influenza virus was isolated in air sampled by the NIOSH sampler. Using the Coriolis sampler, influenza A virus M gene was detected from 14/14 samples at 201–29,888 copies/m³ air (1/14 below LoQ), which were subsequently confirmed as H9 or mixed H5/H9 subtypes. Six H9N2 viruses were isolated from 14 air samples collected by the Coriolis sampler (Table 2).

Influenza A viral RNA was detected from 60% (65/109) environmental swabs (water, faecal droppings and surfaces), with an isolation rate of 18/65. H9 (37/65), H5 (5/65), H7 (2/65), mixed H5/H9 (4/65), mixed H5/H7 (3/65), mixed H7/H9 (1/65) or non-H5/H7/H9 (13/65)

Figure 2

Influenza A virus M gene copy number from particles in air sampled at two separate vendors in a mixed animal market in Guangzhou city, China, July 2014–October 2015



The quantity and subtypes of influenza virus-laden particles at particle sizes at > 4, 1–4 and < 1 µm detected in the air using a NIOSH bioaerosol sampler at two sampling sites (sites B1 and B2) within the market are shown. The horizontal dotted lines indicate the linear range of quantification for influenza A virus M gene by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR) assay. Samples in which virus was isolated after one passage in embryonated eggs are indicated by an asterisk (*).

were further identified by qRRT-PCR (Table 2). H9N2 (n=14), H5N6 (n=2), H5N2 (n=1), and H4N8 (n=1) viruses were isolated. The subtypes detected in samples obtained using the NIOSH bioaerosol sampler and in environmental swabs were not statistically different (p=0.27, Fisher's exact test). Overall, five H5N6 viruses (three mixed with H9N2) and one H5N2 virus were isolated from the air and environmental samples. The H5 isolates belonged to clade 2.3.4.4 with 94.0–99.0% homology to the human H5N6 virus A/Guangzhou/39715/2014 (GISAID: EPI_ISL_180669) [15].

Sampling at a retail market in Guangzhou

This retail market had 10 stalls that sold live poultry. Sampling was performed in one stall of 4 m^2, which held 30–50 birds daily (co-housed chickens, ducks, pigeons, geese and quails). There were no clear all-in/all-out policy or known regular market rest days for disinfection. Sampling at the retail market was conducted from January to October 2015.

Using the NIOSH sampler, influenza A virus M gene was detected by qRRT-PCR from particles›4 µm in 10/10 samples at 9,243–455,714 copies/m^3 air (6/10 below LoQ), particles 1–4 µm in 6/10 samples at 3,130–14,071 copies/m^3 air (4/6 below LoQ) and particles‹1 µm (1/10 samples, 1/1 below LoQ). H9 RNA was predominantly detected while mixed H7/H9 and H5/H9 RNA were also detected by qRRT-PCR. One H9N2 virus was isolated from particles›4 µm among 10 samples positive for influenza A virus M gene.

The viral M gene was detected in 18/36 environmental swabs from drinking water, faecal droppings and surfaces; further subtyping identified H9 RNA (12/18), H7 RNA (2/18), mixed H7/H9 RNA (2/18) and non-H5/H7/H9 RNA (2/18), with one H9N2 virus isolated (Table 2). The subtypes detected by qRRT-PCR from the environmental swabs were not significantly different from those detected in the air samples obtained using the NIOSH sampler (p=0.45, Fisher's exact test).

Sampling at a wholesale poultry market in Hong Kong SAR

This wholesale poultry market served as a temporary holding site for chickens imported from mainland China or raised locally. The chickens stayed for no longer than 48 hours until sold to retail markets, with a first-in/ first-out policy, segregation and strict biosecurity measures. Since 2013, chickens imported from mainland China and those raised locally have been housed separately at different locations.

Sampling was conducted in the area holding local poultry in October and November in 2014 as well as in March, April, July, August, September and October in 2015. At each sampling, NIOSH (n = 2–3) and Coriolis (n = 1–2) samplers were set up and there were varying numbers of chickens (between 50 and 500) in the holding area. Influenza A virus M gene was not detected by qRRT-PCR in any of the 22 NIOSH samples but was detected in 6/13 Coriolis samples at 203–470 copies/m^3 air (3/6 below LoQ). Further subtyping identified H9 (3/6) or non-H5/H7/H9 (3/6) RNA from the M gene-positive samples (Table 2). Furthermore, none of the 39 environmental swabs were positive for the influenza A virus M gene (Table 2). The quantity of influenza A virus-laden particles in the air by the Coriolis sampler at this wholesale live poultry market in Hong Kong SAR (203–470 copies/m^3, M gene-positive rate: 6/13, 3/6 below LoQ) was lower than that for the wholesale live poultry market (310–21,413 copies/m^3, M gene-positive rate: 12/14) or the mixed animal market (201–29,888 copies/m^3, M gene-positive rate: 14/14, 1/14 below LoQ) in Guangzhou city.

Genetic analysis of H9N2 viruses isolated from the live poultry markets

The H9N2 virus was the most frequently isolated subtype from the markets in Guangzhou we sampled, with a total of 58 isolates of H9, H9/H7, or H9/H5 subtypes (Table 2). We performed a phylogenetic analysis of the HA gene of 46 selected H9N2 viruses isolated from the wholesale market (10 air samples, 15 environmental swabs) and the mixed animal market (10 air samples, 11 environmental swabs) in Guangzhou city. The H9N2 viruses isolated from the air and environment from the same market were genetically related. Furthermore, the H9N2 viruses isolated from the wholesale and the mixed animal markets were separately clustered into two clades (Figure 3).

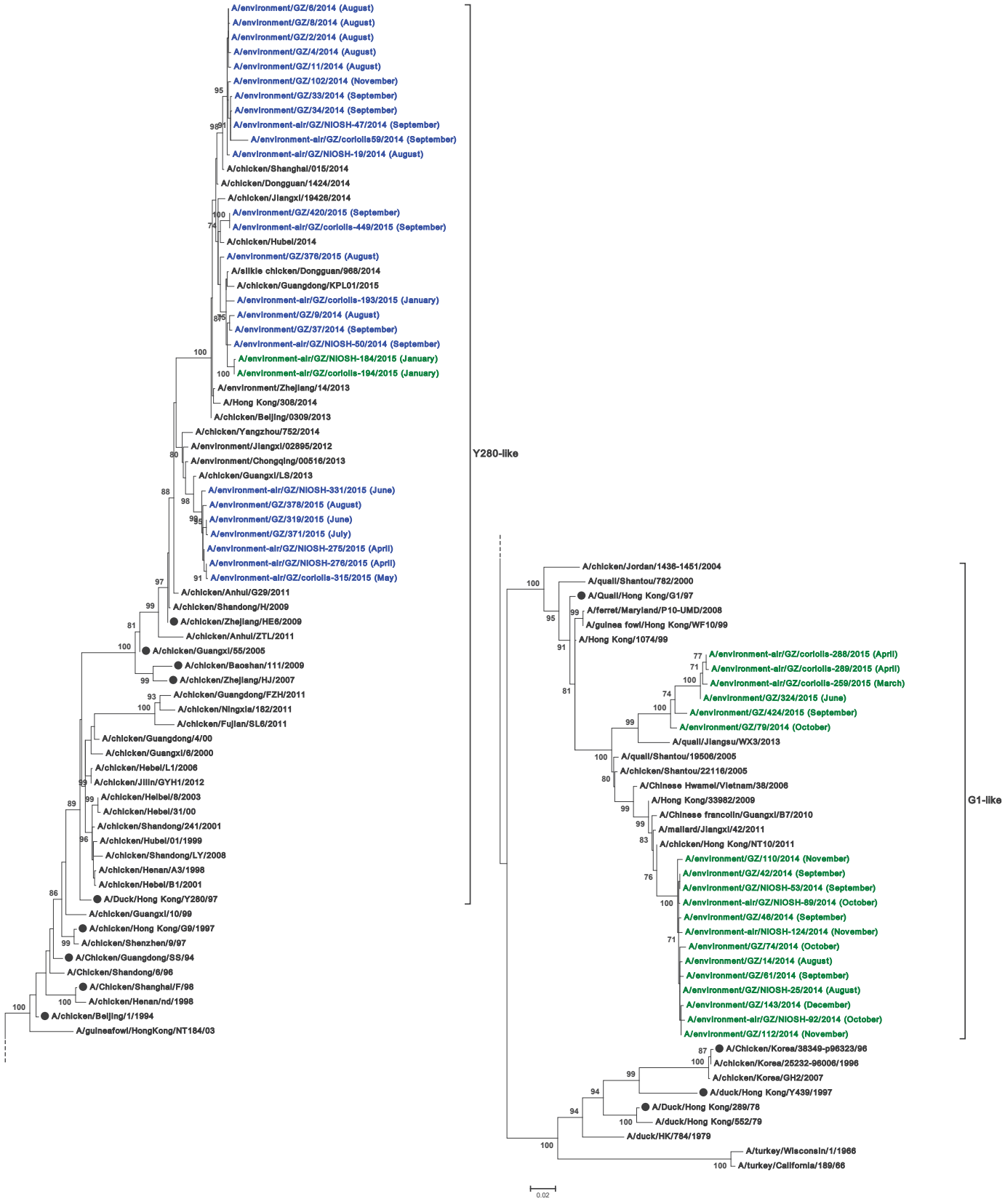
The H9N2 viruses isolated in the wholesale market shared high nucleotide homology (93.5–100%) and all clustered with the A/chicken/Zhejiang/HJ/2007 virus (G57 genotype), which evolved from A/Duck/Hong Kong/Y280/1997 (Y280 genotype) and has become dominant among chickens in China since 2010 [16]. At the mixed animal market, where minor land poultry were sold, the majority of H9N2 isolates (17/19) clustered together with the the A/quail/Hong Kong/G1/1997 (G1-like) virus, with high nucleotide homology (91.4–99.9%), except for two isolates collected in January 2015 by the NIOSH and Coriolis air samplers, which were clustered with the G57 genotype. The G1-like H9N2 viruses have been commonly detected in China since the late 1990s from minor poultry species such as quails and chukar partridges [17,18].

