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Increase in scarlet fever notifications in the United Kingdom, 2013/2014

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Increases in scarlet fever above usual seasonal levels are currently being seen across the United Kingdom. Medical practitioners have been alerted to the exceptional increase in incidence. Given the potential for this to signal a population increase in invasive group A streptococcal disease, close monitoring of invasive disease is essential.

Scarlet fever notifications September 2013 to March 2014

In the United Kingdom (UK), statutory notifications of scarlet fever are submitted to local health protection teams in England, Wales and Northern Ireland. Diagnosis is based on clinical symptoms consistent with scarlet fever, with or without laboratory

confirmation of group A streptococcal (GAS) infection. A total of 3,752 notifications of scarlet fever have been made so far this season (week 37 2013 to week 11 2014) compared with total notifications ranging from 1,565 to 2,868 in the seasons from 2008/09 to 2012/13 (Figure 1). A total of 635 notifications have been received across England, Wales and Northern Ireland in week 11 of 2014.

The increase in scarlet fever has been seen across most parts of the UK with the highest rate of notifications per 100,000 population reported in Cheshire and Merseyside (13.0), East Midlands (11.9), Avon Gloucestershire and Wiltshire (10.6), Thames Valley

FIGURE 1

Weekly notifications of scarlet fever, England, Wales and Northern Ireland, 8 September 2008–16 March 2014

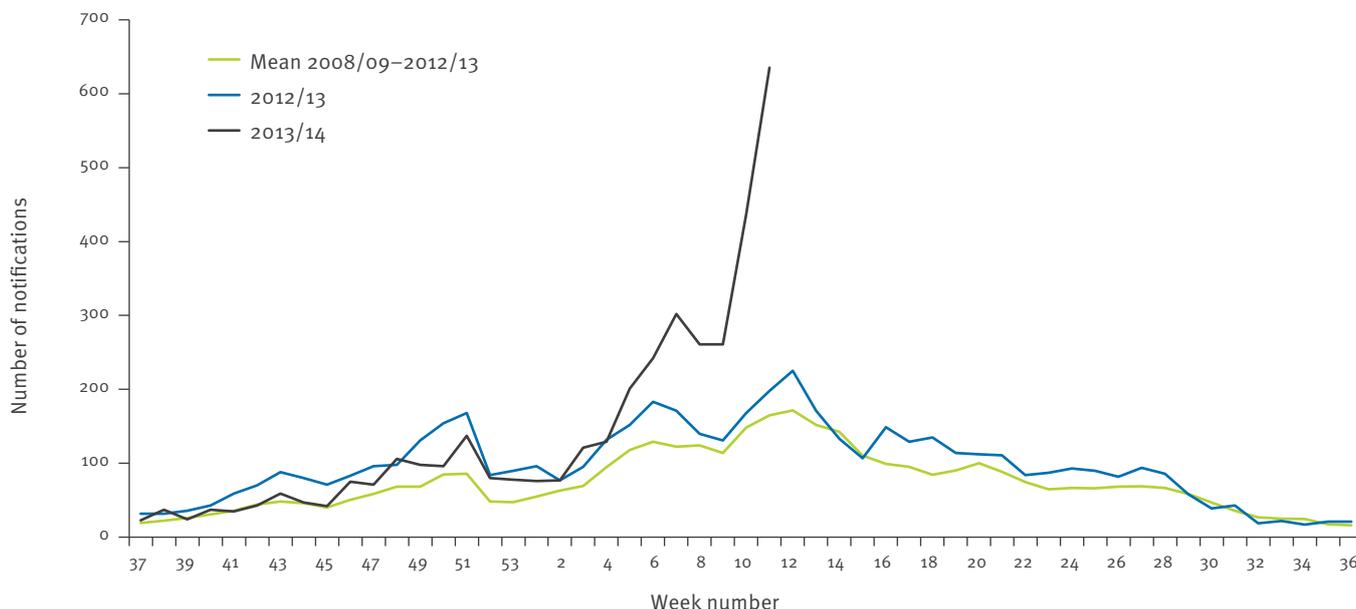
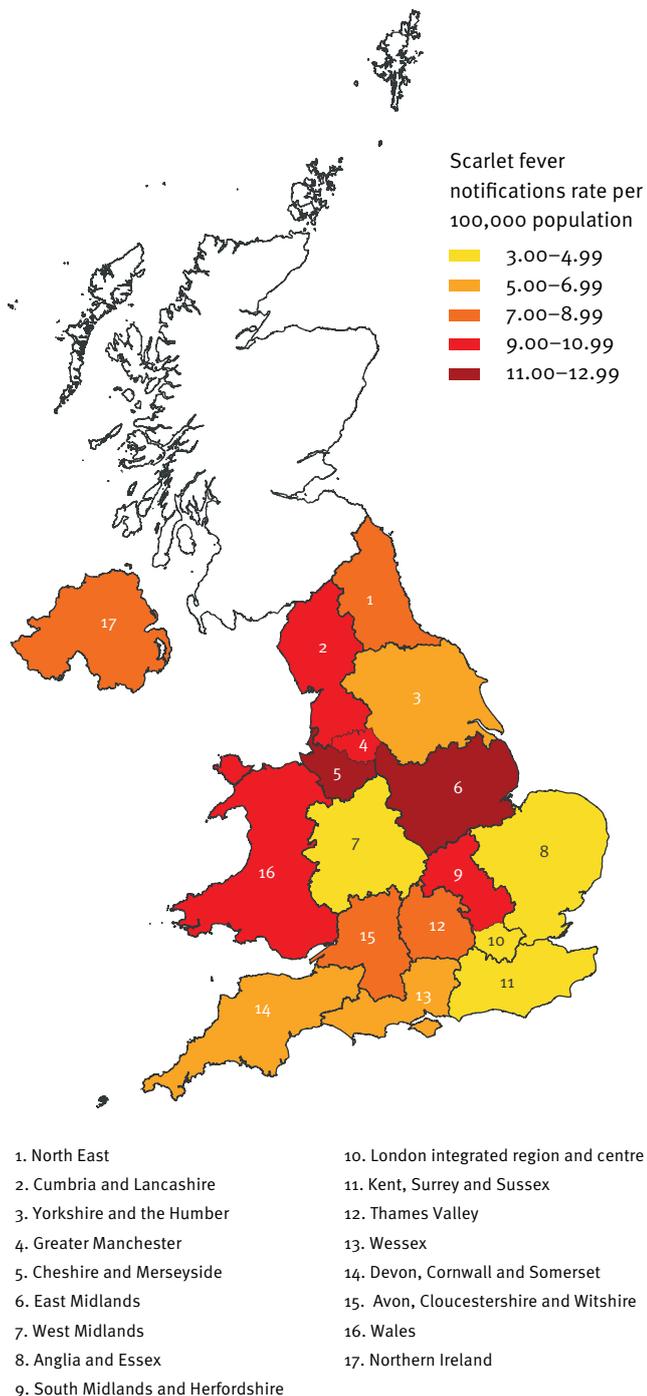


FIGURE 2

Rate per 100,000 population scarlet fever notifications in England, Wales and Northern Ireland, 9 September 2013–16 March 2014



The period in weeks to which the map refers is from week 37 2013 to week 11 2014.

(9.5), North East (9.7) and Northern Ireland (9.2) (Figure 2).

The age and sex distribution of cases notified in England to date for this season remains similar to previous years, with 87% of cases reported in children

under 10 years of age (median: 4 years; range: <1–90 years) and only 5% reported in adults (≥ 18 years-old; 36% male (63/175). Outbreaks in schools have been reported in several parts of the UK [1;2].

Invasive group A streptococcal infection

Cases of invasive GAS (iGAS) infection, defined as the isolation of GAS from a normally sterile site, are identified through national routine laboratory surveillance. Microbiology laboratories report diagnoses electronically to Public Health England (England, Wales and Northern Ireland) and Health Protection Scotland respectively. Reporting of iGAS infection is notifiable in England, Wales and Scotland but not in Northern Ireland. A total of 807 iGAS cases in the UK were identified through routine surveillance between week 37 2013 and week 11 2014, a slight increase on the mean (784 reports) but within the range (692–881 reports) for the same period in the previous four years (Figure 3). The median age of cases was 59 years (range: <1–102 years), similar to previous years. The proportion of iGAS cases who were male, 42% (303/713), was slightly lower than in recent years, 49% to 53% for 2009/10 to 2012/13. Given delays inherent within routine laboratory reporting, further reports for the first few weeks in 2014 can be expected.

Overall, 6% (26/471) of iGAS isolates reported this season (England, Wales and Northern Ireland) were reported as erythromycin-resistant. There have been no reports of penicillin resistance in iGAS isolates in the UK to date.

Assessment of iGAS isolates referred to the Public Health England national reference laboratory in London has not identified any unusual *emm* types so far this season. The proportion of strains identified as *emm3* increased in February (23%; 32/141) when compared to January (16%; 24/152).

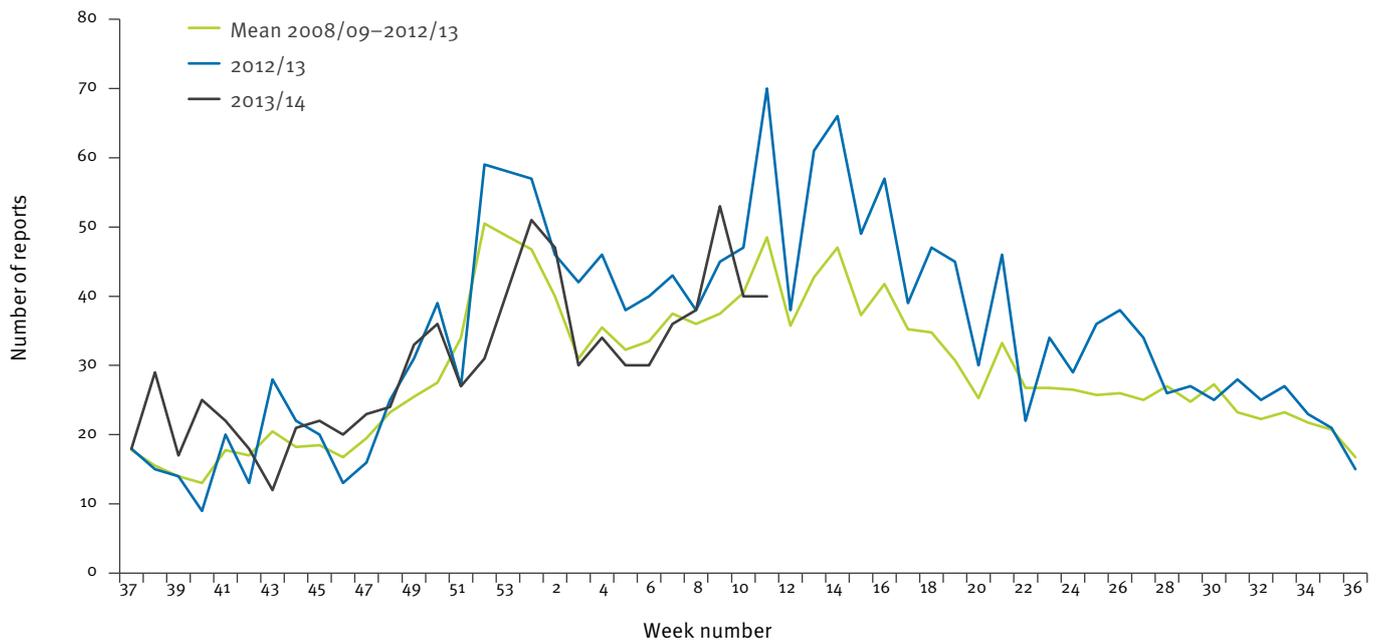
Discussion

Scarlet fever incidence has shown a remarkable increase this season. Previous analysis of scarlet fever notifications in England over the last century suggest cyclical patterns of incidence, with resurgences occurring on average every four years [3]. The last peak year for scarlet fever was 2008/09, with incidence of invasive disease tending to mirror those of superficial manifestations of GAS infection in many but not all years [4]. While the enhanced media coverage and public health alerts may have increased case ascertainment during this season, the escalation prior to this suggests a genuine increase in disease incidence. The reasons behind this increase are unclear but may be attributable to a natural cycle in disease incidence.

