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Research Articles

Detection of Zika virus in Brazilian patients during the first five days of infection – urine *versus* plasma

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Advantages of testing for Zika virus (ZIKV) in urine have been reported, such as the persistence of ZIKV in this type of specimen for up to 20 days after ZIKV disease onset. We investigate 61 patients in the first 5 days post-symptom onset and find more patients testing positive for ZIKV in plasma samples (n=46), than in corresponding urine samples (n=37). For patients respectively testing positive in both plasma and urine (n=28), respective viral loads appeared similar.

Results of recent studies have suggested that after Zika virus (ZIKV) disease onset, the virus persists at higher levels and for a longer period in urine (up to ca 20 days) than in serum (up to ca 5 days) [1,2]. To provide further data, we tested for the presence of ZIKV in the urine and corresponding plasma specimens of 61 patients presenting symptoms of ZIKV disease in Brazil. The samples were collected between 1 and 5 days after symptom onset. During this phase of infection, the proportion of patients testing positive in plasma samples (46/61) appeared to be higher than those testing positive in urine samples (37/61).

Laboratory investigations

Urine and corresponding plasma samples (i.e. from the same patient at the same date) that had been previously collected during the large outbreak in Tuparetama, Brazil, in 2015, were retrospectively analysed in this study. All samples had been obtained within the first 5 days of onset of symptoms, from a total of 61 individuals, who had been diagnosed as having ZIKV disease on clinical and epidemiological grounds. The main symptoms reported for the patients were rash (n=47), fever ≥38.5°C (n=42), headache (n=40), joint pain (n=39), and conjunctivitis (n=37). The median age of patients was 35 years (range: 1–80 years), with the majority being female (n=41). No patient was co-infected with another flavivirus such as dengue at the time of the sample collection, although previous infection with dengue was not known. None of the women were pregnant.

Specimens were investigated by real-time reverse-transcription polymerase chain reaction (RT-PCR), with a published primer set (FP: 5´-GAAGCCCTTGGATTCTTGAACGAGG-3´ and RP: 5´-CGACTCATCTCTTCTAGGACATATCC-3´) [3] and a fluorescein (FAM)/black hole quencher 1 (BHQ1)-labelled Taqman probe, targeting the non-structural protein (NS)5 genetic region of ZIKV (ZIKAp 5'-FAM-GGGAGAGAGAACTCAGGAGGTGG-BHQ1–3`). A sample was considered positive for ZIKV when the mean cycle threshold (*Ct*) value obtained from three parallel real-time RT-PCRs was ≤ 40 cycles.

Zika virus RNA in urine and plasma

Among the 61 patients, more tested positive for ZIKV RNA in plasma (n=46) than in urine (n=37) within the 5 days post-symptom onset, although the difference was not statistically significant (p=0.12; two-tailed test) (Table).

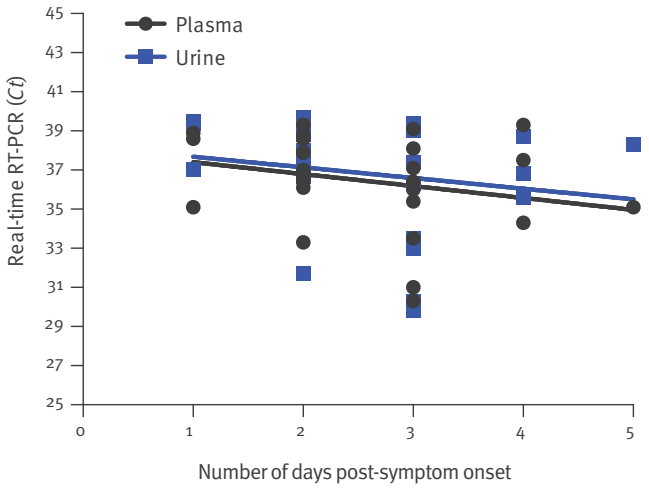
In 28 cases, ZIKV RNA was detected in both types of samples. *Ct* values from plasma and urine samples collected at the same time post-symptom onset did not seem to differ (Figure).

Background and discussion

ZIKV belongs to the genus Flavivirus, a group of RNA viruses transmitted by arthropods. The virus name originates from the Zika Forest of Uganda, where it was first isolated in 1947 from an infected rhesus monkey [4]. In 2007, ZIKV caused an outbreak in Yap State, Micronesia [5]. The outbreak was characterised by relatively mild disease including symptoms such as rash, arthralgia, and conjunctivitis, which are commonly observed upon ZIKV infection [5,6]. Seven years later, the virus appeared in French Polynesia and larger

Figure

Comparison between the cycle threshold (*Ct*) values of plasma and corresponding urine samples from Zika virus infected patients testing positive for both types of samples in the first five days post-symptom onset, Brazil, 2015 (n=28 patients)



RT-PCR: reverse transcription-polymerase chain reaction; ZIKV: Zika virus.

A linear regression analysis was performed by plotting the Ct values of plasma and urine samples versus the time post onset of symptoms using PRISM (Graphpad SoJware Inc., San Diego, California). The Ct values of ZIKV RNA in plasma and urine samples appear similar.

outbreaks were reported in New Caledonia, the Cook Islands, and Easter Island, whereby coincident with the French Polynesia ZIKV outbreak, an increased incidence of neurological complications was observed [7]. The first cases of ZIKV infection in the Americas were detected in Brazil in May 2015 [3,6,8] and, since, the virus spread to other countries in North and South America [9]. In Brazil, concurrent with ZIKV infections, neurological complications were also reported, as well as congenital malformations including microcephaly [10].

Due to cross-reactivity between members of the Flavivirus genus, the serological diagnostic of ZIKV is difficult [11]. Although there are molecular tests based on virus isolation and/or detection of ZIKV RNA during the acute phase of infection, the low-level and short period of viraemia remains a challenge [3]. Recently, Gourinat et al [1] observed that ZIKV RNA in six patients from French Polynesia was detectable at higher levels and for longer periods after symptom onset in urine samples (up to ca 20 days) than in corresponding serum samples (up to ca 5 days). The larger time window post-disease onset of possible ZIKV detection in urine was confirmed by others who investigated returning travellers to the United States (US) [2]. It was further noted that among 55 persons with urine and serum samples obtained within the first 5 days of symptom onset, a higher proportion tested positive for ZIKV RNA in urine

Table 1a

Real-time reverse transcription-polymerase chain reaction results of screening plasma and corresponding urine samples from patients in Brazil, 2015 (n=61 patients)

Sample ID	Sex	Age	*Ct* plasma	*Ct* urine
1 day post-symptom onset				
64	F	5	38.6	37.0
60	M	15	Neg	39.3
77	M	46	38.9	39.4
30	F	26	35.1	39.5
2 days post-symptom onset				
74	F	50	Neg	31.5
18	F	16	36.6	31.7
70	F	50	Neg	32.0
46	F	31	Neg	34.7
61	F	44	36.1	36.7
51	F	45	37.9	36.9
20	F	10	33.3	37.0
40	F	4	38.6	37.1
53	F	19	39.3	37.1
67	F	37	38.8	37.6
31	F	20	36.8	38.0
58	M	46	Neg	38.5
32	F	33	37.0	38.8
11	F	44	37.9	38.8
22	M	8	36.4	39.1
16	F	39	36.7	39.7
57	F	1	26.2	Neg
2	M	37	36.6	Neg
62	F	45	39.2	Neg
65	M	45	Neg	Neg
75	F	10	Neg	Neg
73	F	36	Neg	Neg
3 days post-symptom onset				
39	F	8	39.1	29.8
23	F	80	37.1	30.3
19	M	28	36.0	33.0
43	F	55	38.1	33.5
44	F	35	Neg	35.2
34	F	40	Neg	36.1
69	F	38	31.0	36.3
21	M	42	36.4	37.4
28	F	39	35.4	39.0
54	M	48	30.3	39.2
29	M	28	33.5	39.4
9	F	56	34.1	Neg
13	M	5	35.1	Neg
55	F	10	37.4	Neg
63	F	34	37.5	Neg
76	M	9	38.8	Neg
71	F	62	38.9	Neg

Ct: cycle threshold; F: female; ID: identity; M: male; Neg: negative.

The *Ct* value is the mean of the three *Ct*s of each sample.

Table 1B

Real-time reverse transcription-polymerase chain reaction results of screening plasma and corresponding urine samples from patients in Brazil, 2015 (n=61 patients)

Sample ID	Sex	Age	*Ct* plasma	*Ct* urine
4 days post-symptom onset				
35	M	65	Neg	35.0
7	F	31	37.5	35.6
50	F	70	39.3	36.8
27	F	46	34.3	38.7
47	F	18	Neg	39.3
15	F	43	34.4	Neg
5	F	28	36.5	Neg
17	F	73	36.6	Neg
10	M	34	37.3	Neg
24	M	11	37.4	Neg
37	F	15	Neg	Neg
56	M	13	Neg	Neg
5 days post-symptom onset				
33	F	27	35.1	38.3
1	M	42	33.6	Neg
4	M	3	37.0	Neg
14	M	49	37.2	Neg
6	M	8	37.6	Neg
8	F	36	Neg	Neg

Ct: cycle threshold; F: female; ID: identity; M: male; Neg: negative.

The *Ct* value is the mean of the three *Ct*s of each sample.

than in serum [2]. The findings opened the door for the use of urine for the diagnosis of ZIKV infection [1].

In our study, we focused on the detection of ZIKV in samples from 61 patients collected within the first 5 days of symptom onset. In contrast to the two previous studies mentioned above [1,2], patients in our study had an overall a higher ZIKV detection rate in plasma than in urine during active infection. Moreover, our data do not support the recent finding of higher level of ZIKV RNA in urine than in serum during the acute phase of the disease [1]. The reasons for this discrepancy remain unclear, although this might have been due to the small number of patients investigated in all three studies. Another hypothesis could be that host immune and genetic factors might affect the viral load of ZIKV in distinct body fluids, whereby this may vary among individuals. A similar suggestion has been reported in cases of infection with dengue virus, another flavivirus [12]. In this respect, patients in our study were Brazilian citizens, while the two other studies investigated patients from French Polynesia and travellers returning to the US. Previous infection with other flaviviruses might also alter and/or enhance the replication of ZIKV in specific organs [13]. Information about past dengue infection was however not available for our patients. Finally, beside the small sample size, one limitation of our study was the cross-sectional design that only permitted obtaining data at single time points.

In conclusion, we recommend the simultaneous testing of blood and urine samples in ZIKV infected individuals with focus on plasma samples in the first 5 days of infection. Further investigations will be required to more fully determine factors influencing ZIKV pathogenesis.

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Conflict of interest

None declared.

Authors' contributions

Conceived and designed the experiments: RP, AAEW, SSS. Performed the experiments: RP, JVP, MLS, AAEW, SSS. Data analysis: RP, JVP, MLS, AAEW, SSS. Drafted the manuscript: AAEW, SSS. Critical revision: RP, JVP, MLS. All authors read and approved the final manuscript.

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Research article

High prevalence of anti-hepatitis E virus antibodies among blood donors in central Italy, February to March 2014

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Prevalence of anti-hepatitis E virus (HEV) antibodies is highly variable in developed countries, which seems partly due to differences in assay sensitivity. Using validated sensitive assays, we tested 313 blood donors attending a hospital transfusion unit in central Italy in January and February 2014 for anti-HEV IgG and IgM and HEV RNA. Data on HEV exposure were collected from all donors. Overall anti-HEV IgG prevalence was 49% (153/313). Eating raw dried pig-liver sausage was the only independent predictor of HEV infection (adjusted prevalence rate ratio = 2.14; 95% confidence interval: 1.23–3.74). Three donors were positive for either anti-HEV IgM (n = 2; 0.6%) or HEV RNA (n = 2; 0.6%); they were completely asymptomatic, without alanine aminotransferase (ALT) abnormalities. Of the two HEV RNA-positive donors (both harbouring genotype 3), one was anti-HEV IgG- and IgM-positive, the other was anti-HEV IgG- and IgM-negative. The third donor was positive for anti-HEV IgG and IgM but HEV RNA-negative. HEV infection is therefore hyperendemic among blood donors (80% men 18–64 years-old) from central Italy and associated with local dietary habits. Nearly 1% of donors have acute or recent infection, implying potential transmission to blood recipients. Neither ALT nor anti-HEV IgM testing seems useful to prevent transfusion-transmitted HEV infection.

Introduction

Hepatitis E virus (HEV) is a non-enveloped single-stranded RNA virus of the genus *Hepevirus* in the *Hepeviridae* family. This family contains viruses that infect mammals, including humans, as well as birds and fish. Four major mammalian HEV genotypes (HEV1 to 4) have been identified [1-3]. HEV, once thought to be limited to developing countries, has recently been found also in developed countries of Europe, North America and Asia-Pacific where human autochthonous cases, probably of zoonotic origin, have become prevalent [1,2].

HEV1 and HEV2 infect only humans and are endemic in developing areas of Asia, Africa and Central and South America, where faecal-oral transmission usually occurs through contaminated water and causes both outbreaks and sporadic cases. Clinical disease mainly affects young adults and is severe and associated with excess mortality in pregnant women and in patients with chronic liver disease [1,2].

HEV3 and HEV4 infect humans and various domestic and wild mammals such as pig, wild boar, deer and rodents. HEV strains infecting humans and animals in the same area are usually phylogenetically closely related, supporting zoonotic transmission [1]. HEV3 is ubiquitous, while HEV4 is mainly prevalent in Asia. These genotypes are transmitted by eating contaminated raw or undercooked meat and meat products or shellfish, and by contact with infected animals, causing autochthonous sporadic cases [1,2]. Most HEV3 or HEV4 infections are asymptomatic; clinical disease mainly affects middle-aged and elderly men with underlying illness, and HEV3 may cause chronic hepatitis in immunosuppressed patients [1-3].

In addition to hepatitis, HEV infection also appears to be associated with some extrahepatic manifestations: neurological disorders such as Guillain–Barré syndrome and neuralgic amyotrophy due to peripheral nerve involvement, haematological diseases such as haemolytic anaemia and severe thrombocytopenia, glomerulonephritis and mixed cryoglobulinaemia [4].