Discussion

Influenza viruses are transmitted via different but non-mutually exclusive modes [4]. Infections are mediated via virus-laden particles of various sizes that confer fomite, droplet or airborne transmission [19-21], but the modes of transmission for human zoonotic infections by avian influenza viruses at the human–poultry interface are not well defined. In our study, we determined the quantity, viability, subtype and size of influenza virus-laden particles in the air at three types of live poultry markets in Guangzhou city. Although our study is limited to a small number of markets in

Figure 3

Phylogenetic analysis of the haemagglutinin gene of avian influenza A(H9N2) viruses isolated from a wholesale market and a mixed animal market in Guangzhou, China, July 2014–October 2015 (n=46)



HA: haemagglutinin; G1-like: A/quail/Hong Kong/G1/97-like H9N2 virus; Y280-like: A/Duck/Hong Kong/Y280/97-like H9N2 virus.

The coding sequence of the HA gene (1,093 nucleotides, 115–1,207 nucelotides from ATG) was aligned for the phylogenetic analysis. H9N2 viruses isolated from the wholesale market and mixed animal market in Guangzhou are shown in blue and green, respectively. The black circles indicate the representative strains of distinct H9 lineages and the vaccine strains.

The phylogenetic tree was constructed by maximum likelihood method with bootstrap analysis (n=1,000) using MEGA (version 6.0) software.

Guangzhou city and Hong Kong SAR and the results should be interpreted with caution, we show that viral RNA or viable avian influenza viruses of H5, H7 and H9 subtypes with human zoonotic infection potential are readily detectable in the air, suggesting the feasibility of airborne transmission of avian influenza viruses at the human–poultry interface. Furthermore, human activities, such as operation of de-feathering machines commonly used at live poultry markets in China, may facilitate generation of viable virus-laden particles in the air. In contrast, the negative air sampling results obtained at the wholesale market in Guangzhou on or after market closure day suggest that appropriate interventions may reduce the viral load effectively in the environment. While poultry markets are not common in Europe, the result is consistent with the detection of influenza viral RNA in the air at poultry farms sampled during avian influenza outbreaks in the Netherlands [22]. Our study provides experimental evidence showing that viable avian influenza viruses can be detected in the air where live poultry are kept, which is consistent with previous reports that detected viral RNA and infectious influenza viruses at swine barns or at live pig markets in the United States [23,24]. Although it is difficult to compare our results with those reported previously due to differences in the air samplers used, the concentrations of viral RNA we detected in the air at the live poultry markets were comparable with those detected at the swine barns in the United States in 2011 [24].

Our results suggest that poultry workers in the live poultry markets are constantly exposed to high viral loads in the air and the environment, but human symptomatic infections caused by avian influenza viruses in this population remain uncommon. Excluding the samples collected at the slaughtering area of the Guangzhou wholesale market (site A2) and from the live poultry market in Hong Kong, using the NIOSH bioaerosol sampler, viral RNA or viable virus was identified predominantly from particles › 4 µm (16 viable isolates of 58 samples collected), occasionally from 1–4 µm (1/58), and none from particles ‹ 1 µm (0/58). Previous studies that analysed particle deposition suggest particles ‹ 3 µm are more likely to deposit in the deep lungs [25] where avian influenza viruses with binding specificity for α2,3-linked sialic acids preferentially replicate [26]. In addition, seroepidemiological studies have reported a limited number of cases with low levels of neutralising antibody titres using hemagglutination inhibition assay or neutralisation assay [27-29]; however, the mechanism of cross-protection may be via non-neutralising antibodies or T-cell response. Further studies are needed to evaluate the percentage of subclinical infections and to assess the cross-protective adaptive immune response between poultry workers and the general population.

The H9N2 avian influenza virus ubiquitously present among land-based poultry in China and other countries [30] was the predominant subtype detected from the air and environmental samples in our study. Genetically diverse H9N2 viruses have been shown to possess human-like receptor binding specificity [31], transmission potential among ferrets [32] and have provided the internal genes for the H7N9 or H10N8 viruses that have caused fatal human infections since 2013 [33]. Unlike highly pathogenic viruses of H5 subtype that replicate systematically and cause high mortality, the low pathogenic H9N2 and H7N9 viruses generally do not cause apparent clinical signs in infected poultry [30,34]; this poses a challenge in identifying the infected birds for infection control and facilitates the spread of the H9N2 and H7N9 viruses in live poultry markets. H9N2 and H7N9 viruses are known to replicate more efficiently in the respiratory tract than the gastrointestinal tract of the land-based poultry [34,35], and the highly prevalent H9N2 virus has been the dominant subtype detected in the air at the poultry markets, as shown in the present study. Determining the viral loads and subtypes from oropharyngeal and cloacal swabs from different poultry species may help to understand the effect of viral respiratory tropism versus the quantity of virus-laden particles released in the air. We also observed segregation of species-adapted H9N2 lineages at different markets; further studies should investigate if the segregation is due to repeated re-introduction of a species-adapted virus as a result of selling different species at different markets or if insufficient cleaning of the environment facilitated the persistence and segregation of the H9N2 virus.

Among the three different types live poultry markets in Guangzhou, we noted higher virus isolation rates from air samples collected at the wholesale market and the mixed poultry market than that of the retail market, suggesting the number of poultry sold on site may affect the quantity of viable virus detected in the air. Cleaning practices, such as the market rest day, may have an impact as well. In addition, we noted a higher detection rate and isolation rate from particle›4 µm, regardless of the viral subtype, suggesting that there is no correlation between avian influenza A subtype and virus detection at specific particle sizes. Since the subtypes detected in the air correlate well with the subtypes detected from the environment (water, faecal droppings and surfaces), the prevalence of a subtype in poultry (e.g. H9N2) may be a major contributing factor to the subtype detected in the air; however, other factors including viral tropism in poultry should also be considered. Temperature and relative humidity can affect viral viability and the sizes of virus-laden particles in the air. However, we did not observe a strong impact of temperature and humidity on viral detection at specific particle sizes; a longer observation period and/or frequent sampling will be needed to address this question.

Taken together, our results indicate the possibility of airborne transmission for avian influenza A viruses and may explain some human cases who appear to have acquired H7N9 infection by visiting live poultry

markets but without direct or indirect contact to poultry [8]. Furthermore, the observation that known zoonotic infections have been in people with transient contact with, or passing the vicinity of live poultry markets – rather than those working within them, who are clearly exposed to avian influenza viruses on almost a daily basis – suggests a role for host susceptibility as one of the key determinants of zoonotic infection.

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Conflict of interest

None declared.

Authors' contributions

JZ, CK, JSMP, JW and HY participated in the study design; JZ, XZ, GH, LZ, YS, DG and XZ performed sampling at the poultry markets; JZ and XZ performed the sequence analysis; JZ, XZ, MK, JL, BJC, WGL, JSMP, JW and HY interpreted and analysed the data; JZ, JSMP and HY wrote the manuscript. All authors contributed to the study and have read and approved the manuscript.