It remains possible that the increase or part of the increase is attributable to changes in virulence of circulating strains or increased incidence in particular risk

FIGURE 3

Weekly reports of invasive group A streptococcal infections, United Kingdom, 8 September 2008–16 March 2014



groups. An exceptional increase in scarlet fever incidence in Hong Kong during 2011 and 2012 was attributed to the introduction of a new *emm12* strain [5]. Continued vigilance for the emergence of a novel strain or changes in pattern of clinical disease is essential.

At present, antimicrobial susceptibility results are not indicating any change in antibiotic susceptibility [6]. Analysis of isolates originating from normally sterile sites, which were submitted to the national reference laboratory, has not identified any unusual types circulating, although a slight increase in the proportion of due to *emm3* is currently being observed. Strains harbouring this *emm* type were implicated in the UK rise in incidence in scarlet fever and iGAS infection during 2008/09 [7], and also implicated in population increases in iGAS infection incidence in Ireland in 2013 [8]. While the current rise in *emm3* is slight, it raises some concern given its association with a higher case fatality rate than other *emm* types [9;10]. Rapid assessment of changes in case fatality rate will assist in monitoring any such changes during the current season.

As a result of the current rise in scarlet fever notifications, clinicians, microbiologists and health protection specialists across Europe should continue to be mindful of potential increases in invasive disease and maintain a high index of suspicion in relevant patients, as early recognition and prompt initiation of specific and supportive therapy for patients with iGAS infection can be life-saving. Alerts have been issued by local health protection staff to frontline medical staff in the UK. An unusual pattern of outbreaks of scarlet fever has also been noted in Belgium during this season (K Loens,

personal communication, March 2014). Comparison of strains across the two countries would be beneficial in understanding the current situation in the UK.

Acknowledgments

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Conflict of interest

None declared.

Authors' contributions

All authors reviewed and approved the manuscript. RG drafted the manuscript and analysed the data presented. A Reynolds, B von Wissman extracted and analysed data for Scotland. C Williams and D Thomas extracted and analysed data for Wales. N Irvine and L Doherty extracted and analysed data from Northern Ireland. R Guy and T Lamagni extracted data for England and collated and analysed UK wide data. V Chalker, A Efstratiou, V Chalker and J Coelho were responsible for microbiological analysis of submitted isolates and analysis of typing data. M Chand reviewed all analyses and edited the manuscript. T Lamagni provided oversight to the manuscript submission and Mary Ramsay to associated public health communications.

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Atypical hand, foot, and mouth disease associated with coxsackievirus A6 infection, Edinburgh, United Kingdom, January to February 2014

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In January to February 2014, 16 hand, foot and mouth disease (HFMD) cases were identified in Edinburgh, United Kingdom. All presented with atypical features, with most (n=13) resembling eczema herpeticum or chickenpox. Coxsackievirus A6 (CV-A6) was identified in all the typed cases (n=11). As atypical forms of HFMD associated with CV-A6 are likely to emerge throughout Europe, clinicians should be alert to unusual clinical presentations of HFMD and virologists aware of effective diagnostic testing and *enterovirus* typing methods.

Identification of hand, foot, and mouth disease cases in Edinburgh

Eight patients with rashes and fever were identified by dermatology, paediatric and virology services in Edinburgh, United Kingdom (UK), during January 2014. The first four cases clinically resembled eczema herpeticum (Figure 1) and were identified in previously healthy children under the age of 2 years. They all presented with fever (over 37.5 °C), lethargy and poor appetite. An erythematous papular rash rapidly progressed, affecting the face, trunk and limbs, involving over 10% of the body surface area. This was followed by development of vesicles and bullae; one child also developed erythema multiforme. All four children were hospitalised and initially treated with intravenous aciclovir, based on a presumptive diagnosis of severe, disseminated herpes simplex virus (HSV) infection. Papular eruptions lasted around two weeks. Vesicle fluid specimens taken from these individuals were negative for HSV and varicella zoster virus, but positive for *enterovirus* (EV) RNA [1], confirming the diagnosis of atypical hand, foot and mouth disease (HFMD).

The remaining four patients diagnosed with HFMD in January and a further eight in February also presented with atypical symptoms; four of them clinically

resembled eczema herpeticum and five were suspected of having chickenpox. Molecular typing [2] identified coxsackievirus A6 (CV-A6) in all 11 of the typed 16 cases, including the first four.

Background

HFMD is an acute febrile infection characterised by vesicular exanthema on the hands, feet and oral mucosa, typically occurring in children under the age of 5 years [3]. It is most commonly caused by CV-A16 and EV71 within the species *EV-A*, members of the virus family *Picornaviridae* in the genus *Enterovirus* [3]. Since 2008, HFMD outbreaks in Finland and France have also been associated with other members of species *A enteroviruses*, including CV-A10 and CV-A6 [4-6]. HFMD associated with CV-A6 infection has been described as atypical: vesicular and bullous lesions are often generalised and more widely distributed, including dorsal sides of hands and feet, calves and trunk [7].

There are no published data available on the incidence of HFMD in Scotland or the rest of the UK; there is no active public health surveillance for HFMD and it is not a notifiable infection. Whereas CV-A16, EV71 and CV-A6 have been occasionally detected in clinical samples [8], CV-A6 has been known to circulate in Scotland since 2010, based on our previous environmental surveillance [9].

Retrospective study of cases of atypical hand, foot and mouth disease in Scotland

A review of virology laboratory data from the Specialist Virology Centre in Edinburgh showed that 55 EV RNA-positive cases of clinically suspected HFMD were identified in Edinburgh between January 2010 and February 2014, most of whom (n=39) were diagnosed following admission to hospital; the rest (n=16) were diagnosed by a general practitioner (Figure 2).

FIGURE 1

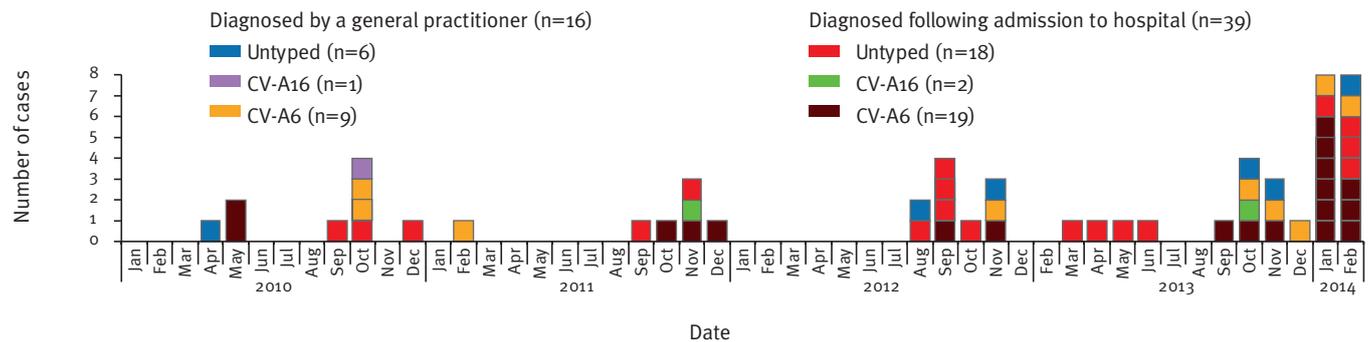
Child with atypical hand, foot and mouth disease associated with coxsackievirus A6 infection, Edinburgh, United Kingdom, January 2014



Erosions, papules and vesicular eruptions resembling eczema herpeticum on the forearm (panel A) and upper arm (panels B and C).

FIGURE 2

Hand, foot and mouth disease diagnoses confirmed by enterovirus PCR, Edinburgh, United Kingdom, January 2010–February 2014 (n=55)



The main aim of this study was to investigate the viral aetiology and presentations of HFMD. All clinically suspected cases of HFMD from whom EV was detected in a vesicle swab sample were included in this study. Cases were defined as having atypical HFMD if CV-A6 was directly identified from the vesicle fluid obtained.

Almost half (25/55) of the EV-positive cases of HFMD were identified within the last six months (September 2013 to February 2014). EV RNA was detected in vesicle fluid specimens by real-time RT-PCR targeting the 5'untranslated region [1]. For genotyping, the VP4 gene was amplified and sequenced as previously described [2]. The VP1 region was amplified using a newly designed nested primers specific for CV-A6 (outer sense: GARGCTAACATYATAGCTCTTGGAGC; inner sense: GACACYGAYGARATY CAACAAACAGC; inner antisense: CGRTRGTTGCAGTGTWGGTTATTGT; outer antisense: CCYTCAARTCHGTGGTGG TTATGCT).

CV-A6 was identified in 29 of the 42 samples typed to date (two samples were typed from one case, otherwise one sample per case): 10 older samples were not available and typing of four newer samples is still ongoing. Three samples were shown to be CV-A16: two of the patients with CV-A16 infection presented with typical HFMD, whereas the third was suspected of having measles.

Most cases (11/16) of atypical HFMD diagnosed since 2013 clustered closely together by phylogenetic analysis of the VP1 gene by neighbour joining [10] (Figure 3), indicating the introduction of a new CV-A6 strain into the UK. Previous cases clustered separately, as did a CV-A6 variant recovered from a cerebrospinal fluid sample from a patient with suspected meningitis in 2007 [8].

Clinical picture and seasonality

Of the 55 EV RNA-positive individuals with clinically suspected HFMD identified in our study, 33 presented

with atypical symptoms. Eruptions observed in areas of previous eczematous dermatitis, including extremities and the trunk, led to initial clinical diagnoses of eczema herpeticum in 14 of the 33 patients with atypical HFMD, consistent with previous reports describing this disease entity as eczema coxsackium [7,11,12]. The remaining 19 of the 33 patients with atypical symptoms were clinically suspected as having chickenpox. In previous study, cutaneous manifestations of CV-A6 -associated atypical HFMD have resembled chickenpox, with vesicles reported to crust in 65% of patients between November 2011 and February 2012 in the United States [13].

In our study, 39 of the 55 EV infections occurred in children under the age of 3 years (Figure 4). One case required treatment in an intensive therapy unit and two were shown to have been systemically infected, with detection of viraemia by PCR. Of the 55 cases, seven were adults aged 30–40 years; 31/55 were male.

In contrast to typical HFMD outbreaks, which occur in the summer and early autumn [3], we saw a cluster of cases (n=24) between October 2013 and February 2014. This is consistent with a previous study from the United States, in which atypical cases of HFMD caused by CV-A6 were seen between November 2011 and February 2012 [13].

Discussion

The number of cases presenting with atypical HFMD and potentially attributable to CV-A6 is likely to be a considerable underestimate of the actual number since surveillance data from Scotland and elsewhere in Europe is limited to EV-infected individuals admitted to hospitals [6,9]. Samples from most cases presenting to general practitioners would not be sent for virological investigation (such investigations are optional) and cases would have probably remained undiagnosed, misdiagnosed or unreported. However, the scale of two nursery outbreaks of HFMD in Edinburgh at the

FIGURE 3

Phylogenetic analysis of complete VP1 sequences of 25 coxsackievirus A6 variants detected in 24 cases of atypical hand, foot and mouth disease^{a,b}, Edinburgh, United Kingdom, January 2010–February 2014



CSF: cerebrospinal fluid; CV: coxsackievirus.

