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MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and *S. Rissen* clones in Portugal, 2011 to 2015

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The *mcr-1* gene was found in 11 isolates from a Portuguese *Salmonella* collection (n = 1,010; 58 serotypes; 2002–15) of clinical samples, foodstuff, food-animals and water. *Mcr-1* has been located on different plasmids (IncX4/IncHI2) in pig-associated multidrug-resistant, copper-tolerant *S.* 1,4,[5],12:i:-/ST34 and *S. Rissen*/ST469 clones from human and pork products since at least 2011. Our data highlight dissemination of *mcr-1* by successful resistant clones in Europe and raise questions about the efficacy of copper-based interventions to reduce colistin use.

Since the description of plasmid-mediated colistin resistance encoded by the *mcr-1* gene in *Enterobacteriaceae* from multiple sources in China [1] and its worldwide dissemination mostly in animal sources [2], the use of polymyxins (colistin) in food-producing animals has been questioned in Europe because it may have an impact on human health [3]. Nevertheless, data on the transmission of *mcr-1*-mediated colistin resistance particularly by clonal expansion are lacking [3,4]. In fact, the *mcr-1* gene has been found in zoonotic food-borne bacteria such as *Salmonella* [2] but the presence of this gene in particular successful resistant clones has not been demonstrated [3]. In this study, we report the presence of the *mcr-1* gene in pig-associated clinically relevant *Salmonella* serotypes and clones recovered from human clinical samples and pork products in Portugal, collected as early as 2011.

Laboratory investigation

We analysed a total of 1,010 *Salmonella* isolates of 58 serotypes from several sources (human clinical cases, food products, food-animal production settings and aquatic environments) and regions of Portugal, collected between 2002 and 2015 (Table 1). The isolates were screened for the *mcr-1* gene by PCR and sequencing, using primers CLR5-F (5'-CGGTCAAGTCCGTTTGTC-3')

[1] and *Mcr1*-Rv2 (5'-CCAGCGTATCCAGCACATT-3') [this study].

The 1,010 isolates comprised the most frequent worldwide *Salmonella* serotypes (n=256 *S. Typhimurium* and n=34 *S. Enteritidis*), but also emerging serotypes (n=436 *S.* 1,4,[5],12:i:- and n=93 *S. Rissen*) or serotypes less frequently detected in European surveillance studies (n=191 isolates from 54 different serotypes). They included all isolates previously characterised [5,6] and recent ones from ongoing surveillance studies (data not shown) covering all serotypes, sampling dates, sources, regions, antibiotic susceptibility phenotypes/genotypes and PFGE types. The isolates positive for *mcr-1* by PCR were further tested for susceptibility to colistin by the proposed broth microdilution method [7] and interpreted according to the European Committee on Antimicrobial Susceptibility Testing [8]. Isolates were also subjected to standard conjugation assays using the recipient strain *Escherichia coli* HB101 [6]. Replicon typing, pMLST, hybridisation experiments (I-Ceul/S1-PFGE nuclease) [5,9] and detection of the insertion sequence element IS*Apl1* was performed in *Salmonella* strains and transconjugants. The presence and location of IS*Apl1* was determined using primers IS*Apl1*-Fw (5'-GTCGCTTTGGACATTGGGAA-3') and IS*Apl1*-Rv (5'-GATTGATGTCTTGGTGTCTCGG-3') designed as part of this study, and CLR5-R (5'-CTTGGTCGGTCTGTAGGG-3') [1]. Clonal relatedness of *Salmonella* strains was assessed by *Xba*I PFGE [5,6] and MLST [10].

Detection of *mcr-1* gene in pig-associated clinically-relevant clones

The *mcr-1* gene was detected in 11 (1.1%) of the 1,010 Portuguese *Salmonella* isolates, recovered from human clinical sources and pork food products from across the country (Table 1, Table 2). This gene had 100% homology with the first published *mcr-1* sequence in an

TABLE 1

Salmonella isolates from different sources by year and presence of the *mcr-1* gene, Portugal, 2002–2015 (n = 1,010)

Source (number of isolates)	Years	Isolates tested for <i>mcr-1</i> (serotype/number of isolates) ^a	<i>mcr-1</i> -positive isolates (serotype/number of isolates) ^a
Human clinical cases (n = 522)	2002–10	258	0
	2011–12	155 (<i>S.</i> 1,4,[5],12:i:-/n = 75)	4 (<i>S.</i> 1,4,[5],12:i:-)
	2013–15	109	0
Food products (n = 413)			
Pork (n = 296)	2002–13	44	0
	2014–15	252 (<i>S.</i> 1,4,[5],12:i:-/n = 130; <i>S.</i> Rissen/n = 23)	7 (<i>S.</i> 1,4,[5],12:i:-/n = 5; <i>S.</i> Rissen/n = 2)
Other ^b (n = 117)	2002–15	117	0
Food production animals (n = 58)			
Pigs/piggeries (n = 54)	2006–08	54	0
Aquacultures (n = 4)	2010–12	4	0
Aquatic environment (n = 17)	2002–11	17	0

^a The serotypes of *Salmonella* isolates are presented only for those among which *mcr-1*-positive ones were detected.

^b Other studied food products comprised: poultry, beef, cow, quail, clam and cooked meals.

Escherichia coli strain from China (GenBank accession number: KP347127) [1], which was further described in diverse other *Enterobacteriaceae* including sporadic *Salmonella* isolates from European countries (France, the Netherlands, Spain, the United Kingdom) [2,11–14]. In most of these studies, detection of *mcr-1* gene was only performed in colistin-resistant isolates. This impairs the determination of its real prevalence because the gene may be silent, as described in one *E. coli* strain [15]. All our isolates carrying the *mcr-1* gene presented a minimum inhibitory concentration (MIC) of 4–8 mg/L for resistance to colistin (Table 2).

During the study period (2002 to 2015), *Salmonella* isolates harbouring the *mcr-1* gene were only recovered between 2011 and 2015 and originated from human clinical sources (0.8%, n = 4/522) and pork products, mostly from slaughterhouses, (2.4%, n = 7/296) (Table 1). Colistin has been widely used in veterinary medicine, particularly in food-producing animals, primarily in pigs [16,17]. The available data from 2004 to 2006 had already shown high use of colistin for food-producing animals in Portugal [18], which is one of the European countries with highest consumption of polymyxins that has been increasing in the last years (2011–13) [3,19]. Taking into account the current picture of colistin use in Portugal, the detection of *mcr-1* in the most recent collections and in pork products is of concern. Nevertheless, data on chronology, current prevalence of the *mcr-1* gene and its evolution in bacteria from animals, food and humans are lacking [3].

The 11 *mcr-1*-positive *Salmonella* isolates belonged to the serotypes *S.* 1,4,[5],12:i:- and *S.* Rissen (Table 2), which have been strongly associated with pig production and caused human infections in Europe [5,6,20–22] including in Portugal [23]. In both cases, we found them associated with particular successful

multidrug-resistant (MDR) clonal lineages, either of the *S.* Rissen/ST469 clone or the *S.* 1,4,[5],12:i:-/ST34 European clone that is currently spreading epidemically in European countries [5,20,22] and has been dominant in our *Salmonella* collection for the last years [5,6,20; unpublished data]. A previous report on *mcr-1* in the clinically relevant *Salmonella* serotype *S.* Typhimurium/ST34 was associated with travel to South-East Asia [13].

Of note, all *S.* 1,4,[5],12:i:- and *S.* Rissen *mcr-1*-carrying isolates were co-resistant to antibiotics used in a human and/or veterinary context and carried diverse metal tolerance genes, remarkably those conferring tolerance to copper (all carrying *pcoD*+*silA* on the chromosome) (Table 2), a feed additive mostly used for pigs or piglets in Europe. The fact that these successful clones presented higher tolerance to copper, as previously demonstrated [6,20], can contribute to their selection and wider expansion with potential repercussions for *mcr-1* transmission.

Location of *mcr-1* gene in diverse plasmid backbones

The *mcr-1* gene was located on two plasmid types, IncX4 (n = 5; 35 kb; 4 transferable) and IncHI2 (n = 6), either of ST4 subtype (n = 3; 200–300 kb; all transferable) or non-typeable (n = 3; 120–125 kb; all non-transferable) and mostly associated with the IS*Apl1* transposable element (Table 2). IncHI2/ST4 and IncX4 plasmids have been widely implicated in the spread of *mcr-1* gene in diverse *Salmonella* serotypes and other *Enterobacteriaceae* in European and non-European countries, both from human and animal sources [2,12–14]. Transferability of the *mcr-1* gene was achieved from *S.* Rissen (n = 1) and *S.* 1,4,[5],12:i:- (n = 6) isolates and was associated with a 32–64-fold increase in the colistin MIC and, in some isolates, with acquisition of

TABLE 2

Characterisation of *Salmonella* isolates recovered from clinical and food samples and carrying the *mcr-1* gene, Portugal, 2011–2015 (n = 11)

Serotype ^a (number of isolates)	Source-origin (number of isolates)	Clone designation; ST(eBG); PFGE-type ^b (number of isolates, source)	Year/ Regions	Antibiotic resistance phenotype/genotype ^c (number of isolates)	Metal tolerance genes (number of isolates) ^d	Plasmid-mediated colistin resistance <i>mcr-1</i>		
						Colistin MIC-mg/L, donor (transconjugant/ number of isolates) ^e	Plasmid type carrying <i>mcr-1</i> gene (pMLST, Kb) ^f (number of isolates)	ISA _{aplI} upstream of <i>mcr-1</i> (number of isolates)
1,4,[5],12:i:- (n=9)	Clinical faeces/blood (n=4)	European clone; ST34(eBG1); C (n=1, 1 hospital), E (n=3, 2 hospitals)	2011–12 North	AMP, (GEN), STR, SUL, TET/ <i>bla</i> _{TEM} , [<i>aac</i> (3)-IV], <i>strA</i> - <i>strB</i> , <i>sul2</i> , <i>tet</i> (B) (n=4)	<i>pcoD</i> + <i>silA</i> + <i>merA</i> + (<i>terF</i>) (n=4)	4–8 (4–8/n=1)	X ₄ (35) (n=1) HI2 (NT, 120–125) (n=3)	No (n=1) Yes (n=3)
	Pork carcass (n=4)	European clone; ST34(eBG1); A (n=1, 1 slaughterhouse), B (n=2, 2 slaughterhouses), F (n=1, 1 slaughterhouse)	2014–15 North, Centre	AMP, (CLO), (CIP, PEF), STR, SUL, TET, (TMP)/ <i>bla</i> _{TEM} , (<i>floR</i>)/(<i>catA</i> - <i>cmiA</i>), (<i>aadA1/aadA2</i>) – <i>strA</i> - <i>strB</i> , <i>sul1-sul3/sul2</i> , <i>tet</i> (A)/ <i>tet</i> (B), (<i>dfrA1</i> / <i>dfrA12</i>) (n=4)	<i>pcoD</i> + <i>silA</i> + (<i>merA</i>) + (<i>terF</i>) (n=4)	4–8 (4–8/n=4)	X ₄ (35) (n=2) HI2 (ST4, 230–300) (n=2)	No (n=2) Yes (n=2)
	Pork meat (n=1)	European clone; STNew/ Single locus variant of ST34; B (n=1, 1 meat production unit)	2015 South	AMP, STR, SUL, TET/ <i>bla</i> _{TEM} , <i>strA-strB</i> , <i>sul2</i> , <i>tetB</i> (n=1)	<i>pcoD</i> + <i>silA</i> + <i>merA</i> + <i>terF</i> (n=1)	4 (4/n=1)	HI2 (ST4, 200) (n=1)	No (n=1)
Rissen (n=2)	Pork carcass (n=2)	ST469(eBG66); N (n=2, 2 slaughterhouses)	2014–15 North	AMP, CLO, STR, SUL, (TET), TMP/ <i>bla</i> _{TEM} , <i>cmiA</i> , <i>aadA1</i> , <i>sul1-sul3</i> , [<i>tet</i> (A)], <i>dfrA1</i> (n=2)	<i>pcoD</i> + <i>silA</i> + <i>merA</i> (n=2)	4 (4/n=1)	X ₄ (35) (n=2)	No (n=2)

AMP: ampicillin; CIP: ciprofloxacin; CLO: chloramphenicol; GEN: gentamicin; MIC: minimum inhibitory concentration; PEF: pefloxacin; pMLST: plasmid multilocus sequence type; STR: streptomycin; SUL: sulfamethoxazole; TET: tetracycline; TMP: trimethoprim.

^a The serotypes of *Salmonella* isolates were determined by classical serotyping, performed at the National Centre of Salmonella (NSA, Lisbon, Portugal) and/or PCR assay for determination of S. 4,[5],12:i:- [5].

^b PFGE types are designated by capital letters and include previously described types [5,6] and types described for the first time in this study. The human clinical isolates (n=4 from four patients) were recovered from three hospitals, and pork products (n=7) were recovered from six slaughterhouses and one meat production unit.

