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Zika virus infection in a traveller returning from the Maldives, June 2015

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We report a Zika virus (ZIKV) infection in a patient with fever and rash after returning to Finland from Maldives, June 2015. The patient had dengue virus (DENV) IgG and IgM antibodies but pan-flavivirus RT-PCR and subsequent sequencing showed presence of ZIKV RNA in urine. Recent association of ZIKV with microcephaly highlights the need for laboratory differentiation of ZIKV from DENV infection and the circulation of ZIKV in areas outside its currently known distribution range.

Case report

A 37-year-old Finnish man returned with his family from a half-a-year work-related stay in the Maldives (in Dhiffushi island, situated in North Malé atoll as the capital Malé) to Finland 16 June 2015, without any stop-overs elsewhere. Two days later he became ill with flu-like prodrome, mild fever and rash in the face and trunk, as well as ocular pain and arthralgia; the symptoms alleviated after a few days. He contacted occupational health and due to suspicion of dengue, a serum sample was taken 24 June, and it was positive for dengue virus IgG (titer 1:1,280, in-house immunofluorescence assay (IFA) test) and IgM (1.9/ cut-off 1.0, Dengue Virus IgM Capture DxSelect ELISA, Focus Diagnostics, USA), but negative for dengue virus (DENV) non-structural (NS) 1 antigen (Dengue NS1 Ag Strip Bio-Rad, France). Along with the serum sample, a urine sample taken on 25 June, the following day, was received for flavivirus RNA detection using a real-time pan-flavivirus NS5 nested RT-PCR [1,2]. RNA from serum and urine samples was extracted using QIAamp Viral RNA Mini Kit (Qiagen).

From the urine (but not the serum) an amplification product was detected and subsequently sequenced (160bp excluding primers, available from the authors upon request). A BLAST search identified the sequence as Zika virus (ZIKV) identical to Asian lineage strains originating from Easter Island 2014 [3], French Polynesia 2013 (GenBank KJ776791), Brazil 2015 (GenBank KU321639) and Thailand 2013 [4] and in phylogenetic analyses the sequence clustered with these strains (Figure). A PCR contamination in the laboratory is further ruled out as no work with ZIKV has ever been conducted, or any positive samples analysed previously in the laboratory - or in the country as a whole.

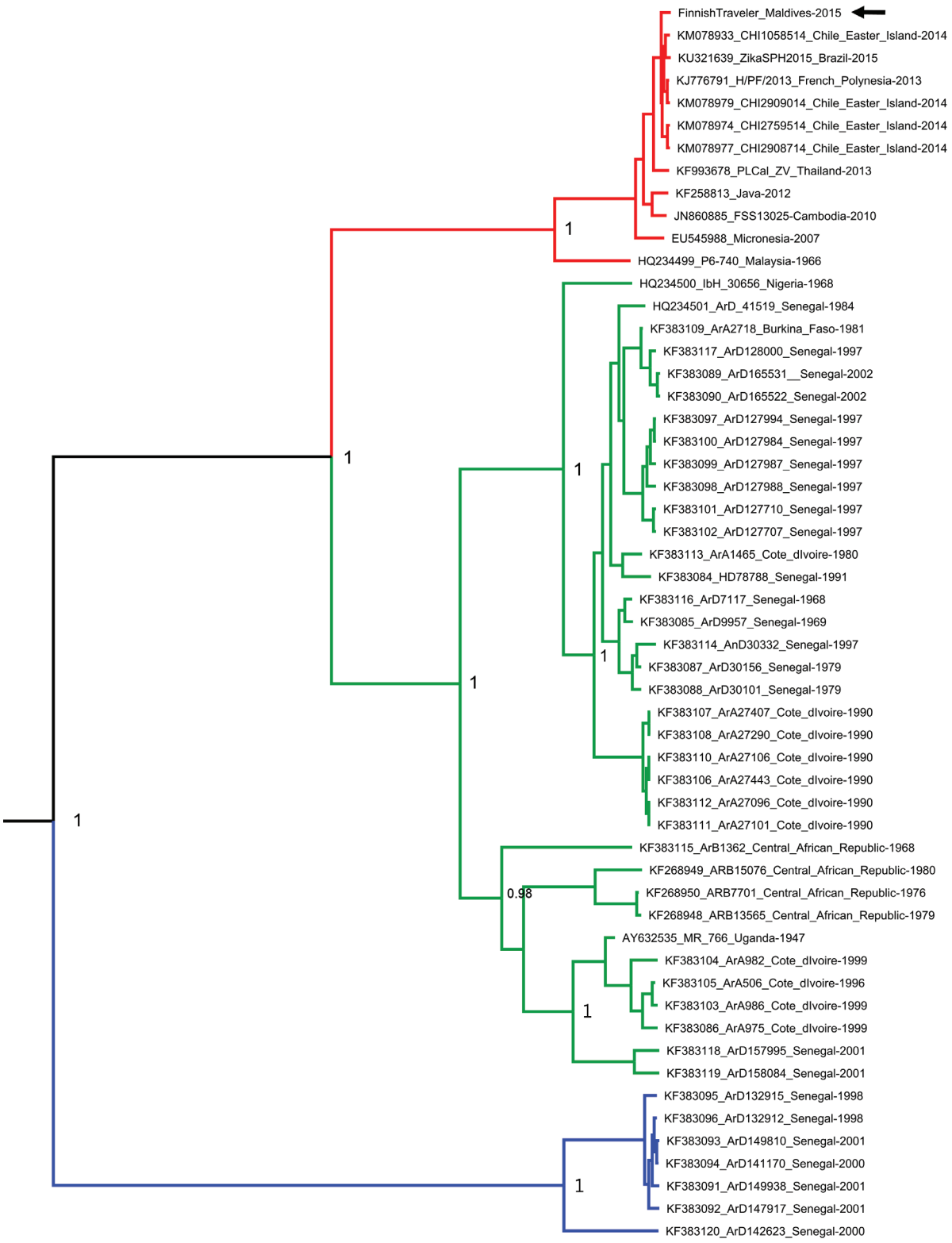
The Asian cluster is shown in red and African clusters as green and blue. Posterior probabilities are shown only for basal nodes. All ZIKV sequences were downloaded from GenBank (5.1.2016) and sequences overlapping the partial NS5 gene sequence were included in the analysis. The sequences were aligned using ClustalW algorithm implemented in MEGA version 6. For the sake of clarity, the identical sequences from Easter Island were removed from the data set. The best-fit substitution model was sought using MEGA version 6. The phylogenetic tree was constructed using Bayesian Monte Carlo Markov Chain (MCMC) method implemented in BEAST version 1.8.0 using Tamura-Nei (TN93+G) model of substitution, strict molecular clock and constant population size demographic model. The Bayesian analysis was run for 50 million states and sampled every 1000 states. Posterior probabilities were calculated with a burn-in of 5 million states and checked for convergence using Tracer version 1.6.

Investigation of family members

Similar disease and mosquitoes were frequently reported in the area at the beginning of the rainy season and generally interpreted as dengue. The patient's wife had experienced a mild febrile illness a couple of weeks before departure. Serum samples obtained on 8 July, two weeks after confirmation of Zika virus in our patient, from the patient's wife and three children all of less than 10 years of age, were negative for flavivirus in pan-flavivirus NS5 nested RT-PCR. The children were also DENV seronegative, but the wife had low positive DENV IgG titer (1:20) and low positivity (1.3/ cut-off 1.0, Dengue Virus IgM Capture DxSelect ELISA, Focus Diagnostics, USA) in DENV IgM test.

Figure

Maximum clade credibility tree of partial Zika virus NS5 sequences



The Asian cluster is shown in red and African clusters as green and blue. Posterior probabilities are shown only for basal nodes. All ZIKV sequences were downloaded from GenBank (5.1.2016) and sequences overlapping the partial NS5 gene sequence were included in the analysis. The sequences were aligned using ClustalW algorithm implemented in MEGA version 6. For the sake of clarity, the identical sequences from Easter Island were removed from the data set. The best-fit substitution model was sought using MEGA version 6. The phylogenetic tree was constructed using Bayesian Monte Carlo Markov Chain (MCMC) method implemented in BEAST version 1.8.0 using Tamura-Nei (TN93+G) model of substitution, strict molecular clock and constant population size demographic model. The Bayesian analysis was run for 50 million states and sampled every 1000 states. Posterior probabilities were calculated with a burn-in of 5 million states and checked for convergence using Tracer version 1.6.

Table

Verified acute Zika virus infections from South/Southeast Asia, 1977–2015

Country of origin	Year	Number of patients	Reference
Indonesia	1977–1978	7	[27]
	2013–2015	2	[31,32]
Cambodia	2010	1	[28]
Philippines	2012	1	[29]
Thailand	2012–2014	10	[4,23,30,33]
Malaysia	2014	1	[22]

Background

ZIKV is a mosquito-borne flavivirus originally isolated in Uganda, 1947 [5]. ZIKV was associated with mild febrile disease and maculo-papular rash in tropical Africa and some areas of South East Asia. Since 2007 ZIKV has caused several outbreaks outside its former distribution area in islands of the Pacific Ocean: in 2007 on Yap island (Federated States of Micronesia) [6] and since 2013–14 in French Polynesia [7,8]. Since 2015, outbreaks have been reported for the first time in South America (Brazil, Columbia) [9,10]. Two lineages of ZIKV, an African (subdivided to West African and East African) and Asian lineage, which emerged in the Pacific and the Americas, respectively, have been identified on the basis of NS5 gene sequences [11].