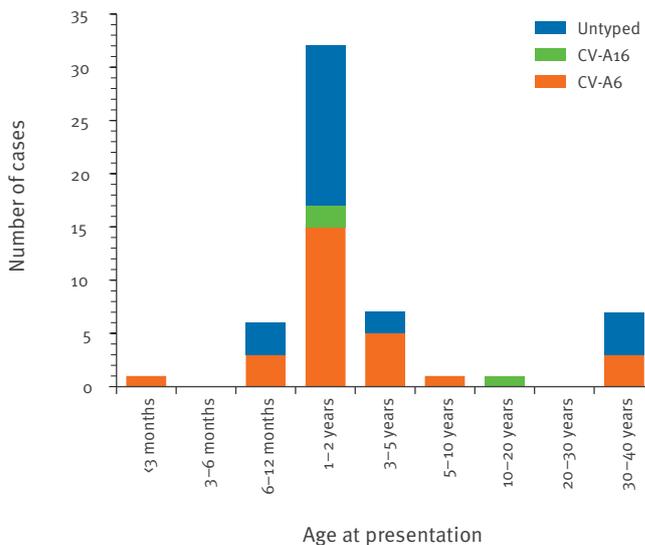
The tree was constructed by neighbour using maximum composite likelihood distances [10]; the dataset was bootstrap resampled 100 times to estimate robustness of groupings; values of 70% or greater shown. All available sequences of CV-A6 in GenBank, downloaded 10 March 2014, were also included (>70% complete; >2% divergent from each other).

^a One patient had two samples sequenced, marked twice in the tree as V2/Ed/UK/2014; one of them originated from blood and the other from vesicle fluid.

^b The remaining four cases were typed based on the VP4 sequences and were thus not included in this analysis.

FIGURE 4

Enterovirus-positive hand, foot and mouth disease diagnoses in Edinburgh, United Kingdom, January 2010–February 2014 (n=55)



CV: coxsackievirus.

The majority of diagnoses (n=39) have been seen in children under the age of 3 years.

beginning of 2014, with 15/45 and 7/40 children under age of 4 years affected, led to notification of the local public health team (data not shown), providing some indication of the extent of its spread in the community. However, these outbreaks were not investigated further and the infections were not laboratory confirmed. These outbreaks, together with the clinical cases reported here since 2010, suggest a change in the clinical severity of CV-A6 infections over the last few years. While CV-A6 clearly circulates locally (being identified as the second most common species A EV in sewage surveillance in Edinburgh in 2010 [9], only one CV-A6 infection (an infant aged under 3 months with meningitis) was identified through routine typing of referred samples during the five years before 2010 [8].

To date, HFMD outbreaks associated with CV-A6 infection have been documented from four countries of the World Health Organization European Region: Finland [4,5], France [6,14], Spain [15] and Israel [16]. However, large outbreaks have also been described in Asia [17–19] and the United States [11,13]. The cases of HFMD associated with CV-A6 infection described here clinically resembled HFMD infections caused by emerging CV-A6 in the United States since 2012 [7,11–13] and Japan in 2013 [20], while other reported HFMD outbreaks caused by CV-A6 were associated with onychomadesis one to two months after onset of the initial symptoms [4,17,18]. It remains to be seen whether our patients develop nail loss.

To the best of our knowledge, this is the first report of CV-A6 infections associated with HFMD in the United Kingdom. Our findings provide evidence of a recent change in severity and clinical presentations of CV-A6 infections in Edinburgh that match reports from elsewhere [7,13,14,20]. CV-A6 infections are underdiagnosed as a result of lack of routine surveillance at the community level and also atypical presenting symptoms and signs. Awareness of the potential clinical extent and variability of this atypical form of HFMD should help to avoid confusion with other skin conditions such as eczema herpeticum and chickenpox. In addition, increasing awareness will help to improve laboratory diagnosis and management of infected children.

When there are institutional outbreaks, additional information regarding atypical HFMD might be required to educate staff and families, and also remind them of appropriate contact precautions for children in nappies. EV PCR testing of vesicle fluid should be recommended in cases where the diagnosis is clinically doubtful, and focused, community-based surveillance as well as formal case investigations should be considered.

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Conflict of interest

None declared.

Authors' contributions

Heli Harvala planned the study, analysed the data and wrote the manuscript. Catriona Sinclair and Olivia Schofield participated in patient care as expert dermatologists, whereas Dylan Broomfield and Nke Nwafor were the pediatricians and Heli Harvala and Kate Templeton the medical virologists involved in the diagnosing of the cases. Eleanor Gaunt and Peter Simmonds did the virus sequencing. Louise Wellington and Lorna Willocks provided the data from NHS Lothian Health Protection. All the authors contributed to manuscript.

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Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012

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In recent years acquired azole resistance in *Aspergillus fumigatus* has been increasingly reported and a dominant mechanism of resistance (TR₃₄/L98H) was found in clinical and environmental isolates. The aim of the present study was to investigate the prevalence of azole resistance in environmental *A. fumigatus* isolates collected in northern Italy. *A. fumigatus* grew from 29 of 47 soil samples analysed. Azole-resistant isolates were detected in 13% (6/47) of the soil samples and in 21% (6/29) of the soil samples containing *A. fumigatus*. High minimal inhibitory concentrations (MIC) of itraconazole (≥ 16 mg/L) and posaconazole (≥ 0.5 mg/L) were displayed by nine isolates from six different soil samples, namely apple orchard (1 sample), rose pot compost (2 samples), and cucurbit yields (3 samples). Seven isolates had a MIC=2 mg/L of voriconazole. Seven of nine itraconazole and posaconazole resistant isolates harboured the same TR₃₄/L98H mutation of *cyp51A*. These findings, together with the occurrence of resistant clinical isolates, suggest that azole resistance should be considered in primary patient care.

Introduction

Aspergillus fumigatus is a filamentous fungus that causes a broad spectrum of diseases. Invasive life-threatening infection affects subjects with compromised immune system, mainly patients with haematological malignancies, recipients of haematopoietic stem cells or solid organ transplantation, or patients under prolonged treatment with steroids. Chronic aspergillosis occurs in patients with pre-existing pulmonary or sinus disease, and locally invasive infection occurs as a result of trauma or surgery.

A. fumigatus is ubiquitous in the soil and in decaying organic matter, and produces asexual spores (conidia) that are continuously dispersed in the air. Most of the patients develop aspergillosis following inhalation of conidia into the alveoli or the upper airways. Triazole antifungals, itraconazole, posaconazole and voriconazole, are increasingly used in the treatment and prophylaxis of aspergillosis [1]. Voriconazole is recommended

as first line treatment for invasive aspergillosis in European and American guidelines [2-5].

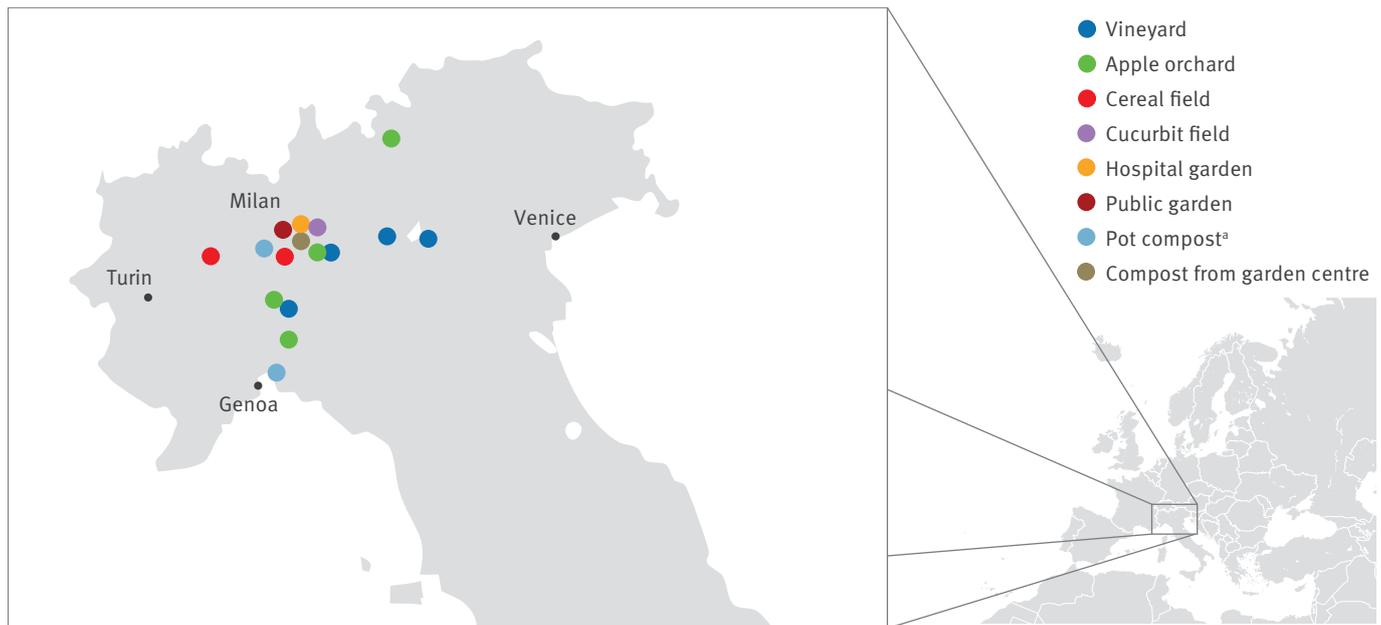
A. fumigatus is generally susceptible to these antifungals. However, in the recent years, azole resistance has been increasingly reported in patients under long-term antifungal treatment and also in azole naïve patients as well as in strains from the environment [1,6-8].

The most common mechanism of azole resistance is an alteration of lanosterol 14- α -demethylase, the key enzyme in the biosynthetic pathway of the ergosterol, a main component of the cell membrane. Different point mutations in the *cyp51A* gene, which encodes this enzyme, have been shown to confer resistance [1,9-11]. A dominant mechanism of resistance involving a 34-bp tandem repeat in the gene promoter region and a substitution of a leucine for a histidine at codon 98 (TR₃₄/L98H) was initially found in clinical and environmental isolates from the Netherlands and a correlation to the use of azoles in the environment was suggested [12].

During the international surveillance of azole resistance in *A. fumigatus* clinical isolates (SCARE Network) intrinsic resistance was observed in four of 209 isolates collected in Italy [13] and the TR₃₄/L98H mutation was detected in all these isolates. The aim of the present study was to investigate the prevalence of azole resistance in *A. fumigatus* environmental isolates collected in northern Italy.

Methods

Environmental sampling was carried out in northern Italy in the period between May 2011 and June 2012. A total of 47 soil samples, namely 12 samples from apple orchard, 12 from cucurbit fields, six from vineyards, five from cereals fields, five from pot composts (including rose and other flower pot compost), three from flowerbeds of public gardens, three from hospital gardens, and one from compost purchased from a commercial garden centre, were examined. The sampling sites are reported in the map (Figure 1). Azole fungicides are

FIGURE 1Map of northern Italy reporting the sampling sites for *Aspergillus fumigatus*, May 2011–June 2012

Only sites where more than one sample could be analysed, are shown.

^a Including rose and other flower pot compost.

used in all the sampled environments, except gardens. No information was available for composts.

The samples were treated according to the method previously described by Snelders et al. [12] with minor modifications. Briefly, 2 g of each sample were suspended in 8 mL of sterile distilled water added of 1% Tween 20 (Sigma, St. Louis USA) and chloramphenicol (0.5 g/L, Sigma) and vortexed. The suspension was stored at room temperature for 30 to 60 min and 100 µL of the supernatant was inoculated on two plates of Sabouraud dextrose agar (SDA, Biolife, Milan, Italy) supplemented with chloramphenicol (0.5 g/L) and on two plates of SDA supplemented with chloramphenicol and itraconazole (4 mg/L, Janssen, Beerse, Belgium). Control plates and itraconazole-containing plates were incubated at 37° C and at 42° C (to limit the fungal growth) and examined after 24, 48, and 72 hours of incubation. All the *A. fumigatus* isolates grown on itraconazole-containing agar and an equal number of isolates grown on control plates were selected and maintained on SDA medium.