^c Antimicrobial susceptibility was evaluated by disc diffusion assay. Variable antibiotic resistance phenotypes and genotypes are presented between brackets; Antibiotic resistance patterns and genes transferred by conjugation are underlined; In two S. 1,4,[5],12:i:- isolates, transfer of genes *strA-strB* and/or *bla*_{TEM} was observed; Some genes were included on class 1 integrons (1,700bp (*dfrA1-aadA1*) or 2,000bp (*dfrA12-oriF-aadA2*); Integrons were located on the chromosome in S. Rissen (n=2) and on the IncHI2/ST4 plasmid in S. 1,4,[5],12:i:- isolates (n=2).

^d Screening for genes encoding tolerance to metals were done by PCR [20]. Metal tolerance genes that were not observed in all the isolates are presented between brackets; Metal tolerance genes transferred by conjugation are underlined. All *pcoD* + *silA* genes were chromosomally located.

^e Recipient strain used in conjugation assays: *Escherichia coli* HB101 (azide sodium, resistant to streptomycin and kanamycin); colistin MIC = 0.125 mg/L).

^f Plasmid types carrying the *mcr-1* gene transferred by conjugation are underlined.

resistance to other antibiotics and metals tolerance genes (Table 2). The fact that successful MDR *S. 1,4,[5],12:i:-* and *S. Rissen* clones have the ability to acquire plasmids carrying the *mcr-1* gene is of concern because colistin resistance may contribute to their further expansion, particularly in the pig reservoir. In addition, those strains could act as reservoir of *mcr-1*-carrying plasmids with a broad host range enhancing colistin resistance transmission for other clinically relevant bacteria sharing the same ecological niche.

Conclusions

This study has evidenced the acquisition of *mcr-1*-carrying plasmids by two clinically relevant MDR and copper-tolerant clones of *S. 1,4,[5],12:i:-* and *S. Rissen*, strongly associated with pork food products and which were dominant in the collection studied. The detection of *S. 1,4,[5],12:i:-* from human infections, already in 2011, is also of note, suggesting long-term dissemination of this resistance gene in humans in Portugal. Finally, the detection of *mcr-1* in copper-tolerant clones raises questions about the efficacy of recently suggested metal-based interventions (e.g. copper) to reduce the use of colistin and contain *mcr-1* dissemination [3].

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Conflict of interest

None declared.

Authors' contributions

JC, LP and PA designed the study and analysed epidemiological, microbiological and molecular data, JC and LC performed the phenotypic and molecular assays, JC and PA wrote the first draft of the manuscript, PA and LP participated in the coordination and concept of the manuscript and revised the final version.

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Detection of Zika virus RNA in whole blood of imported Zika virus disease cases up to 2 months after symptom onset, Israel, December 2015 to April 2016

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Zika virus RNA presence in serum, whole-blood and urine samples from six Israeli travellers symptomatic for Zika virus disease was examined. Whole-blood samples were positive for as late as 2 months (58 days) post-symptom onset, longer than for urine (26 days) and serum (3 days). These findings suggest the utility of whole blood in Zika infection diagnosis.

The ongoing Zika epidemic was recently declared by the World Health Organization as a Public Health Emergency of International Concern [1]. Laboratory diagnosis of Zika virus infection is thus important, but remains however challenging, due to a limited time window of possible virus detection, whereby patients tested more than one to two weeks post-symptom onset are difficult to diagnose [2-4]. There is therefore a need for an accurate diagnostic approach that will prolong the diagnostic period. We examined the utility of whole blood (WB) samples for detecting Zika virus RNA, compared with serum, or urine samples. Our findings suggest that Zika virus-RNA can be found in WB for a longer period than in serum and urine. More importantly, we show that Zika virus RNA can be identified in WB as late as 2 months after the onset of Zika virus disease (ZVD) symptoms.

Zika virus RNA in serum, urine and whole blood

All patients suspected of being infected with Zika virus were diagnosed at the National Center for Zoonotic Viruses, which is part of the Central Virology Laboratory (CVL) of the Israel Ministry of Health and is the reference laboratory for the diagnosis of arboviral infections in Israel. From December 2015 to April 2016, 145 patients returning to Israel after travel to Zika virus endemic areas, were respectively tested for the virus by real-time polymerase chain reaction (qRT-PCR) on different clinical specimens. Altogether 423 tests were

performed; 158 on sera, 135 on WB and 130 on urine samples. Ten samples from six patients were positive for Zika virus RNA (Table).

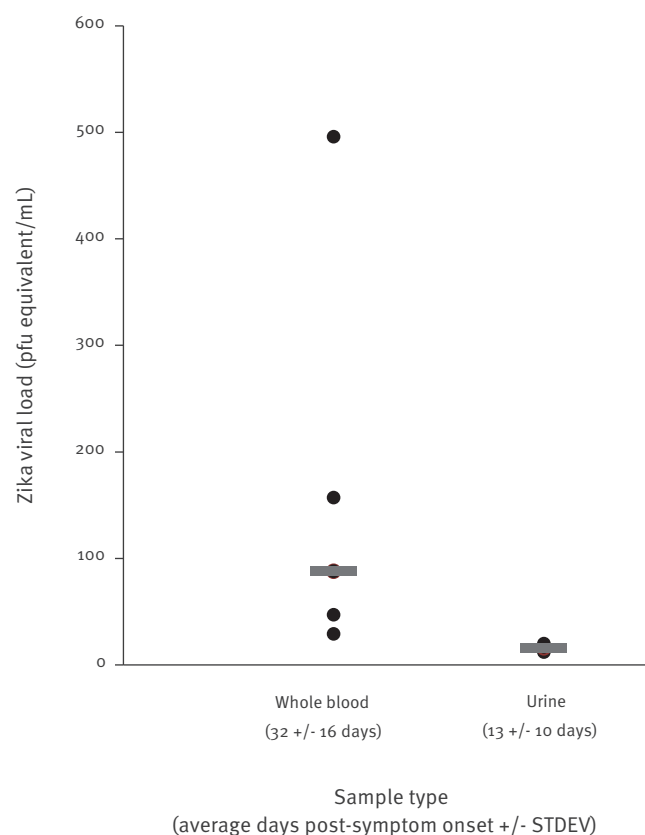
The six patients were aged 3 to 61 years and two of them were male. All had symptoms compatible with ZVD; fever ($\geq 38^{\circ}\text{C}$), rash, conjunctivitis and arthralgia [5]. A serum sample had initially been obtained from each patient, while a follow-up serum sample, as well as urine and WB specimens were only available for five patients.

Testing for the presence of Zika virus RNA in the respective initial serum samples was conducted for all six patients, whereby the qRT-PCR was only positive for one of these samples, which was drawn 3 days post-symptom onset. The other five initial serum samples, which were qRT-PCR negative, were drawn 5 days or more after onset of symptoms. For five initial serum samples, the amount of serum was sufficient to also investigate the presence of Zika virus antibodies by enzyme-linked immunosorbent assay (ELISA). Of these five samples, four were IgM positive, including three who were also IgG positive.

Urine was positive in three samples obtained from three patients 5 to 26 days post-symptom onset, and for these patients a second respective urine sample dated 46 days or later after the start of symptoms was negative. In contrast, all five patients with WB examined, tested positive in a total of six samples obtained 5 to 58 days post-symptom onset (Table). In two patients, WB remained positive for as long as 46 (patient 5) and 58 days (patient 4) after the onset of symptoms (Table). For patient 4, a urine sample was comparatively negative already at 10 days after the symptoms began, while for patient 5, one urine sample obtained 26 days following the start of ZVD was

FIGURE

Zika virus RNA load in samples obtained from infected patients after onset of Zika virus disease, Israel, December 2015–April 2016 (n=6 patients)



Pfu: plaque-forming units; STDEV: standard deviation.

Zika virus load in whole blood (6 samples) and urine (3 samples), as determined by real-time polymerase chain reaction (qRT-PCR). All qRT-PCR negative samples were excluded from viral load analysis. Horizontal bars represent the median viral loads.

positive whereas a second urine sample dated 46 days post-symptom onset was negative.

Considering all urine and WB samples found positive by qRT-PCR in the study, the median amount of Zika virus RNA was 88 plaque-forming units (pfu) equivalents/mL in WB compared with 16 pfu equivalents/mL in urine, despite the fact that the WB was taken at a longer time after symptom onset (Figure), suggesting that the quantity of Zika virus RNA is substantially higher in WB.

Most importantly, sequencing of part of the precursor membrane (prM) and envelope (ENV) genes (327bp) of the Zika genome was achieved from four WB samples. Results showed Zika virus RNA sequences with high similarity to those of strains identified in the likely geographical areas of infection (Asian lineage) [6]. Unfortunately, we were not able to isolate and grow infectious virus in tissue culture.

Laboratory investigations

For Zika virus RNA detection, total nucleic acids were purified from 200 µL (WB and serum), or 1mL (urine) of specimens by using the NucliSENS EasyMAG system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions with minor modifications. Briefly, MS2 Coliphage (10,000 copies/mL) was added to the lysis buffer to control for proper extraction and sample inhibition [7] and external lysis was performed on all samples immediately upon arrival at the CVL (which could be up to 48 hours after a sample was obtained from the patient) to inactivate the virus as recommended by the manufacturer. This was followed by nucleic acid (NA) extraction using the EasyMAG extractor. Extracted NA was eluted in 55 µL elution buffer and stored at -70 °C pending analysis.

The ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, US) was used for the amplification and detection of the MS2 and Zika RNA by qRT-PCR as previously described [7,8]. Briefly, for MS2 qRT-PCR, 5 µL of RNA was added to the AgPath Mastermix (Ambion, Applied Biosystems Inc, Foster City, California), which contained 300nM of each primer and 200nM of probe and 5-carboxy-X-rhodamine succinimidyl ester (ROX) as an internal reference dye, whereas for the Zika qRT-PCR assay, 5 µL of RNA was added and the specific primer and probe set for the ENV protein gene was used as previously described [8]. qRT-PCR was performed under the following conditions: 30 min at 48 °C, 10 min at 95 °C, and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Quantification of Zika virus NA in the tested samples was based on titration of Zika MR-766, which was kindly supplied by R. Lanciotti from the Division of Vector Borne Diseases, Centers for Disease Control and Prevention, Fort-Collins, Co, United States. The limit of detection of the Zika qRT-PCR method was between 10 and 20 pfu equivalents/mL, in WB, urine, and serum specimens.

For sequencing of Zika RNA, a 327 fragment from the prM and ENV genes was amplified by reverse-transcription PCR using primers ZIKV 835 (5'-TTGGTCATGATACTGCTGATTGC-3') and ZIKV 1162c (5'-CCACTAACGTTCTTTTGCAGACAT-3') [8] and sequenced on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, United States) using an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems) [6].

Frozen whole blood (obtained 5, 26, 34 and 58 days post-symptom onset) and urine (obtained 5, 10, and 26 days post-symptom onset) samples were diluted (1:10) in modified Eagle medium (MEM; Biological Industries) supplemented with 2% heat-inactivated fetal bovine serum (FBS), glutamine, non-essential amino acids and penicillin–streptomycin, overlaid on Vero c1008 (E6) for 7 days at 37 °C in 5.0% CO₂ followed by additional 7 days on fresh cells.

TABLE

Zika RNA detection and quantification in samples from patients returning from Zika virus endemic areas, Israel, December 2015–April 2016 (n=6 patients)

Patient number	Probable country of exposure	First set of samples				Second set of samples		
		Serology results (IgM/IgG) ^a	qRT-PCR			qRT-PCR		
			Serum result, days from symptom onset (pfu equivalent/ml)	Urine result, days from symptom onset (pfu equivalent/ml)	WB result, days from symptom onset (pfu equivalent/ml)	Serum result, days from symptom onset (pfu equivalent/ml)	Urine result, days from symptom onset (pfu equivalent/ml)	WB result, days from symptom onset (pfu equivalent/ml)
1	Colombia	ND	Pos 3, (496)	ND	ND	ND	ND	ND
2	Colombia	Pos/Pos	Neg, 5 (NA)	Pos, 5 (16)	Pos, 5 (88)	Neg, 120 (NA)	Neg, 120 (NA)	Neg, 120 (NA)
3	Colombia	Pos/Pos	Neg, 10 (NA)	Pos, 10 (12)	Pos, 34 ^b (157)	Neg, 78 (NA)	Neg, 78 (NA)	Neg, 78 (NA)
4	Vietnam	Pos/Neg	Neg, 10 (NA)	Neg, 10 (NA)	Pos, 58 ^b (88)	Neg, 79 (NA)	Neg, 79 (NA)	Neg, 79 (NA)
5	Dominican Republic	Pos/Pos	Neg, 26 (NA)	Pos, 26 (20)	Pos, 26 (47)	Neg, 46 (NA)	Neg, 46 (NA)	Pos, 46 (29)
6	Mexico	Neg/Neg	Neg, 26 (NA)	Neg, 26 (NA)	Pos, 26 (496)	Neg, 48 (NA)	Neg, 48 (NA)	Neg, 48 (NA)

F: female; M: male; NA: not applicable; ND: not performed due to lack of material; Neg: negative; Pos: positive; pfu: plaque-forming unit; qRT-PCR: quantitative real-time polymerase chain reaction; WB: whole blood.

^a Serology performed by enzyme-linked immunosorbent assay (ELISA; Euroimmun).

^b WB samples were not obtained at the same time as urine and serum samples.