The main transmission occurs in an urban cycle similar as for dengue and chikungunya, with *Aedes* (*Stegomyia*) mosquitoes as vectors [12]. Probable sexual transmission has been associated with ZIKV infection [13] and ZIKV has been isolated, and ZIKV RNA detected from semen samples [14]. Also transplacental transmission of ZIKV during childbirth has been reported in the French Polynesian outbreak [15].

Association with Guillain-Barré syndrome and more recently to an emerging epidemic of congenital microcephaly have increased the public health impact of ZIKV infections [16,17].

Discussion and conclusions

As in our case, the DENV serology may be positive in ZIKV patients due to cross-reactions between other flaviviruses, and the ZIKV-specific RNA detection methods or sequencing are best for confirmation. The RT-PCR positivity from urine but not serum may suggest that urine is better as a sample material for RNA detection of ZIKV, and indeed the viral load in urine has been shown to be higher and detectable for a longer period, as compared to serum [18] in parallel to dengue [19]. Our patient was found negative in DENV NS1 Ag test, which in most DENV patients is positive during the acute phase [20] yet a negative NS1 result does not exclude DENV infection.

The short sequence obtained from the patient confirmed the etiology of the infection as Zika virus and suggested that the virus strain present in Maldives is of the Asian lineage of ZIKV, and indistinguishable within the amplified short fragment from the epidemic strains reported from e.g. Easter Island and Brazil. Yet, as we have so far been able to sequence only a short part of the NS5 gene, more sequence information is evidently needed.

ZIKV is an emerging arbovirus and it seems to fit well to the transmission cycles of DENV and CHIKV [21], which both have been earlier detected from the Maldives. ZIKV infections have earlier been imported from Asia, South America, French Polynesia and the Caribbean to Europe [22-26]. In Asia, there have been no previous verified ZIKV cases anywhere near Maldives (Table).

With this demonstration of ZIKV transmission in the Maldives, it remains to be elucidated if the circulation of ZIKV is already widespread in the area or geographical vicinity, as clinical manifestations of DENV, CHIKV and ZIKV as well as serological test results for DENV and ZIKV may be similar, or a risk of a larger Zika epidemic remains a possible future threat. The most prevalent symptoms associated with ZIKV, based on the reports from cases transmitted in Asia, are fever, rash, gastrointestinal symptoms, conjunctivitis, arthralgia, sore throat, headache and myalgia [4,22,23,27-33]. The recent potential associations of ZIKV with microcephaly and Guillain-Barré syndrome [16,17] highlight the need for ZIKV recognition and detection. The differentiation of DENV and ZIKV infections is a challenge for both clinicians and diagnostic laboratories.

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Conflicts of interest

None declared.

Authors' contributions

Wrote the manuscript: EMK, EH, TS, HKK, MR and OV; performed laboratory investigations: EMK, EH; performed phylogenetic analyses: EH, TS; managed the patient: MR; collected diagnostic and clinical data HKK, MR, OV.

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Multidrug-resistant bacteria in unaccompanied refugee minors arriving in Frankfurt am Main, Germany, October to November 2015

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Many refugees arriving in Germany originate or have travelled through countries with high prevalence of multidrug-resistant Gram-negative organisms. Therefore, all unaccompanied refugee minors (<18 years-old) arriving in Frankfurt am Main between 12 October and 6 November 2015, were screened for multidrug-resistant *Enterobacteriaceae* in stool samples. *Enterobacteriaceae* with extended spectrum beta-lactamases (ESBL) were detected in 42 of 119 (35%) individuals, including nine with additional resistance to fluoroquinolones (8% of total screened), thus exceeding the prevalences in the German population by far.

We report multidrug-resistant *Enterobacteriaceae* in stool samples of unaccompanied refugee minors (<18 years-old) arriving in Frankfurt am Main, Germany, between 12 October and 6 November 2015. Of 119 individuals screened in this study, extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* were found in 42 (35%), including nine with additional resistance to fluoroquinolones (8% of total screened), i.e. 3-multidrug-resistant Gram-negative bacteria (MDR GNB).

Microbiological investigation

All unaccompanied refugee minors arriving without their parents and families in Frankfurt am Main, Germany, from 12 October to 6 November 2015 were screened for multidrug-resistant *Enterobacteriaceae* in stool samples with informed consent of their legal caregivers. The enterobacteria were classified as 3MDR GNB or 4MDR GNB according to the phenotypic definition of the German commission on hospital hygiene and infection prevention (Kommission für Krankenhaushygiene und Infektionsprävention), i.e. *Enterobacteriaceae* resistant against three of four antibiotic groups (penicillins with piperacillin as surrogate substance, cephalosporins with cefotaxime and/or ceftazidime as surrogate substance, and fluoroquinolones with ciprofloxacin as surrogate substance) were part of 3MDR GNB, while bacteria characterised as 4MDR GNB had additional resistance against carbapenems, with imipenem and/or meropenem as surrogate substance [1]. MDR GNB detection was performed by plating stools on ESBL and *Klebsiella pneumoniae* carbapenemase (KPC) chromagar selective media (Mast, Reinfeld, Germany). For identification and susceptibility testing of resistant colonies, matrix-assisted laser desorption ionization (MALDI), Biotyper mass spectrometry (Bruker Daltonics, Bremen, Germany) and VITEK 2 (BioMerieux, Nürtingen, Germany) with Clinical and Laboratory Standards Institute (CLSI) interpretative standards were used [2,3]. ESBL phenotypes were confirmed using double disk synergy testing [4]. Decreased carbapenem susceptibility in *Enterobacteriaceae* was confirmed using Etest and carbapenemase detection was performed using a modified Hodge test [2].

Laboratory findings

Of a total of 119 individuals screened, ESBL-producing *Enterobacteriaceae* were detected in 42 (35%), including nine 3MDR GNB (8% of total screened). No 4MDR GNB was observed. Six (5%) of the 119 refugees reported having a prior antimicrobial therapy, and two (2%) reported a hospital admission during the preceding six months. Among the 42 with ESBL-producing bacteria, two had received prior antimicrobial treatment in the past six months and one had been hospitalised, whereas one of nine refugees colonised with 3MDR GNB reported an antimicrobial treatment, with no hospital stay in this group.

In total, 37 *Escherichia coli* (thereof 9 3MDR GNB) and five *K. pneumoniae* (non-3MDR GNB) were detected. Whereas ESBL-producing bacteria were detected in persons from nearly all of the countries of origin (except Iraq, Iran, Libya, Senegal), 3MDR GNB were found only

Table

Detection of extended spectrum beta-lactamase-producing *Enterobacteriaceae* and thereof multidrug-resistant Gram-negatives in unaccompanied refugee minors arriving in Frankfurt am Main, Germany, 12 October–6 November 2015 (n=119)

Country of origin	Number of persons tested	Number of individuals with ESBL-producing *Enterobacteriaceae*	Number of individuals with 3MDR GNB	Number of individuals with 4MDR GNB
Afghanistan	80	34[a]	7[b]	0
Eritrea	9	1	0	0
Somalia	7	2	1	0
Syria	7	3	0	0
Ethiopia	5	0	0	0
Iraq	4	0	0	0
Pakistan	3	1	1	0
Yemen	1	1	0	0
Other[c]	3	0	0	0
Total n (%)	**119**	**42 (35)**	**9 (8)**	**0 (0)**

ESBL: extended spectrum beta-lactamase; GNB: Gram-negative bacteria; MDR: multidrug-resistant.

[a] 29 *Escherichia coli*, 5 *Klebsiella pneumoniae*.

[b] 7 *E. coli*.

[c] Iran, Libya, Senegal.

in persons coming from Afghanistan, Pakistan, and Somalia (Table).

Discussion and conclusion

There is a dramatic influx of refugees to the European Union under way, with more than 600,000 applications for asylum during the first nine months of 2015 in Germany [5]. Many refugees are coming from countries with high prevalence of multidrug-resistant organisms (MDRO) in hospital and community settings, such as Afghanistan, the Near and Middle East and the North African countries [6]. Additionally, many of the refugees coming from the Near and Middle East have been travelling through countries with high prevalences of MDROs, such as Turkey or Greece [7-9], whereas those coming from Africa are travelling via the 'West-Route', i.e. via Libya and Italy. A current European Centre for Disease Prevention and Control (ECDC) report showed high prevalence of carbapenem resistance and other antimicrobial resistances in Turkey and Greece in the period from 2013 to 2014 [7-9]. On that account, the Robert Koch Institute, Germany, has recommended in October 2015, screening refugees for MDRO on hospital admission in Germany [10]. Preliminary work on screening of 143 refugees admitted to the University Clinic of Frankfurt, Germany has been undertaken [11], however no data have so far been available on MDR GNB prevalences in young healthy refugees.

Here we report the first data on prevalence of 3MDR GNB and ESBL-producing bacteria in unaccompanied refugee minors arriving in the country. ESBL-producing *Enterobacteriaceae* were found in 35% of the individuals included in our study and among these, 3MDR GNB were found in 8% of the total individuals screened. To compare with estimates for the German population, between 2009 and 2012, Valenza et al. had tested 3,344 persons residing in the southern part of Germany, with 6.3% exhibiting ESBL, including 3MDR GNB, which occurred in 1.8% of those tested [12]. The MDR GNB prevalence in the young refugees exceeded these values by four- to fivefold.