Epidemiological studies have reported variable and sometimes unexpectedly high prevalence rates of IgG anti-HEV antibodies in the general population in Europe, ranging from 1.1% to 16.8% in the period between 2004 and 2011, although a decline was observed for example in south-eastern Germany from 1996 to 2011 [5-9]. Rates ranging from 0.4% to 52.5% have been reported among European blood donors in the period between 1993 and 2014, with the highest value observed in the Midi-Pyrénées hyperendemic area in south-western France [10-18]. In Italy, anti-HEV IgG prevalence rates of 1.0–4.3% [19-21] and 0.7–9.1% [19,21-23] were found in the general population (in the period between 1993 and 2011) and in blood donors (in the period between 1993 and 2013), respectively. The large variations in anti-HEV prevalence in different European countries and even within the same country are probably due to several factors such as the performance of antibody assays used in the studies but also dietary habits and occupational exposure [2,10,11].

HEV can be transmitted by blood transfusion [10,24]. Transfusion-transmitted HEV infection has been reported in several countries [25-27], but its true frequency is probably underestimated because it is often asymptomatic and testing of blood donors is infrequent [1,10]. In this study we have assessed the prevalence of HEV infection among blood donors in the Abruzzo region in central Italy by using highly sensitive and validated assays, and we have examined its association with putative risk factors.

Methods

Study population

This study was designed to evaluate the prevalence of and the risk factors for HEV infection among voluntary, unpaid blood donors attending the blood transfusion unit of the San Salvatore Hospital in L'Aquila during February and March 2014. L'Aquila is a city of around 70,000 inhabitants in the Abruzzo region in central Italy.

Serum samples were collected from blood donors who agreed to participate in the study. Participants were administered a questionnaire collecting information on demographics and putative risk factors for HEV infection (professional and recreational activities, contact with domestic or wild animals, eating habits and travel history).

All blood donors provided written informed consent. The study protocol conformed to the Helsinki Declaration and was approved by the Ethics Committee of San Salvatore Hospital.

Serological assays

All serum samples were tested for anti-HEV IgG and IgM antibodies using commercial enzyme-linked immunosorbent assay (ELISA) kits (Wantai, Biologic Pharmacy Enterprise, Beijing, China). Both the IgG and IgM anti-HEV assays use recombinant antigen expressed from the ORF2 region.

Detection and quantitation of hepatitis E virus RNA

Plasma samples from anti-HEV IgG-positive or -negative donors were assembled in minipools of 10 samples (20 µL each for a total volume of 200 µL), or fewer if a full set of 10 was not available, and total RNA was extracted by QIAamp MinElute Virus Spin kit silica columns (Qiagen, Hilden, Germany). Total HEV RNA was extracted individually from each anti-HEV IgM-positive sample.

One half of the extracted RNA was reverse transcribed and HEV RNA amplified using the RealStar HEV RT-PCR kit, version 1.0 (Altona Diagnostics, Hamburg, Germany). This kit includes primers and a probe targeting the ORF3 region of the HEV genome. The sensitivity, reported as 95% limit of detection, was assessed to be 50 IU/mL of HEV RNA. Reactive pools were deconstructed to identify HEV RNA-positive donations by individual HEV RNA testing.

An external standard curve, made from a log dilution series of a HEV RNA World Health Organization (WHO) International Standard (Paul-Ehrlich-Institute, Langen, Germany, code 6329/10) from 5×104 to 5×101 IU/mL was used for estimating the viral load in positive samples.

Sequencing and phylogenetic analysis

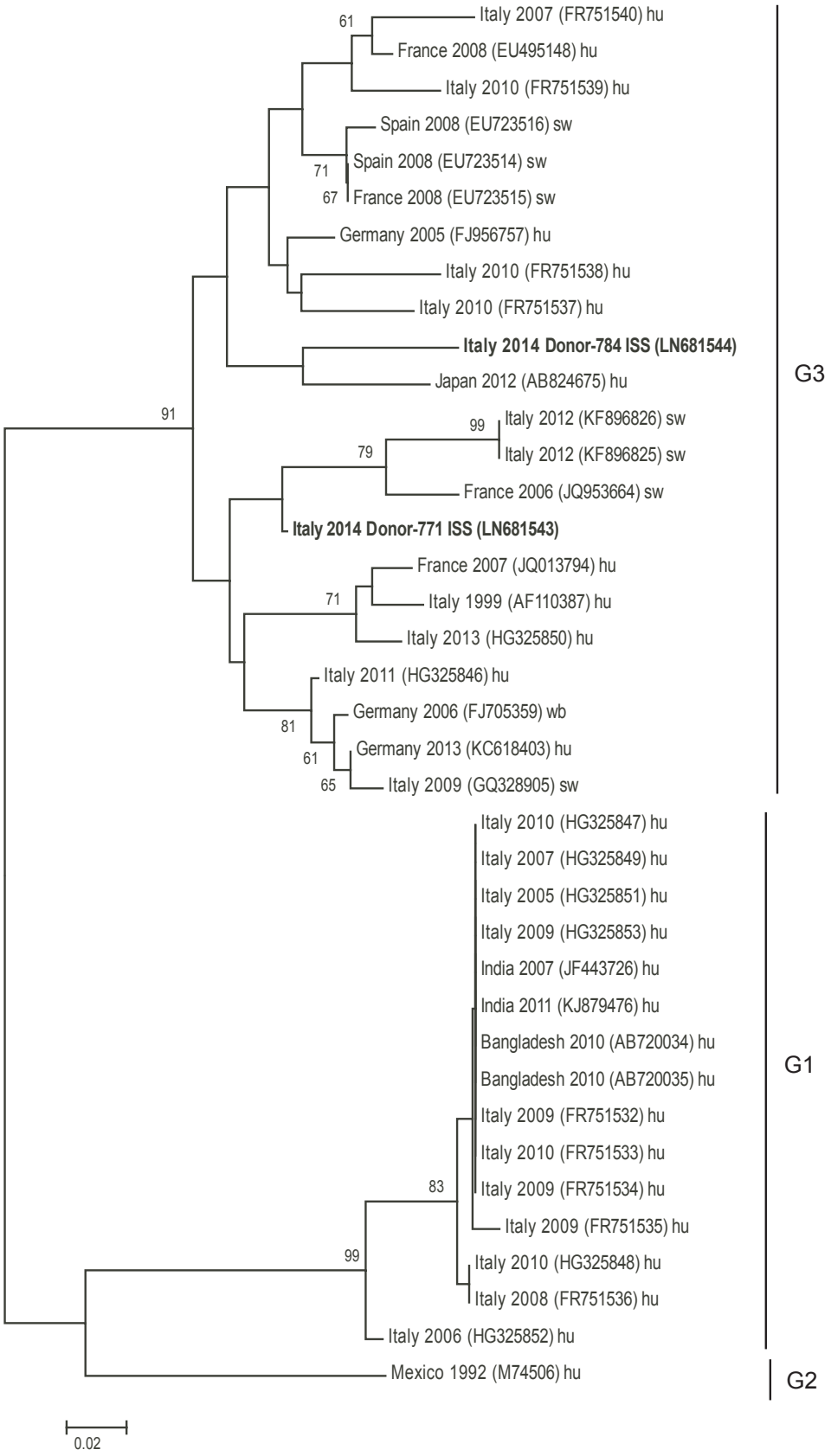
HEV RNA was extracted and amplified by nested RT-PCR using two sets of primers targeting ORF1 (172 bp) and ORF2 (348 bp) as described elsewhere [28,29]. Purified PCR amplicons were subjected to bidirectional automated sequencing. The raw forward and reverse ABI files were assembled into a single consensus sequence using MEGA 6.06 software. The phylogenetic trees were constructed based on the best fit model of nucleotide substitution. The reliability of the phylogenetic trees were determined by bootstrap re-sampling of 1,000 replicates.

Statistical analysis

Prevalence of anti-HEV antibodies (IgG and IgM) and HEV RNA was calculated and the exact binomial distribution was used to calculate 95% confidence intervals (CI). The association between the study variables and HEV infection was estimated by chi-squared test. Factors independently associated with HEV infection were evaluated by a multivariate binomial regression model. All variables with a p value$<$0.20 in univariate

Figure 1

Maximum likelihood phylogenetic tree based on the Tamura-Nei model for ORF1 nucleotide sequences of selected hepatitis E virus strains

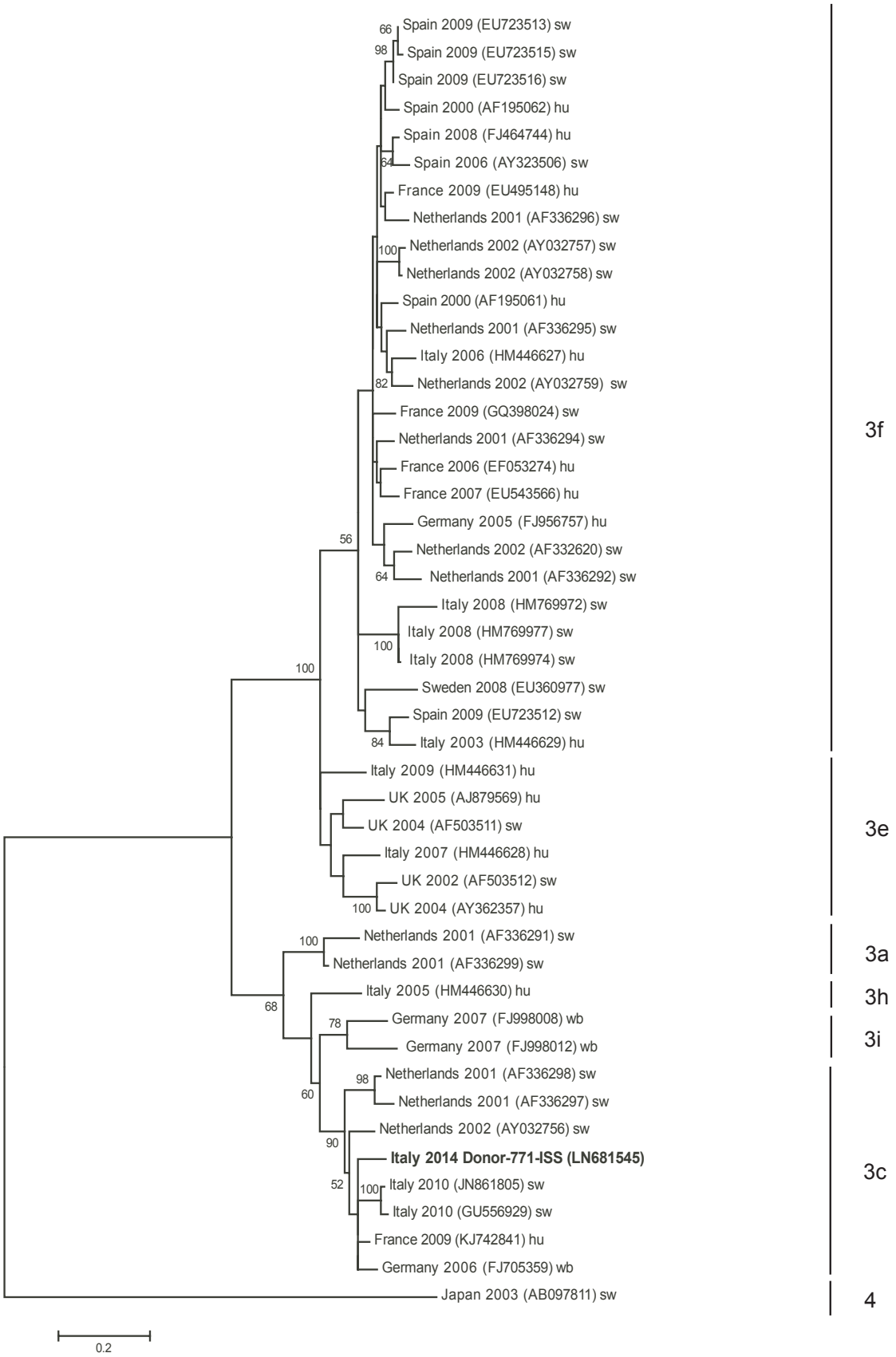


Hu: human; sw: swine; wb: wild boar.

Sequences of hepatitis E virus isolated from blood donors 771 and 784 in Italy in 2014 are shown in bold. The numbers at the nodes indicate bootstrap values ≥ 60%. Sequences are denoted by country, year of isolation (or publication if not available), GenBank accession number in parenthesis, and source.

Figure 2

Maximum likelihood phylogenetic tree based on the Tamura-Nei model for ORF2 nucleotide sequences of selected hepatitis E virus strains



Hu: human; sw: swine; wb: wild boar.

Sequence of hepatitis E virus isolated from blood donor 771 in Italy in 2014 is shown in bold. The numbers at the nodes indicate bootstrap values ≥ 60%. Sequences are denoted by country, year of isolation (or publication if not available), GenBank accession number in parenthesis, and source.