References

1. Neumann G, Kawaoka Y. Transmission of influenza A viruses.Virology. 2015;479-480:234-46. DOI: 10.1016/j.virol.2015.03.009
2. de Graaf M, Fouchier RA. Role of receptor binding specificity in influenza A virus transmission and pathogenesis.EMBO J. 2014;33(8):823-41. DOI: 10.1002/embj.201387442
3. Jones RM, Brosseau LM. Aerosol transmission of infectious disease.J Occup Environ Med. 2015;57(5):501-8. DOI: 10.1097/JOM.0000000000000448
4. Tellier R. Review of aerosol transmission of influenza A virus.Emerg Infect Dis. 2006;12(11):1657-62. DOI: 10.3201/eid1211.060426
5. Lowen AC, Steel J. Roles of humidity and temperature in shaping influenza seasonality.J Virol. 2014;88(14):7692-5. DOI: 10.1128/JVI.03544-13
6. World Health Organization (WHO). Influenza at the human-animal interface. Summary and assessment as of 14 December 2015. Geneva: WHO; 2015. Available from: http://www.who.int/influenza/human_animal_interface/Influenza_Summary_IRA_HA_interface_14_Dec_2015.pdf?ua=1
7. Peiris JSM, Cowling BJ, Wu JT, Feng L, Guan Y, Yu H, et al. Interventions to reduce zoonotic and pandemic risks from avian influenza in Asia. Lancet Infect Dis. 2016;16(2):252-8. DOI: 10.1016/S1473-3099(15)00502-2
8. Liu B, Havers F, Chen E, Yuan Z, Yuan H, Ou J, et al. Risk factors for influenza A(H7N9) disease--China, 2013. Clin Infect Dis. 2014;59(6):787-94. DOI: 10.1093/cid/ciu423
9. Kang M, He J, Song T, Rutherford S, Wu J, Lin J, et al. Environmental Sampling for Avian Influenza A(H7N9) in Live-Poultry Markets in Guangdong, China. PLoS One. 2015;10(5):e0126335. DOI: 10.1371/journal.pone.0126335
10. Indriani R, Samaan G, Gultom A, Loth L, Irianti S, Adjid R, et al. Environmental sampling for avian influenza virus A (H5N1) in live-bird markets, Indonesia. Emerg Infect Dis. 2010;16(12):1889-95. DOI: 10.3201/eid1612.100402
11. Lindsley WG, Schmechel D, Chen BT. A two-stage cyclone using microcentrifuge tubes for personal bioaerosol sampling.J Environ Monit. 2006;8(11):1136-42. DOI: 10.1039/b609083d
12. World Health Organization (WHO). WHO information for molecular diagnosis of influenza virus – update. Geneva: WHO; Mar 2014. Available from: http://www.who.int/influenza/gisrs_laboratory/molecular_diagnosis/en/
13. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses.Arch Virol. 2001;146(12):2275-89. DOI: 10.1007/s007050170002
14. Bogner P, Capua I, Lipman DJ, Cox NJ. others. A global initiative on sharing avian flu data.Nature. 2006;442(7106):981. DOI: 10.1038/442981a
15. Smith GJ, Donis RO. World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5 Evolution Working Group. Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014.Influenza Other Respi Viruses. 2015;9(5):271-6. DOI: 10.1111/irv.12324
16. Pu J, Wang S, Yin Y, Zhang G, Carter RA, Wang J, et al. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. Proc Natl Acad Sci USA. 2015;112(2):548-53. DOI: 10.1073/pnas.1422456112
17. Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong?Proc Natl Acad Sci USA. 1999;96(16):9363-7. DOI: 10.1073/pnas.96.16.9363
18. Xu KM, Smith GJ, Bahl J, Duan L, Tai H, Vijaykrishna D, et al. The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. J Virol. 2007;81(19):10389-401. DOI: 10.1128/JVI.00979-07
19. Cowling BJ, Ip DK, Fang VJ, Suntarattiwong P, Olsen SJ, Levy J, et al. Aerosol transmission is an important mode of influenza A virus spread. Nat Commun. 2013;4:1935. DOI: 10.1038/ncomms2922
20. Milton DK, Fabian MP, Cowling BJ, Grantham ML, McDevitt JJ. Influenza virus aerosols in human exhaled breath: particle size, culturability, and effect of surgical masks.PLoS Pathog. 2013;9(3):e1003205. DOI: 10.1371/journal.ppat.1003205
21. Lindsley WG, Noti JD, Blachere FM, Thewlis RE, Martin SB, Othumpangat S, et al. Viable influenza A virus in airborne particles from human coughs. J Occup Environ Hyg. 2015;12(2):107-13. DOI: 10.1080/15459624.2014.973113
22. Jonges M, van Leuken J, Wouters I, Koch G, Meijer A, Koopmans M. Wind-Mediated Spread of Low-Pathogenic Avian Influenza Virus into the Environment during Outbreaks at Commercial Poultry Farms.PLoS One. 2015;10(5):e0125401. DOI: 10.1371/journal.pone.0125401
23. Choi MJ, Torremorell M, Bender JB, Smith K, Boxrud D, Ertl JR, et al. Live animal markets in Minnesota: a potential source for emergence of novel influenza A viruses and interspecies transmission. Clin Infect Dis. 2015;61(9):1355-62. DOI: 10.1093/cid/civ618
24. Corzo CA, Culhane M, Dee S, Morrison RB, Torremorell M. Airborne detection and quantification of swine influenza a virus in air samples collected inside, outside and downwind from swine barns.PLoS One. 2013;8(8):e71444. DOI: 10.1371/journal.pone.0071444
25. Carvalho TC, Peters JI, Williams RO. Influence of particle size on regional lung deposition--what evidence is there?Int J Pharm. 2011;406(1-2):1-10.
26. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: influenza virus receptors in the human airway. Nature. 2006;440(7083):435-6.
27. Khan SU, Anderson BD, Heil GL, Liang S, Gray GC. A Systematic Review and Meta-Analysis of the Seroprevalence of Influenza A(H9N2) Infection Among Humans.J Infect Dis. 2015;212(4):562-9. DOI: 10.1093/infdis/jiv109
28. Yang P, Ma C, Shi W, Cui S, Lu G, Peng X, et al. A serological survey of antibodies to H5, H7 and H9 avian influenza viruses

amongst the duck-related workers in Beijing, China. PLoS One. 2012;7(11):e50770. DOI: 10.1371/journal.pone.0050770

29. Nasreen S, Uddin Khan S, Azziz-Baumgartner E, Hancock K, Veguilla V, Wang D, et al. Seroprevalence of antibodies against highly pathogenic avian influenza A (H5N1) virus among poultry workers in Bangladesh, 2009. PLoS One. 2013;8(9):e73200. DOI: 10.1371/journal.pone.0073200
30. Sun Y, Liu J. H9N2 influenza virus in China: a cause of concern. Protein Cell. 2015;6(1):18-25. DOI: 10.1007/s13238-014-0111-7
31. Matrosovich MN, Krauss S, Webster RG. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity.Virology. 2001;281(2):156-62. DOI: 10.1006/viro.2000.0799
32. Li X, Shi J, Guo J, Deng G, Zhang Q, Wang J, et al. Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 Avian Influenza viruses. PLoS Pathog. 2014;10(11):e1004508. DOI: 10.1371/journal.ppat.1004508
33. Su S, Bi Y, Wong G, Gray GC, Gao GF, Li S. Epidemiology, Evolution, and Recent Outbreaks of Avian Influenza Virus in China.J Virol. 2015;89(17):8671-6. DOI: 10.1128/JVI.01034-15
34. Pantin-Jackwood MJ, Miller PJ, Spackman E, Swayne DE, Susta L, Costa-Hurtado M, et al. Role of poultry in the spread of novel H7N9 influenza virus in China. J Virol. 2014;88(10):5381-90. DOI: 10.1128/JVI.03689-13
35. Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR. Replication and transmission of influenza viruses in Japanese quail.Virology. 2003;310(1):8-15. DOI: 10.1016/S0042-6822(03)00094-1
36. Squires RB, Noronha J, Hunt V, García-Sastre A, Macken C, Baumgarth N, et al. Influenza research database: an integrated bioinformatics resource for influenza research and surveillance. Influenza Other Respi Viruses. 2012;6(6):404-16. DOI: 10.1111/j.1750-2659.2011.00331.x

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Research article

Hepatitis E in blood donors: investigation of the natural course of asymptomatic infection, Germany, 2011

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Asymptomatic hepatitis E virus (HEV) infections have been found in blood donors from various European countries, but the natural course is rarely specified. Here, we compared the progression of HEV viraemia, serostatus and liver-specific enzymes in 10 blood donors with clinically asymptomatic genotype 3 HEV infection, measuring HEV RNA concentrations, plasma concentrations of alanine/aspartate aminotransferase, glutamate dehydrogenase and bilirubin and anti-HEV IgA, IgM and IgG antibodies. RNA concentrations ranged from 77.2 to 2.19×10^5 IU/mL, with viraemia lasting from less than 10 to 52 days. Donors showed a typical progression of a recent HEV infection but differed in the first detection of anti-HEV IgA, IgM and IgG and seropositivity of the antibody classes. The diagnostic window between HEV RNA detection and first occurrence of anti-HEV antibodies ranged from eight to 48 days, depending on the serological assay used. The progression of laboratory parameters of asymptomatic HEV infection was largely comparable to the progression of symptomatic HEV infection, but only four of 10 donors showed elevated liver-specific parameters. Our results help elucidate the risk of transfusion-associated HEV infection and provide a basis for development of screening strategies. The diagnostic window illustrates that infectious blood donors can be efficiently identified only by RNA screening.