In the Rhine-Main region, Germany, in the 2012 to 2015 period, prevalences for ESBL-producing bacteria and for 3MDR GNB were respectively 7.5% and 3.8% in dialysis outpatients, and 7.7% and 3.8% in patients of rehabilitation clinics, i.e. only slightly exceeding the MDR GNB prevalences in the general population [13,14]. Patients depending on ambulatory care or residing in elderly care homes however, were more frequently colonised with bacteria having an ESBL phenotype or 3MDR GNB, with, in outpatients, 14.4% ESBL-producing bacteria and 7.6% 3MDR GNB, and in nursing home residents, 17.8 to 26.7% ESBL-producing bacteria and 12.3 to 21.3% 3MDR GNB [15-17]. Hence, colonisation with ESBL-producing *Enterobacteriaceae* in the unaccompanied refugee minors was also exceeding rates of bacteria with ESBL in all other patient groups tested in the Rhine-Main region recently, and 3MDR GNB colonisation rates were exceeding those in haemodialysis and rehabilitation patients with regular contact to the German medical system as well.

Prevalence of ESBL-producing *Enterobacteriaceae* in unaccompanied minors was higher than prevalence rates of patients transferred from hospitals abroad to the University Hospital Zurich, Switzerland, from 1 January 2009 to 30 September 2011: of them, 13.9% were found with ESBL-producing bacteria, while 3MDR GNB prevalence was comparable (7.6% refugees compared with 8.1% patients transferred to the university

clinic) [18]. However, prevalence of 3MDR GNB in the unaccompanied minors was still low compared with the data obtained by Reinheimer et al., who tested 143 refugee patients on admission to the University Hospital Frankfurt, Frankfurt/Main, Germany from June to December 2015 and compared the results to data on 1,489 non-refugee patients screened on admission as well. Prevalence of MDR GNB (ESBL-producing bacteria, 3MDR GNB, and 4MDR GNB) in refugee patients was 60.8%, and thus exceeding the prevalence of MDR GNB in non-refugees (16.7%) fourfold [11]. Our sample, however, encompassed only young people, most of them healthy, having fled on their own without their parents or families. This might explain the lower prevalence of MDR GNB in this group compared with that of the refugees on hospital admission. Nevertheless, both data support the demand for surveillance in refugees, not only for communicable disease [19] but also for MDRO [10].

Conflict of interest

None declared.

Authors' contributions

Prof. Heudorf and Dr. Niels Kleinkauf wrote and finalised the paper, Dr. Krackhardt and Mrs. Karathana organised the study. Dr. Zinn was responsible for the analytical results.

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Multidrug-resistant organisms detected in refugee patients admitted to a University Hospital, Germany June–December 2015

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Multidrug-resistant Gram-negative bacteria (MDR GNB) were found to colonise 60.8% (95% confidence interval: 52.3–68.9) of 143 refugee patients mainly from Syria (47), Afghanistan (29), and Somalia (14) admitted to the University Hospital Frankfurt, Germany, between June and December 2015. This percentage exceeds the prevalence of MDR GNB in resident patients four–fold. Healthcare personnel should be aware of this and the need to implement or adapt adequate infection control measures.

Current data provided by Federal Agency of Migration and Refugees, Germany, indicate a dramatic increase in migration, with most people arriving from Syria, Albania, Afghanistan and Iraq [1]. These countries are known as countries with high prevalence for multidrug-resistant Gram-negative bacteria (MDR GNB) (*Enterobacteriaceae*, *Acinetobacter baumannii*) and for meticillin-resistant *Staphylococcus aureus* (MRSA) [2-5]. People from these countries are thus at higher risk of being colonised with such pathogens and adequate infection prevention measures need to be taken to prevent spread in healthcare settings in the countries where they seek refuge. Systematic studies regarding prevalence of multidrug-resistant organisms (MDRO) in refugees are not yet available in the scientific literature. In order to fill this gap, we investigated the prevalence of MDR GNB and MRSA in patients admitted from refugee (REF) accommodations to the University Hospital Frankfurt am Main (UHF), Germany between June and December 2015 and compared it with prevalence in resident patients.

Investigation of prevalence of multidrug-resistant organisms in refugee and resident patients

At UHF, all patients admitted from hospitals in countries with high prevalence or from refugee accommodations are pre-emptively isolated and screened for MDRO on the day of admission. The same algorithm is applied to resident patients with previous treatment in hospitals in countries with high prevalence for MDRO and all patients admitted to intensive/intermediate care units (ICUs/IMCs).

During the study period, REF patients were identified on admission and screened for MDRO by rectal swabs for MDR GNB and nasal swabs for MRSA. MDR GNB screening was undertaken for extended spectrum beta–lactamase (ESBL)-producing *Enterobacteriaceae*, and *Enterobacteriaceae and Acinetobacter baumannii* resistant to piperacillin, any third generation cephalosporin, and fluoroquinolones +/– carbapenems.

Patients admitted to ICU/IMC within the same period were included as comparison group since these patients are routinely screened for MDRO. This group reflects the demographic and epidemiological characteristics of the resident population not admitted from a refugee accommodation (NREF).

Patients admitted from hospitals in countries with high prevalence for MDRO were excluded from the study.

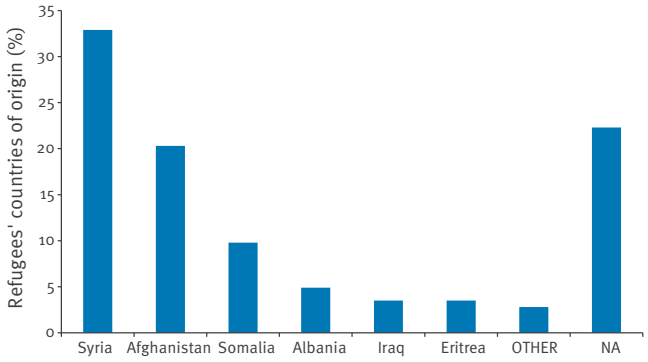
MDRO screening was done in accordance to German infection protection law and the infection control strategy at UHF. Ethical approval was given by Ethics Committee of the UHF.

Laboratory identification of multidrug-resistant bacteria

For detection of MDR GNB, rectal swabs were collected using Amies collection and transport medium (Hain Lifescience, Germany) and streaked onto CHROMagar™ ESBL plates (Mast Diagnostica, Paris, France). Identification of MDR GNB species was done by matrix-assisted laser desorption/ionisation time-of-flight (MALDI–TOF) mass spectrometry. Antimicrobial susceptibility testing was performed using VITEK2 (bioMérieux, Nürtingen, Germany) according to Clinical

Figure

Countries of origin of refugees screened for multidrug-resistant Gram-negative bacteria and meticillin resistant *Staphylococcus aureus*, Frankfurt, Germany June–October 2015 (n=143)



OTHER are Pakistan, Moldavia, Lebanon, or Georgia.
NA: information about country of origin not available.

and Laboratory Standards Institute (CLSI) guidelines [Version M100-S25, 2015] and antibiotic gradient tests (bioMérieux).

Carbapenemase-encoding genes were detected via PCR analysis and subsequent sequencing from carbapenem–resistant *Enterobacteriaceae* including the *bla* genes for carbapenemases NDM, VIM, IMP, OXA–48, and KPC and OXA–23, OXA–24, OXA–51, and OXA–58 for *A. baumannii* [6,7]. Carbapenem–resistant *A. baumannii* isolates were assigned to international clusters by repetitive element sequence based–PCR (DiversiLab®, bioMérieux) [7,8].

For the detection of MRSA, moistened nasal swabs were inoculated on Brilliance MRSA Agar (Oxoid, Wesel, Germany) and identification and antimicrobial susceptibility testing were performed as described above. Clonal identity was determined by *staphylococcal protein A (spa)* typing using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany) [9].

We used the biostatistical data file from University Münster, Germany for statistical analyses [10]. Confidence intervals (CI) were calculated based on binomial distribution and p values (2–tailed) of p≤0.05 were considered statistically significant.

Prevalence in refugee compared with resident patients

Multidrug-resistant Gram-negative bacteria

Between June and December 2015, a total of 143 REF and 1,489 NREF were screened for MDR GNB on day of admission. The average age in the two groups was 21.7 years (range 1-65, SD 16.4) for REF and 64 years (11-94; SD 16.2) for NREF, respectively. Of REF patients 84.6% were male and of NREF 69.4%. REFs' countries of origin were not available for evaluation in 32 cases (22.4%) due to lacking records in patients' files (Figure).

Of the 143 REF samples 60.8% (for 95% CI and patient numbers see Table) were positive for any MDR GNB, significantly exceeding the rate in NREF (16.7%) (p≤0.05).

Among all MDR GNB species, ESBL-producing *Escherichia coli* were detected with higher prevalence in REF than in NREF (23.8 vs 4.9%). Prevalence of ESBL-producing *Klebsiella pneumoniae* exceeded the prevalence in NREF (4.2 vs 0.8%). Prevalence of ESBL-producing *E. coli* with additional resistance to fluoroquinolones was higher in REF than that in NREF (26.6 vs 6.9%) and ESBL-producing *K. pneumoniae* with additional resistance to fluoroquinolones were found in a higher proportion in REF compared with NREF (4.2 vs 1.9%). All results were statistically significant.

One REF carried three MDR GNB organisms: ESBL-producing *E. coli* with additional resistance to fluoroquinolones, ESBL-producing *K. pneumoniae* and carbapenem–resistant *A. baumannii*. Two REF were positive for two MDR GNB organisms each; one carried *E. coli* with additional resistance to fluoroquinolones and ESBL-producing *K. pneumoniae* with additional resistance to fluoroquinolones, one carried ESBL-resistant *E. coli* and ESBL-resistant *E. coli* with additional resistance to fluoroquinolones.

In REF, three carbapenem–resistant isolates were detected: one carbapenem–resistant *K. pneumoniae*, expressing VIM–1 which is frequently detected in southern Europe [11], and two carbapenem–resistant *A. baumannii* (1.4%), harbouring carbapenemases OXA–23 and OXA–24, respectively. Strain typing revealed that these isolates belong to international cluster II which is disseminated worldwide and is frequently associated with OXA carbapenemases [7,8].