Table 1

Prevalence of anti-hepatitis E virus IgG antibodies and univariate and multivariate analysis of factors potentially associated with infection in blood donors, central Italy, 2014 (n = 313)

Characteristics	Tested	HEV IgG+	Univariate analysis			Multivariate analysis		
	N	n (%)	PR	95% CI	p value	APRR	95% CI	p value
Sex								
Female	61	24 (39)	1.00	Ref	Ref	Ref	Ref	Ref
Male[a]	252	129 (51)	1.30	0.84–2.01	0.24	0.87	0.56–1.35	0.52
Age group (years)								
18–34	54	19 (35)	1.00	Ref	Ref	Ref	Ref	Ref
35–44	82	37 (45)	1.28	0.74–2.23				
45–54	102	51 (50)	1.42	0.84–2.41	0.19	1.35	0.77–2.35	0.29
≥ 55[a]	75	46 (61)	1.74	1.02–2.97				
Environment								
Birth in rural area[a]	107	61 (57)	1.28	0.92–1.76	0.14	1.17	0.84–1.64	0.39
Living in rural area	164	84 (51)	1.10	0.80–1.51	0.56	NI	NI	NI
Educational level								
Secondary school	93	48 (52)	1.00	Ref	Ref	NI	NI	NI
High school	166	77 (46)	0.90	0.63–1.29	0.80	NI	NI	NI
Master's degree	54	28 (52)	1.00	0.63–1.60				
Professional exposure								
Work with animals	28	17 (61)	1.27	0.77–2.11	0.35	NI	NI	NI
Contacts with								
Pigs	170	87 (51)	1.10	0.81–1.53	0.53	NI	NI	NI
Wild boar	51	29 (57)	1.20	0.80–1.80	0.37	NI	NI	NI
Other wild animals	37	21 (57)	1.19	0.75–1.88	0.47	NI	NI	NI
Horses	114	53 (47)	0.92	0.66–1.29	0.65	NI	NI	NI
Poultry	194	98 (51)	1.10	0.79–1.52	0.60	NI	NI	NI
Sheep	124	58 (47)	0.93	0.67–1.29	0.67	NI	NI	NI
Cattle	113	62 (55)	1.20	0.87–1.67	0.26	NI	NI	NI
Other animals	80	38 (48)	0.96	0.67–1.39	0.84	NI	NI	NI
Dogs[a]	253	116 (46)	0.74	0.51–1.08	0.12	0.73	0.50–1.07	0.11
Cats	205	100 (49)	1.00	0.71–1.39	0.97	NI	NI	NI
Hobbies								
Hunting	32	20 (63)	1.32	0.83–2.11	0.25	NI	NI	NI
Gardening	207	105 (51)	1.12	0.80–1.58	0.51	NI	NI	NI
Vegetable gardening[a]	200	106 (53)	1.27	0.90–1.80	0.17	1.23	0.87–1.78	0.24
Eating habits								
Pork	312	152 (49)	0.49	0.07–3.48	0.47	NI	NI	NI
Game	249	125 (50)	1.15	0.76–1.73	0.51	NI	NI	NI
Salami	311	151 (49)	0.49	0.12–1.96	0.31	NI	NI	NI
Raw dried pork sausage	311	152 (49)	0.98	0.14–6.98	0.98	NI	NI	NI
Raw dried wild boar sausage	225	115 (51)	1.18	0.82–1.71	0.37	NI	NI	NI
Raw dried pork liver sausage[a]	253	139 (55)	2.35	1.36–4.08	0.002	2.14	1.23–3.74	0.007
Undercooked/raw meat	80	37 (46)	0.93	0.64–1.34	0.70	NI	NI	NI
Consumption of vegetables from own kitchen garden	244	124 (51)	1.21	0.81–1.81	0.36	NI	NI	NI
Frequent consumption of vegetables from own garden	186	98 (53)	1.22	0.87–1.69	0.24	NI	NI	NI
Occasional consumption of vegetables from own garden	59	27 (46)	0.92	0.61–1.40	0.70	NI	NI	NI
Travel								
Travel to non-endemic areas	259	127 (49)	1.02	0.66–1.55	0.93	NI	NI	NI
Travel to endemic areas	118	65 (55)	1.22	0.88–1.68	0.22	NI	NI	NI

APRR: adjusted prevalence rate ratio; CI: confidence Interval; HEV IgG+: donors testing positive for IgG anti-hepatitis E virus antibodies; NI: not included; PR: prevalence ratio.

[a] Variables initially included in the multivariate binomial regression model because of a p value < 0.20 in the univariate analysis.

analysis were initially included in the model, while age and sex were included independently from the p value. A backward selection was then performed and only variables with a p value < 0.10 (by the log-likelihood ratio test) were retained [30]. Statistical analyses were performed using STATA, version 12.

Results

Serological testing and anti-hepatitis E virus prevalence

From February to March 2014, 327 blood donors attended the blood transfusion unit, of whom 313 (81% male; age: 18–68 years, median: 48 years) were suitable for donation and agreed to participate to the study and to complete the questionnaire. Almost all were Italian citizens (99%) and resided in Abruzzo (98%), where 84% of donors were also born.

The overall anti-HEV IgG prevalence was 49% (153/313; 95% CI: 0.43–0.54). As shown in Table 1, prevalence was higher, although not significantly, in men (p = 0.24). Likewise, no statistically significant differences were found for educational level (p = 0.80), professional exposure (work with animals) (p = 0.35) and urban or rural area of birth (p = 0.14) or living (p = 0.56). A high anti-HEV IgG prevalence (> 35%) was observed in all age groups, without significant differences among them (p = 0.19) (Table 1). However, the prevalence increased with age, and people older than 55 years showed the highest rate.

Two of 313 donors (0.6%; 95% CI: 0.08–2.3) were positive for anti-HEV IgM, and both were also positive for anti-HEV IgG (Table 2).

Factors associated with anti-hepatitis E virus IgG positivity

Several potential risk exposures showed in the univariate analysis an association with anti-HEV IgG positivity with a p value < 0.2 (Table 1): birth in a rural area (p = 0.14), contact with dogs (p = 0.12), home vegetable gardening (p = 0.17) and eating raw dried pig liver sausage (p = 0.002). In the multivariate analysis, also adjusting for age and sex, only eating raw dried pig liver sausage was independently associated with anti-HEV IgG positivity (adjusted prevalence rate ratio = 2.14; 95% CI: 1.23–3.74; p = 0.007).

Detection, quantification and genotyping of hepatitis E virus RNA

Three donors (1; 95% CI: 0.20–2.80) showed evidence of acute or recent HEV infection. Two donors (0.6%; 95% CI: 0.08–2.3) were positive for HEV RNA (Table 2). One of them (donor 784) had low-level viraemia (100 IU/mL) and was anti-HEV IgG- and IgM-positive, while the other (donor 771) had 10,000 IU/mL of HEV RNA and was anti-HEV IgG- and IgM-negative. The third one (donor 207), who tested positive for both IgG and IgM, was HEV RNA-negative. These three donors were completely asymptomatic and their blood donation showed normal alanine aminotransferase (ALT) levels. All three were tested again for HEV RNA, anti-HEV IgG and IgM four to eight months later (Table 2). Donor 207 was examined five months later and remained HEV RNA-negative, while donors 784 and 771 had become HEV RNA-negative four and eight months after the first blood sampling, respectively. Anti-HEV IgG and IgM were positive in all samples obtained during the follow-up. Therefore, seroconversion occurred in the donor who was negative at the time of donation while IgG and IgM persisted in the two others.

The phylogenetic analysis performed using partial nucleotide sequences of ORF1 (172 bp) and ORF2 (348 bp) is shown in Figures 1 and 2, respectively. ORF1 sequence was amplified from both HEV RNA-positive donors, while ORF2 amplification was successful only in donor 771, owing to very low-level viraemia in donor 784. The phylogenetic tree based on ORF1 sequences showed that both isolates grouped with HEV3 sequences (Figure 1). The ORF2 based tree indicated that the isolate from donor 771 belonged to subtype 3c and clustered with very high bootstrap values with human and swine 3c sequences from Italy (Figure 2).

Discussion

The observed anti-HEV IgG prevalence is among the highest prevalence rates reported in blood donors from developed countries [11,12,24]. The demographic data and the age distribution of anti-HEV IgG-positive donors suggest that HEV infection is hyperendemic in the Abruzzo region and virus exposure occurs relatively early in life. However, we observed an increasing prevalence with age which is likely to reflect a cumulative exposure to HEV over time.

Such a high anti-HEV IgG prevalence was unexpected, since all studies performed so far among Italian blood donors reported much lower figures (0.7–9.1%) [19,21-23]. Indeed, differences in anti-HEV IgG prevalence seem to be largely due to the different sensitivity and specificity of the assays employed [2,11,13,15] and most previous studies, by now outdated, used assays less sensitive than the assays currently in use. In the present study we used the Wantai IgG ELISA, a validated assay which in various comparative studies proved to be the most sensitive test available for anti-HEV IgG detection, also showing high specificity [2,31,32].

Recent studies using this assay for blood donors in Europe showed varying IgG seroprevalence: 4.6% in Scotland [12], 10% and 16%, respectively, in the north-west and south-west of the United Kingdom (UK) [15,33], 13.6% in Upper Austria [17], 20.0% in Catalonia, Spain [16], 19.8% in Denmark [18], 26.7% in the Netherlands [34], 39.1% in southern France [24] and 52.6% in the Midi-Pyrenees region of south-western France [11].

As these latter studies used the same Wantai IgG ELISA assay, sensitivity and specificity could not be a variable affecting the results. Therefore such differences

Table 2

Serological and virological features in blood donors with acute/recent hepatitis E virus infection, central Italy, 2014 (n = 3)

Donor	Age (years)	Baseline sample						Follow-up sample			
		HEV RNA	HEV RNA load (IU/mL)	HEV IgM (OD)[a]	HEV IgG (OD)[b]	ALT (U/L)	GT	TI (months)	HEV RNA	HEV IgM (OD)	HEV IgG (OD)
771	50	Pos	10,000	Neg (0.000)	Neg (0.001)	13	3c	8	Neg	Pos (0.370)	Pos (2.954)
784	50	Pos	100	Pos (2.466)	Pos (0.423)	10	3	4	Neg	Pos (0.766)	Pos (1.844)
207	46	Neg	NA	Pos (0.602)	Pos (2.785)	16	NA	5	Neg	Pos (0.432)	Pos (2.764)

ALT: alanine aminotransferase; GT: genotype; HEV: hepatitis E virus; HEV IgG: anti-HEV IgM antibodies; HEV IgM: anti-HEV IgM antibodies; NA: not applicable; neg: negative; OD: optical density; pos: positive; TI: time interval.

[a] Cut-off OD value for IgM anti-HEV: 0.260.

[b] Cut-off OD value for IgG anti-HEV: 0.161.

are likely to be genuine, representing true geographical variation in HEV prevalence due to different types and levels of zoonotic exposure. In addition to evident zoonotic exposure, such as professional contact with animals and consumption of contaminated food, other risk factors possibly linked to zoonotic transmission (e.g. ingestion of contaminated water or consumption of shellfish) might play a role in acquiring HEV infection, but their relative importance remains unknown [2]. In the present study, professional exposure to animals was reported by only 9% of blood donors while 54% and 16% reported contact with pigs and contact with wild boar, respectively. This reflects the common habit in the studied area to rear pigs for personal consumption and to hunt boars. However, these three risk factors were not associated with a higher risk of HEV positivity; this seems in contrast with previous studies [2,33], but the high prevalence we observed, mainly related to dietary habits, could have masked the effect of other possible risk factors. In our study, anti-HEV IgG positivity was independently associated with eating raw dried pig liver sausage, a food product widely diffused in the study region. Therefore, it may play a predominant role in HEV transmission, clearly detectable even when analysing a small population. Our data are also in agreement with a study carried out among blood donors from south-western France, where eating uncooked pork liver sausages and being male were independent predictors of anti-HEV prevalence [24]. In addition, cases of an HEV outbreak were linked with eating raw pig liver sausage in south-eastern France [35]. The risk of infection by eating raw pig liver sausages is likely to be higher than other infection modes because liver tissue is the main target of HEV replication and the viral concentration is therefore expected to be very high: an individual is likely to be exposed to a higher viral dose when eating raw liver compared with other raw meat or sausages [35].

We cannot exclude that other risk factors that only played a minor role in our studied population may be more prominent when increasing the sample size. Other studies showed that particular eating habits might explain the spread of infection in a region [2,11,24,35-37]. According to Legrand-Abravanel et al., eating game meat was the only independent predictor of HEV infection in French transplant recipients [36]. Offal and wild boar consumption emerged as unique independent predictors of HEV infection in a German study [37]. Moreover, a recent study in south-western France carried out in a large population, identified offal and eating uncooked pork liver sausages as risk factors [24].

Several studies have shown that HEV circulates widely in Italian pigs. An HEV RNA prevalence of 42–64.6% was found in pigs in northern Italy and the viral sequences belonged to HEV3 (specified in one study as subtypes 3c and 3f) [38,39]. HEV RNA belonging to genotype 3 has been detected in both raw and dried pig liver sausages sold at a grocery store in Rome [40].

Also the two HEV RNA-positive donors in our study harboured HEV3 genotype; one of them, for whom subtyping was successful, belonged to subtype 3c and clustered together with other human and swine strains from Italy [41]. Preparation and consumption of pig liver sausages are widespread throughout the Abruzzo region. However, consumption of pig liver sausage could not by itself explain the high anti-HEV IgG prevalence we saw among blood donors from Abruzzo, because pig liver sausage represents a traditional and widely consumed food also in other regions such as Latium and Molise. Nevertheless, in a seroprevalence study performed during 2009 among 101 blood donors from Latium (89% male; median age: 42 years, range: 20–62 years) we found an overall anti-HEV IgG prevalence of 9% with the same assay employed in this study (data not shown). This finding suggests that other factors in addition to food-borne transmission may have contributed to the high prevalence. Specific geographical and demographic factors, not reported in other studies, may have had a synergistic effect. Firstly, the geographical characteristics of the territory (mostly mountainous, forested and sparsely populated) favoured the uncontrolled expansion of the wild boar population and may have led to contamination of

soil and watercourses. Secondly, almost all study participants were born and lived in Abruzzo. More than half of them lived in rural areas, had a potentially high risk of exposure to HEV-contaminated environment and had similar dietary habits.

In this study, the proportion of HEV RNA-positive blood donors was 0.6% (2/313), to our knowledge one of the highest rates reported among qualified blood donors worldwide [10,12,13,24,31,42,43]. Interestingly, the three donors showing evidence of acute infection were asymptomatic and had normal serum ALT levels. The different patterns of HEV RNA and anti-HEV IgG and IgM observed in each of them suggests they were in three different phases of acute infection (ramp-up phase of viraemia, early control and full control of infection). This picture is corroborated by the follow-up results: HEV RNA clearance occurred in all examined samples, associated with seroconversion or persistence of IgG and IgM antibodies.

These findings have the following implications: Firstly, a small but not negligible proportion of the predominantly male blood donors (nearly 1%) in Abruzzo have evidence of acute/recent HEV infection but are completely asymptomatic and have normal ALT. Secondly, anti-HEV IgM is detectable in only a proportion of viraemic donors, as reported by others [13,42,43]. Thus, infective donations may escape from being identified unless viraemia is examined, and such asymptomatic and viraemic donors are potentially able to cause transfusion-transmitted hepatitis E in blood recipients. Finally, the coexistence of both IgM and HEV RNA is of short duration [13]. Overall, neither ALT measurement nor anti-HEV IgM testing seems appropriate to prevent transfusion-transmitted HEV infection.