Introduction

The hepatitis E virus is a single-stranded RNA virus; there are currently four human pathogenic genotypes 1 to 4 [1]. Genotypes 1 and 2 are hyperendemic in developing countries, restricted to humans, and transmission occurs by the faecal-oral route [2,3]. In industrialised countries, genotypes 3 and 4 are responsible for sporadic cases of HEV infection. However, the incidence of non-travel-associated HEV infections has increased and hepatitis E is now recognised as an emerging and often undiagnosed disease [1,4,5]. The genetic similarity of strains isolated from humans and other mammalian species suggests zoonotic or foodborne transmission [6,7].

Hepatitis E presents asymptomatically or symptomatically. Symptomatic infection presents as an acute, mostly self-limiting hepatitis with clinical characteristics similar to hepatitis A [2]. Clinical manifestations of HEV infections caused by the different genotypes are indistinguishable. Genotype 3 and 4 patients are usually middle-aged and elderly men, whereas genotypes 1 and 2 also cause acute hepatitis in healthy children and adolescents [8]. The pathogenic impact of genotype 1 and 2 and genotype 3 and 4 differ considerably. HEV genotype 1 and 2 infections lead to a high mortality among pregnant women in developing countries (8–20% [9,10]) while no serious infections among pregnant women with genotypes 3 and 4 were described in industrialised countries. HEV genotype 3 and 4 infection proceed asymptomatically in immunocompetent individuals [8], but severe or fatal HEV infections have been observed in individuals with chronic liver disease [11,12], in transplant patients [13,14] and in immunosuppressed individuals [8]. Asymptomatic HEV infection has often been observed in blood donors [15-17], with reported prevalence rates of HEV RNA-positive donors of 1:2,848 (England [18]), 1:1,240 (Germany [17]) and 1:1,761 (the Netherlands [19]).

The progression of viraemia and the serological course of anti-HEV antibodies during clinically apparent HEV infection is well characterised [2,20,21], but so far little is known about the progression of infection in asymptomatic individuals, in whom HEV infection usually remains undetected. Therefore, we conducted a prospective study to characterise the duration of viraemia, the antibody response (IgA, IgM and IgG), and the progression of liver-specific enzymes in 10 HEV genotype 3-infected German blood donors [17].

Methods

Specimens

From July to September 2011, a total of 16,125 individual German blood donors were routinely screened for the presence of HEV RNA by the Uni.Blutspendedienst

Ostwestfalen-Lippe. Their geographical origins were North Rhine-Westphalia, Lower Saxony and Hesse; 57.5% (n = 9,271) were male, with a median age of 33 years (±13; range: 18–72), and 42.5% were female (n = 6,867), with a median age of 32 years (±13; range: 18–71) [17]. The screening recovered 13 HEV RNA-positive donors. Retrospectively, residual plasma samples of one donation preceding and several donations following the initial HEV RNA-positive donation, taken within a short time distance from each other, (Table 1) were available for 10 donors (D1 to D10, all male). The day of the detection of HEV RNA by PCR screening was defined as day 0, but HEV infection is most likely to have occurred before the beginning of our study period. This aspect limits the exact calculation of the diagnostic window between the detection of HEV RNA and anti-HEV antibodies. In addition, the period of detectability of antibodies may have started before the first positive sample and lasted beyond the last positive sample. To take this into account, we calculated two intervals of HEV-RNA positivity: Interval 1 started on the day of the first positive and ended on the day of the last positive sample, whereas interval 2 started at half of the interval between the last negative and first positive sample and lasted until half of the interval between the last positive and first negative sample. The duration of anti-HEV seropositivity was calculated according to interval 2.

All HEV-infected donors underwent pre-donation medical examination and negated current diseases or any known risk factors for viral infection. Post-donation questionnaires to elucidate risk factors for HEV infection were returned by six donors. The study protocol followed the ethical guidelines of the Ruhr University, Bochum, and was approved by the institutional review board. All donors provided informed consent.

RNA extraction and real-time RT-PCR

Total RNA from individual samples was extracted from 500 µl plasma using the NucliSens easyMAG (bioMerieux, Nürtingen, Germany) automated RNA/DNA extraction system. Amplification using the RealStar HEV RT-PCR Kit (Altona Diagnostic Technologies (ADT), Hamburg, Germany) was performed on the Rotor-Gene 3000 system (Corbett Life Sciences, Sydney, Australia). HEV virus titre in positive plasma was quantified using the first World Health Organization (WHO) international standard for hepatitis E virus RNA for NAT-based assays (Paul-Ehrlich institute, Langen, Germany) [22].

Serological testing and measurement of liver-specific parameters

All plasma samples were screened for the presence of HEV-specific antibodies using the recomWell HEV IgM and recomWell IgG immunoassays (quantitative, Mikrogen GmbH, Neuried, Germany) and the Anti-HEV-IgA-ELISA (qualitative, Euroimmun, Lübeck, Germany). Analyses and serostatus interpretation were performed

Table 1

Hepatitis E virus RNA progression in blood donors, Germany, 2011 (n = 10)

Donor	Maximum concentration (IU/mL)	Day[a] with maximum concentration	Maximum concentration in window period (IU/mL)	Distance to last negative sample (days)	Distance to last positive sample (days)	Mean time between serial samples in days (range)	Duration interval 1[b] (days)	Duration interval 2[c] (days)
D1	2.63×10^4	0	5.13×10^3	43	10	5 (3–10)	20	(47)
D2	1.02×10^5	25	1.02×10^5	46	26	11 (5–26)	52	(88)
D3	1.51×10^3	0	No window period	30	8	8 (8)	1	20
D4	4.74×10^4	28	4.74×10^4	9	6	10 (6–15)	42	50
D5	1.86×10^1	0	No window period	>1 year	3	7 (3–11)	11	(195)
D6	1.63×10^4	21	1.63×10^4	7	7	7 (7)	35	42
D7	2.13×10^4	33	2.13×10^4	7	3	6 (3–12)	46	51
D8	2.19×10^5	28	2.19×10^5	28	7	6 (3–12)	52	80
D9	1.36×10^3	7	1.36×10^3	54	42	16 (3–42)	7	(55)
D10	2.48×10^3	21	2.48×10^3	129	38	21 (21)	21	(105)
Range	$1.86 \times 10^1 - 2.19 \times 10^5$	0–33	$1.36 \times 10^3 - 2.19 \times 10^5$	NC	NC	NC	1–52	20–80
Mean	4.38×10^4	20	5.19×10^4	NC	NC	NC	29	49
Median	1.88×10^4	23	1.88×10^4	NC	NC	NC	28	50

NC: not calculated.

[a] Day x post detection of HEV RNA by PCR screening.

[b] Duration interval 1: first positive to last positive sample.

[c] Duration interval 2: starting at half of the interval between the last negative and the first positive sample and ending at half of the interval between the last positive and the first negative sample. Data in parenthesis were excluded from the calculation of mean and median values because the last hepatitis E virus RNA-negative samples went back more than 30 days.

according to the manufacturers' recommendations, results were classified into three categories: (i) no antibodies detectable (<20 U/mL: negative), (ii) evidence for the presence of antibodies (≤20 to ≤24 U/mL: borderline) and (iii) antibodies detectable (>24 U/mL: positive). Results (as the ratio extinction sample/calibrator) of the Anti-HEV-IgA-ELISA were classified as follows: (i) no antibodies detectable (ratio<0.8: negative), (ii) evidence for the presence of antibodies (ratio>0.8 to ≤1.1: borderline) and (iii) antibodies detectable (ratio>1.1: positive).

Comparative testing was performed using the Wantai HEV IgM and IgG ELISA (Sanbio B.V., Uden, the Netherlands), and results were classified into three categories: (i) no antibodies detectable (cut-off<0.9: negative), (ii) evidence for the presence of antibodies (cut-off 0.9–1.1: borderline) and (iii) antibodies detectable (cut-off>1.1: positive). Confirmatory testing with an immunoblot assay was performed on 22 samples using the recomLine HEV-IgM/IgG immunoassay according to the manufacturer's instructions (Mikrogen GmbH, Neuried, Germany). Sample selection included those samples taken at the first positive detection of anti-HEV antibodies and up to two consecutive samples.