In NREF, 16 (1.1%) carbapenem–resistant isolates were detected. Two of these were identified as *K. pneumoniae* expressing OXA–48, one expressed OXA–48 *and* NDM–1, and in seven isolates no specific carbapenemase gene was detected. Carbapenem–resistant *E. coli* isolates were detected in three cases with all of them expressing OXA–48. Carbapenem–resistant *Enterobacter cloacae* strains were detected in two cases with one expressing OXA–48 and one without specific carbapenemase gene detection. Carbapenem–resistant *A. baumannii* was detected in one case; no other carbapenemase than species–specific OXA–51 was detectable.

Meticillin-resistant *Staphylococcus aureus*

Screening for MRSA was performed for 143 REF and 1,170 NREF. In REF, eight MRSA isolates (5.6%; 95%CI: 2.5–10.7) were detected, which significantly exceeded the prevalence in NREF where 14 isolates were positive (1.2%; 95%CI: 0.6–2.0). *Spa* types in REF (n=1 not

Table

Distribution of multidrug-resistant Gram-negative bacteria in refugee compared with resident patients, Frankfurt, Germany July–December 2015

	Patients admitted from refugee accommodations (REF)		Resident patients not admitted from refugee accommodation (NREF)	
Total number of patients	143		1,489	
	% (n)	95% CI	% (n)	95% CI
Positive for MDR GNB[a]	60.8 (87)	52.3–68.9	16.7 (250)	14.9–18.8
Escherichia coli ESBL[b]	23.8 (34)	17.1–31.5	4.9 (73)	3.8–6.1
Escherichia coli ESBL/FQ[b]	26.6 (38)	19.5–34.6	6.9 (104)	5.7–8.4
Escherichia coli Carba	0.0 (0)	0.0–2.5	0.2 (3)	0.04–0.5
	Carbapenemases None		Carbapenemases OXA–48 (3)	
Klebsiella pneumoniae ESBL[b]	4.2 (6)	1.5–8.9	0.8 (12)	0.4–1.4
Klebsiella pneumoniae ESBL/FQ[b]	4.2 (6)	1.5–8.9	1.9 (28)	1.2–2.7
Klebsiella pneumoniae Carba	0.7 (1)	0.02–3.8	0.6 (10)	0.3–1.2
	Carbapenemases VIM–1(1)		Carbapenemases OXA–48 (2) OXA–48 and NDM–1 (1) No carbapenemase detected (7)	
Enterobacter cloacae Ceph/FQ	0.0 (0)	N.a.	0.5 (8)	0.2–1.1
Enterobacter cloacae Carba	0.0 (0)	N.a.	0.1 (2)	0.02–0.5
	Carbapenemases None		Carbapenemases OXA–48 (1) No carbapenemase detected (1)	
Enterobacter aerogenes Ceph/FQ	0.0 (0)	N.a	0.2 (3)	0.04–0.5
Citrobacter freundii Ceph/FQ	0.0 (0)	N.a.	0.3 (4)	0.07–0.7
Klebsiella oxytoca Ceph/FQ	0.0 (0)	N.a.	0.06 (1)	0.002–0.3
Acinetobacter baumannii Ceph/FQ	0.0 (0)	N.a	0.06 (1)	0.002–0.3
Acinetobacter baumannii Carba	1.4 (2)	0.2–0.5	0.06 (1)	0.002–0.3
	Carbapenemases[c] OXA–23 (1) OXA–24 (1)		Carbapenemases[c] No carbapenemase detected (1)	

Carba: resistance to carbapenems; ESBL: extended beta-spectrum lactamase-producing; FQ: resistance to fluoroquinolones; MDR GNB: multidrug-resistant Gram-negative bacteria.

[a] Organism and resistance pattern.

[b] Values in neighboring columns vary statistically significantly (p≤0.05).

[c] Other than species-specific OXA–51.

determined) were t223 (n=2), t386, t790, t852, t1532, and t10343 (n=1 each) and *spa* types in NREF were t003 (n=8), t032 (n=2), t008, t012, t034, and t127 (n=1 each).

Discussion and conclusion

Having protocols in place to control infectious disease transmission in hospitals is good practice to ensure patient safety and should be the focus of preventive efforts. While travel to countries with high prevalence, medical tourism, or contact to local healthcare in such countries have been identified as contributing to the transmission and geographical spread of MDRO worldwide [2,3,5,12], considerations on limiting the spread of MDRO should be expanded to people coming from or passing through countries with high prevalence and seeking refuge, for example in Germany. In terms of hospital infection control strategies, this population may represent a yet unidentified risk in countries with low endemicity.

Our study has revealed a strong link between the status REF and carriage of MDR GNB and MRSA exceeding that of NREF, which might constitute an inherent risk of introduction in another countries healthcare system, such as previously observed in Turkey, where NDM-1-producing *A. baumannii* strains were most likely imported from neighbouring Syria [13]. MRSA *spa* types detected in REF were less common in Germany whereas NREF colonised with MRSA were found to harbour *spa* types known to be most frequent in Germany [14].

According to the guidelines by the infection prevention commission (KRINKO) at the Robert–Koch Institute, Berlin, Germany, isolation is recommended for all hospitalised patients for the following patient groups: patients with MRSA [15] and patients with

carbapenem–resistant MDR GNB [16]. Additionally, in hospital units at high risk for nosocomial transmissions such as ICUs and IMCs, isolation of patients with MDR GNB resistant to cephalosporins and fluoroquinolones and/or MDR GNB with resistance to carbapenems is recommended. In our study the latter would apply to 32.9% of REF versus 10.9% of NREF patients. Taking all KRINKO guidelines (including MRSA) into account, a total of 38.5% of REF and 12.1% of NREF would qualify for isolation. The demonstrated high MDRO prevalence in our REF study population calls for intensive efforts in hospital hygiene to guarantee best medical practice for every single patient.

While it is hardly possible to predict any evolutionary or geographical success of these MDRO strains in German hospitals or elsewhere in Europe, our small-scale investigation provides some evidence for the importance of screening and aligned hygiene measures in patients admitted from refugee accommodations. It is hard to estimate the whether the REF population in the UHF setting is representative of the overall refugee population in Germany. However, a study also conducted in Frankfurt am Main but in unaccompanied refugee minors (‹18 years- old) in refugee centres also found a high prevalence of ESBL-producing *Enterobacteriaceae* (35%) even though lower than that in our study [17].

One limitation of our study may be the pre–selection of NREF. As mentioned above, this group consists of patients admitted to ICUs and IMCs: considering that they are critically ill, possible prior antibiotic treatment might have resulted in increased prevalence of MDRO in the NREF group used for comparison. This might lead to an overestimation of the MDRO prevalence in our NREF group and thus to an underestimation of the difference in MDRO prevalences between REF and NREF patients.

Unfortunately, information regarding refugees' itineraries were not available due to missing records in the patient files and the language barrier on hospital admission. Such information, however, would help to better understand the origin and transmission routes of MDROs. For example refugees might have acquired a particular MDRO during their transit through a country with high prevalence. Future investigations on clonal relatedness and comparisons with major endemic clones should allow to analyse the spread of MDRO in connection with the movements of refugees.

At UHF, all REF are screened for MDRO and pre–emptively isolated on the day of admission. In case of negative screening results, REF are released from isolation. In case of positive MDR GNB and/or MRSA results, REF remain in isolation during their entire stay. Since June 2015, this procedure has been implemented and guarantee best medical practice for every individual patient at UHF, independently of their country of origin.

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Authors declare that this report is exclusively based on epidemiological findings and is not influenced by any political opinion. Authors furthermore confirm that all patients are treated equally and in conditions of best medical care at UHF, regardless of their origin. Stephan Göttig and Volkhard A. J. Kempf were supported by a grant from the Deutsche Forschungsgemeinschaft (DFG–research unit 2251).

Conflicts of interest

The authors declare that they have no competing interests.

Authors' contributions

CR: compiling and interpretation of the data, manuscript writing. VK: interpretation of the data, manuscript writing. SG, MH, TW: generation and interpretation of the data, manuscript writing. FOR: manuscript review. CB: compiling and interpretation of the data, manuscript writing. All authors read and approved the final manuscript.

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Respiratory diphtheria in an asylum seeker from Afghanistan arriving to Finland via Sweden, December 2015

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In December 2015, an asylum seeker originating from Afghanistan was diagnosed with respiratory diphtheria in Finland. He arrived in Finland from Sweden where he had already been clinically suspected and tested for diphtheria. *Corynebacterium diphtheriae* was confirmed in Sweden and shown to be genotypically and phenotypically toxigenic. The event highlights the importance of early case detection, rapid communication within the country and internationally as well as preparedness plans of diphtheria antitoxin availability.

An asylum seeker from Afghanistan was diagnosed with respiratory diphtheria in Finland in December 2015. He arrived in Finland from Sweden where he was already symptomatic with fever and respiratory symptoms and had been suspected for diphtheria. In this report we present details of this case investigation.

Case description

On 26 November 2015, an adolescent male originating from Afghanistan arrived in a transit accommodation centre designated for asylum seekers in Stockholm, Sweden. Prior to this, he had travelled from Afghanistan to Pakistan, then through Iran, Turkey, Greece, Serbia and Germany to Sweden (timeline of events presented in the Figure).