Serological testing for Zika virus was performed by using an ELISA IgM and IgG kit (Euroimmun AG, Germany) which detects antibodies against the Zika nonstructural protein NS1 [9].

Discussion

Zika virus is a mosquito-borne flavivirus that has spread in the last months throughout tropical and sub-tropical areas of the Americas causing a widespread epidemic [10]. As it is strongly suspected that infection with this virus may in some cases result in neurological disorders as well as congenital abnormalities, accurate diagnosis is extremely important [1]. Due to the extensive cross-reactivity between Zika virus and other flaviviruses in serology, which can lead to false positive results, serological assays have substantial limitations [8]. Currently qRT-PCR is the most reliable test, but the period in which Zika virus RNA can be identified after ZVD manifestation is restricted. Following symptom onset, qRT-PCR detects Zika virus RNA for only up to 5 days in serum [4], and up to 10 to 20 days in urine [2]. The use of saliva samples has been recently shown to increase the rate of molecular detection of Zika virus RNA in the acute phase of the disease but did not enlarge the detection period window [3]. Here we find that Zika virus RNA is present for a substantially longer time period in WB than in serum or urine and may even persist for up to 2 months post-symptom onset.

Despite the limited number of samples tested, the study clearly shows the advantage of WB testing. In sera, we could not detect Zika virus RNA more than 5 days after onset of symptoms, and in urine, the latest detection of such RNA post-symptom onset for three of five samples was 26 days. In contrast, WB was Zika virus-RNA positive in all samples collected within the first month post-symptom onset, in three of four

samples in the second month, and in none when testing after 2 months.

The use of WB for flavivirus RNA detection is not new. Several studies, have demonstrated that a large proportion of West Nile virus (WNV) is bound to red blood cells (RBCs) and have shown that WNV RNA concentrations in seropositive donations are 10-fold higher in WB than in plasma and persist for a much longer period of time [11–13]. For diagnostic purposes, we demonstrated very recently that WNV RNA can be identified by qRT-PCR in 86.8% of WB samples but only in 26% of serum samples during acute infection [14]. A higher detection rate in WB compared with serum or plasma has also been described previously for dengue virus [15]. Our current study suggests that Zika virus presents similarities to other flaviviruses in this respect.

Virus isolation was unsuccessful for all WB and urine samples most probably because of the low amount of virus that was present in the samples. However, it is also possible that the virus was not viable due to the time that has passed from symptoms onset until sample collection. Future studies should investigate the possibility, raised by our study that Zika virus could circulate in the blood of infected patients for up to 2 months. Diagnosis of recent Zika virus infection is important even after a patient's full recovery, not only because of the possible risk to the fetus in pregnant women, but also considering reports that sexual transmission of the virus from men to women has likely occurred [16] and the finding of the virus in semen for several weeks following infection [17,18]. Although our study establishes the utility of WB in the routine diagnosis of Zika infection, more studies are required to precisely determine the time frame of presence as

well as the amounts of Zika virus RNA in WB following infection.

Acknowledgements

We thank Robert Lanciotti from the Division of vector Borne Diseases, Centers for Disease Control and Prevention, Fort-Collins, Co, USA for the Zika MR-766 virus and guidance in establishing the qRT-PCR assay and Musa Hindiyeh from the Central Virology Laboratory of the Israel Ministry of Health for the Zika qRT-PCR evaluation.

Conflict of interest

None declared.

Authors' contributions

Y.L. contributed to the study design, performed the molecular experiments, analysed the data, wrote and edited the manuscript, E.M contributed to the study design, edited the manuscript and provided critical review of the manuscript. N.P. and S.H. set up and performed the virus isolation experiments and E.S examined the patients, coordinated the work, analysed the data, contributed to the study design and provided critical review of the manuscript.

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A major outbreak of conjunctivitis caused by coxsackievirus A24, Réunion, January to April 2015

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From January to April 2015, Réunion experienced a major outbreak of acute haemorrhagic conjunctivitis (AHC) caused by coxsackievirus A24, which heavily impacted the healthcare system. According to the general practitioners' (GP) sentinel network, the number of medical consultations due to conjunctivitis during this period was estimated at ca 100,000. This report describes the characteristics of the outbreak, which were obtained through several different yet complementary surveillance systems on the island. These included the network of hospital emergency departments (OSCOUR network), the GPs' sentinel network, an Internet-based population cohort ('Koman i lé') participating in a survey on distinct symptoms including 'red eyes' and the monitoring of eye drop sales. Overall the results of the different surveillance approaches were in good agreement regarding the outbreak dynamic. A peak of patients with conjunctivitis was detected in the first 15 days of March (week 10 and 11), coinciding with increased eye drop sales on the island. Strains recovered from outbreak cases belonged to genotype IV and were most closely related to strains identified in AHC outbreaks in China, Egypt and Japan since 2010. Continued surveillance of AHC in Réunion remains important not only locally, but also because frequent exchanges between the island and mainland France may lead to introduction of this virus in Europe.

Introduction

Réunion is a French overseas territory located in the South-West Indian Ocean (SWIO). It is an island located between Madagascar and Mauritius, with, in 2013, a population of 840,000 inhabitants. Daily exchanges of goods and people occur between Réunion and other islands of the SWIO, which include the Comoros, Madagascar, Mauritius, Mayotte and the Seychelles. Medical facilities in Réunion are similar to those in mainland France, and there are more than 1,000 general practitioners (GP) distributed throughout the

island, as well as four hospitals and six emergency departments (ED).

In tropical countries with high population density, epidemics of viral conjunctivitis occur mainly during the hot and rainy season [1]. They are mostly attributed to adenoviruses and enteroviruses (EV) [2,3]. Enteroviruses are ubiquitous pathogens responsible for a large range of infections [3]. There is no specific antiviral treatment. The two main serotypes of EV associated with acute haemorrhagic conjunctivitis (AHC) are enterovirus 70 (EV-70) and coxsackievirus A24 (CV-A24v). CV-A24v was detected for the first time during an outbreak in Singapore in 1970 [4]. The outbreaks of conjunctivitis caused by CV-A24v are particularly explosive, whereby the disease is highly contagious, and has a very short incubation period (12 hours to 3 days), leading to high attack rates [5]. In 2002, an epidemic of AHC due to CV-A24v occurred in South Korea, involving more than a million cases. The virus then spread in worldwide causing a pandemic [3].

Although a large outbreak of conjunctivitis with more than 12,000 cases was detected in 2012 in Mayotte [6], no outbreak of conjunctivitis in Réunion has been described in the 10 years prior to 2015. At the end of January 2015 however, a cluster of cases of conjunctivitis was detected in the west part of the island in the municipality of Saint-Paul, by a surveillance network that monitors data from emergency departments (ED), i.e. the 'Organisation de la surveillance coordonnée des urgences' (OSCOUR). Further investigations revealed the beginning of an outbreak that spread throughout the island.

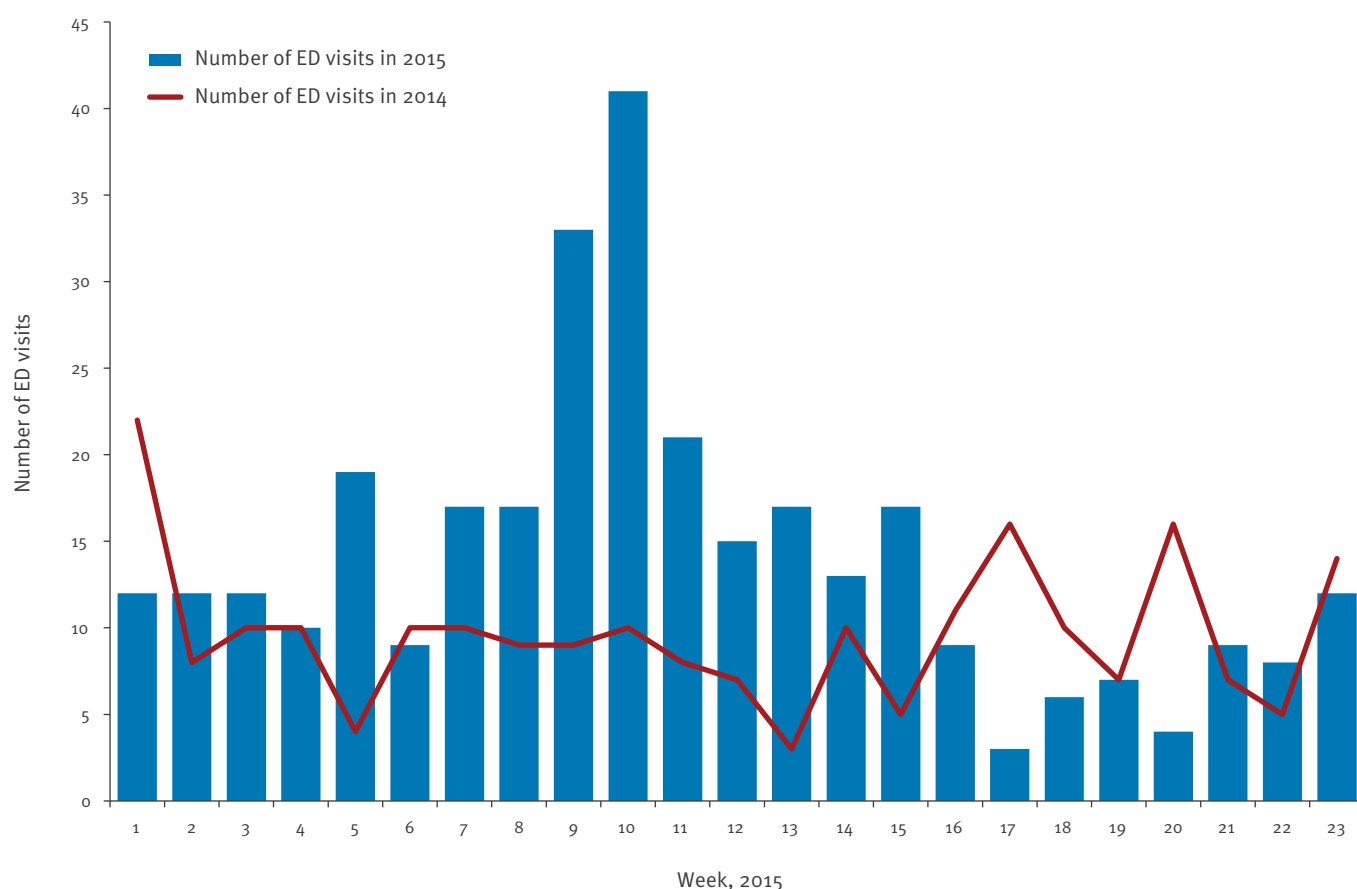
Methods

Epidemiological surveillance

In Réunion, conjunctivitis is monitored routinely by the OSCOUR network [7]. This syndromic surveillance

FIGURE 1

Weekly number of emergency department visits for conjunctivitis, Réunion, 29 December 2014–7 June 2015^a



ED: emergency department; InVs: Institut de veille sanitaire (French Institute for Public Health Surveillance); OSCOUR: Organisation de la surveillance coordonnée des urgences.

a For comparison, weekly number of ED visits for conjunctivitis in the same period of the previous year are provided.

system is based on data collected by the six EDs of the island. Data are collected daily directly from patients' computerised medical files that are filled in during medical consultations [8]. For each ED visit, several variables are collected, including patient age, sex, city of residence and the diagnosis. This allows for analysis by syndromic groups, age groups and geographical areas. The diagnosis is categorised according to the 10th revision of the international Classification of Diseases (ICD-10) [9]. Several indicators are routinely monitored, including the number of ED visits for conjunctivitis (ICD-10 codes B30 and sub-codes, codes H10 and sub-codes, codes H11 and sub-codes). Temporal and spatio-temporal analyses are carried out every day from these data.