Concentrations of glutamate dehydrogenase (GLDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin were measured in plasma samples using the respective enzymatic assays (Abbott Diagnostics Europe, Wiesbaden, Germany) on the Architect ci8200 system (Abbott Diagnostics Europe).

Results

Progression of hepatitis E virus RNA and anti-hepatitis E virus antibodies

The progression of RNA concentration in follow-up samples from infected patients is shown in Figure panel A, and the key observations of HEV RNA progression are summarised in Table 1. HEV viraemia persisted up to 52 days (D2 and D8, interval 1) with considerably different RNA concentrations in individual donors, ranging from 1.86×10^{1} to 2.19×10^{5} IU/mL. High RNA concentrations were observed in the window period ranging from 1.36×10^{3} to 2.19×10^{5} IU/mL. Taking the second interval into account, the duration of viraemia was as long as 20 to 80 days. The maximum viraemia was observed after 20 days, with a mean duration of 29 days for interval 1 and 49 days for interval 2 (Table 1).

Figure panels B–D show the course of anti-HEV IgM, anti-HEV IgG (only results determined by the Mikrogen assay) and anti-HEV IgA. In samples of donor D3, HEV RNA and IgM antibodies were detectable in parallel. Likewise, HEV RNA, IgA and IgG antibodies were detected in parallel in samples of donor D5. This was probably due the fact that HEV infection occurred before the beginning of our HEV screening study period. The progression of anti-HEV IgA and IgM antibodies was virtually equal (Figure, panels B and D). Donor D8 did not have IgA antibodies at any time, and had a very limited increase of IgM antibodies that was only detectable on day 32 after the first detection of HEV RNA. In addition, IgA and IgM antibodies were not detectable in donors D9 and D10, but no samples were available between day 10 and 50 for donor D9 and between day 20 and 60 for donor D10, most probably including the time point where IgA/IgM seroconversion occurred. For the remaining donors, IgA, IgM and IgG antibodies were first detected between days 8 and 42 for IgA and IgM and between days 13 and 59 for IgG (Table 2, results Mikrogen assays).

In four donors (D1, D3, D7 and D8), IgM levels increased before IgG levels, and four donors (D2, D4, D5 and D6) showed a parallel increase of IgA, IgM and IgG. Detection of IgA before IgM was not observed, but IgA antibodies were detectable until the end of the observation period in donor D2 in the absence of IgM antibodies. In contrast, the detection period for anti-HEV IgM was longer than for IgA in donor D1 and donor D3. Three donors had detectable IgM (D3, D4 and D6) or IgA antibodies (D2, D4 and D6) more than 150 days after first detection of HEV RNA. The progression of IgG antibodies in donors D2 and D4 showed an almost equal rapid increase to high values of more than 100 U/mL 35 days after the first detection of HEV RNA (Figure, panel C). Donor D6 demonstrated a prolonged constant IgG increase to values higher than 100 U/mL, while the other donors showed a continuous moderate antibody increase (D1, D7, D8, D9 and D10) or a constant antibody titre (D5). A continuous decrease of anti-HEV IgG antibodies was observed in samples of donor D3.

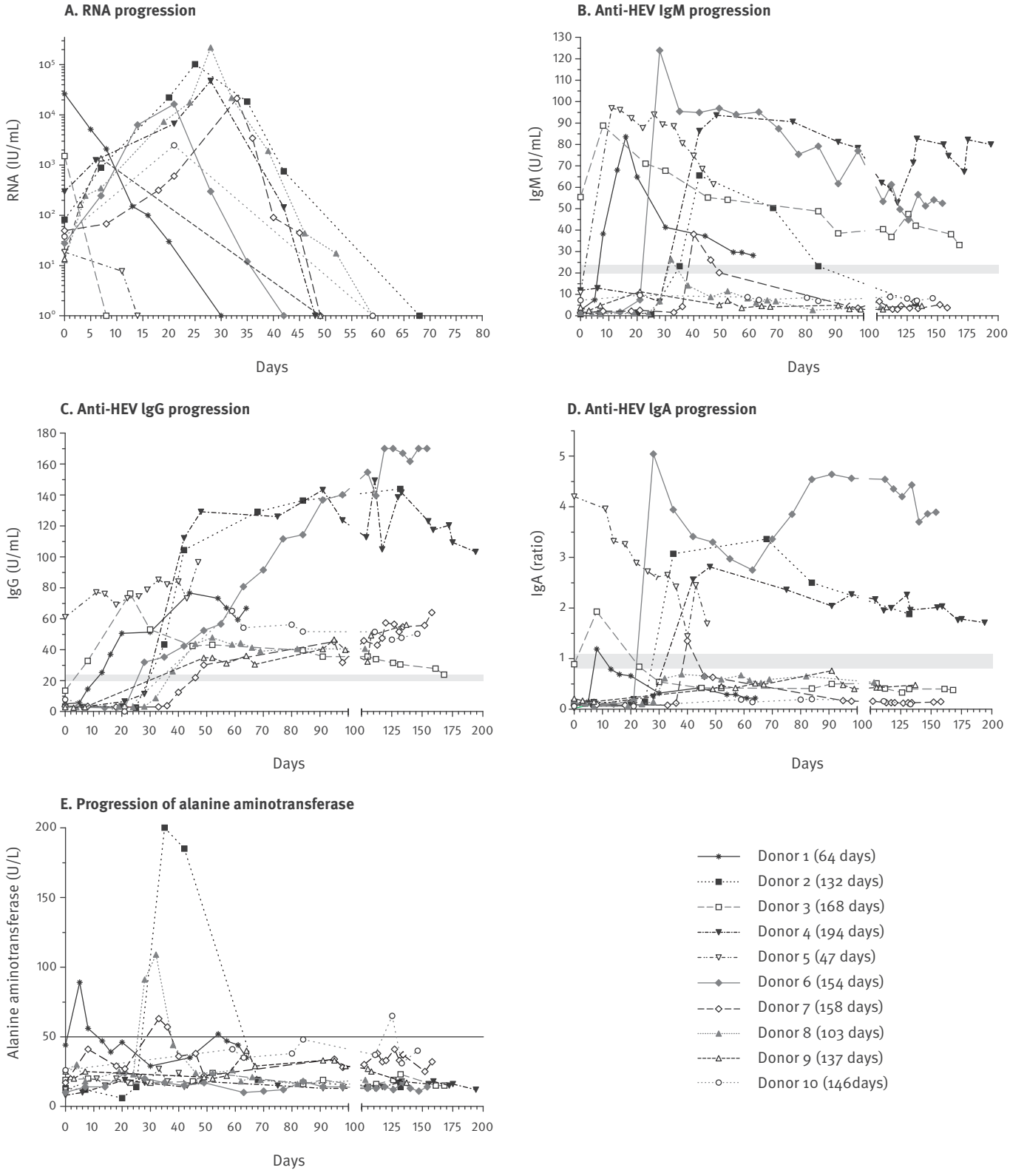
Table 2 further summarises the key observations on the progression of anti-HEV IgA, IgM and IgG. Here we concentrate on the Mikrogen anti-HEV IgM/IgG results; the Wantai results will be described further down. The diagnostic window before the detection of HEV-specific antibodies was up to 42 days for IgA and IgM (D4) and up to 59 days for IgG (D10); the mean values including all donors were 31 days for IgA and IgM and 34 days for IgG. The mean duration of seropositivity was 80 days for IgA antibodies and 69 days for IgM antibodies. The maximum IgM and IgG titres differed considerably between different donors (IgM mean: 71.83 U/mL, range: 26.23–123.9; IgG mean: 108.20 U/mL, range: 47.74–167.64).

Progression of liver specific enzymes

Elevated values of ALT were observed only for five donors (D1, D2, D7, D8 and D10). The ALT values showed a two- to fourfold (D1, D7, D8, D10) and an 11-fold (D2) increase compared with the reference value of 50 U/L. In donor D1, ALT levels showed two peaks, first on day 5, in the period when HEV RNA was detectable, and a second minor peak on day 55 in the absence of detectable HEV RNA. The three donors D2, D7 and D8 had elevated ALT values within the first 40 days after first HEV-RNA detection, with HEV RNA

Figure

Progression of hepatitis E virus RNA, IgM, IgG and IgA antibodies and alanine aminotransferase in blood donors with autochthonous hepatitis E virus genotype 3 infection, Germany, 2011 (n = 10)



The day of the detection of HEV RNA by PCR screening was defined as day 0. Grey-shaded areas (panels B–D): cut-off values of the different serological assays as described in the Methods section. Solid horizontal line (panel E): reference range of 0–50 U/L. Days in brackets after donor legend: time period were samples were taken. Ratio: extinction sample/calibrator.