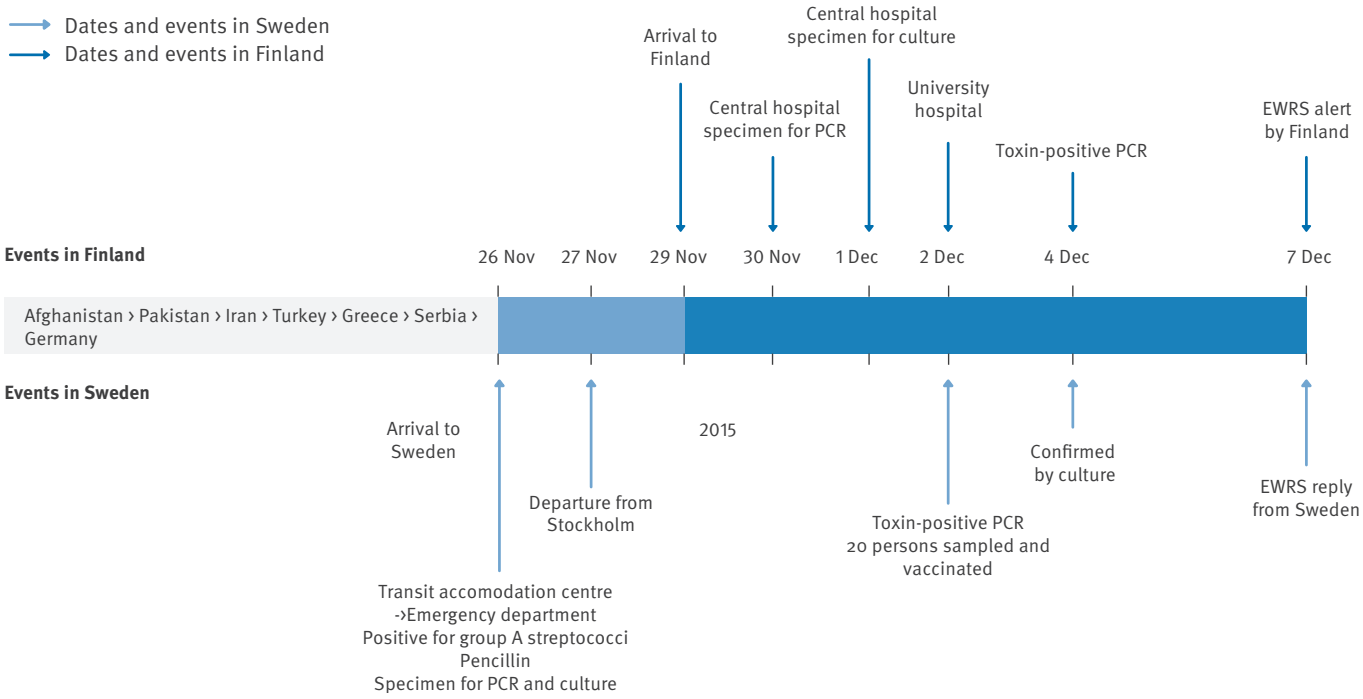
The case showed some symptoms (pharyngitis and fever) while in Germany (further details on duration of stay and locations not known) and upon arrival in Stockholm, he presented with intense pharyngitis, fever and general malaise and was rapidly escorted by the transit centre staff to a nearby emergency department where he stayed overnight. The preliminary diagnosis was tonsillitis positive for group A streptococcus (QuickVue Dipstick Strep A Test, TK Diagnostic, Oxford, United Kingdom). Oral penicillin was prescribed as treatment. As diphtheria was among the differential diagnoses, the attending physician also requested a specimen for throat culture. According to the patient, he had never received any vaccinations. The patient was discharged from the hospital and returned to the asylum accommodation centre. On the following day, 27 November, he left the centre in Stockholm with two other asylum seekers and did not take the prescribed antibiotics along. Their travel destination was not clear at the time but according to the anecdotal evidence, Switzerland, Germany and Finland were mentioned.

On 29 November the asylum seeker arrived in Finland crossing the border in Northern Finland. He had travelled from Stockholm to the north of Sweden by an overnight train from where he moved on to Finland by bus.

When arriving in Finland, he was still unwell and was immediately transferred to a regional central hospital where he was put in isolation as a precautionary measure. Streptococcal tonsillitis and mononucleosis were initially suspected. Subsequently diphtheria was also considered and specimen for PCR amplification (toxin gene) was taken on 30 November and for *Corynebacterium diphtheria* culture on 1 December. On 2 December, the patient was transferred to the nearest university hospital for further treatment.

Figure 1

Timeline of events for the diphtheria case, in an asylum seeker from Afghanistan arriving to Finland via Sweden, November to December 2015



EWRS: Early Warning Response System.

While in hospital, the patient presented with malaise, sore throat and low-grade fever. Membranes in tonsils and soft palate were noticed. Carditis was evident with chest pain, ST-T wave changes in electrocardiograph and considerably elevated cardiac troponin I concentration (70 µg/l, norm: ≤0.04 µg/l). Treatment with intravenous cefuroxime and oral roxithromycin was introduced on 29 November and 20,000 IU diphtheria antitoxin (DAT, Institute of Immunology, Croatia) was given on 2 and 4 December. On 11 December, the patient was assessed to be cured and discharged.

Laboratory findings in Sweden and Finland

On 4 December, the National Institute for Health and Welfare in Finland was alerted by a clinical laboratory the throat swab was positive for *C. diphtheria* toxin gene in the PCR. Isolation of the bacteria was attempted but was not successful. Prior penicillin therapy in Sweden, although incomplete, may have impeded a positive culture in this case.

In Sweden, on 1 December, the local county medical officer in Stockholm was alerted by the local clinical microbiological laboratory of a suspicion of diphtheria based on a positive Maldi-TOF finding of *C. diphtheriae*. The Public Health Agency of Sweden received the strain on the same day and informed on 2 December the local county medical officer of the presence of the diphtheria toxin gene (as analysed by PCR) in the isolate. The strain was Elek-positive confirming suspected toxin-production on 3 December and subsequently biochemical species determination confirmed the isolate to be *C. diphtheriae* and sub species non-gravis [personal communication, Karin Tegmark-Wisell, Public Health Agency of Sweden, December 2015].

On 7 December, Finland posted a message in the European Early Warning and Response System (EWRS) describing the event. Sweden replied the same day disclosing the details about patient's travel itinerary, symptoms and laboratory findings before his arrival in Finland.

Specific and general public health measures in Sweden and Finland

The local public health authorities in Stockholm visited the transit accommodation centre on 3 and 4 December after the suspicion of diphtheria was notified (toxin positivity by PCR). The team examined the premises, obtained throat swabs and vaccinated nearly 20 adolescents (mostly from Afghanistan) out of the ca 30 persons who could have resided in the same room with the case patient. The other persons had already left the centre. None of the surveyed persons were ill and all cultures were negative.

In Finland, the patient was wearing a mask on the bus upon arrival (given by the police at the border) and was rapidly transferred to an isolation room after he crossed the border. Thus, the number of potentially exposed persons was limited. The contact details and

whereabouts of two travel companions from Stockholm were investigated but they could not be contacted.

The National Institute for Health and Welfare in Finland has recommended free of charge vaccination against diphtheria, tetanus (dT), polio (IPV), measles-mumps-rubella (MMR) and influenza as a priority for all adult asylum seekers and refugees with an unknown or incomplete history of vaccination, or lack of protection gained through previous MMR infections. The children are offered the normal national immunisation programme, modified and speeded up as appropriate. As an occupational health measure, the vaccination status of all personnel working at the accommodation centres is checked and dT, MMR, influenza and hepatitis A vaccines are offered when necessary.

Background

Diphtheria is an acute bacterial disease primarily involving the mucous membrane of the upper respiratory tract, skin or seldom other mucous membranes [1,2]. The infection spreads from person to person through respiratory droplets, direct contact with respiratory secretions or from skin lesions. The incubation period is usually two to five days, sometimes up to 10 days. The toxin-mediated disease caused by *C. diphtheria* can be prevented by vaccination, which protects against the effects of exotoxin produced by the bacteria [3].

This patient is the first diphtheria case diagnosed in Finland since 2001. Diphtheria is still endemic or epidemic in many regions of the world, including origin countries for current asylum seekers in Europe [3,4].

Discussion

Except for the present case, no cases of respiratory diphtheria were reported among asylum seekers and refugees since the current European refugee crisis started in 2015. Cases of cutaneous diphtheria were recently reported in refugees from Denmark, Germany and Sweden [5]. These events highlight the possibility of diphtheria among these vulnerable groups and underpins the need of national guidance regarding laboratory capacity for confirming diphtheria infections, raising awareness among clinicians, and early recognition and prompt implementation of prevention and control measures by the public health authorities. Since the exact timeline of the patient´s travel itinerary was unclear, the source of infection and whether it was acquired within or outside the European Union (EU) remains unknown.

Our case further highlights that refugees arriving in the EU are likely not to be fully vaccinated against an array of vaccine-preventable diseases. They are consequently at greater risk of communicable diseases such as diphtheria or measles. In Finland, the public health authorities recommend prompt vaccination of the newly arriving asylum seekers and refugees newcomers, ideally within two weeks of arrival to the country. Due to logistics and resource constrains this target has not yet been achieved in most of the newly opened accommodation centres. As also shown in our case, another challenge to the immunisation activities is the mobility of refugees, not only cross-border but also within the country. The accommodation centre may change several times during the asylum process, which addresses the need for reliable online registry with immunisation data available for all healthcare staff providing services for the refugee population.