The isolates were identified by macroscopic and microscopic morphology on Czapek agar medium (Difco, Becton Dickinson, Buccinasco, Italy) as *A. fumigatus* species complex.

A. fumigatus isolates grown in presence of itraconazole and an equal number of isolates grown on control plates were tested for antifungal susceptibility to itraconazole, posaconazole and voriconazole. Susceptibility testing was performed, within one month from the isolation, by broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [14]. The minimal inhibitory concentration (MIC) of all the azoles was determined visually as the lowest concentration of drug giving a complete inhibition of fungal growth. All tests were performed in duplicate. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as quality control in each test.

Susceptibility testing was also performed using Etest strips (BioMérieux, Bagnoli a Ripoli, Italy) on RPMI 1640 agar supplemented with 2% glucose, according to the manufactory indications. Isolates with MIC of itraconazole and voriconazole >2mg/L and those with MIC of posaconazole >0.25mg/L were considered resistant, according to the EUCAST breakpoints [15-17].

Genomic DNA was extracted from the nine azole-resistant and four susceptible isolates using the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA). Identification of the isolates as *A. fumigatus in sensu stricto* was confirmed by the amplification and sequencing of a portion of the beta-tubulin

TABLE 1

Environmental origin of itraconazole-resistant *Aspergillus fumigatus* isolates, Italy, May 2011–June 2012

Sample source	Period of sampling	Examined samples (n)	Samples with growth of <i>A. fumigatus</i> on control plates (n)	Samples with growth of <i>A. fumigatus</i> on itraconazole medium (n)	<i>A. fumigatus</i> isolates on itraconazole medium (n)	<i>A. fumigatus</i> isolates with confirmed itraconazole resistance (n)
Apple orchard	Oct–Dec 2011	12	3	1	1	1 ^a
Cereal fields	Mar–Apr 2012	5	5	0	0	0
Compost from garden centre	Apr 2012	1	0	0	0	0
Cucurbit fields	May and Sep 2011	12	10	8	28	6 ^b
Hospital gardens	Mar–Jun 2012	3	3	0	0	0
Pot compost	May 2011	5	5	2	29	2 ^c
Public gardens	Apr–May 2012	3	3	0	0	0
Vineyards	Oct 2011	6	0	0	0	0
Total	–	47	29	11	58	9

^a The isolate corresponds to one sample collected in the Lombardy region near the northern Italian border.

^b The isolates were derived from three samples respectively collected in different fields and/or dates near the city of Milan.

^c The isolates were derived from two samples from two different rose pots collected near the city of Genoa.

gene using the primers described elsewhere [9]. The sequences obtained were compared to the sequences present in the GenBank database (www.ncbi.nlm.nih.gov) by basic local alignment search tool (BLAST) analysis and the identification was confirmed if a 99 to 100% sequence identity was observed.

In addition, two further DNA regions, one inside the *cyp51A* gene and one inside its promoter, were sequenced to detect the presence of the point mutation t364a, which leads to the L98H substitution at the protein level, and the 34-bp tandem repeat, both specific for azole resistance. The *cyp51A* gene fragment (1168 bp) was amplified by polymerase chain reaction (PCR) using the two primers, P450-A1 [11] and *Cyp51AR2* (5'-AGTGAATAGAGGAGTGAATCC-3'). PCR was performed in a 50-mL volume containing 10X buffer (10 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl₂; Qiagen, Venlo, Netherlands), 1.5 mM MgCl₂, 200 mM of each of the four deoxynucleotides, 20 pmol of each primer, 2.5 U of Taq polymerase (Qiagen), and 2 mL of genomic DNA. The thermal cycling profile included an initial step at 94°C for 5 min, 30 cycles consisting in denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR product was then sequenced using two forward primers, P450-A1 and *Cyp51AF2* (5'-GACATCTCTGCGGCAATGG-3'), and two reverse primers, *Cyp51AR2* and *Cyp51AR3* (5'-CCATTGCCGAGAGATGTC-3'), to reach the complete sequencing coverage for both strands of the fragment. The *cyp51A* gene promoter was amplified and sequenced using the primers PA5 and PA7 as previously described [10].

All sequences were determined by an ABI PRISM 3100 genetic analyser (Applied Biosystems), electropherograms were analysed by FinchTV software (www.geospiza.com), and sequence alignment was performed using ClustalW algorithm (www.ebi.ac.uk). The *cyp51A* sequence from *A. fumigatus* strain 237 (GenBank accession number: AF338659) was used as wild type reference.

Azole-resistant as well as five susceptible isolates were genotyped by microsatellite analysis using the primers STRAf3A, STRAf3B, STRAf3C, STRAf4A, STRAf4B, and STRAf4C as described elsewhere [18].

Results

A. fumigatus grew in 29 of 47 soil samples (62%), mainly from cucurbit and cereal fields and rose pot composts (Table 1). No *A. fumigatus* was isolated from vineyards and compost from garden centre. A total of 58 isolates grew on itraconazole-containing agar.

Broth microdilution and Etest confirmed itraconazole resistance in nine isolates (MIC ≥16 mg/L). All these isolates were also posaconazole resistant (MIC ≥0.5 mg/L). Seven isolates showed an intermediate susceptibility to voriconazole (MIC =2 mg/L) by broth microdilution method according to EUCAST (Table 2). Etest MIC values were lower but MIC values obtained by broth microdilution and Etest were in the +/- 2 dilution range. The *A. fumigatus* control isolates were susceptible to itraconazole (range of MICs: 0.06 to 0.12 mg/L), posaconazole (range of MICs: ≤0.03 to 0.06 mg/L), and voriconazole (range of MICs: 0.06 to 0.25 mg/L). Identical results were obtained in tests performed in duplicate.

TABLE 2

Results of EUCAST and Etest susceptibility testing, and analysis of mutations in *cyp51A*, Italy, May 2011–June 2012

Isolate number	Sample source	MIC (mg/L) determined by EUCAST			MIC (mg/L) determined by Etest			Mutation in <i>cyp51A</i>
		ITZ ^a	POS ^a	VRC ^a	ITZ ^a	POS ^a	VRC ^a	
11-0087A	Rose pot compost	>16	1	2	>32	1	1	TR34/L98H
11-0088E	Rose pot compost	>16	2	1	16	0.5	1	None
11-0099A	Cucurbit fields	>16	2	2	>32	1	2	TR34/L98H
11-0104A	Cucurbit fields	>16	1	2	>32	0.5	2	TR34/L98H
11-0104B	Cucurbit fields	>16	1	2	>32	0.5	2	F46Y; M172V; N248T; D255E
11-0104D	Cucurbit fields	>16	1	2	>32	1	1	TR34/L98H
11-0317C	Cucurbit fields	>16	2	2	>32	0.5	0.5	TR34/L98H
11-0317D	Cucurbit fields	>16	1	1	>32	0.5	1	TR34/L98H
11-0396	Apple orchard	>16	2	2	>32	0.5	1	TR34/L98H

EUCAST: European Committee on Antimicrobial Susceptibility Testing; MIC: minimal inhibitory concentration.

Isolates with MIC of itraconazole and voriconazole >2mg/L and those with MIC of posaconazole >0.25mg/L by broth microdilution method according to EUCAST, were considered resistant [15-17]. Data obtained by EUCAST and Etest differed, however results in a +/- 2 dilution range are considered acceptable.

^a The azoles used for susceptibility testing were ITZ (itraconazole), POS (posaconazole), VRC (voriconazole).

FIGURE 2

Alignment of three amino acid sequences derived from the *Cyp51A* genetic sequence from itraconazole susceptible and resistant isolates, Italy, May 2011–June 2012

11-0087C (S)	MVPMLWLTAYMAVAVLTAILLNVVYQLFFRLWNRTEPPMVFHWV P FLGSTISYGIDPYKF	60
11-0099A (R)	*****	60
11-0104B (R)	***** Y *****	60
11-0087C (S)	FFACREKYGDIFTFILLGQKTTVYLGVOGNEFILNGK L KDVNAEEVYSPLTTPVFGSDVV	120
11-0099A (R)	***** H *****	120
11-0104B (R)	*****	120
11-0087C (S)	YDCPNSKLMQKFKIKYGLTQSALESHVPLIEKEVLDYLRDSPNFQGS S GR M DISAAMAE	180
11-0099A (R)	*****	180
11-0104B (R)	***** V *****	180
11-0087C (S)	ITIFTAARALQGQEVRSKLTAEFADLYHDLDKGFTPINFMLPWAPLPHNKKRDAAHARMR	240
11-0099A (R)	*****	240
11-0104B (R)	*****	240
11-0087C (S)	SIYVDI I NQRRLDG D KDSQKSDMIWNLMNCTYKNGQQVPDKEIAHMMITLLMAGQHSSSS	300
11-0099A (R)	*****	300
11-0104B (R)	***** T ***** E *****	300
11-0087C (S)	ISAWIMLRLASQPKVLEELYQEQLANLGPAGPDGSLPPLQYKDLDKLPFHQHVIRETLRI	360
11-0099A (R)	*****	360
11-0104B (R)	*****	360
11-0087C (S)	HSSIH	365
11-0099A (R)	*****	365
11-0104B (R)	*****	365

An asterisk (*) indicates that the amino acid in the sequence is identical to that of the sequence presented at the top of the alignment. The sequence 11-0087C is derived from an itraconazole susceptible (S) isolate, while the sequences 11-0099A, with the resulting L98H mutation, and 11-0104B, with other resulting amino acid substitutions, are from respective itraconazole resistant (R) isolates.

TABLE 3

Allelic profiles at six microsatellite loci of itraconazole resistant (n= 8) and susceptible (n=5) environmental isolates, Italy, May 2011–June 2012

Isolate	R/S	Fragment size as bp (number of repeats)					
		STRAF _{3A}	STRAF _{3B}	STRAF _{3C}	STRAF _{4A}	STRAF _{4B}	STRAF _{4C}
11-0087A	R	292 (60)	164 (10)	78 (5)	182 (9)	185 (9)	185 (10)
11-0088E	R	190 (27)	169 (12)	80 (6)	231 (20)	184 (9)	175 (7)
11-0099A	R	310 (66)	158 (8)	78 (5)	179 (8)	185 (9)	185 (10)
11-0104A	R	323 (70)	158 (8)	86 (8)	179 (8)	185 (9)	175 (7)
11-0104B	R	161 (18)	162 (9)	108 (15)	201 (13)	184 (9)	175 (7)
11-0104D	R	179 (23)	160 (9)	78 (5)	183 (9)	184 (9)	185 (10)
11-0317C	R	203 (31)	164 (10)	75 (4)	186 (10)	180 (8)	224 (20)
11-0396	R	310 (66)	158 (8)	80 (6)	178 (8)	184 (9)	185 (10)
11-0023B	S	212 (34)	164 (10)	194 (42)	196 (12)	184 (9)	175 (7)
11-0034A	S	212 (34)	156 (7)	80 (6)	182 (9)	180 (8)	242 (26)
11-0036C	S	222 (37)	164 (10)	80 (6)	196 (12)	175 (7)	162 (5)
11-0087C	S	190 (27)	162 (9)	118 (18)	182 (9)	196 (12)	162 (5)
11-0104E	S	219 (36)	169 (12)	80 (6)	185 (10)	192 (11)	181 (9)

R: itraconazole resistant; S: itraconazole susceptible.

The nine itraconazole- and posaconazole-resistant isolates were recovered from pot composts (2 isolates from 2 different rose pots from Genoa), from apple orchard (1 isolate from an apple orchard near the northern border of Italy), and from cucurbit fields (6 isolates). These last six isolates were from three different samples, 11-0099, 11-0104 and 11-0317, collected in different fields and/or dates.