Although the presence of HEV RNA in blood donors is not a rare event, only a few cases of transfusion-transmitted hepatitis E have been reported in developed countries [25-27] and such cases have never been reported in Abruzzo or elsewhere in Italy. It is possible that the vast majority of transfusion-transmitted infections remain asymptomatic and unrecognised, or that transmission is hampered by low infectious dose and/or high anti-HEV titre in blood donation as well as immunity of the recipient, especially important in high prevalence regions [44].

We were able to trace two recipients who had received red blood cell concentrates from the two viraemic donors identified by us. These recipients remained uninfected, without markers of HEV infection, up to seven months after transfusion and were then lost to follow-up.

Some important issues need to be addressed before considering implementation of HEV nucleic acid testing in blood screening. These include: the duration of HEV viraemia, the minimal infectious dose, the possible protective effect of anti-HEV antibodies in donors and recipients (e.g. nearly half of the Abruzzo population, including blood recipients, has anti-HEV IgG), the true incidence of symptomatic and asymptomatic acute infections in the general population, and the frequency of chronic HEV infection in blood recipients.

Published data about all these issues are scarce. In Italy, notification of acute hepatitis E to the Italian surveillance system for acute viral hepatitis (SEIEVA) began only in 2007, and from 2007 to 2014, only 145 cases of acute infection were reported [45]. This low annual incidence markedly disagrees with the anti-HEV IgG prevalence found in our study and also in previous Italian studies [19-23,46-48]. However, virtually all acute viral hepatitis cases reported to SEIEVA are symptomatic (and hospitalised), while most cases of HEV3 and HEV4 acute infection are subclinical or completely asymptomatic [1,2] and thus pass unrecognised. It is also possible that the discrepancy may, at least in part, be due to under-reporting to the surveillance system because of low awareness of healthcare professionals.

Some limitations of this study need to be discussed. We are aware that the sample of blood donors analysed by us was small. However, this limited size made a complete virological analysis possible at affordable cost (all donors were tested for anti-HEV IgG and IgM and HEV RNA) as well as an evaluation of risk factors for infection. Our findings cannot be representative of the Italian population of blood donors because the study was limited to a restricted geographical area. However, a similar epidemiological scenario could exist in other countries with similar dietary habits and should be the object of further studies.

In addition, our study sample enrolled only 19% women, lower than the annual percentage (30%) of female blood donors in Italy [46]. However, our study covered a period of only two months and this short period has probably reduced the chance to enrol female blood donors who have a longer inter-donation interval than men. Several studies have shown that reasons for exclusion or long inter-donation intervals are more frequent for women, e.g. lower body weight, lower iron levels, lower blood pressure, minor transient health problems, pregnancy and breastfeeding [49,50].

Conclusion

Anti-HEV IgG prevalence among the predominantly male blood donors from Abruzzo was high and associated with eating raw dried pig liver sausage. HEV RNA was detected in two donors, who harboured HEV3 and were completely asymptomatic, without ALT abnormalities. More data are required before considering implementing HEV nucleic acid testing in blood donors. Recommendations for blood donors and immunocompromised patients against eating undercooked pork meat would help reduce the risk of HEV infection and chronic liver disease.

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Conflict of interest

None declared.

Authors' contributions

Spada E and Ciccaglione AR participated in study's design, data analysis, manuscript drafting and supervision of the study; Lucarelli C and Taliani G contributed to data analysis, interpretation of data and helped manuscript drafting; Pezzotti P performed statistical analysis and assisted in study design, analysis and interpretation of data; Bruni R, contributed to study design and assisted in the analysis and interpretation of the data; Chionne P, Madonna E, La Rosa G and Pisani G performed laboratory analysis and contributed to the interpretation of data. Marcantonio C, Dell'Orso L, Ragone K and Tomei C contributed to samples and data collection. All authors read and critically revised the first as well as subsequent drafts to this manuscript and approved the final version.

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Research article

Emerging aspergillosis by azole-resistant *Aspergillus fumigatus* at an intensive care unit in the Netherlands, 2010 to 2013

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The prevalence of invasive aspergillosis (IA) at the intensive care unit (ICU) is unknown and difficult to assess since IA also develops in patients lacking specific host factors. In the Netherlands, increasing azole-resistance in *Aspergillus fumigatus* complicates treatment of patients with IA. The aim of this study was to determine the prevalence of IA by azole-resistant *A. fumigatus* at the ICU among patients receiving antifungal treatment and to follow their clinical outcome and prognosis. A retrospective cohort study was conducted in a university hospital ICU from January 2010 to December 2013. From all patients who received antifungal treatment for suspected IA, relevant clinical and microbiological data were collected using a standardised questionnaire. Of 9,121 admitted ICU-patients, 136 had received antifungal treatment for suspected IA, of which 38 had a positive *A. fumigatus* culture. Ten of the 38 patients harboured at least one azole-resistant isolate. Resistance mechanisms consisted of alterations in the cyp51A gene, more specific TR_{34}/L98H and TR_{46}/T289A/Y121F. Microsatellite typing did not show clonal relatedness, though isolates from two patients were genetically related. The overall 90-day mortality of patients with IA by azole-resistant *A. fumigatus* and patients with suspicion of IA by azole-susceptible isolates in the ICU was 100% (10/10) vs 82% (23/28) respectively. We conclude that the changing pattern of IA in ICU patients requires appropriate criteria for recognition, diagnosis and rapid resistance tests. The increase in azole resistance rates also challenges a reconsideration of empirical antifungal therapy.

Introduction

Azole resistance is an emerging problem in *Aspergillus* infections caused by *Aspergillus fumigatus*, with increasing reports of azole treatment failure [1-6]. Although azole resistance can develop during azole therapy, exposure to azole compounds used in the environment appears to contribute to a greater extent [1,2,7]. Surveillance studies increasingly report geographical spread of azole resistance in environmental and clinical *A. fumigatus* isolates, including in Europe, Asia, the Middle East, Africa and most recently North and South America [4,8-10]. In contrast, surveillance studies from the Netherlands revealed azole resistance to be endemic, with dominance of the TR34/L98H and TR46/Y121F/T289A cyp51A-gene mediated resistance mechanisms [3]. These studies were based on routinely screening for the presence of azole resistance in all *Aspergillus* spp. isolates cultured from clinical samples in five university hospitals, irrespective of the clinical relevance of the culture result [3]. Although these surveys provide insight in the overall prevalence of azole resistance in culture-positive patients, the implications of azole resistance for specific patient groups remain unknown. In recent years, new categories of patients, lacking specific host factors as defined by the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG), such as intensive care unit (ICU) patients, are increasingly recognised for their susceptibility to develop IA. Thus the reported prevalence of invasive aspergillosis (IA) has increased at the ICU [11-13]. As information on IA by azole-resistant *A. fumigatus* in the ICU is scarce, we performed a retrospective cohort study in order to investigate the prevalence of azole resistance and its implications for patient treatment.

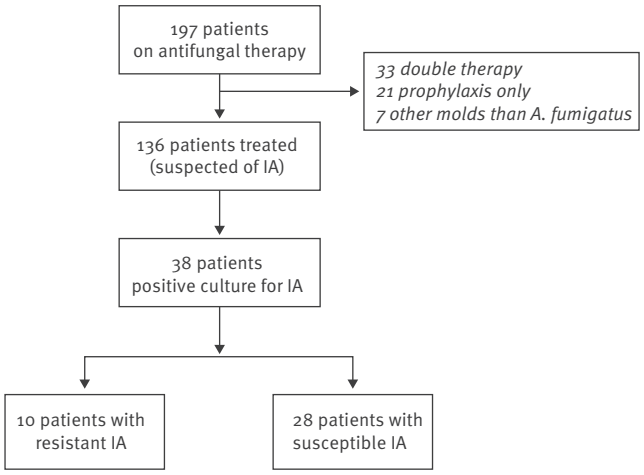
Methods

Study population

A retrospective cohort study was conducted in a 33-bed tertiary university hospital ICU in the Netherlands. Patients were identified by running a query for the period from January 2010 to December 2013 as those prescribed with voriconazole, conventional amphotericin B (c-AmB) or liposomal amphotericin B (L-AmB). Patients receiving mold-active drugs as prophylaxis only or antifungal therapy for non-*Aspergillus* fungal

Figure 1

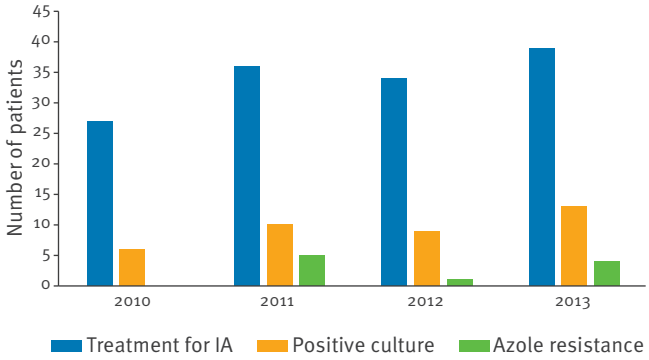
Flow-chart of patient selection to investigate the prevalence of invasive aspergillosis by azole-resistant *Aspergillus fumigatus* in the intensive care unit of a university hospital, Netherlands, 2010–2013 (n=38 selected patients)



IA: invasive aspergillosis.

Figure 2

Distribution of patients treated for suspected invasive aspergillosis in the intensive care unit of a university hospital, Netherlands, 2010–2013 (n=136 patients)



IA: invasive aspergillosis.

infections were excluded. Furthermore, patients suspected for IA, but where *A. fumigatus* could not be grown, were excluded as well. Thus, the study population consisted of ICU patients who were treated for suspected IA and in whom *A. fumigatus* was cultured, since resistance testing can only be done on cultured isolates.

Ethical consideration

Due to the retrospective design and report on anonymous patient data, ethical approval was not required.

Diagnosis of invasive aspergillosis

All patients with a clinical syndrome suspected for IA underwent computed tomography (CT)-scanning and bronchoscopy for histological and microbiological examinations, unless clinical respiratory condition did not allow interventions or transportation. In addition to the bronchoalveolar lavage (BAL) or lung biopsy, supplementary samples were occasionally available for galactomannan test or culture, such as serum, tracheal aspirate or sputum. Antifungal treatment was administered to all patients with specific CT lesions (among others halo sign) and a positive culture or *Aspergillus* biomarker in serum or BAL. Since CT lesions are only present in a minority of the ICU population [14], patients with a high clinical suspicion of IA (based on host characteristics and exclusion of an alternative diagnosis) and a positive respiratory or serum sample were treated as well. Following local and national guidelines, first choice treatment was voriconazole. When azole resistance was suspected or severe abnormal liver function tests were present, L-AmB or c-AmB were prescribed.

Data collection

Electronic patient records (EZIS-Chipsoft, *i*MD*soft*, GLIMS*)* were searched by the investigators (AitV, AR, JvP). Data regarding host factors, CT reports, microbiology results, mortality and use of prior antifungal therapy in the past 6 months were extracted from the hospital information system using a pre-assessed questionnaire. The EORTC/MSG criteria were applied to categorise patients as proven IA, probable IA or unclassified [15]. Since these criteria have been reported as less suitable for ICU patients, the ICU-criteria of Blot et al. were also used to classify patients with proven IA, putative IA or colonisation [16].

Microbiology

BAL fluids were tested for galactomannan (Platelia Aspergillus EIA, Bio-Rad, the Netherlands) using the recommended cut-off index of 0.5 for positivity. Direct microscopy was performed using the optical brightener calcofluor white on the sediment of the BAL fluid. Cultures for yeast and fungi were performed using standard techniques [17], including use of a selective Sabouraud dextrose agar incubated for 1 week at 35°C. If the culture was positive, *Aspergillus* species were identified to the species complex level using macroscopic and microscopic morphology. In addition, the ability of the fungus to grow at 48 °C was used to identify *A. fumigatus* species complex.

From cultures yielding *A. fumigatus*, up to four colonies were subcultured on a four-well agar plate to screen for azole resistance. In three wells the agar was supplemented with itraconazole (4 mg/L), voriconazole (1 mg/L), or posaconazole (0.5 mg/L). The fourth well served as growth control. Growth was assessed after 24 and 48 hours of incubation and any growth on the agars supplemented with azoles was indicative of possible azole resistance. These isolates were further analysed at the Mycology Reference Laboratory (Radboud

University Medical Centre, Nijmegen), including molecular identification, azole resistance phenotype and resistance genotype [3]. The mimimum inhibition concentration (MIC) was determined using the European Committee on Antimicrocial Susceptibility Testing (EUCAST) broth microdilution reference method [18]. Azole resistance was defined as a MIC-value›2 mg/L for itraconazole and/or voriconazole and/or a MIC-value›0.25 mg/L for posaconazole [18].

Microsatellite typing was retrospectively performed on available isolates by the Mycology Reference Laboratory (Nijmegen), as described previously [19]. In summary, six short tandem repeats (STR) were selected and amplified in two multiplex polymerase chain reactions (PCR)s. The repeat numbers of the six markers were analysed by Bionumerics version 4.6, software (Applied Maths, Kortrijk, Belgium). The summed absolute distance between two multilocus variable-number tandem repeat analysis (MLVA)-typed isolates is the summed tandem-repeat differences (STRD) at six markers. Isolates with an STRD≤10 were defined as genetically related, irrespective of the number of differing loci. Clonal complexes were defined as by an STRD≤2 provided that isolates were single-locus variants or double-locus variants of one another.

Endpoints

The primary endpoint was the prevalence of IA by azole-resistant *A. fumigatus* at the ICU. Secondly, we investigated patient characteristics coinciding with resistant IA, such as pre-treatment with azoles, underlying illness, and IA probability according to EORTC/MSG criteria [15]. Moreover, 90-day mortality and cause of death were assessed. Furthermore, we compared ICU prevalence of IA by azole-resistant *A. fumigatus* with the prevalence in the same period in the rest of the hospital.

Data analyses

Descriptive statistics were used for primary analysis and assessment of clinical relevance. A formal risk assessment with multivariate analyses could only be performed in the presence of enough events, in order to prevent false positive results.