Table 2

Occurrence of anti-hepatitis E virus antibody classes (IgA, IgM and IgG) and first detection and duration of IgM and IgG seropositivity in two serological assays, blood donors, Germany, 2011 (n = 10)

		IgA			IgM							IgG				
					Mikrogen	Wantai	Mikrogen	Wantai	Mikrogen	Wantai	Mikrogen	Mikrogen	Wantai	Mikrogen	Wantai	Mikrogen
Donor	Mean time between samples in days (range)	First positive detection on day x[a]	Duration[b] and detection interval in days (range)	Maximum concentration on day x[a]	First positive detection on day x[a]	First positive detection on day x[a]	Duration[b] and detection interval in days (range)	Duration[b] and detection interval in days (range)	Maximum concentration on day x[a]	Maximum concentration on day x[a]	Maximum concentration (U/mL)	First positive detection on day x[a]	First positive detection on day x[a]	Maximum concentration on day x[a]	Maximum concentration on day x[a]	Maximum concentration (U/mL)
D1	6 (3–14)	8	5 (8)	8	8	8	›55 (8 –›61)	›55 (8–›61)	16	16	83.61	13	8	44	44	76.66
D2	17 (7–44)	35	102 (35 –›132)	68	35	35	›54 (35 –›84)	›54 (35 –›84)	42	42	65.55	35	35	132	132	143.9
D3	17 (7–44)	8	13 (8)	8	0	0	›168 (0 –›168)	›52 (0 –›52)	8	8	88.82	8	0	23	30	76.26
D4	11 (3–27)	42	159 (42 –›194)	48	42	42	159 (42 –›194)	80 (42–111)	48	48	93.62	42	42	111	130	149.39
D5	4 (3–11)	0	47 (0 –›47)	11	0	0	›47 (0 –›47)	›47 (0 –›47)	0	0	96.95	0	0	47	47	96.61
D6	7 (6–8)	28	130 (28–›154)	28	28	28	130 (28 –›154)	130 (28 –›154)	28	28	123.91	28	28	›119	›119	167.64
D7	8 (3–46)	40	6 (40)	40	40	40	10 (40–46)	34 (40 –›49)	40	40	38.07	49	40	158	158	63.96
D8	7 (3–21)	ND	ND	ND	32	28	7 (1)	23 (28–46)	32	32	26.23	39	39	52	52	47.74
D9	11 (3–21)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	49	49	132	›137	56.05
D10	19 (4–38)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	59	59	59	59	65.03
Range	NC	8–42	5–159	8–68	8–42	8–42	7–159	23–130	16–48	16–48	26.23–123.9	13–59	8–42	44–158	30–158	47.74–167.64
Mean[c]	NC	31	80	38	31	30	69	63	34	34	71.83	34	32	103	89	108.20
Median[c]	NC	35	102	40	34	32	55	55	36	36	74.58	37	37	115	86	110.30

NC: not calculated; ND: no detection of antibodies.

[a] Day x post detection of HEV RNA by PCR screening.

[b] Duration: starting at half of the interval between the last negative and first positive sample and ending at half of the interval between last positive and first negative sample.

[c] IgA: donors D3, D5, D8, D9 and D10 were excluded from the calculation of mean and median values. IgM/IgG: donors D3, D5, D9 and D10 were excluded from the calculation of mean and median values. Donors were excluded either because no antibodies were detected (IgA: D8, IgM/IgG: D9 and D10) or because all samples were taken ≥ 30 days from each other (D3 and D5).

detected at the same time. AST and GLDH values followed the progression of ALT in these three donors, all other donors had normal AST and GLDH values (Figure and data not shown). Total bilirubin was within the reference range for all donors (data not shown).

Comparison of the diagnostic window using different serological assays

We further compared the timing of the first detection of different antibody classes during the window period when only HEV RNA was detectable and the duration of seropositivity of HEV-specific IgM and IgG antibodies using two different serological assays (Table 2). For IgM and IgG antibodies, the diagnostic window differed depending on the assay used (Table 2), with a mean of 31 days (IgM) and 34 days (IgG) for the Mikrogen assay and a mean of 30 days (IgM) and 32 days (IgG) for the Wantai assay. In addition, the duration of IgM seropositivity depended on the serological assay: the Mikrogen assay had a longer detection period than the Wantai assay (mean: 69 days and 63 days, respectively) with a range of with 23 to 130 days (Wantai) vs seven to 159 days (Mikrogen). Overall, the Wantai assay showed a higher sensitivity than the Mikrogen assay and often detected IgM or IgG seropositivity at least one sampling point earlier (IgM: D8, IgG: D1, D3 and D7, Table 2).

Samples taken at the first positive IgM and/or IgG detection by the two different assays and up to two consecutive samples were further analysed by immunoblot (Table 3). Borderline results were counted as positive. The Mikrogen ELISA, Wantai ELISA and immunoblot revealed concordant IgM results for 12 samples and concordant IgG results for 15 samples. For two IgM and two IgG samples, only the Wantai ELISA gave positive results. In eight IgM samples and five IgG samples, both ELISAs gave positive results but the interpretation of the immunoblot was negative.

Discussion

HEV viraemia in symptomatic cases usually lasts from four to six weeks but can remain more than 100 days in some cases [23]. Liver enzyme values reach a peak about six weeks post exposure before decreasing towards normal levels by week 10 [20]. The typical serological course of an HEV infection shows an initial rise in short-lived anti-HEV IgM after three to four weeks that decline to baseline levels within three to six months, followed by an increase of IgG which remains detectable for up to 15 years [2,20,21]. However, the knowledge about the natural course of HEV infection in asymptomatic HEV-infected individuals is limited.

The clinically asymptomatic cases analysed in this study represent the preselection of apparently healthy individuals voluntarily donating blood and lacking physically detectable symptoms of infection. The retrospective character of this study limited the availability of consecutive samples from the same donor taken less than 30 days apart and the accuracy of the calculated durations (viraemia, seropositivity). The observed differences in the sensitivity of the serological assays further influenced the calculation of the diagnostic window. For example, it has been shown that the performance of anti-HEV IgG assays strongly influences the estimation of hepatitis E seroprevalence [24]. The progression of HEV RNA in a Japanese cohort of 15 patients with acute symptomatic hepatitis E was largely comparable with what we observed in our study [25]. In contrast to our results, anti-HEV IgA and IgM (first detection: day 8–42) and IgG antibodies (first detection: day 13–59) in the Japanese cohort were detectable in symptomatic cases in parallel to the presence of HEV RNA at first sampling [25], pointing towards an earlier onset of viraemia in the patients without symptoms. Accordingly, anti-HEV IgA and IgM remained detectable until the end of the observation period in symptomatic cases in the Japanese cohort while two different progressions were observed in the asymptomatic cases in our study. Antibodies in some asymptomatic cases showed the same persistence as in symptomatic cases, whereas antibody levels in other asymptomatic cases continuously decreased and reached undetectable levels. Furthermore, we observed IgM positivity for a significantly longer period compared with the Japanese cohort with seropositivity (longer than 100 days in D3, D4 and D6). However, these differences between symptomatic and asymptomatic cases could be related to the performance of the ELISAs used. There is no consensus on whether immunoblot assays (rather than ELISAs) are needed in order to detect anti-HEV antibodies accurately. The immunoblot results in our study did not add informative value; the immunoblot provided negative results for samples with divergent results in the two different ELISAs, most probably because of inferior sensitivity.

Unexpectedly, anti-HEV IgG antibodies declined under detectable levels in samples from donor D3. Previous studies have shown that the period when anti-HEV IgG remains detectable can vary individually from six months to 14 years, but HEV IgG antibodies have also been shown to disappear [26-28]. Remarkably, a rise in liver-specific enzymes was observed only in four of 10 asymptomatic individuals, although high viral loads were detected in plasma. The elevation of ALT may have been missed in donors D9 and D10 because of the long delay of 42 and 38 days between two samples, respectively, but for the other eight donors, samples within the first 50 days after detection of HEV viraemia were taken at average intervals of less than 10 days.