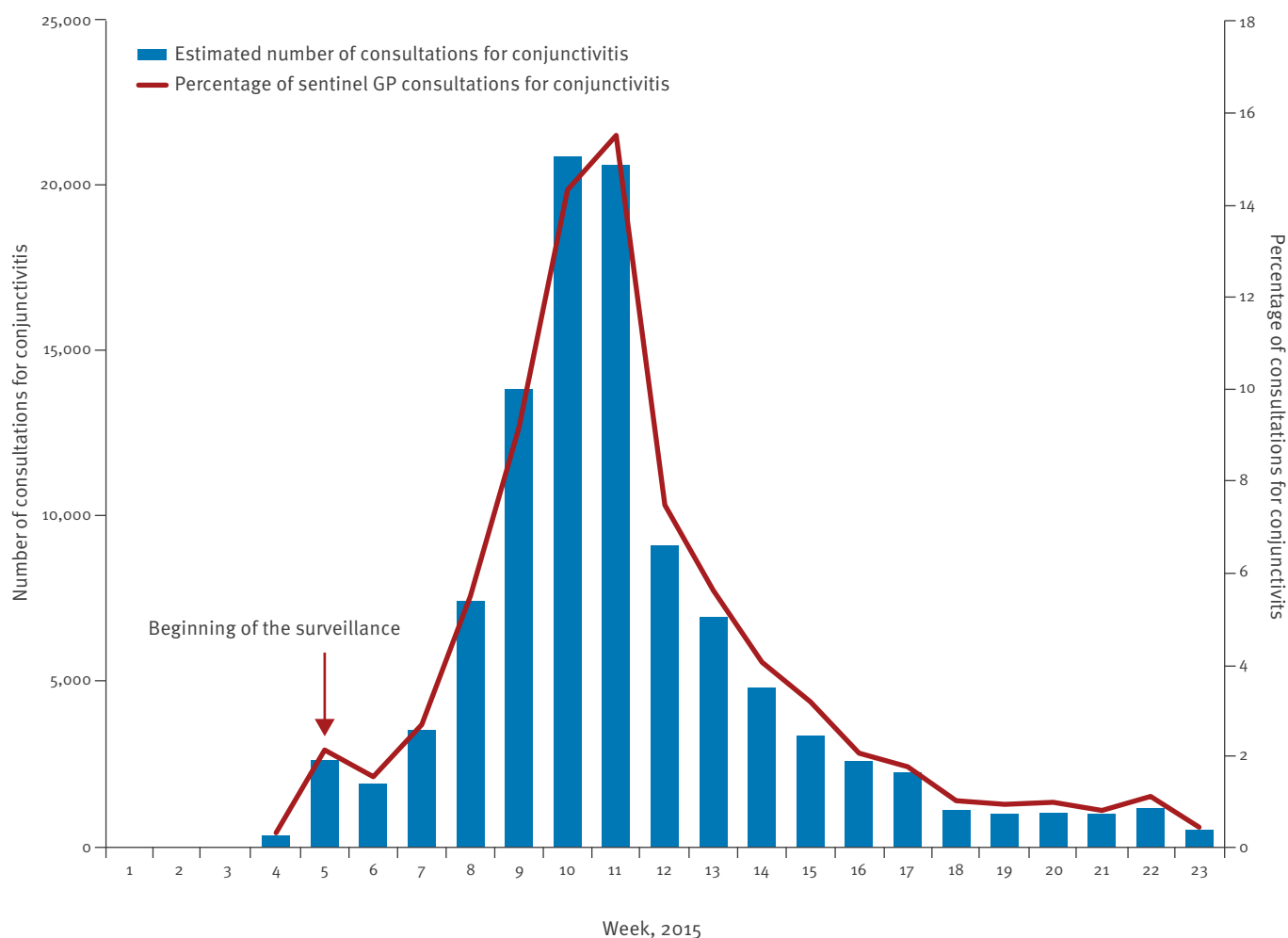
On 27 January 2015, data from the OSCOUR network allowed to detect a cluster of conjunctivitis cases in the municipality of Saint-Paul during week 4 (20 to 27 January 2015). The same day, six GPs of this municipality were interviewed by phone and they confirmed an

outbreak. On 28 January 2015, after interview, several sentinel GPs reported that the outbreak had spread to the whole island. In this context, from the end of January (week 5) routine surveillance was enhanced by specific surveillance of conjunctivitis using data from sentinel GPs and a sentinel population network.

The sentinel GPs' network is based on 56 volunteer GPs located throughout the island, who report to the regional office of the French Institute for Public Health Surveillance (Cire OI) the weekly number of consultations for acute respiratory infections and acute diarrhoea and the total number of consultations [10,11]. These GPs are easily mobilised at the onset of any health event on the island and they can be asked to add specific items to their weekly report. At the beginning of the week 5, the Cire OI suggested to the sentinel GPs to report, in addition to usual indicators, the weekly number of consultations for conjunctivitis according to the following case definition: inflammation of the eye with burning sensation or watering eyes

FIGURE 2

Estimation of the weekly number of consultations carried out by general practitioners for conjunctivitis, Réunion, 29 December 2014–7 June 2015 (n = ca 104,403)



GP: general practitioner.

The estimation of weekly consultations for conjunctivitis = $A/(B/C \times D)$ where: A is the weekly number of sentinel general practitioner's (GP) consultations for conjunctivitis; B is the weekly number of sentinel GP's consultations all causes; C is the weekly number of consultations carried out by all GPs and reimbursed by the national health insurance centre of Réunion (CGSS); D is the coverage of CGSS data in general population (72%).

or lacrimal secretion or light sensitivity. To estimate the total number of consultations for conjunctivitis on the whole island, data from the GPs were extrapolated using data from the national health insurance centre of Réunion (Caisse Générale de Sécurité Sociale; CGSS) [11,12]. Each week, the CGSS sent by email to Cire OI, the aggregated number of medical consultations and home visits carried out in the prior week (week -1) by GPs and reimbursed by CGSS. The transmitted data concerned 72% of the population of the island.

Since April 2014, the Cire OI pilots an experimental surveillance system based on the use of the Internet-based cohort 'Koman i lé'. Individual volunteers aged over 18 years with Internet access and living in Réunion are included. In total, 350 adult volunteers contribute

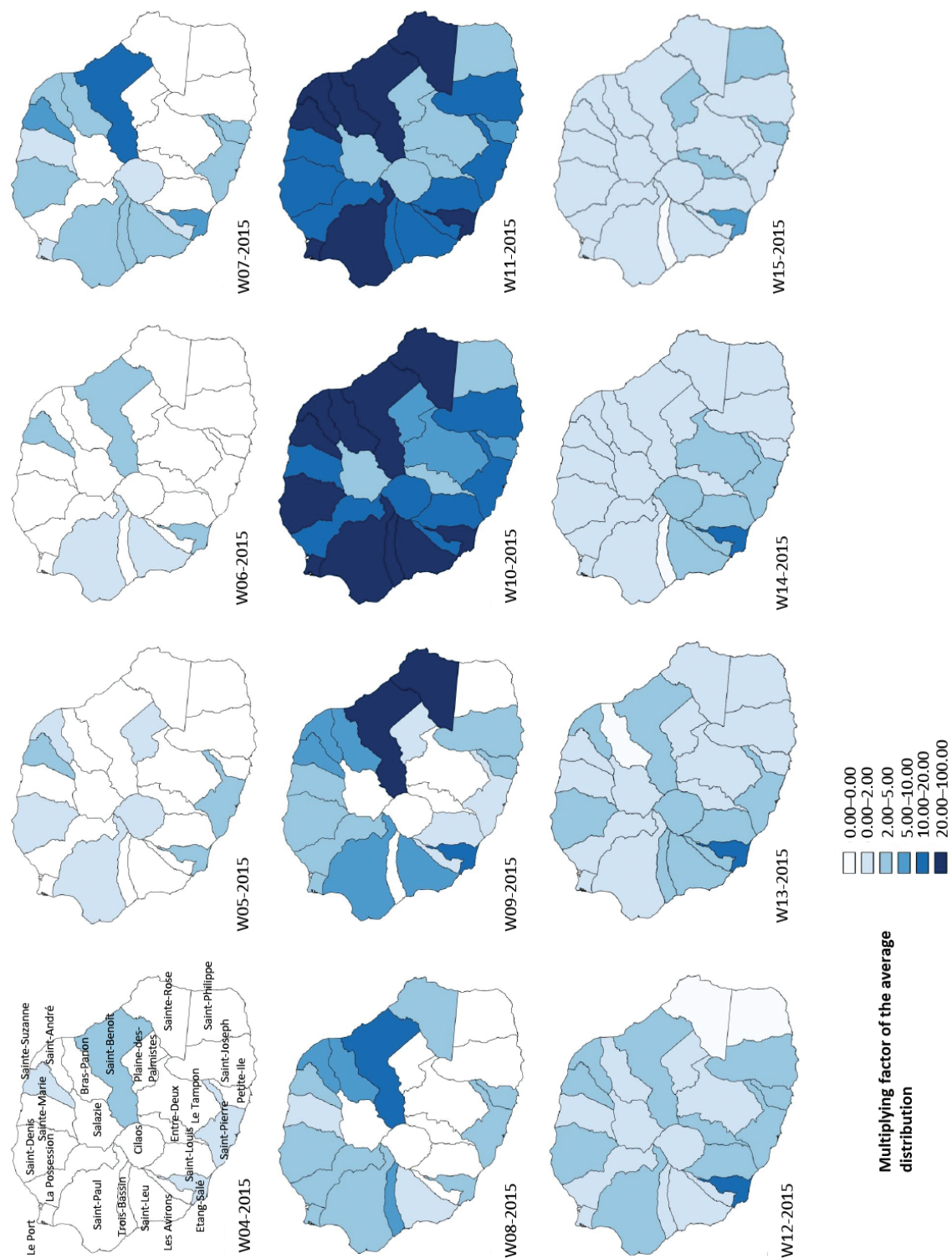
to this surveillance system. They fill in anonymously a short survey every week asking if they had any of 17 symptoms during the previous week [13]. Each week for each symptom, the percentage of participants who presented the symptom compared with all participants who answered the questionnaire is calculated. This allows monitoring trends of different symptoms. Among these symptoms, one is 'red-eyes'.

Data from these three surveillance systems were analysed by the Cire OI.

Furthermore, to assess the impact on the population, the use of collyrium was monitored in real time. A supplier of some 40% (100/250) of the pharmacies on the island sent to the Cire OI its data on eye drop sales

FIGURE 3

Trends of collyrium sales by municipality, Réunion, 19 January–12 avril 2015 (n=24 municipalities)



from the beginning of 2015. For each municipality on the island, collyrium sales for weeks 4 to 15 were compared with the average collyrium sales in weeks 2 and 3, before the outbreak.

Virological screening of conjunctival specimen

In order to determine the aetiology of this outbreak, sentinel GPs were encouraged to collect swabs from conjunctivitis cases. As a first step, twenty-six conjunctivitis specimens were analysed by the hospital laboratory of Saint-Denis using a real-time polymerase chain reaction (RT-PCR) detecting adenoviruses (Adenovirus R-gene, bioMérieux) and a multiplex PCR detecting enteroviruses (Respifinder Smart 22, Pathofinder).

Molecular typing of enterovirus positive samples and phylogenetic analyses

Among the conjunctivitis specimens testing positive for enteroviruses, ten randomly selected samples were sent to the National Reference Centre for Enteroviruses (Lyon, France) for further characterisation. Partial viral protein 1 (VP1) coding sequences were determined using the Nix method [14] and identified as CV-A24v strains using Basic Local Alignment Search Tool (BLAST) software [15]. For phylogenetic analysis, a nt alignment (330 bp) including nine of the 10 VP1 sequences determined from conjunctival specimens (one sequence of shorter length was excluded), thirty selected CV-A24 VP1 sequences from clinical strains representative of the genotypes I, II, III and IV and the prototype CV-A24 strain (Joseph) was performed. Genetic distances were calculated with the Tamura–Nei model of evolution. The tree was constructed by the neighbour-joining method using molecular evolutionary genetics analysis (MEGA)5 and validated using 1,000 bootstrap pseudo-replicates. The nine VP1 sequences determined in this study, and included in the phylogenetic analysis, were deposited in GenBank database (accession numbers: KR399980–KR399986; KR399988; KR478685). Two of those were deposited as part of a complete genome sequence (see below).

Metagenomic sequencing and de novo assembly of consensus sequences

Metagenomic sequencing was attempted on three conjunctival swab specimens. Viral nucleic acid enrichment was performed by a combination of centrifugation (10 min at 6,000 x g), filtration (at 0.45 µm) and nuclease treatment using 0.1 U µL⁻¹ Turbo DNase (Life Technologies) as described before [16]. Nucleic acid extraction was then performed with a NucliSens easyMAG magnetic bead system (BioMérieux). cDNA synthesis was performed as described before [17] with some modifications. Reverse transcription was performed with 50 ng of random hexamers (New England Biolabs) and 200 U of SuperScript III reverse transcriptase enzyme (Life Technologies) followed by RNA digestion with 2 U of RNase H (Life Technologies). After the reverse transcription reaction, second strand synthesis was performed with 5 U Klenow fragment (3' – 5' exo-) (New England Biolabs) and 1 µg of random

hexamers. cDNA was purified and concentrated using a DNA Clean and Concentrator column (Zymo Research) and the totality of the specimen was used to prepare sequencing libraries using NEBNext Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England Biolabs) with 12 PCR cycles. The three barcoded libraries were pooled at equimolar concentrations and sequenced on an Ion Torrent PGM instrument (Life Technologies) with an Ion 318 Chip v2. Ion Torrent data was de-multiplexed and quality-trimmed using Ion Torrent Suite software (version 4.4.2). Reads were then assembled using MIRA (v. 4.0.1) [18] and contigs were further merged via V-Fat [19]. Two of the three resulting consensus sequences were deposited into GenBank database (KR399988; KR478685). Consensus sequences were aligned with all complete CV-A24 sequences available in GenBank and potential recombinations were assessed by using the bootscanning method implemented in SimPlot [20].

Results

Outbreak detection

At the end of January 2015, the spatio-temporal analysis of data from the OSCOUR network allowed to detect a cluster of conjunctivitis cases in the municipality of Saint-Paul that had occurred during week 4 (20 to 27 January) 2015. GPs in the area, as well as the sentinel GPs in the whole island were interviewed. They confirmed an increase in cases and highlighted that it concerned the whole island. Several practitioners also reported a clinical picture compatible with a highly contagious viral infection.