Results

During the study period, 197 prescriptions of voriconazole, c-AmB or L-AmB were identified in the ICU. Thirty-three patients received both voriconazole and c-AmB or L-AmB and appeared twice in the search. Twenty-eight patients were excluded; 21 patients received antifungal prophylaxis, and seven patients were treated for proven fungal infection due to yeast or fungi other than *A. fumigatus*. Consequently, a total of 136 patients were treated for suspected IA, representing 15 patients per 1,000 admissions at the ICU. A culture for *A. fumigatus* had been attempted for all 136 patients. Of these, 38 (28%) had a positive culture for *A. fumigatus* and were analysed further (Figure 1).

Azole resistance prevalence

Azole-resistant isolates were not recovered in 2010, but in the two following years a total of 10 culture-positive patients with resistant isolates were identified (Figure 2). Azole resistance was present in 10 of the 38 culture-positive patients (26%), which corresponds to 7% (10/136) of all patients receiving antifungal treatment in the ICU from January 2010 to December 2013, including culture-negative patients. In all other departments in the hospital, of all patients treated for suspected IA, *A. fumigatus* was cultured in 25 patients. Six of these patients had an azole resistant strain, yielding an azole resistance prevalence of 24% (6/25) (95 % confidence interval (CI): 7–41%), which was seemingly comparable to that in the ICU.

Clinical characteristics of *Aspergillus fumigatus* culture positive patients in the intensive care unit

No differences were found in patient characteristics between the 10 patients with IA by azole-resistant *A. fumigatus* when compared with 28 patients with susceptible isolates (Table 1). Our low number of events (n=10) unfortunately prevented us from doing multivariable analysis.

Underlying disease

In the entire study group encompassing 38 patients with suspected IA in the ICU, 16 underwent a haematopoietic stem cell transplantation and four suffered from haematological malignancy, but were not yet transplanted (Table 1). Three patients had chronic immunosuppressive therapy for solid organ transplants. Other host factors included autoimmune disease (n=6), history of pulmonary disease (n=6: cancer, chronic obstructive pulmonary disease (COPD), irradiation) and the admission diagnoses of the remaining three patients were heart failure, acute liver failure, and severe disseminated infection with *Strongyloides stercoralis*.

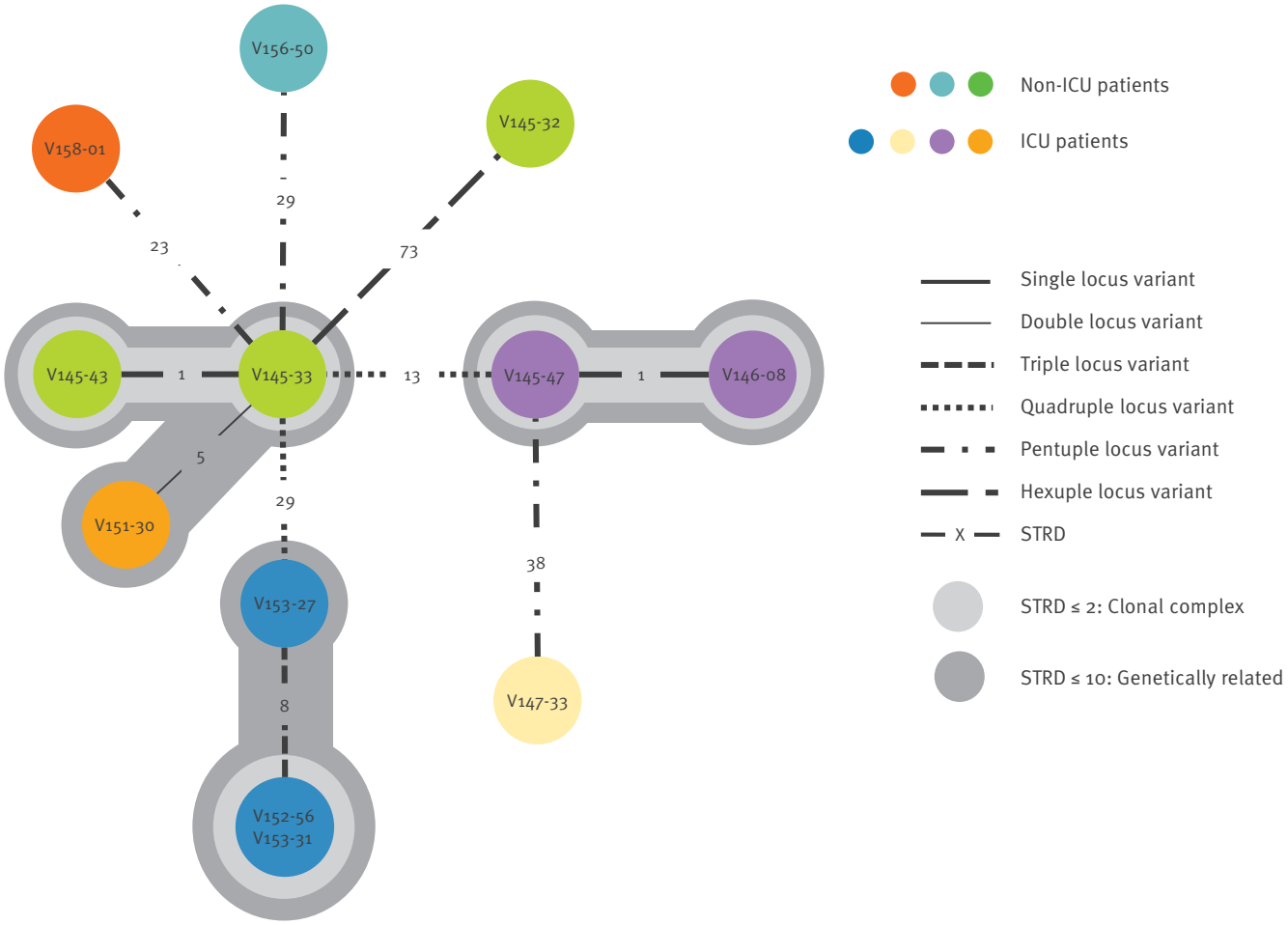
Diagnosing invasive aspergillosis using international criteria

Of 28 patients with an azole-susceptible *A. fumigatus* infection, 15 were classified as probable IA, while proven IA was only established in two. Eleven of 28 patients with an azole-susceptible strain did not fulfil EORTC/MSG criteria of proven, probable or possible IA, most often due to lack of host factor, inconclusive CT scan or inability to perform a CT scan. Of 10 patients with an azole-resistant A. *Fumigatus*, five patients remained unclassified according to the EORTC/MSG criteria; in three cases, CT scans showed unspecific changes and two patients were not stable enough to be transported to the CT scan, but did show bilateral pulmonary infiltrates on the chest X-ray. The other five qualified as probable IA. Overall 22 of 38 patients could be classified according to EORTC/MSG.

Except for one patient, who was on 'Extra Corporeal Membrane Oxygenator' (ECMO) therapy for heart

Figure 3

Microsatellite typing of azole-resistant *Aspergillus fumigatus* isolates, in a university hospital, Netherlands, 2013 (n=7 patients)



ICU: intensive care unit; STRD: sum of tandem repeat difference.

failure, applying the recently published ICU criteria of Blot et al. [16], allowed classification of all patients with an *A. fumigatus* positive culture. ECMO therapy hampers the assessment of *A. fumigatus*-related clinical signs and symptoms (fever, dyspnoea, worsening of respiratory insufficiency), since they are regulated by the device itself. According to this system, a total of two patients, both infected with an azole-susceptible strain, had proven IA. A further twenty-one patients with an azole-susceptible strain qualified as having putative IA as well as all 10 patients with azole-resistant *A. fumigatus*. Only four patients, all with an azole-susceptible *A. fumigatus* isolate, were classified as colonised with *Aspergillus* according to Blot's criteria. For one of these (with a BAL), post-mortem results indicated angio-invasive aspergillosis. For the other three patients, difficult mechanical ventilation prohibited the performance of BAL. After intubation, a tracheal aspirate with a positive *A. fumigatus* culture was however available for these three patients. EORTC criteria would classify one of them as probable invasive pulmonary aspergillosis, while post-mortem findings in another were consistent with angio-invasive aspergillosis. In the fourth patient the diagnosis allergic broncho-pulmonary aspergillosis (ABPA) was made.

Azole prophylaxis

The relative amount of patients receiving prior treatment with azoles appeared higher in the group developing IA due to resistant *A. fumigatus*. The low number of events however prohibited a formal statistical risk assessment (Table 1).

Microbiological characteristics of *Aspergillus fumigatus* culture-positive patients at the intensive care unit

Galactomannan in bronchoalveolar lavage fluids

Overall of the 136 patients treated in the ICU on suspicion of IA, galactomannan testing was performed for 124, mostly on BAL fluids. In 82 of these 124 patients antigen testing was positive.

Table 1

Characteristics of patients with isolates of *Aspergillus fumigatus* susceptible or resistant to azoles, in the intensive care unit of a university hospital, the Netherlands, 2010–2013 (n=38 patients)

Characteristics	Azole-resistant N = 10 n	Azole-susceptible N = 28 n
Mean age in years (range)	52 (24–69)	58 (1–85)
Underlying disease		
Haematological malignancy/stem cell transplant	6	14
Solid organ transplant	1	2
Othera	3	12
Prophylaxis in prior 6 months		
None	3	14
Azoles	6	11
Treatment for IA		
Voriconazole	6	17
Amphotericin	4	4
Switch from azole to amphotericin	0	7
BAL results		
BAL available	10	23b
Galactomannan positive	9	20
EORTC/MSG diagnosis		
Proven	0	2
Probable	5	15
Unclassified	5	11
Diagnosis according to Blot[c]		
Proven	0	2
Putative	10	21
Colonisation	0	4

BAL: bronchoalveolar lavage; EORTC/MSG: European Organization for Research and Treatment of Cancer/Mycoses Study Group; IA: invasive aspergillosis.

[a] This group comprised patients with auto-immune disease (n=6), pulmonary disease (n=6), heart failure (n=1), acute liver failure (n=1), and severe disseminated infection with *Strongyloides stercoralis* (n=1).

[b] BAL was not performed in six patients: two of these patients underwent biopsy, one patient had a positive serum galactomannan and the clinical condition of the three remaining patients did not allow BAL.

[c] One patient on the Extra Corporeal Membrane Oxygenator (ECMO) - device could not be classified.

In 38 of the 136 patients *A. fumigatus* could be cultured. A BAL was not performed in five of the 38 culture-positive patients, since a lung biopsy was available for one patient, serum galactomannan was positive in another neutropenic patient and clinical condition did not allow interventions in three patients. In 33 patients who underwent BAL, 29 had samples positive by galactomannan testing.

Time to detection of resistant strain

In most of the 10 ICU patients found with a resistant strain, the resistant strain was cultured before admission, or shortly after admission at the ICU, whereby the time after ICU admission that it took to be aware of the resistance had a median 1 day and a range of 0 to 20 days. In the six patients with azole-resistant strains at the non-ICU departments, resistant strains could also be cultured shortly after hospital admission (median 4 days; range: 1–71 days) (Table 2).

Resistance mechanisms and microsatellite typing

A cyp51A-mediated azole resistance mechanism was found in all ICU patients (Table 2). Microsatellite typing was available for resistant isolates of seven patients (4 from ICU population, 3 from non-ICU departments), all cultured in 2013 (Figure 3). Isolates of unique patients belonged to identical clonal complexes, although for one patient the presence of two genetically unrelated strains was revealed. These strains differed also phenotypically, with isolates either susceptible or with varying degrees of resistance. Only two patients, one hospitalised in the ICU the other elsewhere, shared a genetically related isolate (Figure 3; STDR < 10), but we did not find an epidemiological link between these patients.

Outcome

The 90-day mortality in 10 patients with an IA by azole-resistant *A. fumigatus* was 100% (with 90% within 30 days after ICU admission), compared with 82% (n=23)

in the 28 patients with a suspected azole-susceptible IA. Given that among the latter, one patient on ECMO was neither classifiable by Blot nor EORTC/MSG methods, and one patient, who was classified as colonised according to Blot might have actually been colonised (since he was diagnosed with BCPA), the 90-day mortality in azole-suceptible IA-group would be 88 % (23/26).

In the group of patients infected with azole-resistant strains, cause of death was IA in all patients, except one. This patient's clinical course was most likely unfavourable due to acute liver failure after a very recent liver transplantation. Of 28 patients infected with azole-susceptible *A. fumigatus,* the cause of death was attributable to IA in seven patients, whereas five patients died of respiratory failure caused by multiple pathogens and 11 patients died of other causes than IA (heart failure, sepsis, liver failure).

In four of 10 patients infected with azole-resistant *A. fumigatus*, azole resistance was detected after the patient had deceased. Hence, only six of 10 patients with a resistant isolate were treated with L-AmB, of which four received treatment for over 2 weeks (Table 1).

Discussion

Our study shows a very high prevalence (10/38) of azole-resistant *A. fumigatus* in patients suspected of IA in the ICU of a university hospital in the Netherlands. This prevalence is even higher (10/33) among patients with putative or proven IA according to the Blot criteria, whereby patients classified as 'colonised' with *A. fumigatus* are excluded. Both prevalence estimates nevertheless appear much higher than those reported in two previously published large Dutch surveillance studies [1-3], where, irrespective of the clinical relevance of the culture result, all *Aspergillus* spp. isolates cultured from clinical samples in five university hospitals were routinely screened for the presence of azole resistance. In the first study covering the 2007 to 2009 period, azole resistance was found in 4.6% (82/1,782) of *A. fumigatus* isolates [3], while in the second study, between 2009 and 2011, this increased to 6.8% (63/921) of such isolates [2].

Prevalence of azole resistance in specific patient groups has only been recently investigated. A published report from another academic centre in the Netherlands, revealed a high incidence of azole resistance (16.2%) in 105 high-risk patients with IA, with an even higher value in the haematology population (25%) and 10% in ICU patients [20]. Similarly, a high azole-resistance rate of 29.6% was demonstrated in *A. fumigatus* isolates from haematological stem cell transplant patients in Germany [6]. Our study also shows high prevalence of resistance in other departments of our hospital.