There is an ongoing debate about HEV genotype 3 and 4 infection and blood safety. Published reports of HEV infections transmitted by contaminated blood products [29,30] and of the detection of HEV genotypes 3 and 4 in plasma fractionation pools [31] and blood donors [15-17] suggest that transfusion transmission of HEV is probably not uncommon, with many undiagnosed subclinical infections [15,16]. In a recent study by Hewitt et al., transmission of HEV genotype 3 via contaminated

Table 3

Hepatitis E virus-specific antigens in selected samples with different detection of anti-hepatitis E virus antibodies, Wantai vs Mikrogen ELISA, blood donors, Germany, 2011 (n = 8)

Donor (sex, age in years)	Day	Anti-HEV	Mikrogen	Wantai	Immunoblot[a]							
					O2N (1)		O2C (4)		O2M (1)	O3 (2)		Σ (interpretation)
					Gt1	Gt3	Gt1	Gt3		Gt1	Gt3	
D1 (M, 27)	5	IgM	Negative	Negative	–	–	–	–	–	–	–	0 (negative)
		IgG	Negative	Negative	–	–	–	–	–	–	–	0 (negative)
	13	IgM	Positive	Positive	+/–	+	+	–	–	+/–	+/–	5 (positive)
		IgG	Positive	Positive	–	–	+/–	+/–	–	–	–	0 (negative)
D2 (M, 37)	35	IgM	Borderline	Positive	+++	++	+/–	+/–	–	–	–	1 (negative)
		IgG	Positive	Positive	+	+/–	–	+	–	+++	++	7 (positive)
D3 (M, 26)	0	IgM	Positive	Positive	+/–	+	+	+/–	–	–	–	5 (positive)
		IgG	Negative	Positive	–	+/–	+/–	+/–	–	–	–	0 (negative)
	8	IgM	Positive	Positive	+/–	+	+	+/–	–	–	–	5 (positive)
		IgG	Positive	Positive	–	+/–	+/–	+	–	–	–	4 (positive)
D4 (M, 53)	42	IgM	Positive	Positive	–	–	–	+/–	–	–	–	0 (negative)
		IgG	Positive	Positive	–	–	+	+	–	+	+	6 (positive)
	48	IgM	Positive	Positive	–	–	–	+/–	–	–	–	0 (negative)
		IgG	Positive	Positive	–	–	+	+	–	+	+	6 (positive)
D5 (M, 26)	0	IgM	Positive	Positive	+/–	+/–	+	–	–	+	–	6 (positive)
		IgG	Positive	Positive	–	+	+	++	–	+++	+/–	7 (positive)
	11	IgM	Positive	Positive	–	–	+/–	+/–	–	+/–	–	0 (negative)
		IgG	Positive	Positive	–	+	+	++	–	+++	+/–	7 (positive)
D6 (M, 27)	21	IgM	Negative	Negative	–	–	–	–	–	–	–	0 (negative)
		IgG	Negative	Negative	–	–	–	–	–	–	–	0 (negative)
	28	IgM	Positive	Positive	+/–	+/–	+	–	–	+++	+++	6 (positive)
		IgG	Positive	Positive	–	–	–	+/–	–	++	+++	2 (negative)
	35	IgM	Positive	Positive	+/–	+/–	+/–	+/–	–	+++	+++	2 (negative)
		IgG	Positive	Positive	+	+/–	+/–	+	–	++	+++	7 (positive)
D7 (M, 22)	40	IgM	Positive	Positive	+	+	+/–	+/–	–	+	–	3 (borderline)
		IgG	Negative	Positive	+	+/–	–	–	–	+/–	–	1 (negative)
	46	IgM	Positive	Positive	+/–	+/–	–	–	–	+/–	–	0 (negative)
		IgG	Borderline	Positive	++	–	–	+/–	–	–	–	1 (negative)
	49	IgM	Borderline	Positive	+/–	+/–	–	–	–	+/–	–	0 (negative)
		IgG	Positive	Positive	++	+/–	–	+/–	–	+/–	–	1 (negative)
D8 (M, 26)	32	IgM	Positive	Positive	–	+/–	+/–	–	–	–	–	0 (negative)
		IgG	Negative	Negative	+/–	+/–	–	–	–	–	–	0 (negative)
	39	IgM	Negative	Positive	–	+/–	+/–	+/–	–	–	–	0 (negative)
		IgG	Positive	Positive	+/–	+/–	+/–	+/–	–	+/–	–	0 (negative)
	46	IgM	Negative	Positive	–	–	–	–	–	–	–	0 (negative)
		IgG	Positive	Positive	+/–	+/–	+	++	–	+/–	–	4 (positive)
D9 (M, 21)	49	IgM	Negative	Negative	NT	NT	NT	NT	NT	NT	NT	NT
		IgG	Positive	Positive	–	+/–	+	+	–	–	+/–	4 (positive)
	52	IgM	Negative	Negative	NT	NT	NT	NT	NT	NT	NT	NT
		IgG	Positive	Positive	–	+/–	+	+	–	–	+/–	4 (positive)
D10 (M, 20)	59	IgM	Negative	Negative	+/–	+/–	–	–	–	–	–	0 (negative)
		IgG	Positive	Positive	++	+/–	+	++	–	–	–	5 (positive)
	63	IgM	Negative	Negative	–	–	–	–	–	–	–	0 (negative)
		IgG	Positive	Positive	++	+/–	+	++	–	–	–	5 (positive)

HEV: hepatitis E virus; M: male; NT: not tested.

[a] O2N, O2C, O2M, O3 (Gt1/Gt3: genotype 1 and 3): highly purified recombinant HEV antigens provided by the manufacturer; numeric score in parenthesis. –: no reaction; +/–: very weak intensity (‹ cut-off); +: weak intensity (= cut-off); ++: strong intensity (› cut-off); +++: very strong intensity. Interpretation: ≤ 2: negative; 3: borderline; ≥ 4: positive; only reactions with intensities higher than + were included in the interpretation. Numeric scores of antigens were summed up for final interpretation: once for samples with +, ++ or +++, and only once per antigen if Gt1 and Gt3 or both reacted. Calculation example for sample D2 IgG: 1 × score 1 (O2N Gt3 positive) + 1 × score 4 (O2C Gt3 positive) + 1 × score 2 (O3 Gt1 and Gt3 positive)

blood was demonstrated in 42% of transfusion recipients [18]. The clinical course (asymptomatic, mild hepatitis or acute liver failure) and severity of HEV infection in transfusion recipients are variable, most probably depending on predisposition or immune status. The vast majority of HEV genotype 3 and 4 infections are most likely to result in an asymptomatic course [32] but, for instance, chronic manifestations of HEV genotype 3 infection in immunosuppressed persons can become important in industrialised countries [33]. Feray et al. concluded that transfusion of blood products not screened for HEV RNA is associated with the risk of chronic infection in immunocompromised patients [34]. Nevertheless, the clinical relevance of transfusion-associated HEV infection is insufficiently understood and more data are needed regarding the duration of viraemia, the infective dose, the role of anti-HEV in the recipient and the frequency of clinically apparent transfusion-transmitted HEV infection [35]. Our results on the progression of HEV viraemia illuminate at least one of these questions. To our knowledge, neither the length of HEV window periods nor the course of HEV viraemia during window periods in blood donors have been studied so far. The observed high level viraemia during window period infection could represent an underestimated risk of HEV transmission.

Post-donation questionnaires returned by six donors did not reveal a potential source of HEV infection. None of the infected donors had travelled within two months before the HEV-positive donation. The consumption of pork meat was described by five of the six donors. The number of returned questionnaires in our study is too small for a statistically significant analysis. We currently perform routine HEV blood donor screening and ask those with positive results to answer a questionnaire.

Conclusion

We observed a diagnostic gap between the detection of high viral loads and the detection of anti-HEV antibodies, independently of the antibody class (IgA, IgM or IgG), in our cohort of clinically asymptomatic HEV-infected blood donors. The progression of viraemia and anti-HEV immunoglobulins was comparable to symptomatic cases, but a rise in liver-specific enzymes was infrequent in our blood donor cohort. Asymptomatic HEV infection make NAT screening methods necessary to detect infection and avoid transfusion of contaminated blood donations. However, the majority of infections are transmitted via the zoonotic or food-borne route. It is therefore important to focus public health measures both on blood safety and also on other infection routes for patients at risk, including immunosuppressed patients.

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Conflict of interest

None declared.

Authors' contributions

T. Vollmer and J. Dreier designed the study, analysed and interpreted the data and draft the manuscript, J. Diekmann and M. Eberhardt collected the data and revised the manuscript critically, C. Knabbe designed the study and revised the manuscript critically. All authors contributed to drafting the text and approved the manuscript.