In general, refugees do not pose a threat to general population in Europe with respect to communicable diseases and they are themselves most vulnerable [6]. Since 1995, the vaccination coverage of Finnish children against diphtheria has been 94% to 99% [7]. However, there are no official estimates of the decennial dT booster vaccination coverage among the adults and elderly persons. It is likely that at least a part of the adult population will be at risk for diphtheria and other vaccine preventable diseases. This emphasises the need for a vaccination policy that guarantees lifelong protection against significant infectious diseases threats.

The patient was treated with DAT which was available at the adjacent university hospital. The batch of DAT used had expired in March 2014 but was previously evaluated by the Finnish Medical Agency to be still safe and efficacious for use in emergency situation (personal communication, Pertti Sormunen, Director of Pharmaceutical Wholesale, National Institute for Health and Welfare, December 2015). The potency of the DAT was tested according to the European Pharmacopoeia monograph for Diphtheria Antitoxin (intradermal challenge in guinea pigs) by an authorised laboratory in February 2014. The diphtheria potency was still more than three times over the minimum level of 1,000 IU/ml. No abnormal toxicity was detected.

The rapid administration of DAT is crucial for a successful treatment effect [3]. However, a large number of EU Member States do not have stockpiles and many countries have difficulties in having rapid access to DAT, a problem highlighted in a recent case of diphtheria in Spain [8]. Many countries ceased manufacturing DAT following the significant decline in incidence of the diphtheria after the mass vaccination campaigns in Europe [4]. The lack of DAT availability in the EU is worrying and needs urgent attention in the light of this event. Possibilities for joint procurement of DAT in the EU/European Economic Area (EEA) should be assessed as suggested by the European Centre for Disease Prevention and Control (ECDC) [8].

Prompt communication between clinicians, microbiology laboratories and public health authorities is crucial in the effective public health response. The patient was found PCR-positive for toxin gene on 2 December in Sweden (but yet not confirmed by culture) two days before the clinical laboratory in Finland alerted

the national public health institute about the positive PCR finding for the toxin gene. Swedish authorities were not fully aware of the travel route and thus not able to directly alert Finnish authorities before arrival in Finland. However, due to the apparent problems in DAT availability in the EU and the importance of rapid public health measures for treating and controlling diphtheria, we propose that any suspected or probable case among highly mobile migrant populations, is communicated through the EWRS as early as possible.

*Authors' correction

The name of Micael Widerström was corrected upon request of the authors on 1 February 2016.

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Conflict of interest

None declared.

Authors' contributions

JS was the officer on duty during the event, JS designed the study, coordinated the data collection and wrote the manuscript. TS contributed to the data collection and drafted the timeline figure. MW led the Swedish response and data collection. HK and UK were the treating physicians in Finland and provided the clinical details of the case. ET provided laboratory input, TP reviewed and the manuscript. MK reviewed the manuscript. MS reviewed the manuscript. OL designed the study, coordinated data collection and wrote the manuscript.

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Research article

In-season and out-of-season variation of rotavirus genotype distribution and age of infection across 12 European countries before the introduction of routine vaccination, 2007/08 to 2012/13

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The EuroRotaNet surveillance network has conducted rotavirus genotype surveillance since 2007 in 16 European countries. Using epidemiological and microbiological data from 39,786 genotyped rotavirus-positive specimens collected between September 2007 and August 2013, we assessed genotype distribution and age distribution of rotavirus gastroenteritis (RVGE) cases in and out of peak season in 12 countries which were yet to implement routine rotavirus vaccination. In multinomial multivariate logistic regression, adjusting for year, country and age, the odds of infection caused by genotype-constellation 2 DS-1-like stains (adjusted multinomial odds ratio (aM-OR) = 1.25; 95% confidence interval (CI): 1.13–1.37; p<0.001), mixed or untypable genotypes (aM-OR = 1.55; 95% CI: 1.40–1.72; p<0.001) and less common genotypes (aM-OR = 2.11; 95% CI:1.78–2.51; p<0.001) increased out of season relative to G1P[8]. Age varied significantly between seasons; the proportion of RVGE cases younger than 12 months in the United Kingdom increased from 34% in season to 39% out of season (aM-OR = 1.66; 95% CI: 1.20–2.30), and the proportion five years and older increased from 9% in season to 17% out of season (aM-OR = 2.53; 95% CI: 1.67–3.82). This study provides further understanding of the rotavirus ecology before vaccine introduction, which will help interpret epidemiological changes in countries introducing or expanding rotavirus vaccination programmes.

Introduction

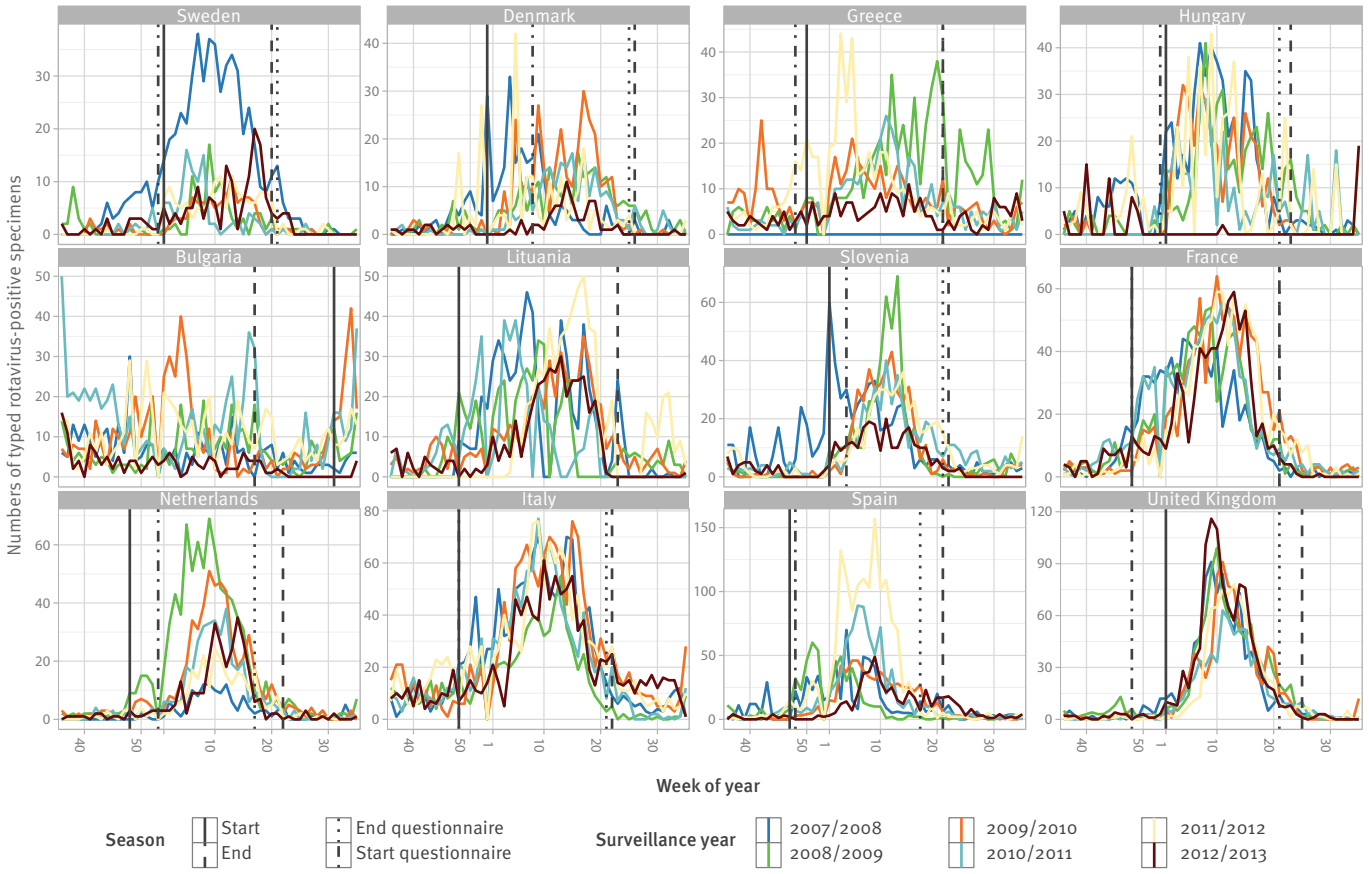
Rotavirus is the most common cause of acute gastroenteritis in children under five years of age, causing an estimated 450,000 deaths per year worldwide, with over 90% of deaths occurring in developing countries [1]. In high-income countries, rotavirus infections result in few deaths but still constitute a substantial healthcare burden and can cause severe morbidity [2,3]. There are eight groups of rotaviruses defined by the middle capsid antigen [4]; the majority of rotavirus gastroenteritis (RVGE) in humans is caused by group A rotaviruses.

Group A rotavirus genotypes are typically further classified into G and P types, based on sequence diversity of the genes encoding the outer viral proteins VP7 (glycoprotein) and VP4 (protease-sensitive protein), respectively [5]. Furthermore, whole genome sequencing has allowed rotavirus strains to be classified into genotype constellations based on a common genomic backbone in which the genotypes of nine of the 11 genes are conserved, while G and P types may vary. Human rotaviruses typically belong to the Wa-like or the DS-1-like genotype constellations [6].

Two oral vaccines, the two-dose monovalent vaccine (Rotarix, GlaxoSmithKline Biologicals, Belgium) and the three-dose pentavalent vaccine (RotaTeq, Merck, United States), have been introduced into a number

Figure 1

Number of rotavirus specimens typed per week by country and surveillance year, 12 European Union countries, September 2007–August 2013 (n = 39,786)



The season legend shows lines for the start and the end of the rotavirus season as defined by the median threshold method and reported through the questionnaire.

of countries worldwide since their licensure in 2006. Eight European Union countries have included rotavirus vaccines in their routine childhood immunisation schedules and several other countries make the vaccine available through state or private sector healthcare [7].