Outbreak description

Emergency department visits for conjunctivitis

The peak of emergency department visits for conjunctivitis was reached in week 10 (first week of March). The first increase of ED visits in 2015 was visible in week 5, when the beginning of the epidemic was detected. From week 1 to week 17, a total of 277 ED visits for the disease were recorded on the whole island, against 162 over the same period in 2014 (Figure 1).

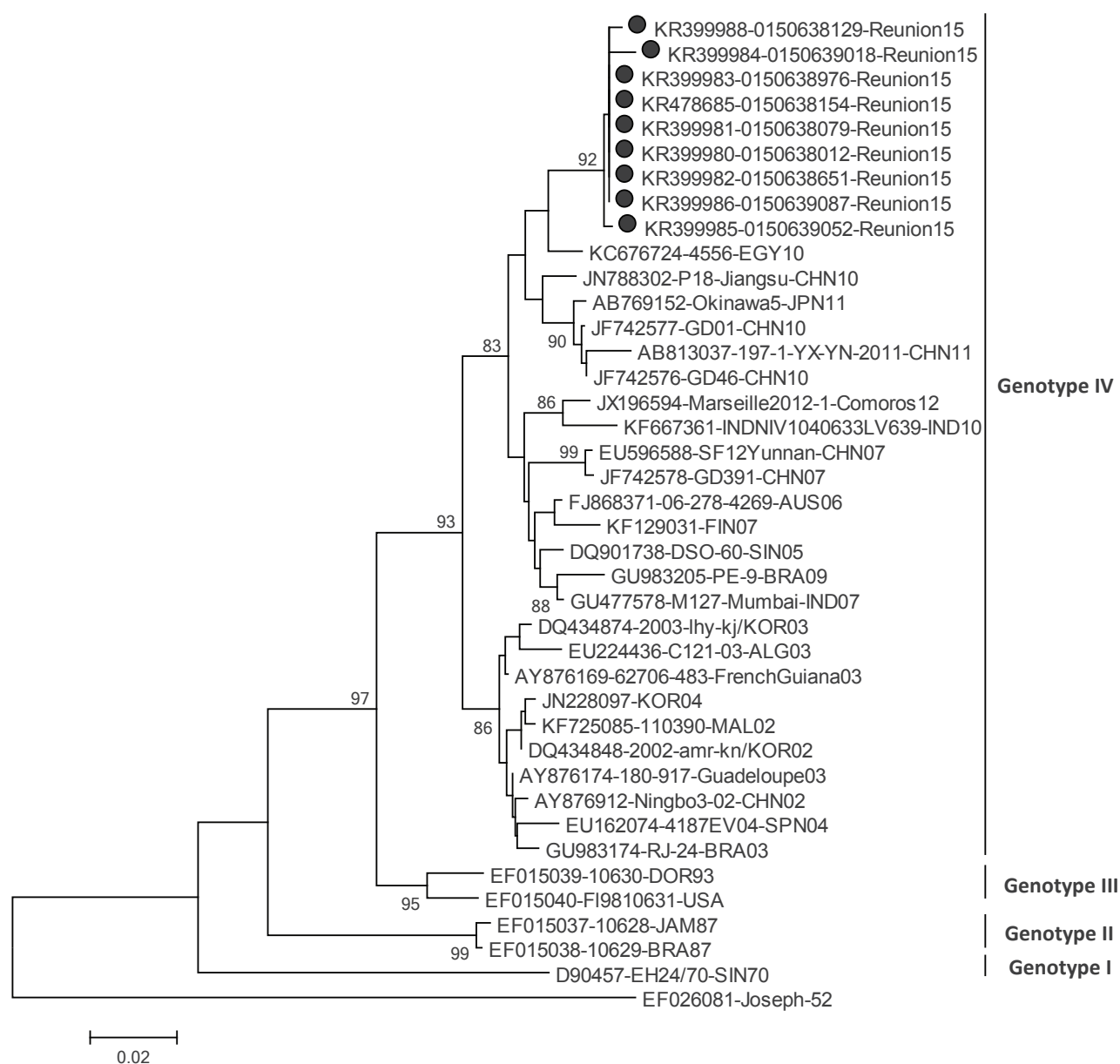
The ratio male/female was 1.4 (160/117). The analysis by age group showed that 44% (122/277) of patients who presented to the ED for conjunctivitis were under 20 years of age, and 55% (152/277) were under 30 years-old (Table). The repartition by age of the patients was not statistically different compared with 2014: 48% (78/162) under 20 years and 58% (94/162) under 30 years.

Consultations for conjunctivitis of sentinel general practitioners

The beginning of the outbreak in week 5 was confirmed by the GPs' network. Their reports showed that the outbreak had spread throughout the island in late January and ended in week 17 (end of April) (Figure 2). The outbreak lasted 12 weeks. The epidemic peak was reached

FIGURE 4

Phylogenetic analysis, based on coxsackievirus A24 partial viral protein 1 coding sequences, of strains recovered in the outbreak of acute haemorrhagic conjunctivitis, Réunion, 2015



CV-A24v: coxsackievirus A24 variant.

Phylogenetic analysis based on partial VP1 coding sequences (330 bp) of CV-A24v strains. The analysis included nine VP1 sequences determined in this study from conjunctival swab specimens collected during the outbreak in Réunion (shown by a dot), thirty selected sequences from clinical strains representative of the genotypes I, II, III and IV and the prototype CV-A24 strain (Joseph). Genetic distances were calculated with the Tamura–Nei model of evolution. The tree was constructed by the neighbour-joining method using molecular evolutionary genetics analysis (MEGA)5 and validated using 1,000 bootstrap pseudo-replicates. The nine VP1 sequences determined in this study and included in the phylogenetic analysis were deposited in GenBank database (accession numbers: KR399980–KR399986; KR399988; KR478685).

in week 11, one week later than in the ED. For each of the weeks 10 and 11, more than 20,000 consultations for conjunctivitis were estimated, and the whole island was affected. The total number of consultations for conjunctivitis on the whole island during this outbreak was estimated to be ca 100,000. The epidemic curve shows a highly contagious outbreak.

Results from 'Koman i lé'

From week 8 to week 10, the percentage of participants reporting red eyes was between 7% (7/104) in week 8 and 8% (8/106) in weeks 9 and 10. In contrast this percentage was 3% (3/108) in week 7. Overall among adults having declared that they had red eyes during this period, 22% (5/23) consulted a GP.

TABLE

Distribution by age groups of patients visiting the emergency department for conjunctivitis, Réunion, 29 December–26 April 2015 (n=277)

Age group (in years)	n (% among emergency department visits for conjunctivitis)
0–9	97 (35)
10–19	25 (9)
20–29	30 (11)
30–39	36 (13)
40–49	45 (16)
50–59	22 (8)
≥60	21 (8)

Data from the supplier

Data from the supplier of the pharmacies showed that the sales of eye drops increased rapidly on the whole island (Figure 3). Sales were multiplied by twenty in 11 municipalities during week 10 and in eight municipalities during week 11. Almost all municipalities of the island were affected. However, coastal municipalities were more impacted than municipalities in the centre of the island, which are located in mountainous areas and less populated.

Laboratory investigations

Of the twenty-six swabs collected by the sentinel GPs, twenty-three of them tested positive by RT-PCR for enteroviruses and negative for adenoviruses. Ten randomly selected samples were sent to the National Reference Centre for Enteroviruses for further characterisation. CV-A24v belonging to genotype IV was identified in all the samples by sequencing of the VP1 coding region (Figure 4). The 10 VP1 partial nt sequences shared a 99.1 to 100% nt identity and had 95.1 to 97.9% nt similarity with sequences from genotype IV isolates previously identified in AHC outbreaks in Egypt (2010) [21], Japan (2011) [22], China (2007 and 2010) [23–25], Brazil (2009) [26], India (2007) and Singapore (2005) [27] (Figure 4). They also grouped in a larger cluster containing the VP1 sequence from a genotype IV isolate detected in a traveller returning from Comoros to France in 2012 (Figure 4; accession number JX196594). Two whole genome sequences were generated by a metagenomic approach and showed a 97.2–97.3% similarity with sequences from Japanese strains detected in 2011 in Okinawa [22]. Similarity plots provided no evidence of recombination (data not shown).

Discussion

Between January and April 2015, a major outbreak of AHC occurred in Réunion, being the first of such outbreaks ever described on the island. The outbreak was caused by strains of CV-A24v belonging to genotype IV. In a phylogenetic analysis, nt sequences of the

outbreak strains grouped with other strains identified since 2002 in AHC outbreaks in Africa, America and Asia. Within this group, the sequences from this study were most closely related to those of recent AHC outbreaks (i.e. China 2010, Egypt 2010 and Japan 2011), which belong to a sub-cluster that might have emerged between 2008 and 2009 [22].

During the same period as the Réunion outbreak, an outbreak of conjunctivitis also occurred in Mauritius, but the only viral aetiology identified was adenovirus. Then, in early March, similar outbreaks began in Madagascar and the Seychelles but no data are available on the causative agents. Three years prior, from February to May 2012, Mayotte had experienced an outbreak of acute conjunctivitis, most likely caused by a CV-A24v closely related to the isolates associated with the Réunion outbreak [6,28].

Because outbreaks of AHC can reappear and due to the frequent exchanges between Réunion and mainland France, there is a recurring risk of further exportation of the virus to France and other European countries through returning travellers. The exchanges between the SWIO islands are also important but surprisingly the two concomitant conjunctivitis outbreaks in Mauritius and Réunion were due to different viruses. This highlights the need for specific laboratory investigations in each island even when similar outbreaks occur in the same period.

In Réunion, information about the outbreak was broadcasted to the population [29], by the local media (radio, television and websites) together with advice on how to avoid infection. For example, following good hygiene practices was recommended, such as washing hands regularly with soap and water, avoid rubbing eyes and using clean water or a disposable tissue to rinse eyes. The first epidemiological report was broadcasted on 30 January 2015 and the last on 30 April 2015. In total, nine weekly epidemiological reports were published.

This study shows the usefulness of several complementary surveillance systems. It also underscores the importance of cooperation between different health professionals, which were quickly mobilised. The different surveillance systems produced consistent results. Indeed, the OSCOUR network, the sentinel GP network and the sentinel population network ‘Koman I lé’ showed the same outbreak dynamic. Moreover, the most important sales of collyrium occurred in weeks 10 and 11, which corresponds to epidemic peaks visible by the three other surveillance systems.

Nevertheless, each of these systems presents strengths and limits.

The main strength of the OSCOUR network is that it enabled early detection of the outbreak because the data are collected, sent and analysed on a daily basis. Second, several variables are collected for each

patient, including age and city of residence, which allows for analysis by age groups, and geographical areas. Moreover, data from the six EDs of the island are transmitted daily since 2009, so comparisons are possible with previous years. The limitation of the OSCOUR network is that it is not the most appropriate surveillance system for monitoring conjunctivitis because few patients go to ED for this pathology. OSCOUR is able to detect community outbreaks but not to assess their range or to monitor their evolution.

The sentinel GPs' network is particularly suitable for monitoring conjunctivitis, this pathology not usually being severe. Moreover, the data from this network, coupled with those from the national health insurance contributed to estimate the impact of the epidemic in terms of number of consultations and to follow its dynamics. Even if this network is less reactive than the OSCOUR network because the data are transmitted once a week, activation of GPs for the surveillance of conjunctivitis was adequate to monitor the outbreak. Nevertheless, no historical data are available to make comparisons with previous years. As this network saw only people seeking medical care for their conjunctivitis, people using self-medication or using traditional phytotherapy, who are numerous on the island, were not taken in account in the assessment of the outbreak burden. Indeed, the outbreak was probably more important than estimated by the GPs' network.

The sentinel population 'Koman i lé' is a new project directly involving the population, including people not consulting their doctors when they are sick. The weakness is that this system is not representative for the population of Réunion. Indeed, only adults can participate, and the elderly are not much involved because they do not use the Internet much. Moreover, some municipalities are still under-represented. At the current stage of development of 'Koman i lé', it is not possible to extrapolate the results of this system to the general population and to use it to evaluate the real burden of an epidemic. In the future, if the number of participants increases, it will be possible to better monitor health events in the community.

The surveillance based on sales of collyrium provides data stratified by municipalities, shows the spatiotemporal dynamic. However, this monitoring is not exhaustive because the supplier who sends its data does not provide for all pharmacies of the island. Nevertheless, data were congruent with those of the others systems and were sufficient to describe the dynamics of the epidemic in the island.

Despite some weaknesses of the surveillance systems, the analyses of all of these complementary sources of data allowed to describe and characterise the outbreak. Information produced by this surveillance system enabled to health authorities to adapt prevention actions to the dynamic of the outbreak.

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Conflict of interest

None declared.

Authors' contributions

All authors contributed to the interpretation of the results, the revision of the draft manuscript and approved the final version. Nadège Marguerite contributed to the epidemiological analyses and wrote the manuscript. Elise Brottet, Frédéric Pagès, Pascal Vilain and Laurent Filleul contributed to the epidemiological analyses and to the writing of the manuscript. Marie-Christine Jaffar-Bandjee was responsible for the viral laboratory analyses. Isabelle Schuffenecker and Laurence Josset conducted the characterisation of the CVA24v strains, performed the phylogenetic analyses and contributed to the writing of the manuscript.