Although no known risk factors for developing IA by azole-resistant *A. fumigatus* were apparent, one could speculate that patients with IA who are transferred to the ICU are those whose condition deteriorates despite voriconazole treatment in the hospital wards, and thus are more likely to carry a resistant strain. Resistance rates could be overestimated due to the use of antifungal therapy at the time BAL is performed, because azole-susceptible strains are less likely to grow under mold-active therapy. Furthermore, as tertiary stem cell and solid organ transplant centre, our patient population is probably not representative for most ICUs in other hospitals. The conclusions from this study, which are also based on small numbers of patients, therefore cannot be applied to other ICUs with a higher number of immunocompetent patients.

Recognition of IA and start of empirical treatment remains difficult at the ICU. First of all, diagnostic criteria for patients with suspected IA are suboptimal. As demonstrated in this study, EORTC/MSG consensus definitions for IA are not generally applicable for ICU-patients: only 22 of 38 culture-positive patients could be classified. Second, new categories of ICU patients susceptible to invasive pulmonary aspergillosis, are increasingly reported [11,12,21]. Since these new categories of ICU patients lack the host-factors defined by EORTC/MSG consensus definition, new criteria have been proposed for ICU-patients [11,16,21]. Blot et al. suggested a different algorithm that appears to be more suitable, although some patient categories at risk, for instance liver failure, COPD, and solid organ cancer, are not included in these diagnostic criteria [11,16]. When applying the criteria of Blot to our group of 38 ICU patients, 37 of the patients could be categorised, though in two patients, classified as 'colonisation', post-mortem findings were congruent with angio-invasive aspergillosis, and one would be diagnosed probable IA, if one would use EORTC/MSG criteria, illustrating the restraints of this scoring system. Hence, development of better clinical criteria for diagnosing IA, especially in ICU patients, is warranted [16].

Multiple mechanisms can be considered with regard to the development of azole resistance in *A. fumigatus*. In our study we found a short interval from admission to detection of a resistant strain (median 3 days, mean 8 days; range: 0–71days). Moreover, molecular typing, did not reveal genetically-related strains, suggesting different sources of infection, instead of an outbreak of azole-resistant *A. fumigatus* or patient-to-patient transmission. This could imply that acquirement of resistant strains could have occurred before hospital admission. Some reports suggests an increase of prevalence of resistant *A. fumigatus* strains in the environment [2,22]. The use of fungicides in the agricultural sector might contribute to this phenomenon [1]. Other reports suggest development of azole resistance during therapy. A review regarding this subject reports a median time for development of resistance under therapy of 4 months, with a range of 3 weeks to 23 month [23]. In our study the number of events was too small to rule out prior treatment or prophylaxis with azoles

Table 2

Characteristics of patients infected with azole-resistant *Aspergillus fumigatus* in a university hospital, Netherlands, 2010–2013 (n=16)

Patient number[a]	Predisposing condition	EORTC classification	Time in days from admission to finding a culture of resistant *A. fumigatus*	Underlying lung disease	Neutropenia at admission	Azole pre-treatment	MIC[b] (mg/L)			Resistance mechanism
							Itra.[b]	Vori.[b]	Posa.[b]	
ICU population										
1	Liver transplant	Unclassified	4	No	No	No	16	16	1	TR_{34}/L98H
2	Autoimmune hepatitis (prednisone)	Unclassified	0	No	No	No	>16	>16	1	TR_{46}/T289A/Y121F
3	Allogeneic SCT	Unclassified	1	No	No	NA	>16	>16	1	TR_{46}/T289A/Y121F
4	Allogeneic SCT	Probable	20	No	No	No	16	16	1	TR_{34}/L98H
5	Haematological malignancy	Probable	1	No	No	No	16	16	1	TR_{46}/T289A/Y121F
6	Allogeneic SCT	Probable	0	No	No	>6 months	>16	8	1	TR_{34}/L98H
7	NSCLC (prednisone)	Probable	0	NSCLC	No	No	16	4	0.5	TR_{34}/L98H
8	COPD (prednisone)	Unclassified	6	COPD	No	No	>16	>16	1	TR_{34}/L98H
9	Allogeneic SCT	Probable	0	No	Yes	>3 months	16	8	1	TR_{34}/L98H
10	Allogeneic SCT	Unclassified	5	No	No	11 days	16	8	2	TR_{34}/L98H
Non-ICU population										
1	Allogeneic SCT	Probable	71	Bronchiectasis	No	No	16	0.5	8	NA
2	Waldenström's macroglobulinaemia	Unclassified	8	No	No	No	>16	0.5	4	NA
3	COPD (prednisone)	Unclassified	3	COPD GOLD stage IV	No	No	>16	4	>16	NA
4	Allogeneic SCT	Probable	1	No	No	>4 weeks	16	1	16	NA
5	Autoimmune hepatitis (prednisone)	Probable	3	Influenza	No	No	2	1	>16	TR_{46}/T289A/Y121F
6	Autoimmune vasculitis (prednisone)	Probable	4	ANCA positive vasculitis	Yes	No	>16	0.5	>16	Not detected

ANCA: antineutrophil cytoplasm antibodies; COPD: chronic obstructive pulmonary disease; GOLD: Global Initiative for Chronic Obstructive Lung Disease; ICU: intensive care unit; EORTC: European Organization for Research and Treatment of Cancer; MIC: minimum inhibitory concentration; NA: not available; NSCLC: non-small cell lung carcinoma; SCT: stem cell transplant.

[a] The patient number is assigned consecutively within each patient population (i.e. ICU or non-ICU) so patients belonging to separate population groups but with the same number are different patients.

[b] MICs were evaluated for itraconazole (itra.), voriconazole (vori.), or posaconazole (posa.).

as a risk factor. Finally, other studies suggest the concomitant presence of both susceptible and resistant strains in patient samples [24], resulting in a selection of resistant strains during therapy. It is not unlikely that all of the mechanisms mentioned above contribute to an observed increase of resistance in patients with *A. fumigatus* infections, making it difficult to predict which patients are carriers of a resistant strain.

Additionally, identifying IA with azole-resistant *A. fumigatus* is still dependent on culture-positivity and technical facilities. From the 136 patients initially suspected of IA in the ICU, 124 underwent the galactomannan test, which was positive for 82 (66%). In contrast *A. fumigatus* could only be successfully cultured in 28% (38/136) of patients suspected of IA in the ICU. In this culture positive group BAL galactomannan was more frequently positive (29/33). Hence a negative culture may not necessarily mean the absence of IA.

Currently, the presence of resistance can be established only in culture-positive patients. In culture-positive patients, detection of azole resistance is often delayed, since MIC-testing of *A. fumigatus* is not routinely performed in clinical microbiology laboratories. Reference methods such as the EUCAST method require a mature culture and testing results are available 5 to 7 days after the culture has become positive. Screening of isolates with four-well plates has been shown to be useful and gives results within 24 to 48 hours, but this method is not commercially available [1,3].

Direct molecular testing for azole resistance in patients with high clinical suspicion and a positive antigen testing of BAL fluid appears to be the most promising method for early detection of azole-resistant *A. fumigatus* [25-27]. However, there are several technical issues to be resolved, such as the presence of single copy gene mutations of the cyp51A gene limiting the sensitivity of PCR detection. The most frequent found azole resistance mechanism in *A. fumigatus* is a mutation in the target protein [2,5]. The cyp51A gene encodes lanosterol 14α-demethylase that catalyses a step in the biosynthetic pathway of ergosterol (an essential

cell membrane component of filamentous fungi). Both TR34/L98H, conferring cross-resistance to voriconazole and posaconazole and TR46/T289A/Y121F, conferring high level voriconazole resistance, were present in our patients' isolates. These findings in the ICU are in line with surveillance studies in the Netherlands, indicating that environmental azole resistance mechanisms (i.e. TR34/L98H and TR46/Y121F/T289A) are the most common cause of azole resistance, accounting for 80 to 90% of resistance mechanisms [2].

High resistance rates urge us to explore alternative (empirical) treatment strategies for IA [28]. These include first-line treatment with a polyene or with combination therapy. The clinical experience with these alternative treatment options is very limited, but animal models showed similar efficacy of L-AmB in IA by azole-susceptible and azole-resistant isolates, independent of the underlying resistance mechanism [29]. The combination of voriconazole and anidulafungin has also been shown to be effective [30]. MIC-guided high dosing of azoles is still under investigation, but is probably of limited use due to adverse events [31,32].

In conclusion, we observed very high rates of azole resistance *A. fumigatus* in culture-positive ICU patients with IA and very high mortality rates among such patients. Generalising our results requires caution, though recent reports suggest that similar trends are observed among other patient groups [20,25]. There is an urgent need to identify molecular markers for resistance in *A. fumigatus* other than the cyp51A-gene in order to enable the development of rapid molecular tools. Development of adequate diagnostic criteria for early diagnosis and reliable classification remains a challenge in IA, especially in the ICU setting.

Conflict of interest

None declared.

Authors' contributions

Judith van Paassen contributed to design, acquisition of clinical data, data analysis, writing and revising of the manuscript, and takes responsibility for the integrity of the data and the accuracy of the data analysis, and serves a first author. Anne Russcher contributed to study design, acquisition of relevant microbiological data, data analysis and writing and revising the manuscript. Astrid in 't Veld - van Wingerden contributed to acquisition of relevant clinical data, data analysis and writing and revising the manuscript. Paul Verweij contributed to acquisition of microbiological data regarding resistance, and writing and revising the manuscript. Eduard Kuijper contributed to study design, acquisition of relevant microbiological data, data analysis, and writing and revising the manuscript.

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Research article

Influenza epidemiology, vaccine coverage and vaccine effectiveness in children admitted to sentinel Australian hospitals in 2014: the Influenza Complications Alert Network (FluCAN)

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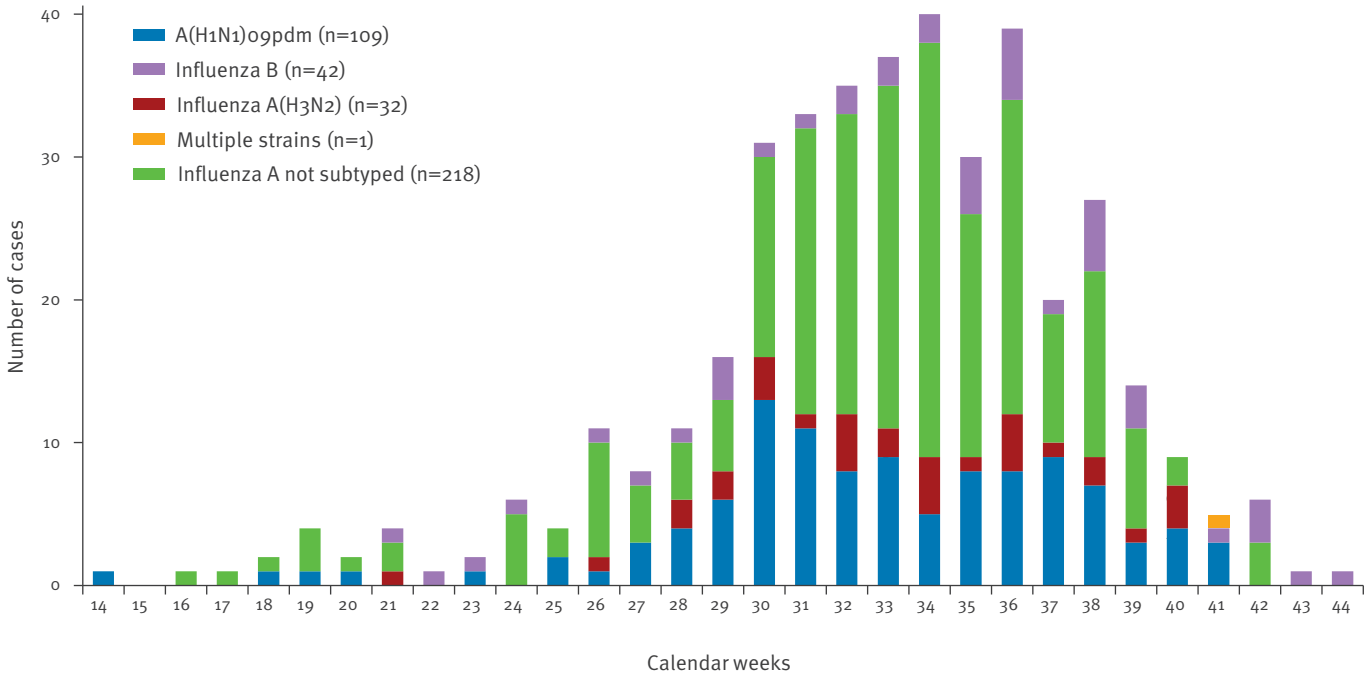
The Influenza Complications Alert Network (FluCAN) is a sentinel hospital-based surveillance programme operating in all states and territories in Australia. We summarise the epidemiology of children hospitalised with laboratory-confirmed influenza in 2014 and reports on the effectiveness of inactivated trivalent inactivated vaccine (TIV) in children. In this observational study, cases were defined as children admitted with acute respiratory illness (ARI) with influenza confirmed by PCR. Controls were hospitalised children with ARI testing negative for influenza. Vaccine effectiveness (VE) was estimated as 1 minus the odds ratio of vaccination in influenza positive cases compared with test-negative controls using conditional logistic regression models. From April until October 2014, 402 children were admitted with PCR-confirmed influenza. Of these, 28% were aged < 1 year, 16% were Indigenous, and 39% had underlying conditions predisposing to severe influenza. Influenza A was detected in 90% of cases of influenza; influenza A(H1N1)pdm09 was the most frequent subtype (109/141 of subtyped cases) followed by A(H3N2) (32/141). Only 15% of children with influenza received antiviral therapy. The adjusted VE of one or more doses of TIV for preventing hospitalised influenza was estimated at 55.5% (95% confidence intervals (CI): 11.6–77.6%). Effectiveness against influenza A(H1N1)pdm09 was high (91.6% , 95% CI: 36.0–98.9%) yet appeared poor against H3N2. In summary, the 2014 southern hemisphere TIV was moderately effective against severe influenza in children. Significant VE was observed against influenza A(H1N1)pdm09.