Monitoring the emergence of novel rotavirus genotypes and the potential for genotype replacement and genetic drift is an essential activity of surveillance. This has become more important since the introduction of rotavirus vaccination, as there was some early evidence in countries such as Australia, Brazil and Belgium that vaccination may have contributed to changes in the predominant genotypes, although these changes may also have been the result of natural variation [8,9]. The EuroRotaNet surveillance network, established in 2007 and including 16 countries, has been monitoring rotavirus genotype diversity and year-to-year genotype fluctuations across Europe for eight years [10,11]. Critically, the availability of substantial genotyping and epidemiological data for EuroRotaNet countries provides a baseline for genotype diversity and the epidemiology of RVGE cases before vaccine introduction. Therefore, while year-to-year differences in genotypes in Europe have been described previously [11,12], this paper reports in-peak season and out-of-season variation of rotavirus genotypes and age of infection for 12 European countries before the introduction of routine vaccination.

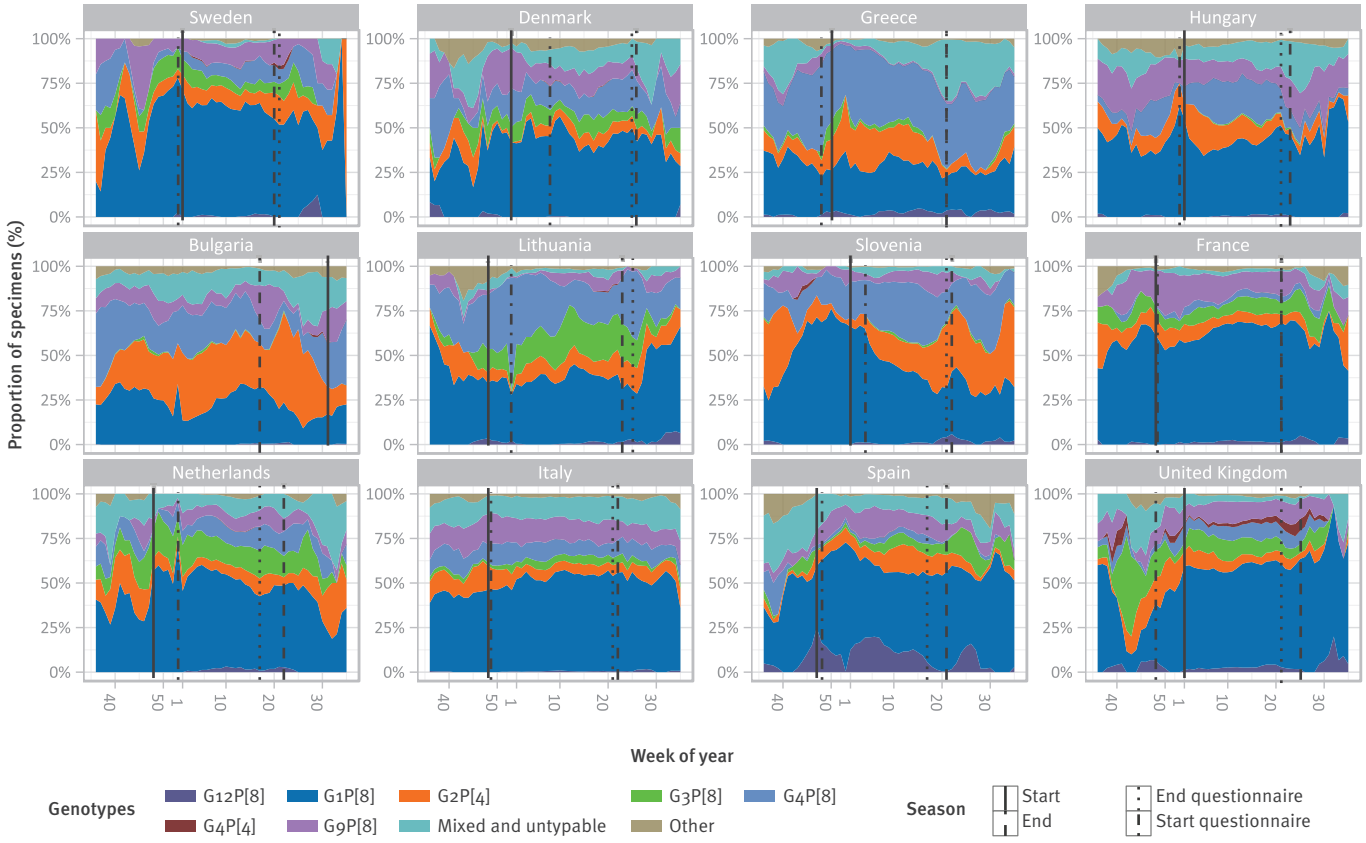
Methods

EuroRotaNet

The EuroRotaNet surveillance network was initiated in 2007 and includes 16 countries: Austria, Belgium, Bulgaria, Denmark, Finland, France, Germany, Greece, Hungary, Italy, Lithuania, the Netherlands, Slovenia, Spain, Sweden and the United Kingdom (UK). Data from typed rotavirus-positive faecal specimens is linked to case epidemiological information by participating laboratories and uploaded to a secure web-accessible EuroRotaNet database. The data contained in the EuroRotaNet dataset has been described previously [10,11].

Figure 2

Number of rotavirus specimens collected in season and out of season, by country, 12 European Union countries, September 2007–August 2013 (n = 39,786)



Case numbers were smoothed using a four-week moving average before conversion to proportions. The season legend shows vertical lines for the start and end of the rotavirus season, defined, respectively, by the median threshold method and through the questionnaire.

Study area

Twelve countries from EuroRotaNet were included in the study. Data from Austria, Belgium, Finland and Germany were excluded from the analysis because rotavirus vaccination was either routine or widespread (›35%) in these countries during the study period [13].

Samples

Study samples included rotavirus-positive faecal samples from mostly sporadic gastroenteritis cases; if associated with outbreaks, only a single sample per outbreak was submitted for routine diagnostic testing at sentinel participating EuroRotaNet laboratories and typed using standardised G and P typing methods [12,14]. Diagnostic testing protocols varied between countries [12,14].

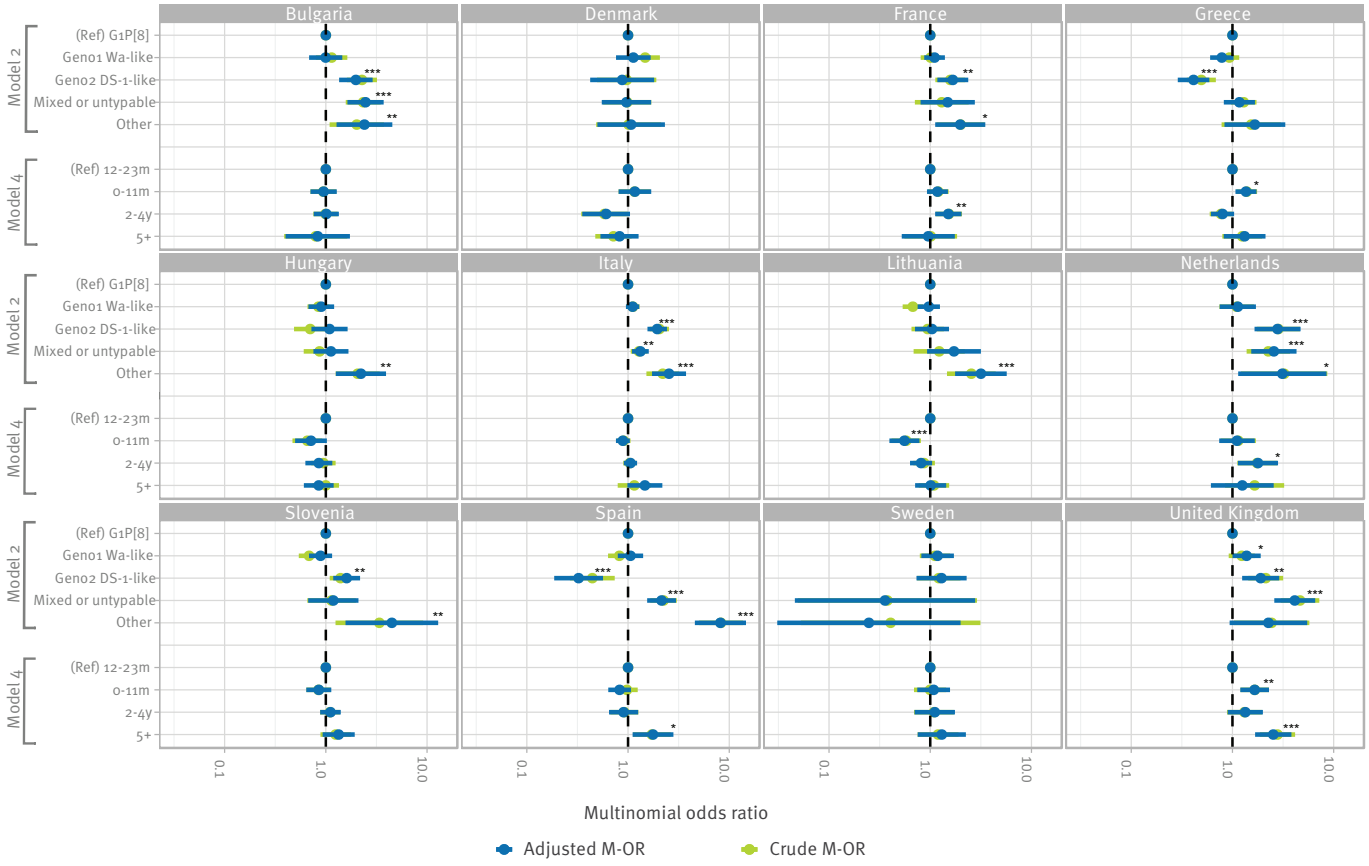
Data and survey

Details on case age, sex and country, specimen collection date and rotavirus genotyping results for a total of six rotavirus seasons spanning September 2007 to August 2013 were included in this study. Greece joined EuroRotaNet in 2008; therefore, for Greece only five rotavirus seasons were included in the analysis, spanning September 2008 to August 2013.