All nine resistant isolates were identified as *A. fumigatus sensu stricto* by amplification of a fraction of the beta-tubulin gene.

Sequence analysis of *cyp51A* gene showed the t364a point mutation, which results in the L98H substitution, combined with the 34-bp tandem repeat in the promoter region, in seven of nine itraconazole- and posaconazole-resistant isolates (Table 2). The TR₃₄/L98H alteration was neither detected in the four azole susceptible isolates used as control nor in the resistant isolates 11-0088E and 11-0104B. No mutation in the *cyp51A* gene was found in the recovered sequence of 11-0088E. The other resistant isolate (11-0104B) lacking the mutation leading to the L98H substitution had four other inferred amino acid changes at codons 46, 172, 248 and 255, as reported in Figure 2.

Eight of nine resistant isolates (one was no more vital) and five additional susceptible environmental isolates were genotyped by microsatellite analysis. The results showed that all the isolates presented genotypes different from each other (Table 3).

Discussion

Acquired resistance to azoles develops in response to exposure of fungi to azole compounds in patients and in agricultural settings and is favoured by long duration of exposure to these compounds and high numbers of reproducing fungi [1,19,20]. Acquisition of resistance in patients is characterised by a variety of resistance mechanisms. The dominance of the TR₃₄/L98H resistance mechanism in unrelated clinical isolates in a large Dutch culture collection suggested that isolates with this mechanism might be present in the environment, favoured by azole fungicides used in agriculture [20]. The detection of the TR₃₄/L98H resistance mechanism also in Italy, in four *A. fumigatus* isolates from two azole-naïve patients among the 209 isolates tested in the SCARE project [13] and in an additional isolate from an azole-exposed patient, lead to investigate the presence of azole-resistant *A. fumigatus* in the environment in our country.

Nine of the 58 isolates obtained by screening soil samples collected in northern Italy on an itraconazole containing medium were confirmed to be resistant to itraconazole and posaconazole by MIC determined with broth microdilution and Etest. Seven of these nine had also a reduced susceptibility to voriconazole when tested with broth microdilution. The discrepancy between the numbers of isolates on the screening medium containing itraconazole at a concentration of 4 mg/L and the numbers of confirmed resistant isolates could be attributed to trailing of organic compounds present in the soil.

Azole-resistant *A. fumigatus sensu stricto* were detected in 13% (6/47) of the soil samples collected and in 21% (6/29) of the soil samples confirmed as containing *A. fumigatus*. These results are in agreement with the data from other European countries: resistant isolates were detected in 8% of soil samples and in 11% of samples containing *A. fumigatus* in Denmark [8] and in 20.4% of *A. fumigatus* positive soil samples in the Netherlands [12]. Due to the limited number of samples it was impossible to note a seasonal variation

Molecular analysis showed the presence of the TR₃₄/L98H resistance mechanism in seven of nine resistant isolates. This resistance mechanism has also been reported in clinical isolates from several European and Asian countries [1,7,9,13,21-25]. To explain the prevalence of this resistance mechanism it was hypothesised that TR₃₄/L98H isolates may have an advantage with respect to fitness compared to isolates with other mutations [6]. Other point mutations (in codons 46, 172, 248, 255) were detected in one of the remaining resistant isolate. These mutations have been reported by other authors, and were found in both azole-susceptible and resistant strains [7,26,27]. No mutation was observed in the sequenced *cyp51A* gene of the other resistant isolate. These two resistant isolates would suggest the existence of other mechanisms of resistance.

As shown by molecular typing, all the resistant isolates were characterised as different strains confirming that resistance does not arise by a clonal expansion of a mutant but it is likely induced by the selective pressure resulting from the presence of azoles in the environment.

In conclusion this study provides evidence that azole-resistant *A. fumigatus* are detected in the environment also in Italy, in soil or composts exposed to azole fungicides, and confirms the presence of the TR₃₄/L98H mutation in several countries in Europe. Susceptibility testing of filamentous fungi is not routinely carried out in most medical microbiology laboratories, however the risk for patients to acquire multi-azole-resistant strains from the environment could have a serious impact on the management of life-threatening invasive infections. Finally, the possible selection of resistant fungal pathogens should lead applying azoles in agriculture with caution.

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Conflict of interest

None declared.

Authors' contributions

Prigitano Anna, Venier Valentina, Cogliati Massimo, Esposto Maria Carmela contributed to the planning of the research, performed sampling and laboratory tests, and contributed to the manuscript preparation. De Lorenzis Gabriella contributed to microsatellite analysis tests and contributed to the manuscript preparation. Tortorano Anna Maria planned the research and prepared the manuscript.

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Decreasing incidence and changes in serotype distribution of invasive pneumococcal disease in persons aged under 18 years since introduction of 10-valent and 13-valent conjugate vaccines in Portugal, July 2008 to June 2012

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The 10-valent pneumococcal conjugate vaccine (PCV10) became available in Portugal in mid-2009 and the 13-valent vaccine (PCV13) in early 2010. The incidence of invasive pneumococcal disease (IPD) in patients aged under 18 years decreased from 8.19 cases per 100,000 in 2008–09 to 4.52/100,000 in 2011–12. However, IPD incidence due to the serotypes included in the 7-valent conjugate vaccine (PCV7) in children aged under two years remained constant. This fall resulted from significant decreases in the number of cases due to: (i) the additional serotypes included in PCV10 and PCV13 (1, 5, 7F; from 37.6% to 20.6%), particularly serotype 1 in older children; and (ii) the additional serotypes included in PCV13 (3, 6A, 19A; from 31.6% to 16.2%), particularly serotype 19A in younger children. The decrease in serotype 19A before vaccination indicates that it was not triggered by PCV13 administration. The decrease of serotype 1 in all groups, concomitant with the introduction of PCV10, is also unlikely to have been triggered by vaccination, although PCVs may have intensified and supported these trends. PCV13 serotypes remain major causes of IPD, accounting for 63.2% of isolates recovered in Portugal in 2011–12, highlighting the potential role of enhanced vaccination in reducing paediatric IPD in Portugal.

Introduction

Introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) led to changes in the circulating serotypes of *Streptococcus pneumoniae* and also often to decreases in the incidence of invasive pneumococcal disease (IPD) worldwide [1–3]. Two new pneumococcal conjugate vaccine (PCV) formulations are now

commercially available and are used for children [4]. A 10-valent formulation (PCV10) including, in addition to the PCV7 serotypes, serotypes 1, 5 and 7F and a 13-valent conjugate vaccine (PCV13), including all PCV10 serotypes plus serotypes 3, 6A and 19A. The few available early reports point to the effectiveness of these expanded valency vaccines against the serotypes included in their formulations [4–8].

In Portugal, PCVs are not included in the national immunisation plan. Had they been, they would have been offered free of charge. Nevertheless, since 2001, when the vaccine became available, there has been a steady increase in PCV7 uptake bought privately, without any reimbursement, reaching 75% of children aged 2 years or under in 2008 [9]. PCV10 became available for childhood vaccination in mid-2009 and PCV13 in early 2010. Soon after PCV13 became available, according to sales data, this vaccine was mostly used (data not shown).

In previous studies, we showed that significant changes in the serotypes causing IPD in children followed PCV7 availability in Portugal [9,10] and that there was evidence for a herd effect in the adult population [9,11,12]. Serotypes 1 and 7F, both included in PCV10 and PCV13, and serotype 19A, included in PCV13, emerged as major causes of paediatric (persons aged under 18 years) IPD in the post-PCV7 period in Portugal [10]. Given the limited information on the efficacy of PCV10 and PCV13, this study aimed at documenting the potential effects of vaccination on serotype distribution, antimicrobial resistance and incidence of paediatric IPD in Portugal from July (week 26) 2008 and June (week 25) 2012.

TABLE 1

Population under 18 years during the study period, Portugal, 2008–12

Calendar year	Number of persons per age group				Total
	0–11 months	12–23 months	2–4 years	5–17 years	
2008	103,746	101,339	318,186	1,454,385	1,977,656
2009	98,759	103,011	311,641	1,444,426	1,957,837
2010	100,492	96,995	302,067	1,429,777	1,929,331
2011	95,703	99,519	294,727	1,412,241	1,902,190
2012	92,651	97,591	295,215	1,405,356	1,890,813

Methods

Bacterial isolates

Since 2007, the Portuguese Group for the Study of Streptococcal Infections and the Portuguese Study Group of Invasive Pneumococcal Disease of the Paediatric Infectious Disease Society have monitored pneumococcal invasive infections in Portugal. During the study period, this involved the microbiology laboratories and paediatric departments of 61 hospitals throughout Portugal. The network includes centres covering the entire country, including all referral hospitals and most centres where microbiological diagnostic services are available. All centres included in the study reported during the entire period.

A case of IPD was defined as a person from whom an isolate of *S. pneumoniae* was recovered from a normally sterile body site (not including middle ear fluid) or from whom pneumococcal DNA was detected in cerebrospinal fluid (CSF) or pleural fluid. Isolates recovered up to 2008 were previously characterised [9,10,13]. Isolates recovered from patients aged under 18 years between July 2008 and June 2012 were included in the present study. Epidemiological years were defined as spanning from week 26 of one year to week 25 of the following year.

Only one isolate from each patient was considered. All strains were identified as *S. pneumoniae* by colony morphology and haemolysis on blood agar plates, optochin susceptibility and bile solubility. The *lytA* gene was used to identify pneumococci in CSF or pleural fluid.

Incidences were calculated based on the entire Portuguese population of the relevant age groups using data available from the Instituto Nacional de Estatística [14] (Table 1), using the population data of the first calendar year of each epidemiological year. The calculation assumes that all IPD cases were treated at the 61 hospitals in our network. Four age groups were considered: infants aged less than 12 months, children aged 12–23 months, children aged from two to four years and children and adolescents aged from five years to less than 18 years.

Serotyping and antimicrobial susceptibility testing

Serotyping was performed by the standard capsular reaction test using the chessboard system [15] and specific sera (Statens Serum Institut, Copenhagen, Denmark). Serotypes were classified into vaccine serotypes, i.e. those included in PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F), the additional three found in PCV10 (addPCV10: 1, 5, 7F), the additional three found

TABLE 2Cases of invasive pneumococcal disease and available *Streptococcus pneumoniae* isolates from patients aged under 18 years, Portugal, July 2008–June 2012^a

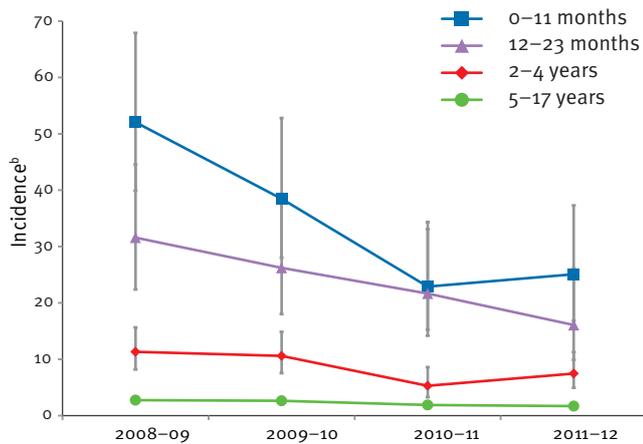
Epidemiological years ^a	Number of cases ^b /available isolates, by age group				Total
	0–11 months	12–23 months	2–4 years	5–17 years	
2008–09	54/49	32/26	36/25	40/33	162/133
2009–10	38/35	27/23	33/21	38/35	136/114
2010–11	23/20	21/19	16/13	27/25	87/77
2011–12	24/17	16/16	22/19	24/16	86/68
Total	139/121	96/84	107/78	129/109	471/392

^a From week 26 of one year to week 25 of the following year.