Introduction

Influenza is a common respiratory viral infection that affects up to 5–10% of the population each year [1]. Previous studies demonstrate that young children have the highest rate of hospitalisation [2]. A national sentinel surveillance programme for severe influenza was established in Australia in 2009, primarily to monitor hospitalisations in adults with confirmed influenza: the Influenza Complications Alert Network (FluCAN). Given the significant burden of disease in young children and the important role that children play in introducing and spreading influenza virus in the household and the community [3], paediatric influenza surveillance provides public health authorities with important and timely information on disease severity in the early phase of the winter respiratory virus season. Hospital-based sentinel surveillance enables

Figure 1

Date of admission in children hospitalised with confirmed influenza, epidemiological cohort, Influenza Complications Alert Network, Australia, April to October 2014 (n=402)



detailed information on the severity of illness to be collected, and complements community- and primary care-based surveillance systems. Comprehensive nationwide clinical data were collected from Australian children admitted to six tertiary paediatric hospitals during the pandemic in 2009 [4]. However, from 2010 to 2013, insufficient numbers of children were prospectively enrolled in existing surveillance programs to ascertain paediatric seasonal influenza activity and severity in Australia. Two tertiary paediatric hospitals (from the separate Paediatric Active Enhanced Disease Surveillance network (PAEDS) [5]) were included in the existing FluCAN sentinel system in 2014.

The Australian Technical Advisory Group on Immunisation (ATAGI) recommends influenza vaccination in all children 6 months and older, yet in 2014, influenza vaccine was only provided free of charge under the National Immunisation Programme (NIP) for children with comorbidities that predispose them to severe outcomes following influenza infection [6]. In Western Australia, a state funded programme has provided free influenza vaccine to all children between 6 months and 5 years of age from 2008 [7-9]. Four brands of inactivated unadjuvanted trivalent influenza vaccine (TIV) were available for use in Australian children: more than 80% of vaccine administered to children in Australia was Vaxigrip or Vaxigrip junior (Sanofi-Pasteur Pty Ltd; personal communication, Brynley Hull, October 2015). Live attenuated and quadrivalent influenza vaccines were not available in Australia in 2014.

Previous studies have demonstrated that inactivated influenza vaccine is protective against influenza [10, 11], yet have concluded that insufficient evidence exists to confirm the effectiveness in the very young. The Western Australian Influenza Vaccine Effectiveness (WAIVE) study has previously estimated vaccine effectiveness (VE) of TIV in children aged 6 to 59 months attending a paediatric emergency department against any laboratory-confirmed influenza at 64.7% (95% confidence interval (CI): 33.7–81.2%) [7]. Insufficient numbers of hospitalised children have been enrolled in this and similar paediatric VE studies to generate robust estimates against hospitalisation. Cowling et al. estimated VE against hospitalisation with laboratory-confirmed influenza to be 61.7% (95% CI: 43.0–74.2%) in Hong Kong (2009–2013) [12].

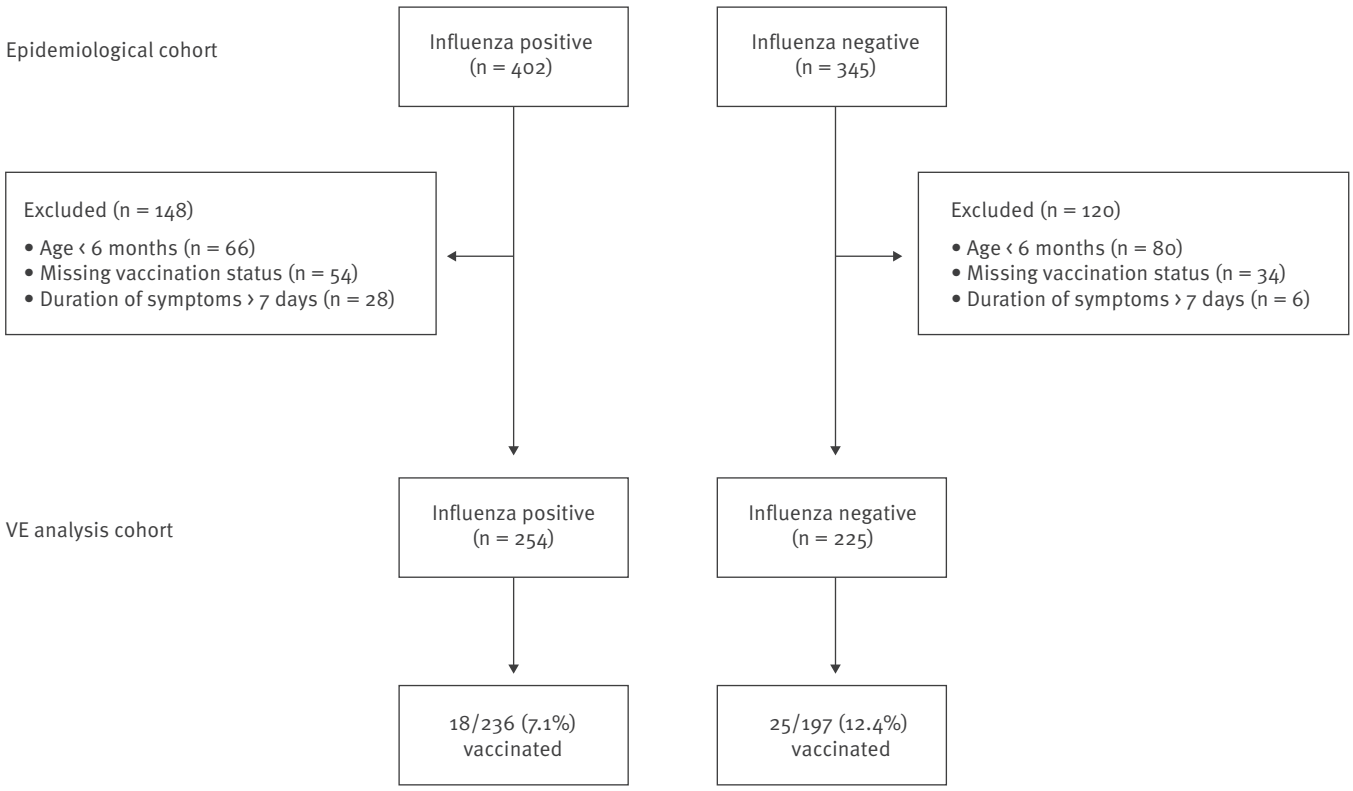
With nearly 70,000 notifications of laboratory-confirmed influenza, the incidence of disease in 2014 was high compared with previous seasons [13]. Virological surveillance of circulating strains suggested influenza A(H1N1)pdm09 predominated across most jurisdictions throughout the season, but influenza A(H3N2) was predominant in New South Wales and the Australian Capital Territory [14]. In this report, we describe the epidemiology of hospitalisation in children with confirmed influenza and report on VE estimates for the 2014 southern hemisphere inactivated TIV.

Methods

FluCAN is a national hospital-based sentinel surveillance system [15]. In 2014, surveillance was expanded to include two large specialty paediatric hospitals:

Figure 2

Flowchart of children included in epidemiological and vaccine effectiveness cohorts, Influenza Complications Alert Network, Australia, April to October 2014 (n=747)



VE: vaccine effectiveness.

Children's Hospital at Westmead (New South Wales) and the Princess Margaret Hospital for Children (Western Australia). In addition, paediatric cases from the other 15 participating sites were also included: Canberra Hospital (ACT), University Hospital Geelong (VIC), Princess Alexandra Hospital (QLD), Cairns Base Hospital (QLD), and Alice Springs Hospital (NT) contributed cases. Ethics approval has been obtained at all participating sites, at Monash University and the Australian National University.

An influenza case was defined as a paediatric patient (<16 years) admitted to hospital with an acute respiratory illness (ARI) and with influenza confirmed by PCR. Influenza testing was initiated by clinicians based on clinical indications and local guidelines. All influenza cases were confirmed using real-time reverse transcriptase PCR assays using standard primers. All tests were performed in local or referral laboratories accredited by the National Association of Testing Authorities. An ARI was defined by the presence of new respiratory symptoms including cough and rhinorrhoea. A hospital admission was defined as requiring inpatient care outside of the emergency department.

Under FluCAN, surveillance is conducted during the southern hemisphere influenza season (i.e. April to October with follow up continuing to the end of November each year). Admission to an intensive care unit (ICU), including high dependency unit (HDU), was also recorded. The presence of risk factors predisposing to severe outcomes following influenza infection including ethnicity (Indigenous or non-Indigenous Australian) and the presence of underlying conditions (hereafter referred to as comorbidities) was ascertained from the patient's medical record [6]. Comorbidities assessed included congenital heart disease, chronic respiratory and neurological disorders, immunocompromising conditions or immunosuppression, Down syndrome and chronic illnesses such as diabetes mellitus and renal failure [6].

We examined factors associated with ICU admission and the length of hospital stay (LOS) using multivariable regression. Factors associated with ICU admission were determined using a logistic regression model, with factors retained in the multivariable model if $p<0.20$. Factors associated with LOS were modelled using a linear regression, as the mean duration of stay was the parameter of interest. Standard errors were estimated using bootstrapping (1,000 replicates) to correct for heteroskedasticity.

Table 1

Demographic characteristics of children hospitalised with confirmed influenza, epidemiological cohort, Influenza Complications Alert Network, Australia, April to October 2014 (n=402)

	Influenza type				Total influenza positive cases
	A(H1N1A(H1N1)09pdm	A(H3N2)	A not subtyped	B	
Number of children	109	32	218	42	402[a]
Age group					
Neonate<28 days	2 (1.8%)	0 (0.0%)	5 (2.3%)	0 (0.0%)	7 (1.7%)
Infant 28 days to 1 year	29 (26.6%)	9 (28.1%)	60 (27.5%)	8 (19.0%)	107 (26.6%)
1–5 years	40 (36.7%)	16 (50.0%)	94 (43.1%)	15 (35.7%)	165 (41.0%)
5–9 years	23 (21.1%)	5 (15.6%)	34 (15.6%)	12 (28.6%)	74 (18.4%)
10–16 years	15 (13.8%)	2 (6.3%)	25 (11.5%)	7 (16.7%)	49 (12.2%)
Male	62 (56.9%)	16 (50.0%)	107 (49.1%)	24 (57.1%)	209 (52.0%)
Indigenous	9 (8.3%)	3 (9.4%)	48 (22.0%)	3 (7.1%)	63 (15.7%)
Hospital					
Alice Springs	0 (0.0%)	0 (0.0%)	43 (19.7%)	5 (11.9%)	48 (11.9%)
Canberra	15 (13.8%)	14 (43.8%)	0 (0.0%)	2 (4.8%)	31 (7.7%)
Cairns Base	4 (3.7%)	0 (0.0%)	10 (4.6%)	3 (7.1%)	17 (4.2%)
Children›s Hospital, Westmead	0 (0.0%)	0 (0.0%)	135 (61.9%)	16 (38.1%)	151 (37.6%)
Geelong Hospital	0 (0.0%)	0 (0.0%)	21 (9.6%)	0 (0.0%)	22 (5.5%)
Princess Alexandra	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.4%)	1 (0.2%)
Princess Margaret	90 (82.6%)	18 (56.3%)	9 (4.1%)	15 (35.7%)	132 (32.8%)

[a] One child with disease due to multiple subtypes included in total

Estimation of vaccination coverage and effectiveness

Vaccination status was obtained from the medical record, by parental report and confirmed, in children<7 years of age, on the Australian Childhood Immunisation Register (ACIR). In those 10 years and older, 'fully immunised' was defined by receipt of one dose of 2014 TIV more than 2 weeks before presentation. In children age<10 years, 'fully immunised' was defined as either (i) two doses of TIV at least 21 days apart and at least 2 weeks before presentation or (ii) one dose of TIV at least 2 weeks before presentation and receipt of at least one TIV dose in a previous year [6]. 'Partially vaccinated' children were those aged<10 years receiving only one dose of vaccination in 2014 without receipt of TIV in previous years. 'Unvaccinated' children where those not receiving TIV in 2014 or receiving the vaccine less than 2 weeks before presentation.

Vaccination coverage was estimated in patients›6 months of age admitted with ARI who tested negative to influenza by PCR. We used an incidence density test negative design to estimate VE, where controls were selected from influenza-test negative subjects with ARI tested contemporaneously with a case: controls could be test-negative for all pathogens or have an alternative pathogen detected [16-18]. VE was estimated as 1 minus the odds ratio (OR) of vaccination in influenza-positive cases compared with test-negative control patients using methods previously described [15,19]. Only children›6 months of age and tested within 7 days of admission were included in VE estimates. A conditional logistic regression model using influenza case status as the dependent outcome was constructed from influenza vaccination and adjusted for potential confounders (age group<2 years and comorbidities). The regression was stratified on site, except for the models that considered VE against H1N1 due to small numbers. Models that included more age groups (<1 year, 1–4 years, 5–9 years and≥10 years,) and Indigenous status as adjusting variables were considered in sensitivity analyses. In addition, VE estimates excluding children with duration of symptoms of›7 days (as opposed to restriction the analysis to who underwent testing within 7 days) were performed. These adjustments had minimal effect (<3%) on VE estimates and thus were dropped from the final model. Analyses were performed using Stata 13 for Windows (College Station, Texas, US).

Results

During the period 3 April to 31 October 2014, 402 children were admitted with PCR-confirmed influenza to seven of 17 sentinel hospitals, including 283 admissions to the two specialist paediatric hospitals, and 119 admissions to five non-specialist hospitals (Table 1). The peak rate of admission was in late August (Figure 1). Of these 402 children, 114 (28%) were<1 year of age, 63 (16%) were Indigenous Australians, and 155 (39%) had underlying comorbidities (Table 1; Table 2).