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Antibiotic prescribing and expenditures in outpatient adults in Greece, 2010 to 2013: evidence from real-world practice

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We provide a representative analysis of antibiotic prescribing, identify factors associated with broad-spectrum antibiotic prescribing and assess the costs associated with antibiotic use in adult outpatients in Greece. Outpatient antibiotic prescriptions for patients older than 19 years between 2010 and 2013 in Greece were extracted from the IMS Health Xponent database. Prescribing rate and total cost for prescribed antibiotics were calculated. Multivariate logistic regression was used to identify factors related to broad-spectrum antibiotic prescribing. More than 20 million antibiotics were prescribed during the study period, an annual rate of 768 prescribed antibiotics per 1,000 adults. Overall, 33.5% of antibiotics were prescribed for acute respiratory tract infections (ARTIs) for which antibiotics are often not indicated. Macrolides (29.9%), cephalosporins (26.9%) and fluoroquinolones (21.0%) were the most commonly prescribed antibiotic classes. The majority (89.0%) of antibiotics were broad-spectrum. Antibiotic expenditures were approximately EUR 185 million during the study period. Factors associated with broad-spectrum prescribing included older patient age, specialty pulmonologists or otorhinolaryngologists, training in eastern Europe, diagnosis of ARTI, acute diagnosis, and first episode of disease. Broad-spectrum antibiotic prescribing for ARTIs is common in adult Greek outpatients and frequently inappropriate. These data indicate the need for initiatives aiming to control antibiotic prescribing.

Introduction

Antibiotics are among the most frequently prescribed medications in adult outpatients in European countries such as Greece [1]. There is evidence that a number of antibiotic prescriptions are dispensed for viral infections, for which antibiotics provide no benefit [2]. Such

prescriptions indicate an overuse of antibiotics, a common and alarming problem for many countries, potentially resulting in redundant drug spending, increased risks of adverse effects and development of antimicrobial resistance [3-5]. The latter is a rapidly growing global health problem [6-8].

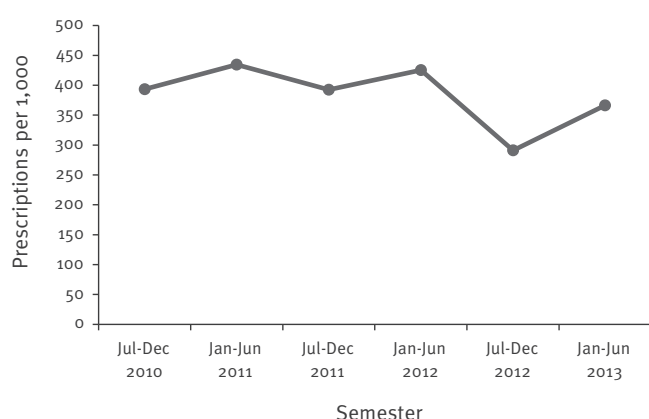
In addition, over the last decades, the use of broad-spectrum antibiotics has increased dramatically, contributing to further antibiotic resistance [9-11]. There are instances where antibiotics may be indicated, but instead of prescribing narrow-spectrum antibiotics, broad-spectrum antibiotics are inappropriately preferred [12].

Antibiotic resistance and increased healthcare costs are thought to be a considerable problem in Greece, which has one of the highest rates of antibiotic consumption and resistance among European countries [13,14]. During the last few years, Greece has experienced a significant financial crisis, resulting in healthcare budgetary constraints. Hence, avoiding unnecessary costs should be a top priority for policymakers. A thorough understanding of antibiotic prescribing patterns can help policy makers identify areas where potentially unnecessary costs can be avoided. Although data on antibiotic prescribing patterns are available for several European countries [15-22], such data are limited for Greece [13].

The aim of this study was to analyse antibiotic prescribing in adult outpatients and to identify factors associated with broad-spectrum antibiotic prescribing in Greece. The secondary objective was to assess the overall and class-specific antibiotic costs in Greece.

FIGURE 1

Trend of prescribed antibiotics per 1,000 persons per six months, Greece, July 2010–June 2013



Methods

Data source and design

In order to obtain data for the analysis we used the Intercontinental Marketing Services (IMS) Health Xponent (XPO) Greece database. This database contains longitudinal representative data of prescribing activity in the community, on the basis of prescriptions given by a random cluster sample, by specialty and region, of 625 physicians (2.5% of all doctors in the outpatient setting in Greece). Physicians, systematically exchanged during the study period, filled in a form for every patient contacted, regardless of contact type (i.e. personal visit or telephone) for seven consecutive days, including the weekend. IMS data directly link antibiotic prescriptions to the clinical indication (ICD-9 codes) and provide diagnosis-related characteristics such as recurrence, the existence of co-diagnoses and the type of diagnosis (i.e. acute or chronic). Patient characteristics such as age, sex and type of insurance were also obtained from the database in addition to physician characteristics including specialty, training location, age and sex. We extracted data for all prescriptions given to patients older than 20 years between July 2010 and June 2013.

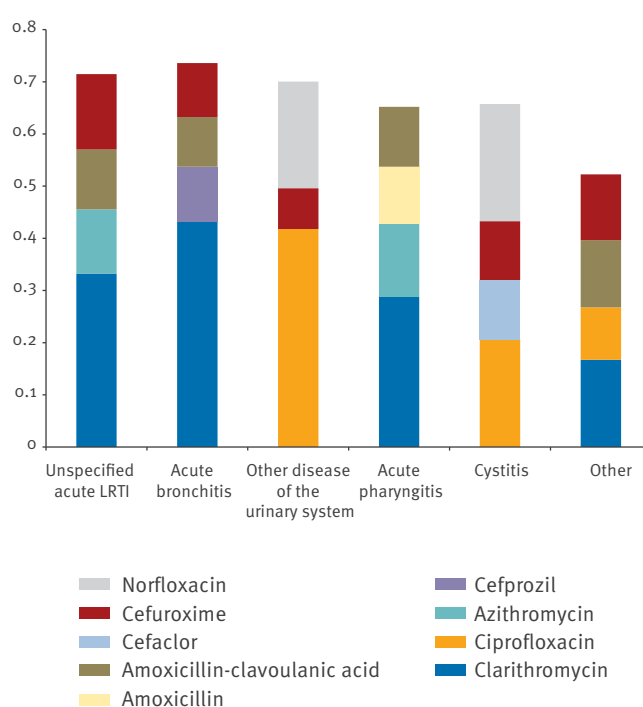
Consultations in Greece are mainly performed via private physicians. Antibiotic prescribing data are presented by specialty of the prescriber: general practitioners (GPs), the main antibiotic prescribers for adults, gynaecologists, pulmonologists, urologists, otorhinolaryngologists and all other specialties (cardiologists, endocrinologists, gastroenterologists, neurologists, orthopaedists, dermatologists and rheumatologists).

Antibiotic classification and duration of therapy

Antibiotics were categorised in two groups. Narrow-spectrum antibiotics included narrow-spectrum penicillins (pivmecillinam, penicillin, amoxicillin), first

FIGURE 2

Most frequently prescribed antibiotics by clinical diagnosis, Greece, July 2010–June 2013



LRTI: lower respiratory tract infection.

generation cephalosporins, tetracyclines and sulfonamides; broad-spectrum antibiotics included broad-spectrum penicillins (amoxicillin/clavulanic acid), macrolides, second and third generation cephalosporins and quinolones. This classification has been used in previously published research [12]. Moreover, the duration of therapy was calculated using the total prescribed dose (number of packs) with the daily dose and dose frequency as recommended by physicians.

Diagnostic categories

Clinical diagnoses were provided by physicians (tonsillitis, sinusitis, etc.). If the physician could not decide on a specific diagnosis, they had to mention the basic symptoms (chest pain, headache, etc.). The 10 most common clinical diagnoses for which antibiotics were prescribed were used in the analysis, whereas the rest of diagnoses were grouped in the category 'other'. Moreover, to estimate the inappropriate prescription of antibiotics, acute respiratory tract infections (ARTIs) for which antibiotics are rarely or not indicated were identified based on the definition used in a previously published survey [12], so that our results are directly comparable to this previous study. To be more specific, abnormal sputum, allergy, asthma, bronchitis (acute and not otherwise specified), chronic bronchitis, chronic sinusitis, cough, dyspnoea, haemoptysis, influenza, laryngitis/tracheitis, nasopharyngitis, stridor, unspecified ARTIs and viral pneumonia

TABLE 1

Antibiotic prescribing rates in adult outpatients, Greece, July 2010–June 2013

Age (years)	Number of prescribed antibiotics (millions)	Greek population 2011 ^a	Prescribed antibiotics per 1,000 persons per year (95% CI)	Treatment duration mean (SD)
20–29	3.3448	1,350,868	825 (824.51–826.28)	9.14 (4.87)
30–39	4.0870	1,635,304	833 (832.47–834.09)	9.08 (4.26)
40–49	3.6150	1,581,095	762 (761.34–762.92)	9.18 (4.12)
50–59	2.9617	1,391,854	709 (708.56–710.17)	9.16 (4.05)
≥ 60	6.0165	2,734,621	733 (732.85–734.02)	9.01 (4.04)
Total	20.025	8,693,742	768 (767.45–768.13)	9.09 (4.25)

CI: confidence interval.

SD: standard deviation

^a Based on census 2011 [30].

were considered as diagnoses for which antibiotics are rarely or not indicated.

Costs

The total antibiotic expenditures for adult outpatients from the perspective of the National Health Insurance were calculated based on the total dose (number of packs) of each antibiotic as well as the corresponding reimbursed drug prices. These prices were obtained from the reimbursed drug list published by the Ministry of Health, reduced by the patient's co-payment (25%). Costs data reflect the year 2013.

Statistical analysis

All collected variables were analysed descriptively. Nominal variables were analysed using frequencies and percentages. Treatment duration was presented as median and interquartile range (IQR), since it was not normally distributed. In order to calculate the antibiotic prescribing rates, we used the number of prescriptions and 2011 census denominators from the Hellenic Statistical Authority [30].

Chi-squared tests of independence were performed in order to evaluate the independence between broad- and narrow-spectrum antibiotic prescription and patient, physician and diagnosis characteristics. In addition, we performed simple and multivariate logistic regressions to identify factors associated with broad-spectrum antibiotic prescribing. In the multivariate logistic regression model, we entered the factors found to be significantly associated with broad-spectrum antibiotic prescribing at a univariate level. These results are presented as odds ratios (OR) with 95% confidence intervals (95% CI). The significance level was 0.05 (two-sided). Bonferroni correction was applied to adjust for multiple comparisons. Time series analysis was conducted to assess the antibiotic prescription trend over time. Finally, non-parametric statistic tests (i.e. Mann–Whitney and Kruskal–Wallis) were used to assess the association between selected patient,

physician and diagnosis characteristics and treatment duration. The analysis was performed using SPSS 20.

Results

Antibiotic prescribing patterns

During the study period, an estimated 20 million antibiotic regimens were prescribed in Greece to adult outpatients, resulting in an annual rate of 768 prescribed antibiotics per 1,000 adults (95% CI: 767.45–768.13), with a median duration therapy of eight days (IQR: 6.0–10.5) (Table 1). Prescribing rates were higher among adults younger than 40 years (830 prescriptions/1,000 adults; 95% CI: 829.12–830.31) compared with adults 40 years and older (736 prescriptions/1,000 adults; 95% CI: 735.11–736.22), while no statistically significant difference was detected in treatment duration across the age groups ($p = 0.330$). Details on prescribing rates stratified by 10-year age bands are presented in Table 1. Time trend analysis suggested a slight decline in antibiotic prescribing rate during the last two semesters of the study period, although no statistically significant trend was detected ($p = 0.742$) (Figure 1).