^b Case numbers comprise patients from whom pneumococci were isolated from a normally sterile site or pneumococcal DNA was detected in cerebrospinal fluid or pleural fluid.

FIGURE 1

Incidence of invasive pneumococcal disease in persons aged under 18 years, Portugal, July 2008–June 2012^a



The bars represent 95% confidence intervals for the incidence estimates.

^a Epidemiological years: from week 26 of one year to week 25 of the following year.

^b Number of cases per 100,000 in specified age group.

in PCV13 (addPCV13: 3, 6A, 19A) and non-vaccine serotypes (NVT).

Etest strips (AB Biodisk, Solna, Sweden) were used to determine the minimal inhibitory concentrations (MICs) for penicillin, cefotaxime, ceftriaxone and levofloxacin. We opted to use the Clinical and Laboratory Standards Institute (CLSI) guidelines because these were the standards in use in Portugal during the study period. In 2008, the CLSI changed the recommended breakpoints to those currently used to interpret MIC values [16]. Unless otherwise stated, we have used the CLSI-recommended breakpoints before 2008 [17] as epidemiological breakpoints that allow comparison with previous studies.

Isolates were further characterised by determining their susceptibility to erythromycin, clindamycin, vancomycin, linezolid, tetracycline, trimethoprim-sulfamethoxazole and chloramphenicol by the Kirby-Bauer disk diffusion technique, according to the CLSI recommendations and interpretative criteria [16]. Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin. Simultaneous resistance to erythromycin and clindamycin defines the MLS_B phenotype (resistance to macrolides, lincosamides and streptogramin B) while non-susceptibility only to erythromycin defines the M phenotype.

Statistical analysis

Simpson's index of diversity (SID) and respective 95% confidence intervals (CIs) was used to measure the serotype diversity [18]. The SID measures the probability that two isolates sampled at random will not share the same serotype. The Cochran–Armitage test (a modification of the Pearson chi-squared test to incorporate a suspected ordering in the effects of the categories of the second variable) was used for trends with the false discovery rate (FDR) correction for multiple testing [19]. A p value of less than 0.05 was considered significant for all tests.

Results

Isolate collection

Between July 2008 and June 2012, a total of 471 cases of IPD were reported in Portugal. Their distribution by age and epidemiological year is shown (Table 2) and the annual incidence of IPD by age group is presented (Figure 1). Although starting from very disparate values, IPD incidence decreased significantly in all age groups when comparing 2008–09 with 2011–12 (0–11 months, $p < 0.001$; 12–23 months and 2–4 years, $p = 0.002$; and 5–17 years, $p = 0.003$; all robust after FDR) (Table 3).

For the majority of IPD cases ($n = 430$), pneumococci were isolated from a normally sterile site. Only 41 cases involved solely the identification of pneumococcal DNA in CSF or pleural fluid: for these cases, no capsular serotype information is available. Of the 430 IPD cases from whom *S. pneumoniae* bacteria were recovered, 392 isolates were available for further characterisation. The remaining 38 isolates were lost before reaching the central laboratory for characterisation. Available isolates were recovered from blood ($n = 335$, 85.5%), CSF ($n = 39$, 9.9%), pleural fluid ($n = 15$, 3.8%) and peritoneal fluid ($n = 3$, 0.8%).

Serotype distribution

We detected 39 different capsular types as well as non-typable isolates among the 392 available isolates (Figure 2). The most frequent, which accounted for 57% ($n = 225$) of isolates from all analysed IPD cases in the study period, were serotypes 1 ($n = 74$, 18.9%), 19A ($n = 72$, 18.4%), 7F ($n = 43$, 11.0%), and 14 ($n = 36$, 9.2%). The proportion of IPD cases caused by PCV7 serotypes remained relatively constant, at around 21%. However, the fraction of IPD cases that could have been potentially prevented by PCV10 and PCV13 decreased significantly during the study period, from 59.4% to 47.1% ($p = 0.008$) and from 91.0% to 63.0% ($p = 0.024$), respectively. This was accompanied by an increase in serotype diversity when comparing 2008–09 (SID = 0.846, CI_{95%}: 0.810 to 0.882) with 2011–12 (SID = 0.957, 95% CI: 0.939–0.975).

In order to estimate the incidence of IPD due to individual serotypes, the cases for which no isolate was available ($n = 79$) were assumed to have the same serotype distribution as that found among isolates from

TABLE 3

Incidence of invasive pneumococcal infections caused by *Streptococcus pneumoniae* serotypes included in conjugate vaccine formulations by age group, Portugal, July 2008–June 2012^a

Age group	Serotype group	Incidence ^b (95% confidence intervals)			
		2008–09	2009–10	2010–11	2011–12
0–17 years	PCV7	1.75 (1.26–2.44)	1.41 (0.97–2.04)	0.82 (0.51–1.34)	1.10 (0.72–1.69)
	PCV10	4.98 (4.09–6.07)	3.66 (2.90–4.61)	2.01 (1.47–2.75)	2.17 (1.60–2.94)
	PCV13	7.50 (6.38–8.81)	5.27 (4.34–6.39)	3.10 (2.41–3.99)	2.87 (2.21–3.74)
	NVT	0.69 (0.41–1.17)	1.68 (1.20–2.36)	1.41 (0.97–2.05)	1.65 (1.16–2.33)
	All serotypes	8.19 (7.06–9.55)	6.95 (5.92–8.22)	4.51 (3.68–5.56)	4.52 (3.66–5.58)
5–17 years	PCV7	0.33 (0.14–0.79)	0.38 (0.17–0.85)	0 (0–0.27)	0 (0–0.27)
	PCV10	2.17 (1.53–3.07)	1.65 (1.11–2.46)	0.83 (0.47–1.46)	1.17 (0.72–1.88)
	PCV13	2.67 (1.95–3.65)	2.10 (1.48–3.00)	1.51 (0.99–2.30)	1.17 (0.72–1.88)
	NVT	0.08 (0.02–0.41)	0.53 (0.26–1.06)	0.38 (0.17–0.86)	0.53 (0.26–1.07)
	All serotypes	2.75 (2.02–3.74)	2.63 (1.92–3.61)	1.89 (1.30–2.75)	1.7 (1.14–2.53)
2–4 years	PCV7	1.36 (0.55–3.38)	2.02 (0.94–4.33)	0.41 (0.08–2.00)	2.36 (1.14–4.88)
	PCV10	9.5 (6.67–13.55)	8.07 (5.47–11.9)	3.67 (2.05–6.55)	3.14 (1.67–5.92)
	PCV13	11.31 (8.17–15.66)	9.58 (6.71–13.69)	4.07 (2.35–7.07)	5.11 (3.10–8.42)
	NVT	0 (0–1.21)	1.01 (0.35–2.90)	1.22 (0.46–3.26)	2.36 (1.14–4.88)
	All serotypes	11.31 (8.17–15.66)	10.59 (7.54–14.87)	5.30 (3.26–8.60)	7.46 (4.93–11.3)
12–23 months	PCV7	12.15 (7.00–21.08)	7.98 (4.08–15.61)	6.84 (3.25–14.38)	7.03 (3.41–14.52)
	PCV10	14.57 (8.80–24.14)	11.40 (6.48–20.04)	6.84 (3.25–14.38)	7.03 (3.41–14.52)
	PCV13	27.93 (19.37–40.29)	20.51 (13.44–31.32)	13.67 (8.03–23.27)	9.04 (4.76–17.19)
	NVT	3.64 (1.37–9.71)	5.70 (2.59–12.53)	7.98 (4.00–15.92)	7.03 (3.40–14.52)
	All serotypes	31.58 (22.37–44.57)	26.21 (18.02–38.13)	21.65 (14.16–33.1)	16.08 (9.9–26.12)
0–11 months	PCV7	12.75 (7.48–21.71)	7.70 (3.83–15.45)	8.01 (4.07–15.78)	7.38 (3.58–15.18)
	PCV10	21.24 (14.04–32.16)	10.99 (6.12–19.76)	9.15 (4.85–17.28)	8.85 (4.57–17.15)
	PCV13	43.55 (32.57–58.23)	21.99 (14.48–33.38)	12.59 (7.30–21.69)	14.75 (8.81–24.71)
	NVT	8.50 (4.44–16.26)	16.49 (10.19–26.68)	10.30 (5.65–18.77)	10.32 (5.59–19.08)
	All serotypes	52.05 (39.9–67.90)	38.48 (28.04–52.81)	22.89 (15.25–34.34)	25.08 (16.85–37.31)

NVT: non-vaccine serotypes; PCV: pneumococcal conjugate vaccine.

^a Epidemiological years: from week 26 of one year to week 25 of the following year.

^b Number of cases per 100,000 in specified age group.

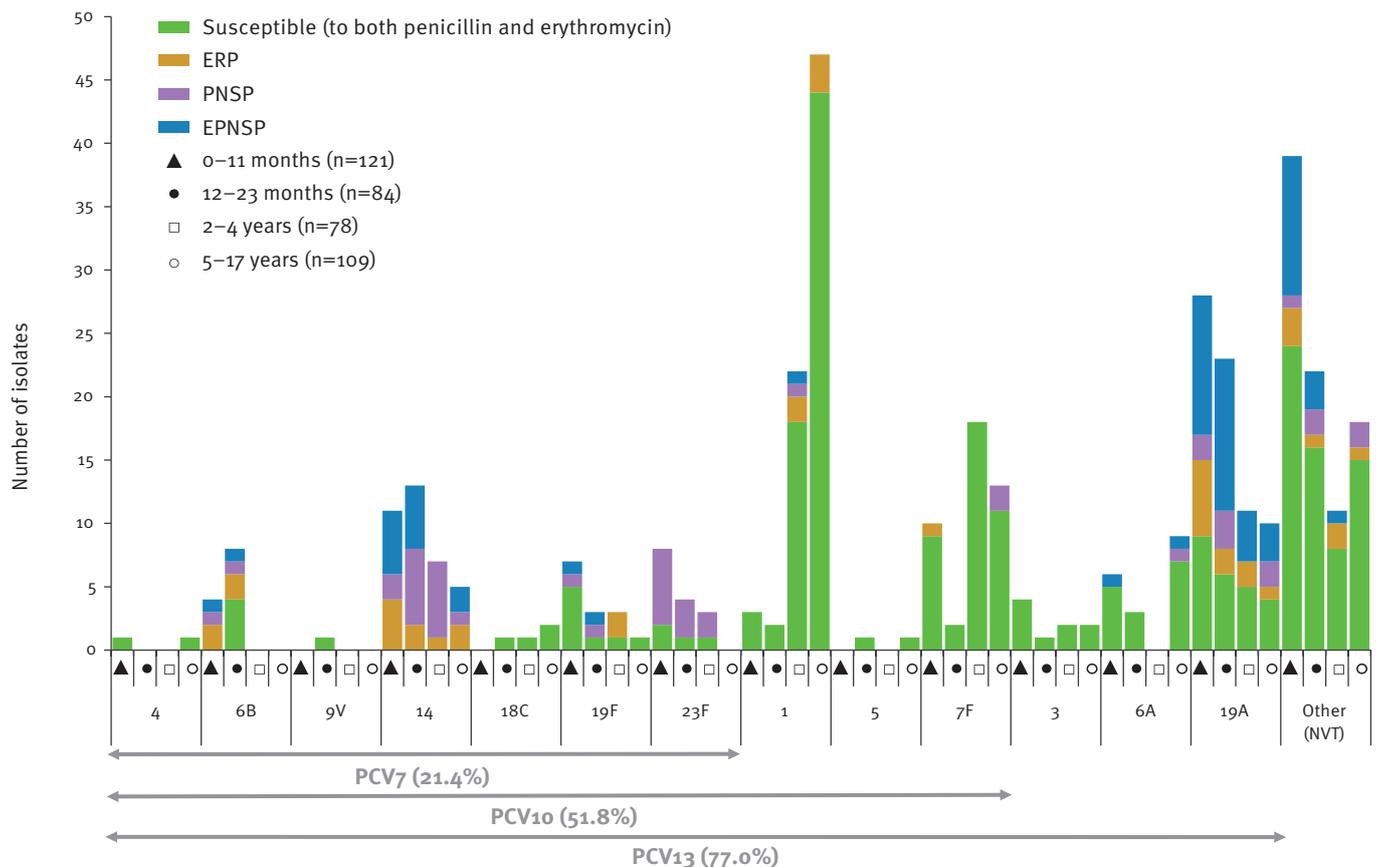
the same epidemiological year and age group. The overall incidence of IPD in all age groups due to PCV7, addPCV10 and addPCV13 serotypes decreased during the study period ($p=0.026$, $p<0.001$ and $p<0.001$ respectively, robust after FDR) while that of cases with NVT increased ($p=0.027$, also robust after FDR) (Figure 3A, Table 3). While IPD incidence due to the serotypes included in the conjugate vaccines decreased, these were still a significant cause of IPD in all age groups, accounting for 62.3% ($n=43$) of the isolates in 2011–12. When considering individual serotypes (for which at least five isolates were detected), the incidence of IPD due to serotypes 1 ($p<0.001$), 19A ($p<0.001$), 7F ($p=0.024$) and 14 ($p=0.015$) decreased and increases in serotype 10A ($p=0.001$) were noted. However, only the decrease in serotypes 1 and 19A incidence and the increase in 10A incidence were supported after adjustment by FDR.