Table 2

Risk factors, severity and outcomes in children hospitalised with confirmed influenza, epidemiological cohort, Influenza Complications Alert Network, Australia, April to October 2014 (n=402)

	Not admitted to ICU	Admitted to ICU	Total
Total	**356**	**46**	**402**
Age group			
Neonate<28 days	5 (1.4%)	2 (4.3%)	7 (1.7%)
Infant 28d - 1 year	93 (26.1%)	14 (30.4%)	107 (26.6%)
1–4 years	147 (41.3%)	18 (39.1%)	165 (41.0%)
5–9 years	68 (19.1%)	6 (13.0%)	74 (18.4%)
10–15 years	43 (12.1%)	6 (13.0%)	49 (12.2%)
Smoking	4 (1.1%)	2 (4.3%)	6 (1.5%)
Others smoking in the household	21 (5.9%)	5 (10.9%)	26 (6.5%)
Chronic medical comorbidities	127 (35.7%)	28 (60.9%)	155 (38.6%)
Chronic respiratory disease	38 (10.7%)	12 (26.1%)	50 (12.4%)
Prematurity	33 (9.3%)	12 (26.1%)	45 (11.2%)
Chronic cardiac disease	21 (5.9%)	3 (6.5%)	24 (6.0%)
Diabetes	4 (1.1%)	1 (2.2%)	5 (1.2%)
Chronic neurological disease	26 (7.3%)	7 (15.2%)	33 (8.2%)
Chronic renal disease	10 (2.8%)	4 (8.7%)	14 (3.5%)
Immunosuppressed	35 (9.8%)	4 (8.7%)	39 (9.7%)
Chronic liver disease	7 (2.0%)	3 (6.5%)	10 (2.5%)
Genetic abnormality	28 (7.9%)	10 (21.7%)	38 (9.5%)
Inborn error of metabolism	4 (1.1%)	3 (6.5%)	7 (1.7%)
Chronic aspirin use	4 (1.1%)	0 (0.0%)	4 (1.0%)
Residential care	1 (0.3%)	1 (2.2%)	2 (0.5%)
Influenza vaccination	11/242 (4.5%)	4/35 (11.4%)	15/277 (5.4%)
Influenza subtype			
A(H1N1)09pdm	92 (25.8%)	17 (37.0%)	109 (27.1%)
A(H3N2)	24 (6.7%)	8 (17.4%)	32 (8.0%)
A not subtyped	199 (55.9%)	19 (41.3%)	218 (54.2%)
B	40 (11.2%)	2 (4.3%)	42 (10.4%)
multiple strains	1 (0.3%)	0 (0.0%)	1 (0.2%)
Mortality	0/317 (0.0%)	1/41 (2.4%)	1/358 (0.3%)

Presentation and treatment

In 395 patients with influenza where the duration of symptoms was known, the median duration of symptoms before admission was 3 days (interquartile range (IQR): 2, 5 days). Only 64 (15%) of patients with influenza, received oseltamivir; of these, 24 patients were known to have received oseltamivir within 48 hours of symptom onset.

Admission to intensive care

Of all influenza cases, 40 (10%) were initially admitted to intensive care (ICU) and a further six (1%) patients were subsequently transferred to ICU after initial admission to a general ward. The presence of comorbidities was associated with intensive care admission: OR 2.80 (95% CI: 1.49–5.27, p=0.001). Influenza B appeared associated with a lower risk of admission to ICU but this difference was not statistically significant: OR 0.36 (95% CI: 0.08–1.53, p=0.16). In a multivariate model, only the presence of one or more comorbidity was associated with ICU admission (Table 3).

Outcome

The mean LOS of all patients was 3.7 days. The presence of comorbidities was associated with an increase in mean hospital length of stay of 2.6 days. Other factors associated with prolonged length of stay included ICU admission and being Indigenous but these differences were not statistically significant (data not shown). The duration of hospital stay was similar in patients that received antivirals within 48 hours of symptom onset (median: 2.5 days; IQR: 2, 6 days), compared with those who received antivirals more than 48 hours after symptom onset (median: 4 days; IQR: 1, 7 days) and who did not receive antivirals (median: 2 days; IQR: 1, 3 days).

One in-hospital death was reported, in a 13-year-old boy with no known comorbidities.

Table 3

Factors associated with admission to intensive care in patients hospitalised with confirmed influenza, epidemiological cohort, Influenza Complications Alert Network, Australia, April to October 2014 (n=402)

Variable	Crude OR (95% CI)	p value	AOR (95% CI)	p value
Infant<12 months	1.40 (0.73, 2.69)	0.306	1.86 (0.94, 3.69)	0.076
Medical comorbidities	2.80 (1.49, 5.27)	0.001	3.20 (1.66, 6.16)	0.001
Indigenous Australian	0.79 (0.32, 1.94)	0.603	NI	NA
Influenza type				
Influenza A	1 (referent)		1 (referent)	
Influenza B	0.36 (0.08, 1.53)	0.166	NI	NA

AOR: adjusted odds ratio; CI: confidence interval; NA: not applicable; NI: not included in final model; OR: odds ratio.

Vaccine coverage

Vaccine coverage for all children>6 months of age, as shown in Figure 2, was low. Of the 225 children who tested negative for influenza within 7 days of onset of illness, 28 children had received at least one dose of vaccine in 2014 (estimated full or partial vaccine coverage: 12.4%). Eighteen children were regarded as fully vaccinated (estimated full coverage: 8.0%) Of those with comorbidities (eligible to receive influenza vaccine under the NIP), only 16 of 89 children had received at least one dose of vaccine in 2014 (estimated full or partial coverage: 18.0%), of whom only nine children were regarded as fully vaccinated (estimated full coverage: 10.1%).

Vaccine effectiveness

In children aged>6 months, the crude VE of full or partial vaccination (i.e. children who received at least one dose of vaccine in 2014) was estimated as 48.8% (95% CI: 1.1–73.5%; Table 4). After adjusting for age group and comorbidities, the adjusted full/partial VE was estimated as 55.5% (95% CI: 11.6–77.6%). VE differed by infecting strain (Table 4) with poor VE against circulating influenza A(H3N2) noted. Only one child with A(H1N1) infection was partially vaccinated with no vaccine breakthrough cases in fully vaccinated children identified: adjusted fully/partial VE estimate for A(H1N1) was 91.6% (95% CI: 36.0–98.9%).

In children aged>6 months, the crude VE based on children who were regarded as fully vaccinated in 2014 was estimated as 30.5% (95%CI -45.7 to 66.8%). After adjusting for age group (age<2 years), and chronic medical comorbidities, the adjusted VE was estimated as 41.1% (95% CI: -26.7 to 72.6%).

Discussion

Inclusion of two tertiary paediatric hospitals (from the separate Paediatric Active Enhanced Disease Surveillance network; PAEDS [5]) into the existing FluCAN sentinel system has allowed us to report on influenza in 3,400 hospitalised children and adults in 2014 (unpublished data), inclusive of metropolitan and regional hospitals, specialist paediatric and adult hospitals and hospitals in tropical and subtropical regions. By collecting data on control patients with ARI who tested negative for influenza, vaccine coverage (particularly in vulnerable patients) and VE against severe influenza can also be accurately estimated [20]. Here we report the first significant VE estimate against hospitalised influenza in Australian children.

In 2014, we recorded over 400 paediatric admissions in the FluCAN system. When compared with children with influenza requiring hospitalisation in 2009 (n=601 across six paediatric hospitals), a number of similarities and differences were identified. In both cohorts, more than 50% of children did not have any underlying comorbidities, highlighting that healthy children form a significant proportion of those requiring hospital admission. Indigenous Australians are at increased risk of hospital admission with influenza; national hospitalisation discharge data indicate that indigenous children aged <5 years are hospitalised more than twice as frequently with influenza compared with their non-indigenous peers [21]. This finding has prompted the inclusion of Indigenous children<5 years of age as eligible for NIP-funded influenza vaccination from 2015 onwards. The higher proportion of indigenous children enrolled in this study in 2014 (16.0% vs 4.5% in 2009) needs to be interpreted with caution as recruitment from sites with sizable indigenous populations (e.g. Alice Springs Hospital) occurred in 2014 and not in 2009. The proportion of Indigenous children with influenza in the study (excluding those admitted to Alice Springs Hospital) was 6.5% (23/354). This is compared with the national average of 4.4% [22].

For all children, similar outcomes were observed in 2014 compared with 2009, respectively: 11.4% and 9.9% of children were admitted to ICU, and mortality was 0.3% and 0.9% respectively. Despite the availability of free vaccine through the NIP for children with comorbidities from 2010, uptake of seasonal TIV in those at greatest risk has not significantly changed since 2009: in 2014 only 21.0% of controls with comorbidities were vaccinated compared with 18.4% in 2009 [4]. Another striking difference is the infrequent use of antiviral medications in 2014 compared with the pandemic year, 2009 (15% vs 47%). The effectiveness of

Table 4

Estimated vaccine effectiveness against hospitalisation with influenza in children aged > 6 months (vaccine effectiveness cohort), Influenza Complications Alert Network, Australia, April to October 2014

Strains	Number of cases and controls				Unadjusted VE (95% CI)	Adjusted VE[a] (95% CI)
	Vaccinated cases	Unvaccinated cases	Vaccinated controls	Unvaccinated controls		
Vaccinated cases inclusive of fully and partially vaccinated children						
All strains[b]	18	236	28	197	48.8% (1.1%, 73.5%)	55.5% (11.6%, 77.6%)
H1N1	1	72	28	197	90.2% (26.9%, 98.7%)	91.6%[c] (36.0%, 98.9%)
H3N2	13	90	28	197	6.2% (-110.7%, 58.2%)	-4.0% (-138.9%, 54.7%)
B	2	22	28	197	66.0% (-163.3%, 95.6%)	65.0% (-179.4%, 95.6%)
Vaccinated cases inclusive of fully vaccinated cases only						
All strains[b]	15	236	18	197	30.5% (-45.7%, 66.8%)	41.1% (-26.7%, 72.6%)
H1N1	0	72	18	197	100%	100%[c]
H3N2	11	90	18	197	3.5% (-154.1%, 63.4%)	-13.6% (-204.1%, 57.6%)
B	2	22	18	197	47.3% (-317.0%, 93.3%)	51.5% (-294.4%, 94.0%)

CI: confidence intervals; VE: vaccine effectiveness.

[a] adjusted for age›2 years, and comorbidities

[b] Inclusive of patients with untyped influenza A infection, H1N1, H3N2 and influenza B.

[c] 1 patient with A(H1N1) was partially vaccinated and none fully vaccinated. Non-conditional logistic regression used

oseltamivir in children and adults with influenza has recently been debated following meta-analyses by Jefferson et al. and Dobson et al. with conflicting methods, results and conclusions [23,24]. Data pooled by Jefferson et al. demonstrates that oseltamivir reduces the length of symptoms by 29 hours (95% CI: 12 to 47 hours; p=0.001) at the expense of increased rates of vomiting in children [23]. Despite no appreciable difference in complications or hospitalisation being noted, the numbers of children in both the intervention and control arms of these analyses are very small. Given the current evidence, oseltamivir is most likely to benefit patients at high risk of hospitalisation and patient with influenza requiring hospitalisation [25]. Future work should focus on ways to improve both vaccine uptake and antiviral use, particularly among children with comorbidities or other risk factors for severe influenza.

VE estimates are now generated using test-negative design in multiple populations to guide vaccine strain choice. Existing southern-hemisphere systems and VE studies have either focused on children (and adults) presenting for outpatient or emergency care [7,26,27] or enrolled insufficient numbers of children to generate robust estimates for hospitalised influenza in children, particularly in any single influenza seasons [9,26,27]. The addition of large paediatric sites to the FluCAN network, has enabled calculation of VE estimates against hospitalised influenza for children aged‹16 years in a single season. Moreover, the VE point estimate (55.5% (95% CI: 11.6–77.6%)) is comparable to that observed in hospitalised adults (51.5% (95% CI: 41.6–59.7%), unpublished data), albeit with less precision. Restricting the estimate to those fully vaccinated resulted in a lower point estimate (41.1% (95% CI: -26.7–72.6%)) but given the small numbers of vaccinated cases and controls and wide confidence intervals, this result needs to be interpreted with caution, Similar differences in VE between different influenza strains were also observed (data not shown). The addition of data from more paediatric hospitals, or over subsequent seasons, would assist in providing VE estimates against specific influenza strains and in subgroups of interest, for example the children aged 6 months to 2 years in whom data on VE is sparse.

There are a number of limitations to this study. The decision to test was left to the treating clinician using local guidelines. The impact of this is expected to be small as influenza tests are routinely recommended for infection control purposes in children requiring hospital admission with acute respiratory symptoms. It remains possible that the decision to test might have been influenced by vaccination status. As in all observational studies, a biased estimate of VE may result from unmeasured confounding or misascertainment of vaccination status or outcome. Case ascertainment was likely incomplete due to the underutilisation of influenza laboratory testing by treating clinicians, despite the diagnosis of influenza having implications for infection control and antiviral use in hospitals.

Delayed presentations or secondary bacterial pneumonia may be associated with false negative influenza tests as the influenza infection may be cleared at the time of presentation. Influenza subtyping was not available for the majority (55%) of patients, thereby limiting our ability to determine the relative burden of influenza A types and calculate accurate VE estimates by strain. Furthermore, the antigenic characteristics of influenza viruses from cases was not performed and as such we are unable to determine the relatedness of circulating strains with influenza strains included in the 2014 seasonal vaccine. The inability to determine vaccination status in all children was a limitation although no significant differences were noted when influenza status and risk factors of those with known vaccination status were compared with children with unknown vaccination status (data not shown). Low vaccine uptake was a major limitation impacting on our ability to more precisely calculate VE.

In summary, we describe more than 400 children hospitalised with seasonal influenza in Australia, of whom 10% required ICU admission. Influenza A was detected in 90% of cases with influenza A(H1N109)pdm the most frequent subtype. Vaccine uptake in those with and without comorbidities remains poor. Use of influenza antivirals in children is infrequent. TIV appeared moderately effective against hospitalisation with any influenza in 2014, but was more effective against the influenza A(H1N109)pdm subtype.

The FluCAN network and PAEDS group

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Conflict of interest

Dr Blyth has received salary supported from a WA Health / Raine Medical Clinical Research Fellowship.

Authors' contributions

Drs Blyth, Macartney, Hewagama, Senenayake, Friedman, Simpson and Upham supervised recruitment of children at their respective sites. Drs Cheng, Kotsimbos and Kelly established the FluCAN network. Drs Blyth and Cheng undertook the analysis and drafted the manuscript. All authors reviewed the manuscript prior to submission.

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