The majority of prescribed antibiotics (89.0%) were broad-spectrum; no difference was detected in treatment duration between broad- and narrow-spectrum antibiotics. The most frequently prescribed antibiotics were macrolides (29.9%), second and third generation cephalosporins (26.9%), fluoroquinolones (21.0%), and penicillins (15.4%, a quarter of which were narrow spectrum). Clarithromycin (19.8%), cefuroxime (11.8%), ciprofloxacin (11.6%), amoxicillin/clavulanic acid (10.8%) and cefprozil (7.6%) made up 61.6% of all prescribed antibiotics. Trimethoprim/sulfamethoxazole was rarely prescribed, accounting for 1.6% of total prescriptions (Table 2). With respect to treatment duration, it was found that the duration of macrolide therapy was significantly longer than cephalosporin therapy (median duration: 10.5 days vs 7.0 days). Clarithromycin and ciprofloxacin were the antibiotics prescribed with the longest duration (median: 10.5 days and 10.0 days,

TABLE 2

Antibiotic prescribing patterns and expenditures in adult outpatients, Greece, July 2010–June 2013

	Number of prescribed antibiotics ($\times 10^6$)	Proportion of prescribed antibiotics	Expenditures (in million EUR)	Proportion of total expenditures	Treatment duration median (IQR)
Total	20.025		184.6		8 (6–10.5)
Antibiotic class					
Macrolides	5.9833	29.9%	60.0	32.5%	10.5 (7–10.5) _{a,b,c}
Cephalosporins	5.3781	26.9%	53.6	29.0%	7 (6–12) ^a
Fluoroquinolones	4.1991	21.0%	58.2	31.5%	10 (7–10) _{a,b,c}
Penicillins	3.0761	15.4%	6.1	3.3%	8 (5–8)
Tetracyclines	1.0153	5.1%	3.4	1.8%	8 (8–12) _{a,c,d}
Trimethoprim/sulfamethoxazole	0.3144	1.6%	1.0	0.5%	10 (5–10) ^a
Other beta-lactams excluding penicillins and cephalosporins	0.0591	0.3%	2.4	1.3%	10 (5–10)
Antibiotic^m					
Clarithromycin	3.9661	19.8%	45.3	24.5%	10.5 (10.5–10.5) ^e
Cefuroxime	2.3724	11.9%	21.7	11.7%	7 (7–14) ^f
Ciprofloxacin	2.3312	11.6%	28.1	15.2%	10 (10–10)
Amoxicillin/clavulanic acid	2.1681	10.8%	5.1	2.8%	8 (4–8) ^g
Cefprozil	1.5234	7.6%	14.7	8.0%	6 (6–12) ^f
Azithromycin	1.4300	7.1%	10.8	5.9%	6 (4.5–8) ^h
Cefaclor	1.1882	5.9%	10.9	5.9%	8 (4–8) ^g
Broad vs narrow spectrum					
Narrow spectrum	2.1975	11.0%	5.1	2.7%	8 (6–12)
Broad spectrum	17.8275	89.0%	179.6	97.3%	8 (7–10.5)
Diagnosis					
Unspecified acute LRTI	2.4491	12.2%	23.8	12.9%	8 (7–10.5) ⁱ
Acute bronchitis	2.3552	11.8%	22.4	12.1%	10 (7–10.5) ⁱ
Other disease of urinary system than cystitis	2.2742	11.4%	23.2	12.5%	10 (7–12)
Acute pharyngitis	0.8450	4.2%	5.6	3.1%	7 (6–10.5) ^j
Cystitis	0.8250	4.1%	6.9	3.7%	7 (6–10) ^k
Acute sinusitis	0.7881	3.9%	9.1	4.9%	10 (7–10.7)
Acute tonsillitis	0.7420	3.7%	5.9	3.2%	8 (7–10.5)
Other chronic obstructive pulmonary disease	0.6320	3.2%	6.7	3.7%	8 (7–10.5)
Pneumonia, organism unspecified	0.6030	3.0%	8.7	4.7%	10 (7–10.5)
Inflammatory disease of prostate	0.5911	3.0%	12.2	6.6%	10 (10–16) ^l
Other	7.9203	39.6%	60.2	32.6%	8 (6–10.5)

IQR: interquartile range; LRTI: lower respiratory tract infection.

Broad-spectrum antibiotics included broad-spectrum penicillins (amoxicillin/clavulanic acid), macrolides, second and third generation cephalosporins and fluoroquinolones.

^a $p < 0.001$ compared with penicillins, after Bonferroni correction.^b $p < 0.001$ compared with other beta-lactams excluding penicillins and cephalosporins, after Bonferroni correction.^c $p < 0.001$ compared with cephalosporins and trimethoprim/sulfamethoxazole, after Bonferroni correction.^d $p < 0.001$ compared with macrolides, after Bonferroni correction.^e $p < 0.001$ compared with ciprofloxacin, after Bonferroni correction.^f $p < 0.001$ compared with ciprofloxacin and clarithromycin, after Bonferroni correction.^g $p < 0.001$ compared with cefprozil, cefuroxime, ciprofloxacin and clarithromycin, after Bonferroni correction.^h $p < 0.001$ compared with all antibiotics except for cefuroxime, after Bonferroni correction.ⁱ $p < 0.001$ compared with 'other dysfunction of urinary system', 'acute pharyngitis', 'cystitis', 'acute sinusitis' and 'inflammatory disease of prostate', after Bonferroni correction.^j $p < 0.001$ compared with all, except for cystitis, after Bonferroni correction.^k $p < 0.001$ compared with all, except for acute pharyngitis, after Bonferroni correction.^l $p < 0.001$ compared with all others, after Bonferroni correction.^m Data are presented only for the most frequently prescribed antibiotics.

respectively) in contrast to cefprozil and azithromycin that were prescribed for significantly shorter durations (Table 2).

Antibiotic expenditures

Throughout the whole study period, the total expenditures for antibiotics were EUR 184.6 million; EUR 66.5, 64.0 and 54.5 million during the first, second and third year of study respectively. Cephalosporins, macrolides and fluoroquinolones made up 93.1% of the total antibiotic expenditures. Clarithromycin alone accounted for almost one quarter (24.5% or EUR 45.3 million) of the total antibiotic spending, followed by ciprofloxacin (15.2% or EUR 28.1 million) and cefuroxime (11.7% or EUR 21.7 million). Although amoxicillin/clavulanic acid was the fourth most frequently prescribed antibiotic, less than 3% (EUR 5.1 million) of the total antibiotic expenditures were attributed it (Table 2).

Antibiotic prescribing across selected clinical diagnoses

The three most common clinical diagnoses for which antibiotics were prescribed in adult outpatients were, unspecified acute lower respiratory tract infections (LRTIs) (12.2%), acute bronchitis (11.8%) and other diseases of urinary system than cystitis (11.4%), followed by acute pharyngitis (4.2%) and cystitis (4.1%). One third of all antibiotics (33.5%) were prescribed for diagnoses for which antimicrobials are rarely or not indicated.

The most commonly prescribed antibiotics by clinical diagnosis are presented in Figure 2. Notably, clarithromycin was the most commonly prescribed antibiotic for unspecified acute LRTIs, acute bronchitis and for acute pharyngitis (33.2%, 43.2% and 28.7%, respectively). Ciprofloxacin was the most frequently prescribed antibiotic for cystitis and other diseases of the urinary system (20.7% and 41.8%, respectively) followed by norfloxacin (22.5% and 20.4%, respectively).

Factors associated with broad-spectrum antibiotic prescribing

Multivariate analysis revealed that adults older than 30 years were more likely to receive broad-spectrum antibiotics. In addition, pulmonologists and otorhinolaryngologists as well as physicians trained in eastern Europe (e.g. Bulgaria, Hungary or Romania) were more likely to prescribe broad-spectrum antibiotics compared with gynaecologists, urologists and doctors trained in Greece. However, physicians trained in these countries only contributed 5.5% of the overall prescribed antibiotics. Other diseases of the urinary system were associated with higher rates of broad-spectrum antibiotic prescribing compared with unspecified acute LRTIs, after adjustment for possible confounders. Furthermore, acute pharyngitis and other diagnoses were associated with lower rates of broad-spectrum antibiotic prescribing compared with unspecified acute LRTIs. Finally, broad-spectrum antibiotics were less likely to be prescribed for recurrent

and chronic illnesses compared with first episode and acute diseases (Table 3).

Discussion

We aimed to assess the antibiotic prescription patterns in adult outpatients in Greece, as well as expenditures and potential factors associated with broad-spectrum antibiotic prescribing.

We found a high annual rate of antibiotic prescribing, but this seemed to decline over the studied period. The majority (89%) of antibiotics prescribed were broad-spectrum with a median duration of therapy of eight days. Almost one-third of all prescriptions were for ARTIs for which antibiotics are rarely or not indicated. The cost analysis revealed considerable healthcare expenditures for antibiotics.

The overall annual rate of 768 prescribed antibiotics per 1,000 adults that we found is much higher in comparison with countries such as Sweden (388 prescriptions/1,000 persons) [23], and similar to the United States (US) (833 prescriptions/1,000 persons) [24]. Perhaps the most alarming finding in our study was that 89% of all antibiotics prescribed were broad-spectrum, with patients older than 60 years being the most common recipients (91.4% of antibiotic prescriptions). Shapiro et al. have recently reported high prescription rates of broad-spectrum antibiotics (61% of total prescriptions) in the US, the majority of which (64%) were given to patients older than 60 years [12]. In contrast, the most common antibiotics prescribed in Sweden were narrow-spectrum penicillins (30% of total prescriptions) [23]. It is a well-known fact that in many cases, broad-spectrum antibiotics are prescribed instead of narrower-spectrum antibiotics, especially for the treatment of bacterial ARTIs [9,11,23]. In this context, the Hellenic Centre for Disease Control and Prevention has already taken initiatives to inform the public about the consequences of antibiotic misuse/overuse and antimicrobial resistance and prepared instructions on the rational use of antibiotics [31].

Concerning the distribution of antibiotics, our study showed extensive use of macrolides, cephalosporins and fluoroquinolones, at higher percentages than in other countries. In the US, the top five list of prescribed antibiotics included azithromycin, amoxicillin, amoxicillin/clavulanic acid, ciprofloxacin and cefalexin in 2010 [24], which is similar to our results. In Switzerland, amoxicillin and amoxicillin/clavulanic acid were the most used antibiotics for outpatients, with fluoroquinolones and macrolides the second and third most prescribed antibiotics in 2007 [15]. In Germany most common antibiotics consumed were penicillin/amoxicillin, followed by tetracyclines and newer macrolides (roxithromycin, azithromycin and clarithromycin) in 2003 [17]. It is unlikely that this variability in prescribing can be explained by differences in the epidemiology of ARTI. The rates of common ARTI are likely to be similar rather than different across countries in

TABLE 3A

Differences in prescription of broad-spectrum antibiotics in adult outpatients, based on patient, physician and diagnosis characteristics, Greece, July 2010–June 2013

	Broad spectrum (proportion of prescribed antibiotics)	p value	Crude OR (95% CI)	Adjusted OR (95% CI)
Patient characteristics				
Age (years)		<0.001		
20–29	84.6%		1.00	1.00
30–39	87.8% ^a		1.31 (1.15–1.49)	1.18 (1.03–1.36)
40–49	89.0% ^a		1.47 (1.27–1.69)	1.09 (0.94–1.26)
50–59	91.0% ^{a,b}		1.84 (1.57–2.15)	1.17 (0.99–1.39)
≥ 60	91.4% ^{a,b}		1.93 (1.69–2.20)	1.12 (0.97–1.31)
Sex		<0.001		
Female	87.4%		1.00	-
Male	91.3%		1.52 (1.38–1.67)	-
Physician characteristics				
Specialty		<0.001		
General practice	93.1%		1.00	1.00
Pulmonology	94.4% ^c		1.25 (1.04–1.50)	1.47 (1.21–1.79)
Otorhinolaryngology	95.4% ^c		1.56 (1.25–1.95)	2.50 (1.97–3.16)
Gynaecology	72.4% ^{c,d,e}		0.20 (0.17–0.22)	0.21 (0.18–0.24)
Urology	87.0% ^{c,d,e}		0.50 (0.43–0.58)	0.62 (0.52–0.74)
Other	79.1% ^{c,d,e}		0.28 (0.24–0.33)	0.36 (0.31–0.43)
Training location (self-identified)		<0.001		
Greece	88.5% ^f		1.00	1.00
Eastern Europe ^g	92.9%		1.71 (1.35–2.16)	1.53 (1.18–1.99)
Western Europe ^h	89.8% ^f		1.14 (1.02–1.28)	0.81 (0.71–0.94)
Age		<0.001		
<40	90.7% ⁱ		1.00	1.00
41–50	90.7% ⁱ		1.00 (0.82–1.22)	1.07 (0.86–1.33)
51–60	88.7% ⁱ		0.80 (0.67–0.96)	0.85 (0.70–1.05)
≥ 60	87.9%		0.74 (0.62–0.90)	0.78 (0.64–0.97)
Sex		0.979		
Female	89.0%		1.00	-
Male	89.0%		0.99 (0.86–1.15)	-

CI: confidence interval; LRTI: lower respiratory tract infection; OR: odds ratio.
Statistically significant results are shown in bold.

^a $p < 0.001$ compared with age group 20–29 years, after Bonferroni correction.

^b $p < 0.001$ compared with age group 30–39 years, after Bonferroni correction.

^c $p < 0.001$ compared with general practice, after Bonferroni correction.

^d $p < 0.001$ compared with pulmonology, after Bonferroni correction.

^e $p < 0.001$ compared with otolaryngology, after Bonferroni correction.

^f $p < 0.001$ compared with group Eastern Europe, after Bonferroni correction.

^g The tree most common countries were Bulgaria, Hungary and Romania. However, physicians trained in these countries only contributed 5.5% of the overall prescribed antibiotics.

^h The tree most common countries were Germany, Italy and the United Kingdom.

ⁱ $p < 0.001$ compared with age group ≥ 60 years, after Bonferroni correction.

^j $p < 0.001$ compared with age group 41–50 years, after Bonferroni correction.

the European Union. Regarding resistance patterns, for pharyngitis, group A streptococci remain universally susceptible to penicillin.

Variations in the incidence of infections, educational and cultural background could be some of the reasons for the observed differences in antibiotic use between countries. Also, each country's treatment guidelines

and pharmaceutical markets may influence doctors' prescribing behaviour [25,26].

A comparison of our findings with the guidelines of the Hellenic Centre for Disease Control and Prevention for the ARTIs revealed that physicians in Greece seem to have partially adopted the national guidelines in terms of prescribed antibiotics and therapy duration.