In contrast to the continual decreases in the number of isolates of serotype 1 and 19A during the study period, serotype 7F decreased only in 2010–11 after the introduction of PCV10 (Table 4). These resulted in decreases in the proportion of isolates of addPCV10 from 37.6% in 2008–09 to 20.6% in 2011–12 and of addPCV13 from 31.6% in 2008–09 to 16.2% in 2011–12.

The overall changes in IPD incidence are the result of very different dynamics in the various age groups (Figure 3 and Table 3). In the youngest age group (0–11 months), IPD incidence due to the additional serotypes included in the expanded valency conjugate vaccines decreased (addPCV10, $p=0.006$; addPCV13, $p<0.001$, both robust after FDR) while the incidence due to PCV7 serotypes and NVTs did not change significantly. A similar situation occurred in those aged 12–23 months, but here only the addPCV13 ($p=0.004$) was robust after FDR.

FIGURE 2

Streptococcus pneumoniae isolates expressing serotypes present in conjugate vaccines causing invasive pneumococcal infections, Portugal, July 2008–June 2012 (n=392)



EPNSP: isolates presenting both erythromycin resistance and penicillin non-susceptibility; ERP: erythromycin-resistant isolates; NVT: non-vaccine serotypes; PNSP: penicillin non-susceptible isolates.

The number of isolates expressing each serotype in each of the age groups considered is shown. The serotypes included in each of the conjugate vaccines are indicated by the arrows and the percentage of the total number of isolates expressing the serotypes included in each of the vaccines is indicated. A total of 27 NVT were detected, representing 90 isolates as follows: 10A, 15A and 23B (n=7 each); 22F (n=6); 8, 15B, 15C, 21 and 24F (n=5 each); 25A (n=4); 9N, 11A, 20, 24A, 33A and non-typable (n=3 each); 6C, 16F, 29, 33F and 35F (n=2 each); 7C, 12B, 18B, 28A, 34 and 45 (n=1 each).

In patients aged 2–4 years, there was no significant change in IPD incidence due to addPCV13, but there was a substantial decrease in IPD incidence due to the addPCV10 serotypes ($p < 0.001$) accompanied by an increase in IPD incidence due to NVTs ($p = 0.007$), both supported after adjustment by FDR. In the older age group (5–17 years), in which no individuals were vaccinated with PCV10 or PCV13, the decreases in IPD incidence due to addPCV10 and addPCV13 serotypes were not statistically significant: the only significant change was the decrease in IPD incidence due to PCV7 serotypes ($p = 0.006$, robust after FDR).

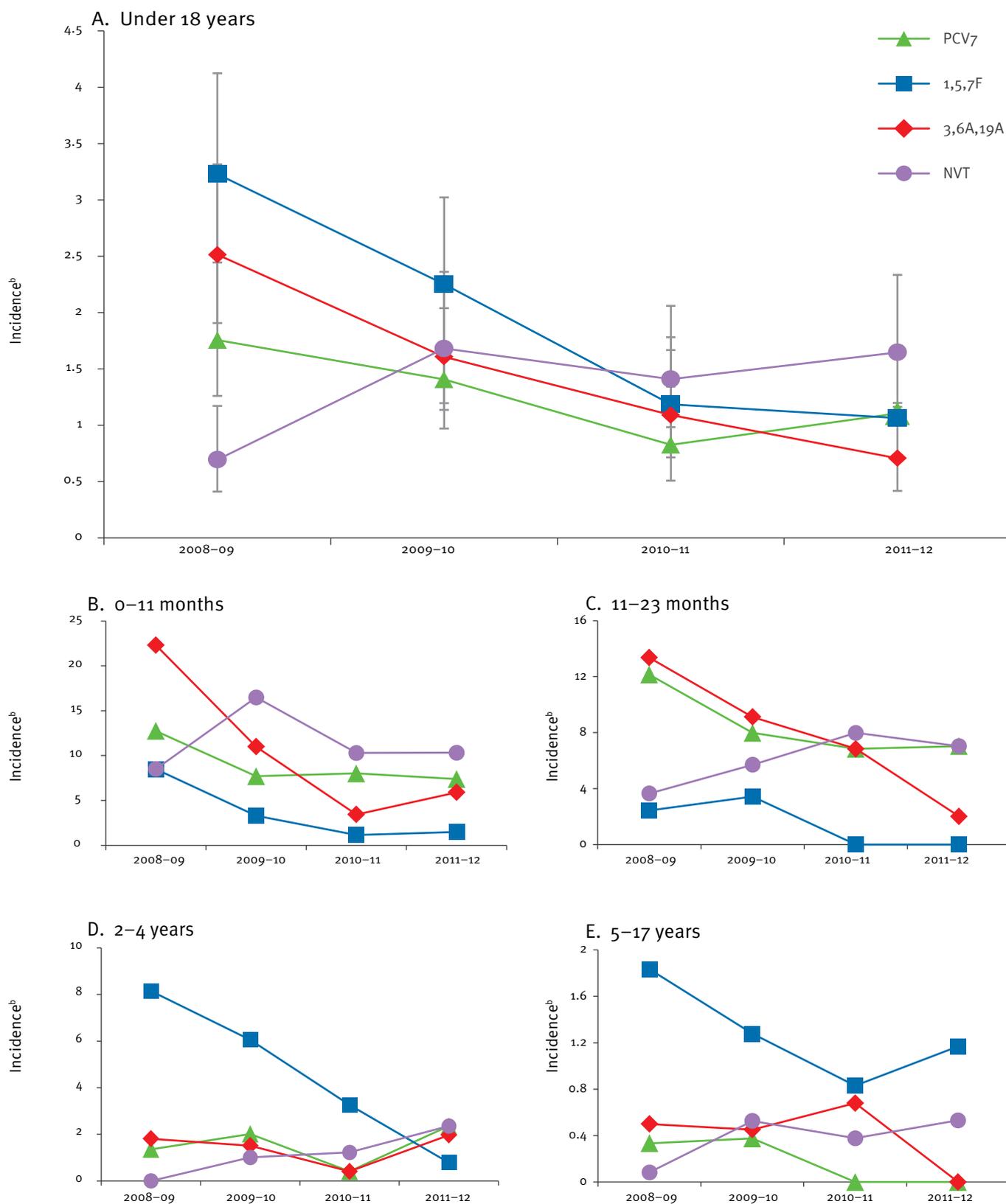
Antimicrobial susceptibility

Resistance to the tested antimicrobials is summarised in Figure 2 and Table 5. Overall, 110/392 isolates (28.1%) were non-susceptible to penicillin (PNSP): 82 (20.9%) expressed low-level resistance and 28 (7.1%)

high-level resistance [17]. Considering the current CLSI breakpoints for parenteral penicillin [16], 16/39 isolates from CSF would have been considered resistant and three isolates (0.8%) from 353 non-meningitis cases would have been considered non-susceptible, all intermediately resistant. Resistance to erythromycin (ERP) was found in 103/392 isolates (26.0%), of which 78 isolates (75.7%) expressed the MLS_B phenotype and 25 (24.3%) the M phenotype. All isolates were susceptible to levofloxacin, vancomycin and linezolid. The simultaneous expression of erythromycin resistance and penicillin non-susceptibility (EPNSP) was found in 64/392 (16.3%) of the isolates. Within the study period, there was a modest decrease in ERP (when comparing 36/133 (27.1%) in 2008–09 with 16/68 (23.5%) in 2011–12) and a more noticeable decrease in PNSP (when comparing 45/133 (33.8%) in 2008–09 with 14/68 (20.6%) in 2011–12).

FIGURE 3

Incidence of invasive pneumococcal disease due to vaccine serotypes in patients aged under 18 years, Portugal, July 2008–June 2012^a



NVT: non-vaccine serotypes; PCV7: 7-valent pneumococcal conjugate vaccine.

^a Epidemiological years: from week 26 of one year to week 25 of the following year.

^b Number of cases per 100,000 in specified age group.

TABLE 4

Serotypes of the isolates responsible for invasive pneumococcal disease in patients aged under 18 years, Portugal, July 2008–June 2012^a (n=392)

Serotype	Number of isolates			
	2008–09	2009–10	2010–11	2011–12
1	33	20	14	7
3	2	3	1	3
4	1	0	0	1
5	2	0	0	0
6A	5	5	5	3
6B	5	2	2	3
7F	15	15	6	7
9V	0	0	1	0
14	13	12	6	5
18C	0	3	0	1
19A	35	19	13	5
19F	5	2	3	4
23F	5	4	2	4
NVT	12	29	24	25
Total	133	114	77	68

NVT: non-vaccine type.

^a Epidemiological years: from week 26 of one year to week 25 of the following year.

Together, serotypes 19A and 14 contributed greatly to ERP (62/103, 60.2%) and PNSP (64/110, 58.2%) (Figure 2). Serotypes included in PCV10 represented 33/46 (71.7%), 21/39 (53.8%) and 17/64 (26.6%) of PNSP, ERP and EPNSP, respectively, while serotypes included in PCV13 constituted 41/46 (89.1%), 32/39 (82.1%) and 49/64 (76.6%), respectively.

Discussion

The most important finding of our study is the decrease in incidence of IPD in all age groups analysed. Moreover, the changes in distribution of the serotypes of pneumococci causing IPD in Portugal that accompanied the introduction of PCV7 [9,10] have continued with the introduction of PCV10 and PCV13. Overall, IPD incidence due to the serotypes included in the PCVs decreased (Table 3 and Figure 3A): this affected particularly the additional serotypes included in PCV10 and PCV13 and to a lesser extent the serotypes included in PCV7. The incidence of IPD means that the actual number of isolates per serotype in each epidemiological year is small (Table 4). This is even more marked when stratifying by age group and in the last years of the study, due to the decrease in IPD incidence.

The reasons behind the persistence of PCV7 serotypes as causes of IPD may be multifactorial [12,20].