References

1. Adlhoch C, Kaiser M, Pauli G, Koch J, Meisel H. Indigenous hepatitis E virus infection of a plasma donor in Germany. Vox Sang. 2009;97(4):303-8. DOI: 10.1111/j.1423-0410.2009.01211.x PMID: 19555366
2. Purcell RH, Emerson SU. Hepatitis E: an emerging awareness of an old disease.J Hepatol. 2008;48(3):494-503. DOI: 10.1016/j.jhep.2007.12.008 PMID: 18192058
3. Guthmann JP, Klovstad H, Boccia D, Hamid N, Pinoges L, Nizou JY, et al. A large outbreak of hepatitis E among a displaced population in Darfur, Sudan, 2004: the role of water treatment methods. Clin Infect Dis. 2006;42(12):1685-91. DOI: 10.1086/504321 PMID: 16705572
4. Dalton HR, Kamar N, Izopet J. Hepatitis E in developed countries: current status and future perspectives.Future Microbiol. 2014;9(12):1361-72. DOI: 10.2217/fmb.14.89 PMID: 25517900
5. Rutjes SA, Lodder WJ, Lodder-Verschoor F, van den Berg HH, Vennema H, Duizer E, et al. Sources of hepatitis E virus genotype 3 in The Netherlands. Emerg Infect Dis. 2009;15(3):381-7. DOI: 10.3201/eid1503.071472 PMID: 19239749
6. Krumbholz A, Mohn U, Lange J, Motz M, Wenzel JJ, Jilg W, et al. Prevalence of hepatitis E virus-specific antibodies in humans with occupational exposure to pigs. Med Microbiol Immunol (Berl). 2012;201(2):239-44. DOI: 10.1007/s00430-011-0210-5 PMID: 21773797
7. Matsuda H, Okada K, Takahashi K, Mishiro S. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar.J Infect Dis. 2003;188(6):944. DOI: 10.1086/378074 PMID: 12964128
8. Kamar N, Dalton HR, Abravanel F, Izopet J. Hepatitis E virus infection.Clin Microbiol Rev. 2014;27(1):116-38. DOI: 10.1128/CMR.00057-13 PMID: 24396139
9. Boccia D, Guthmann JP, Klovstad H, Hamid N, Tatay M, Ciglenecki I, et al. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. Clin Infect Dis. 2006;42(12):1679-84. DOI: 10.1086/504322 PMID: 16705571
10. Christensen PB, Engle RE, Hjort C, Homburg KM, Vach W, Georgsen J, et al. Time trend of the prevalence of hepatitis E antibodies among farmers and blood donors: a potential zoonosis in Denmark. Clin Infect Dis. 2008;47(8):1026-31. DOI: 10.1086/591970 PMID: 18781880
11. Dalton HR, Hazeldine S, Banks M, Ijaz S, Bendall R. Locally acquired hepatitis E in chronic liver disease.Lancet. 2007;369(9569):1260. DOI: 10.1016/S0140-6736(07)60595-9 PMID: 17434400
12. Péron JM, Bureau C, Poirson H, Mansuy JM, Alric L, Selves J, et al. Fulminant liver failure from acute autochthonous hepatitis E in France: description of seven patients with acute hepatitis E and encephalopathy. J Viral Hepat. 2007;14(5):298-303. DOI: 10.1111/j.1365-2893.2007.00858.x PMID: 17439518
13. Kamar N, Selves J, Mansuy JM, Ouezzani L, Péron JM, Guitard J, et al. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N Engl J Med. 2008;358(8):811-7. DOI: 10.1056/NEJMoa0706992 PMID: 18287603
14. Legrand-Abravanel F, Kamar N, Sandres-Saune K, Garrouste C, Dubois M, Mansuy JM, et al. Characteristics of autochthonous hepatitis E virus infection in solid-organ transplant recipients in France. J Infect Dis. 2010;202(6):835-44. DOI: 10.1086/655899 PMID: 20695798
15. Baylis SA, Gärtner T, Nick S, Ovemyr J, Blümel J. Occurrence of hepatitis E virus RNA in plasma donations from Sweden, Germany and the United States.Vox Sang. 2012;103(1):89-90. DOI: 10.1111/j.1423-0410.2011.01583.x PMID: 22220775
16. Ijaz S, Szypulska R, Tettmar KI, Kitchen A, Tedder RS. Detection of hepatitis E virus RNA in plasma mini-pools from

blood donors in England.Vox Sang. 2012;102(3):272. DOI: 10.1111/j.1423-0410.2011.01554.x PMID: 21957873
17. Vollmer T, Diekmann J, Johne R, Eberhardt M, Knabbe C, Dreier J. Novel approach for detection of hepatitis E virus infection in German blood donors.J Clin Microbiol. 2012;50(8):2708-13. DOI: 10.1128/JCM.01119-12 PMID: 22675127
18. Hewitt PE, Ijaz S, Brailsford SR, Brett R, Dicks S, Haywood B, et al. Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. Lancet. 2014;384(9956):1766-73. DOI: 10.1016/S0140-6736(14)61034-5 PMID: 25078306
19. Hogema BM, Molier M, Slot E, Zaaijer HL. Past and present of hepatitis E in the Netherlands.Transfusion. 2014;54(12):3092-6. DOI: 10.1111/trf.12733 PMID: 24889277
20. Dalton HR, Bendall R, Ijaz S, Banks M. Hepatitis E: an emerging infection in developed countries.Lancet Infect Dis. 2008;8(11):698-709. DOI: 10.1016/S1473-3099(08)70255-X PMID: 18992406
21. Kamar N, Bendall R, Legrand-Abravanel F, Xia NS, Ijaz S, Izopet J, et al. Hepatitis E. Lancet. 2012;379(9835):2477-88. DOI: 10.1016/S0140-6736(11)61849-7 PMID: 22549046
22. Baylis SA, Blümel J, Mizusawa S, Matsubayashi K, Sakata H, Okada Y, et al. World Health Organization International Standard to harmonize assays for detection of hepatitis E virus RNA. Emerg Infect Dis. 2013;19(5):729-35.
23. Blood Advisory Committee. Topic I: Hepatitis E virus (HEV) and blood transfusion safety. 104th Meeting; 20 Sep 2012 Rockville; 2012. Available from: http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/bloodvaccinesandotherbiologics/bloodproductsadvisorycommittee/ucm319542.pdf
24. Wenzel JJ, Preiss J, Schemmerer M, Huber B, Jilg W. Test performance characteristics of Anti-HEV IgG assays strongly influence hepatitis E seroprevalence estimates.J Infect Dis. 2013;207(3):497-500. DOI: 10.1093/infdis/jis688 PMID: 23148290
25. Takahashi M, Kusakai S, Mizuo H, Suzuki K, Fujimura K, Masuko K, et al. Simultaneous detection of immunoglobulin A (IgA) and IgM antibodies against hepatitis E virus (HEV) Is highly specific for diagnosis of acute HEV infection. J Clin Microbiol. 2005;43(1):49-56. DOI: 10.1128/JCM.43.1.49-56.2005 PMID: 15634950
26. Bendall R, Ellis V, Ijaz S, Thurairajah P, Dalton HR. Serological response to hepatitis E virus genotype 3 infection: IgG quantitation, avidity, and IgM response.J Med Virol. 2008;80(1):95-101. DOI: 10.1002/jmv.21033 PMID: 18041018
27. Khuroo MS, Kamili S, Dar MY, Moecklii R, Jameel S. Hepatitis E and long-term antibody status.Lancet. 1993;341(8856):1355. DOI: 10.1016/0140-6736(93)90873-F PMID: 8098491
28. Mansuy JM, Peron JM, Bureau C, Alric L, Vinel JP, Izopet J. Immunologically silent autochthonous acute hepatitis E virus infection in France.J Clin Microbiol. 2004;42(2):912-3. DOI: 10.1128/JCM.42.2.912-913.2004 PMID: 14766888
29. Dreier J, Juhl D. Autochthonous hepatitis e virus infections: a new transfusion-associated risk?Transfus Med Hemother. 2014;41(1):29-39. DOI: 10.1159/000357098 PMID: 24659945
30. Huzly D, Umhau M, Bettinger D, Cathomen T, Emmerich F, Hasselblatt P, et al. Transfusion-transmitted hepatitis E in Germany, 2013. Euro Surveill. 2014;19(21):20812. DOI: 10.2807/1560-7917.ES2014.19.21.20812 PMID: 24906377
31. Baylis SA, Koc O, Nick S, Blümel J. Widespread distribution of hepatitis E virus in plasma fractionation pools.Vox Sang. 2012;102(2):182-3. DOI: 10.1111/j.1423-0410.2011.01527.x PMID: 21806631
32. Beale MA, Tettmar K, Szypulska R, Tedder RS, Ijaz S. Is there evidence of recent hepatitis E virus infection in English and North Welsh blood donors?Vox Sang. 2011;100(3):340-2. DOI: 10.1111/j.1423-0410.2010.01412.x PMID: 21392024
33. Pischke S, Behrendt P, Bock CT, Jilg W, Manns MP, Wedemeyer H. Hepatitis E in Germany--an under-reported infectious disease.Dtsch Arztebl Int. 2014;111(35-36):577-83.PMID: 25249359
34. Féray C, Pawlotsky JM, Roque-Afonso AM, Samuel D, Dhumeaux D. Should we screen blood products for hepatitis E virus RNA?Lancet. 2014;383(9913):218. DOI: 10.1016/S0140-6736(14)60072-6 PMID: 24439737
35. Juhl D, Baylis SA, Blümel J, Görg S, Hennig H. Seroprevalence and incidence of hepatitis E virus infection in German blood donors.Transfusion. 2014;54(1):49-56. DOI: 10.1111/trf.12121 PMID: 23441647

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