TABLE 3B

Differences in prescription of broad-spectrum antibiotics in adult outpatients, based on patient, physician and diagnosis characteristics, Greece, July 2010–June 2013

	Broad spectrum (proportion of prescribed antibiotics)	p value	Crude OR (95% CI)	Adjusted OR (95% CI)
Diagnosis related characteristics				
Diagnosis		<0.001		
Unspecified acute LRTI	96.0%		1.00	1.00
Acute bronchitis	94.9%		0.76 (0.58–1.02)	0.82 (0.62–1.08)
Other disease of the urinary system than cystitis	95.2%		0.81 (0.61–1.07)	1.51 (1.12–2.02)
Acute pharyngitis	86.2% ^k		0.25 (0.19–0.34)	0.22 (0.16–0.29)
Cystitis	87.3% ^l		0.28 (0.21–0.37)	0.79 (0.58–1.08)
Other	85.4%		0.24 (0.19–0.29)	0.48 (0.38–0.61)
Recurrence		<0.001		
First episode	90.0%		1.00	1.00
Recurrence	85.7% ^m		0.67 (0.60–0.75)	0.76 (0.64–0.90)
Not specified	87.9% ^m		0.81 (0.72–0.91)	1.10 (0.96–1.27)
Type of diagnosis		<0.001		
Acute	90.3%		1.00	1.00
Chronic	81.1% ⁿ		0.46 (0.41–0.52)	0.62 (0.52–0.74)
Not specified	84.9% ⁿ		0.60 (0.51–0.71)	0.85 (0.70–1.05)

CI: confidence interval; LRTI: lower respiratory tract infection; OR: odds ratio.

Statistically significant results are shown in bold.

^k $p < 0.001$ compared with all, except for cystitis and other, after Bonferroni correction.

^l $p < 0.001$ compared with all, except for acute pharyngitis, after Bonferroni correction.

^m $p < 0.001$ compared with first episode, after Bonferroni correction.

ⁿ $p < 0.001$ compared with acute diseases, after Bonferroni correction.

Specifically, we found that clarithromycin (i.e. macrolides) was the most commonly prescribed antibiotic for the treatment of acute pharyngitis, tonsillitis, LRTIs and chronic obstructive pulmonary disease, which is in agreement with national guidelines. On the other hand, it was noticeable that second generation cephalosporins such as cefprozil and cefuroxime were also commonly prescribed antibiotics for the management of acute pharyngitis, acute tonsillitis and LRTIs, although are not recommended by KEELPNO guidelines. With respect to treatment duration, our findings indicate that the median of therapy duration is close to that recommended by national guidelines for the vast majority of diagnoses.

We identified several factors that were related to broad-spectrum antibiotic prescribing. The strongest factors were patient's age over 60 years, male sex and physician's specialty of pulmonology/otorhinolaryngology, which were additive. Factors associated with lower rates of broad-spectrum antibiotic choice included physician's training in Greece or western Europe compared with eastern Europe, older age of the physician, a diagnosis other than ARTI and a recurrent and/or chronic condition. There is no clear explanation for the fact that physicians who were trained in eastern Europe were more likely to prescribe broad-spectrum antibiotics.

However, we can hypothesise that the reason may be differences in medical education regarding antibiotic use, resistance and stewardship or differences in national policies and guidelines regarding antibiotic prescribing in these countries. It needs to be noted that this group constituted only a small sample of the total.

The total antibiotic expenditures for adult outpatients in Greece between 2010 and 2013 were approximately EUR 185 million, accounting for almost the 2% of the total public pharmaceutical expenditures in Greece during the study period. According to our study, one third of prescriptions were given for diagnoses that rarely or do not require antibiotic therapy, which means that approximately EUR 62 million could have been saved, no small sum considering the financial crisis that Greece has recently experienced.

Overall, our study indicated that antibiotic overprescribing is a problem in Greece. Patient pressure to prescribe them antibiotics and inadequate knowledge around appropriate utilisation of antibiotics are some of the factors leading to antibiotic overuse [25,27].

Potential limitations of this study include the possibility that although doctors were randomly selected, our

sample may not be representative of the total population of physicians. Second, patients in Greece can acquire antibiotics from pharmacists without a prescription [28]. The data from the Eurobarometer on antimicrobial resistance indicate that 15% of the Greek survey responders obtained their last course of antibiotics without prescription [29]. Third, we did not have access to prescribing data from hospitals and emergency departments, which could lead to an underestimation of the prescribing rates. Fourth, prescribing data may not accurately represent actual antibiotic consumption, since patient compliance with treatment is unknown. Fifth, direct assessment of appropriate antibiotic prescribing is difficult, as the IMS Health XPO database does not provide information on patient symptoms, severity of disease, drug allergies or whether or not a test was performed to identify bacteria or viruses. Sixth, the duration of antibiotic therapy was calculated indirectly by using available data on the number of prescribed packs, daily dose and dose frequency. As such, we cannot be sure that this was an accurate estimation of the actual treatment duration. Seventh, although it would be interesting to distinguish between different antibiotic classes within the broad-spectrum category and assess specific factors associated with them, the small sample size did not allow that. Finally, it should be noted that the costs related to antibiotic use throughout the whole study period may be underestimated as we used in our calculations the drug prices as published at the end of the year 2013 and it is known that there was a sharp reduction in drug prices in Greece during the period from 2010 to 2013.

Conclusion

We observed a high rate of antibiotic prescribing in adult outpatients in Greece, one-third of which were for diagnoses that rarely or do not require antibiotic therapy. Broad-spectrum antibiotics accounted for 89% of the total antibiotic prescriptions. The reasons for extensive use of broad-spectrum antibiotics need to be studied in depth. We have identified potential targets for antimicrobial stewardship including broad-spectrum prescribing for most ARTIs as well as specific practitioner characteristics. Broad implementation of programmes targeting outpatient care settings should be a public health priority for Greece, with the final aim to reduce antimicrobial resistance and the financial burden.

Conflict of interest

None declared.

Authors' contributions

All authors contributed extensively to the work presented in this paper. All authors discussed the results and implications and commented on the manuscript at all stages. G.K designed the study. G.K and E.K carried out the statistical analysis of the data. E.G-K categorised the diagnoses and

edited the manuscript. G.K, E.K and G.M wrote the manuscript. T.E.Z supervised the study and edited the manuscript.

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Letter to the editor: *Escherichia coli* harbouring *mcr-1* gene isolated from poultry not exposed to polymyxins in Brazil

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To the editor: The recent paper by Fernandes et al. [1] described the presence of the *mcr-1* gene in *Escherichia coli* from pigs and poultry in Brazil. The authors stated that microbiology laboratories worldwide should be aware of *mcr-1* isolates resistant to polymyxins in patients living in or returning from Latin American countries and highlighted that the *mcr-1* gene dissemination results from polymyxins' misuse as growth promoter in food animals. In view of the concerning spread of antibiotic resistance, we screened *E. coli* isolates obtained from a poultry slaughterhouse in southern Brazil with official reports on antibiotic use.

Poultry rectal swabs were collected between August and October 2015. A total of 340 chickens farmed in Brazil and belonging to 17 batches were included in the study. All poultry had received bacitracin, narasin and nicarbazin during a first period of life (between the 2nd and the 18th day) and avilamycin and salinomycin during a second period (between the 20th and 35th day); the chickens of batches 10 and 11 had also received doxycycline during a total of 3 days, in the second period of life. Poultry included in this study were not exposed to polymyxin during their entire life (around 40 days).

A total of 343 isolates were evaluated by polymerase chain reaction (PCR) for the *mcr-1* gene [2] and 10 (3%) were positive. The *mcr-1* gene was confirmed by sequencing the PCR amplicon. The *mcr-1* positive isolates were obtained from 10 different chickens belonging to three batches from three different breeders. The polymyxin B minimum inhibitory concentrations (MIC) of the 10 *mcr-1* positive isolates were 2 mg/L (8 isolates), 1 mg/L and 0.25 mg/L and they could be classified as susceptible to polymyxin B, according to the European Committee on Antimicrobial Susceptibility

Testing (EUCAST) (resistance >2 mg/L). In contrast, most reports indicate that the *mcr-1* gene is usually found in isolates presenting resistance to polymyxins [2-6].

The *mcr-1* positive isolates were submitted to DNA macrorestriction typing by pulsed-field gel electrophoresis (PFGE) and five isolates, from the same batch, proved to be clonally related while the other five isolates were unrelated. Conjugation experiments with the *E. coli* J53 were successful for two *mcr-1* positive isolates which confirmed that the *mcr-1* gene was located in a plasmid. The transconjugants presented positive results by PCR for the *mcr-1* gene and had a polymyxin B MIC of 2 mg/L.

According to Brazilian law, all slaughterhouses must submit in advance to the Federal Inspection Service of the Ministry of Agriculture, the bulletin of health of each batch of animals to be slaughtered. It is of note that the chickens evaluated in this study have received antibiotics as growth promoters, but polymyxins were not included among these compounds. This goes against the hypothesis that the emergence of the *mcr-1* gene is linked to the use of polymyxins in animal feed in Brazilian livestock [1] and suggests that others compounds or factors may also be involved in the selection of this gene.

Finally, the fact that the *mcr-1* was originally described in China and thereafter in several other countries including Europe indicates that this gene is already widespread in the world. Therefore, isolates with *mcr-1* should be considered in any patient, regardless of whether they were living in or returning from Latin America or not.

*Authors' correction

On 3 August, the Acknowledgements section was modified to include Laurent Poirel and acknowledge his provision of an mcr-1 positive control.

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Conflict of interest

None declared.

Authors' contributions

Conceived the project: AFM; Managed sample collection: SAML, ASM, AFM; Performed laboratory investigations: SAML, DLM, LSN, VMLC; Drafted the article: DLM; Revised the article: APZ, ALB, AFM.

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Authors' reply: *Escherichia coli* harbouring *mcr-1* gene isolated from poultry not exposed to polymyxins in Brazil

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To the editor: The foregoing letter by Lentz et al. examined the occurrence of the *mcr-1* gene in *Escherichia coli* isolates recovered from chicken cloacal swabs collected between August and October of 2015, from a poultry slaughterhouse in southern Brazil [1], providing valuable additional data on the epidemiology of this novel gene. Of 343 animals screened, 10 (3%) different chickens belonging to three flocks from three different breeders were found with *mcr-1* positive *E. coli* isolates [1]. None of these chickens had been reportedly exposed to polymyxins (as growth promoter) [1]. The authors therefore considered their findings as contradicting the plausible hypothesis that the emergence of the *mcr-1* gene is linked to the use of polymyxins in animal feed in Brazilian livestock [2], suggesting that others compounds or factors may also be involved in the selection of this gene.

With regard to this interpretation however, we put forward several points that might be taken into account. Indeed, the investigation by Lentz et al. was conducted as a prospective and short three-month period study, whereby the use of antibiotics other than polymyxins to promote growth might have been part of a transitory change in local agricultural practices, which is not reflected in the whole country. Furthermore, the chickens were only tested for *mcr-1*-harbouring bacteria after 35 days of life, so it is not known if they already had acquired *E. coli* with this gene at a younger stage. For example, if the *mcr-1* gene had been detected already at one day of age, this could have suggested vertical transmission from breeder flocks, as well as the capacity of *mcr-1* positive strains to survive the hatchery process[3]. In addition, retrospective use of colistin, in the studied breeder flocks, was not raised. So, although the authors state that polymyxins were not employed as a growth promoters throughout the study period, the possible use of colistin in the past

years, along the poultry production chain, cannot be ruled out. This could explain the polymyxin susceptibility (i.e. polymyxin B minimum inhibitory concentration: MIC_{≤2} mg/L) exhibited by *mcr-1* positive *E. coli* strains found in the study [1]. In fact, the persistence of a resistance gene may be related to the stability of the plasmid in its host, where the expression of resistance is normally silent until it is induced by antibiotic pressure [4,5]. Moreover, antibiotic-resistant bacteria may also be acquired from external sources, and potentially transferred to current animals, from animals kept at the same location during the previous farming cycle ('carry-over') [3]. In brief, we believe that studies conducted to evaluate the presence of *E. coli* harbouring *mcr-1* gene in poultry not exposed to polymyxins should be preferably addressed in experimental farm settings where antimicrobial exposure is well controlled.

Regardless, valuable information in the letter by Lentz et al. was the identification of more *mcr-1* positive *E. coli* in southern Brazil, which is worrisome, since strains of *E. coli* carrying *mcr-1* have been previously identified in food-producing animals from Minas Gerais, São Paulo, Paraná and Santa Catarina states [2]. Recently, we have also identified the first colistin-resistant *mcr-1* positive *E. coli* isolate from a human infection in Rio Grande do Norte State, north-eastern Brazil (data not shown; GenBank accession number: CP015977). Thus, although data on MCR-1 are currently few, there is supportive evidence that *E. coli* strains carrying *mcr-1* genes are widespread in Brazil in both humans and animals. Currently, to optimise the performance of farming, use of colistin sulfate is allowed within the levels recommended by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) in diets of food-producing animals [6].

In summary, these results should encourage greater restrictions of colistin in farming systems. Furthermore, the emergence of *mcr-1* positive *E. coli* isolates and their potential spread require very close monitoring and surveillance.

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Conflict of interest

None declared.

Authors' contributions

MRF, QM, FE and NL wrote the letter.

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