TABLE 5

Antimicrobial resistance of *Streptococcus pneumoniae* isolates responsible for invasive pneumococcal disease in patients aged under 18 years, Portugal, July 2008–June 2012^a (n=392)

Antibiotic	Number of resistant isolates (%) ^b			
	0–11 months (n=121)	12–23 months (n=84)	2–4 years (n=78)	[5–17 years (n=109)
PEN ^c	43 (35.5)	38 (45.2)	15 (19.2)	14 (12.8)
MIC ₉₀	0.75	1.5	0.75	0.064
MIC ₅₀	0.023	0.032	0.016	0.016
CTX	4 (3.3)	7 (8.3)	1 (1.3)	2 (1.8)
MIC ₉₀	0.75	0.75	0.5	0.125
MIC ₅₀	0.023	0.032	0.023	0.023
CRO	6 (5.0)	4 (4.8)	0 (0.0)	2 (1.8)
MIC ₉₀	0.75	1	0.5	0.125
MIC ₅₀	0.032	0.032	0.032	0.023
ERY	46 (38.0)	29 (34.5)	15 (19.2)	13 (11.9)
CLI	34 (28.1)	23 (27.4)	13 (16.7)	8 (7.3)
CHL	6 (5.0)	4 (4.8)	0 (0.0)	4 (3.7)
SXT	27 (22.3)	21 (25.0)	16 (20.5)	15 (13.8)
TET	29 (24.0)	21 (25.0)	11 (14.1)	13 (11.9)

CHL: chloramphenicol; CLI: clindamycin; CRO: ceftriaxone; CTX: cefotaxime; ERY: erythromycin; LEV: levofloxacin; MIC: minimal inhibitory concentration; PEN: penicillin; SXT: trimethoprim-sulfamethoxazole; TET: tetracycline.

^a Epidemiological years: from week 26 of one year to week 25 of the following year.

^b Unless otherwise specified.

^c Number of isolates and percentage of penicillin non-susceptible isolates is indicated.

Chief among those could be the slower uptake and lower vaccination coverage in Portugal when compared with countries where PCV7 was introduced in the national immunisation plan [1,2]. As with serotype 19A (see below), the high proportion of resistant isolates expressing PCV7 serotypes, particularly to penicillin and macrolides, could be an important factor in their persistence. Serotype 14, which is known to represent more virulent clones [21], remains the most important PCV7 serotype in IPD. In contrast to our data, a recent study in Portugal found the virtual elimination of PCV7 serotypes from nasopharyngeal carriage, with the exception of serotype 19F, a serotype already associated with carriage before the introduction of PCVs [22].

The serotype dynamics underpinning the changes in IPD incidence were different in the various age groups. In the youngest children (under 2 years), including those vaccinated with either PCV10 or PCV13, there were decreases in IPD caused by the serotypes included in the PCVs (Figure 3, B and C). While a reduction in

incidence due to addPCV10 could be expected in 2009–10, since the PCV10 vaccine was available from the outset of the epidemiological year, the reduction in incidence due to addPCV13 occurred in spite of PCV13 becoming available only in early 2010. IPD incidence due to PCV7 serotypes decreased slightly, but not significantly: these serotypes remain important causes of IPD in spite of more than a decade of PCV7 use.

The 2–4 years age group started including children potentially vaccinated with at least one dose of PCV10 or PCV13 in 2010–11. In this age group, there was a strong decrease in the IPD incidence due to addPCV10 serotypes already in 2009–11 (Figure 3D), whereas no significant change was seen in incidence due to addPCV13. In the older age group, (5–17 years), the decreases in IPD incidence caused by addPCV10 occurring between 2008–09 and 2010–12 and those due to addPCV13 serotypes in 2011–12 were not statistically significant. Taken together, our data suggest that there may be different reasons for the decreases in the incidence due to the various serotypes included in both PCV10 and PCV13 and those included only in PCV13.

Limited data available from observational studies in different geographical regions have documented decreases in IPD incidence due to PCV13 serotypes following PCV13 introduction and of serotypes 1 and 19A in particular [6,7]. A field study showed clear effectiveness of PCV13 against serotype 19A IPD [4], while another study demonstrated reduced nasopharyngeal acquisition of both serotypes 1 and 19A [8].

Decreases in the proportion of isolates expressing serotypes 1 and 5, included in both PCV10 and PCV13, and of serotype 6A, included only in PCV13, were noted in the adult (≥ 18 years) population in Portugal between 2009 and 2011 [12]. In contrast, the proportion of isolates expressing serotype 19A, also included only in PCV13, did not change significantly as a cause of IPD in the adult population in the country [12]. The herd effect that is known to occur due to PCV7, and now also expected for PCV10 and PCV13, is predicted to be delayed relative to the effects on the vaccinated children [2,9,23]. It is therefore unlikely that the changes seen in adults, and now in older unvaccinated paediatric age groups, can be attributed to PCV use. These data emphasise the importance of unexplained temporal trends in the distribution of pneumococcal serotypes [24] as potential confounders in observational studies such as ours. Similar to our observations, a vaccine trial in Alaska saw decreases in the incidence of PCV13 serotype IPD even before a direct effect of vaccination would be expected [20].

Although marked fluctuations in the incidence of serotype 1 IPD have been known to occur, no such changes have been documented in IPD due to serotype 19A, one of the most important serotypes that emerged in the post-PCV7 era and a serotype that remains a stable cause of IPD in adults in Portugal [10,12,25]. Another

important difference between isolates expressing these two serotypes is their antimicrobial resistance. While serotype 1 isolates are mostly susceptible to the antimicrobials most frequently used to treat pneumococcal infections, serotype 19A isolates are frequently resistant to penicillin and the macrolides [10,24,25], a characteristic that has been maintained throughout the study period (Figure 2).

Our data argue that factors other than vaccination, such as unrelated temporal trends, triggered the decrease in the incidence of the two serotypes that had become the leading causes of paediatric IPD in the post-PCV7 years: serotypes 1 and 19A (Table 4) [10]. The reason for these changes remains unknown and may be different for these two serotypes. However, even if the observed serotype changes were not triggered by vaccination, PCV use might have reinforced them.

Although the decreases in incidence of PCV13 serotypes were partly offset by an increase in incidence of NVT IPD, the overall result was a substantial reduction in the incidence of paediatric IPD. An increase in NVT IPD incidence was noted in most age groups, although only in the 2–4 year-olds was this increase statistically significant. The decrease in the incidence of cases due to PCV13 serotypes resulted in multiple serotypes becoming more prominent causes of IPD, leading to increased serotype diversity of IPD cases. Serotype 10A was the only NVT serotype rising significantly in an overall analysis. However, this serotype was not among the most frequent NVT serotypes detected in a carriage study [22] nor was it found to be particularly invasive [21], raising the possibility that this will not be a sustained increase.

The decrease in overall PNSP during the study period reflected primarily the decrease of serotype 19A. When comparing with previous data [10], resistance to penicillin and cefotaxime remained unchanged in all age groups and there was a modest decline in ceftriaxone resistance. On the other hand, resistance to erythromycin and clindamycin rose in the youngest children (aged < 2 years) but remained approximately constant in the older age groups. Serotype 19A and the PCV7 serotypes, particularly serotype 14, remained the most important serotypes in terms of resistance, suggesting a potential influence of PCVs in the dynamics of antimicrobial resistance.

Since the criterion for identification of an IPD case is the isolation of pneumococci from a normally sterile body site or the identification of pneumococcal DNA in cerebrospinal fluid (CSF) or pleural fluid, and this is almost exclusively done in hospital laboratories, we believe that few cases of laboratory-confirmed IPD would have been diagnosed outside of our network. The active nature of the surveillance and the involvement of a large number of hospitals covering the entire country offer further reassurance that our surveillance system identified most cases. We cannot guarantee that

the serotype distribution of the cases where isolates were unavailable followed the serotype distribution of available isolates, but we consider that the approach adopted minimises potential bias and describes the actual situation. However, the small proportion of cases with unknown serotype information ensures that our extrapolation does not affect the results.

In spite of substantial decreases in PCV13 IPD incidence in all age groups (Table 3), these serotypes remain the most important causes of IPD, being responsible for 63.2% of IPD cases in patients under 18 years in 2011–12 (Table 4). The persistence of these serotypes, including the PCV7 serotypes that have been subject to vaccine pressure for more than a decade, suggests that this could be due to the relatively modest vaccination coverage in Portugal. Coverage peaked around 2008 at 75% but declined to 63% in 2012 (data not shown), a lower coverage than in countries where PCVs are in the national immunisation plan and highlighting the potential benefits of increasing vaccination coverage. Although the study period is probably too close to vaccine introduction to expect a herd effect in older unvaccinated children, the relatively lower vaccination coverage may also compromise the extent of this effect. This may reduce the overall benefits of vaccination, including the potential protection of infants younger than 90 days [26] who are not currently protected by direct vaccination. Continued surveillance will monitor the extent of PCV13 success by evaluating the capacity of PCV13 serotypes to persist in spite of vaccination and in documenting the changes in antimicrobial resistance that accompany the changing serotypes and the emergence of any NVT replacement serotypes.

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Conflict of interest

JMC research grants administered through his university and honoraria for serving on speakers bureaus of Pfizer, Gilead and Novartis. MR honoraria for serving on the speakers bureau of Pfizer and for consulting for GlaxoSmithKline.

Authors' contributions

JMC, MJB and MR developed the design for the study; MJB, JMC and the Portuguese Group for the Study of Streptococcal Infections and the Portuguese Study Group of Invasive Pneumococcal Disease of the Paediatric Infectious Disease Society coordinated the collection of the surveillance data; SIA, ANH and JPL characterised the isolates; JMC, SIA and MR analysed the data; JMC and MR drafted the manuscript; all co-authors reviewed and contributed to the final version of the manuscript.

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European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2012 published

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On 25 March, EFSA and ECDC jointly published ‘The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2012’ [1]. The report features *Salmonella*, *Campylobacter*, *E. coli* indicator bacteria and *Staphylococcus aureus*.

Salmonella and *Campylobacter* are the most common causes of zoonotic foodborne infections. According to the report, antimicrobial resistance was detected commonly in isolates from human cases as well as from food-producing animals and food in the European Union (EU). Almost half of the isolates from clinical cases were resistant to at least one antimicrobial and 28.9% were multidrug-resistant.

Levels of clinical resistance in *Salmonella* spp. isolates from humans to critically important antimicrobials in human medicine, such as cefotaxime and ciprofloxacin, were relatively low, and co-resistance across the countries that submitted data was very low. *Salmonella* spp. isolates of food and animal origin from fattening turkeys, broiler meat, turkeys and broilers of *Gallus gallus*, showed the highest resistance to ciprofloxacin varying from 46.0 % to 86.2 % in the reporting countries.

In *Campylobacter* spp. isolates from human cases, clinical resistance to common antimicrobials was frequently detected. Very high proportions of *Campylobacter* spp. isolates (47.4% EU average) were resistant to ciprofloxacin, with increasing trends observed in several countries. High to extremely high resistance to fluoroquinolones was also observed in *Campylobacter* isolates of food and animal origin such as *Gallus gallus* and broiler meat, pigs and cattle (ranging from 32.0 % in isolates from pigs to 82.7 % in isolates from broiler meat).

Among indicator (commensal) *E. coli* isolates from animals, resistance to ampicillin, streptomycin sulfonamides and tetracyclines, was commonly reported in

Gallus gallus and pigs (29.5 %–54.7 %) and levels were lower in cattle (24.5 %–30.6 %). Resistance to ciprofloxacin and nalidixic acid was highest among *E. coli* isolates from *Gallus gallus* and lower in pigs and cattle.

Antimicrobial resistance in humans was determined using clinical breakpoints, whereas epidemiological cut-off values (ECOFFs) were used to interpret the food and animal data. The use of ECOFFs aims to detect early resistance development to enable actions to be taken before the bacteria become impossible to treat. The use of different types of interpretive criteria limits the direct comparison of resistance data obtained for humans and food/animals.

The levels of clinical resistance to antimicrobials in humans showed a great variability across participating countries, partly due to the use of different methods and criteria for interpreting data. To obtain improved data quality and comparability in the future, ECDC is launching the EU protocol for harmonised monitoring of antimicrobial resistance in *Salmonella* and *Campylobacter* isolates from humans [2].

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