Data for each of the 12 countries were pooled for the study period. Age groups of cases (0–11 months, 12–23 months, 2–4 years and ≥ 5 years) were constructed using date of birth and date of specimen collection. Genotypes were categorised as 'G1P[8]', 'genotype-constellation 1 (Wa-like: G3P[8], G4P[8], G9P[8] and G12 P[8])', 'genotype-constellation 2 (DS-1-like: G2P[4] and G8P[4])', 'mixed and untypable', and less common genotypes were combined under the category 'other'. Although G1P[8] is considered part of genotype-constellation 1 (Wa-like), we grouped it separately because of its high prevalence across Europe [12]. A derived binary variable was constructed to denote weeks within a country's peak season and non-peak rotavirus seasons, and was developed by pooling country-specific weekly specimen frequencies over the study period to calculate the overall median weekly specimen frequency. We used the country-specific median value as a threshold for defining the start and end of the peak rotavirus season over the study period. Consequently, a weekly specimen frequency greater than or equal to the median identified weeks as in-season and a weekly frequency less than the median identified weeks as out-of-season. A consecutive period of two weeks

Figure 3

Country-stratified crude and adjusted multinomial odds ratios for genotypes occurring out of season vs in season (model 2; n = 39,786) and for age group (model 4; n = 39,007), 12 European Union countries, September 2007–August 2013



Multinomial odds ratios (M-OR) for out-of-season occurrence were estimated with multinomial logistic regression with the outcome variable genotype (G1P[8] as the reference group) (model 2) or age group (12–23-month-olds as the reference group) (model 4). Models were additionally adjusted for surveillance year (September–August) and either age group or genotype.

* p<0.05, ** p<0.01, ***p<0.001.

Blue line: adjusted M-OR; green line: crude M-OR; Geno1 Wa-like: genotype-constellation 1 Wa-like; Geno2 DS-1-like: genotype-constellation 1 DS-1-like.

above or below the threshold was required to identify the beginning and end of a season to ensure season identification was robust to stochastic fluctuations.

To identify additional detail on country-specific in-season and out-of-season testing practices, we constructed a brief semi-structured questionnaire using SelectSurvey.Net [15]. The questionnaire was distributed to EuroRotaNet collaborators by email in July 2014. The questionnaire included questions on duration of rotavirus season within the country, typical diagnostic testing practices, identification of changes to testing practices during the study period (including dates of any changes), positivity rate and proportion of positive samples typed. The questionnaire also asked for details on any age restrictions to testing or other algorithms that may have influenced testing and whether these may have changed between rotavirus seasons.

Statistical analysis

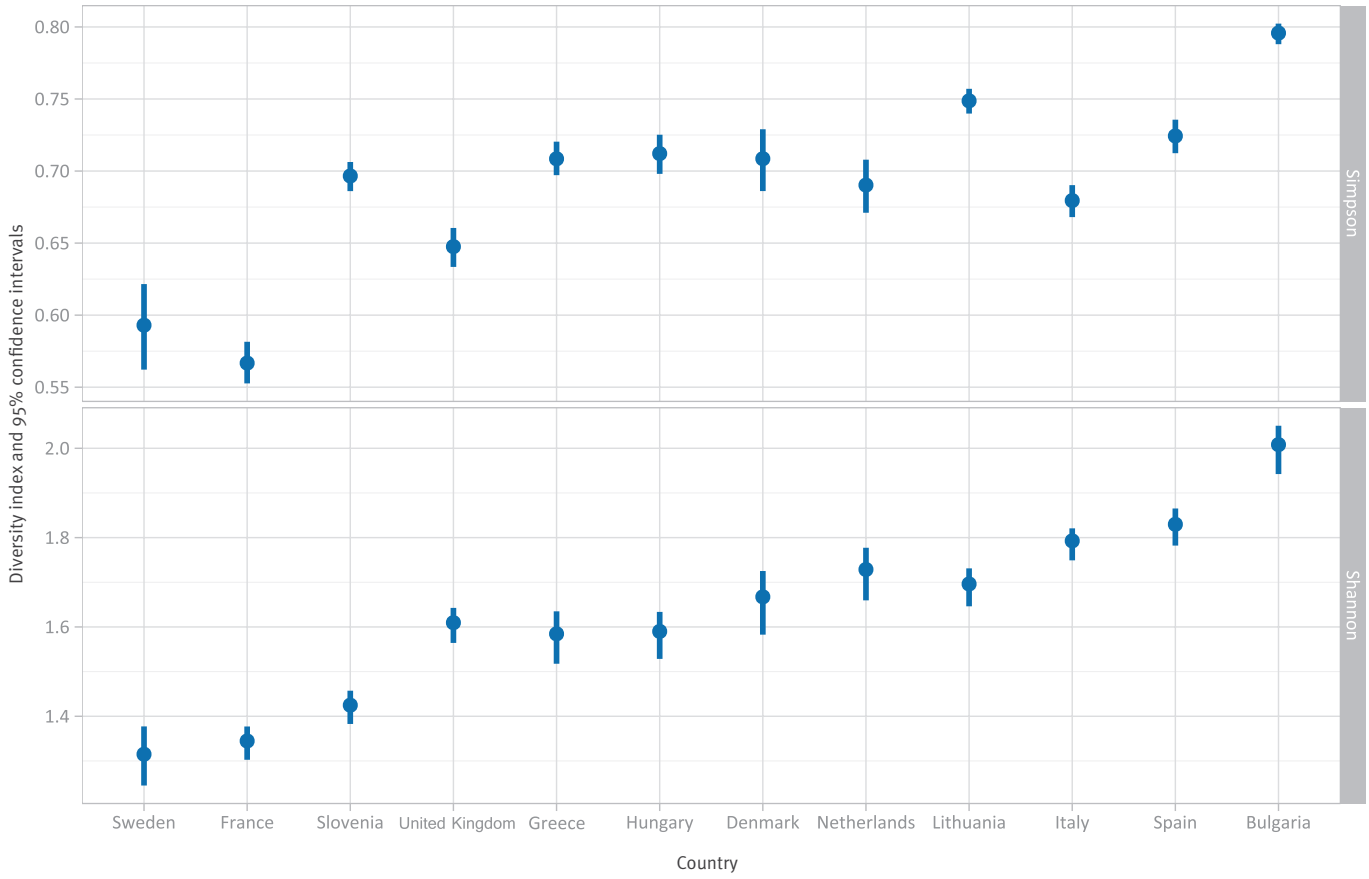
Models relating genotypes and age of cases to season

To investigate differences in circulating genotypes and age of cases out of season vs in season, we fitted a series of mixed-effect multinomial logistic regression models with the two main outcomes: genotype group and age group of cases. Model fitting was based on variables identified a priori and used categorical variables for genotype group (reference group: G1P[8]), age group of the case (reference group: 12–23-month-olds), surveillance year (September to August) and country, and the binary season indicator was the covariate term of interest. The following adjusted models were then fit:

Genotype full model (model 1): genotype as the outcome variable; season, age group of case and surveillance year as covariates; and a random intercept for country.

Figure 4

Rotavirus genotype diversity measured using Shannon's index and Simpson's index of diversity, with 95% confidence intervals, by country, 12 European Union countries, pooled September 2007–August 2013 (n = 39,786)



Genotype country-stratified model (model 2): model 1 but without a random intercept for country; effectively a series of country-specific multinomial logistic regressions.

Age group full model (model 3): age group of cases as the outcome variable; season, genotype and surveillance year as covariates; and a random intercept for country.

Age group country-stratified model (model 4): model 3 but without a random intercept for country; effectively a series of country-specific multinomial logistic regressions.

Each model was initially run as a univariate analysis including only the binary season indicator as the covariate term of interest. Multinomial odds ratios (M-OR; also referred to as RR ratios), 95% confidence intervals (CI) and the associated p values for season were calculated from the Wald test. Results were considered significant at $p < 0.05$. In supplementary analyses, mixed-effects multinomial logistic regression investigated the relationship between age group and genotype group regardless of season, therefore model 1 was re-run excluding season as a covariate (model 5).

Strain diversity

Rotavirus genotype diversity in the 12 European countries studied was compared using two established biodiversity indices, Simpson's index of diversity and Shannon's index [16]. Simpson's index of diversity (D) represents the probability that two randomly chosen rotavirus genotypes will have different G and P types and is calculated as $1 - \lambda$, where $\lambda = \Sigma(p_i^2)$ and p_i is the proportional abundance of a genotype *i*. Shannon's index (H') quantifies the uncertainty in predicting the rotavirus genotype identity of an individual sample that is taken at random from the dataset and is calculated as $H' = -\Sigma(p_i \times \ln(p_i))$. Confidence intervals were estimated using bootstrap resampling methodology and differences in season and out of season were compared for each country.

United Kingdom representativeness test

Linear regression was used to assess the representativeness of the seasonality of genotyped rotavirus data in comparison to all confirmed laboratory reports of rotavirus infection in the UK. The regression takes the form, $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$, where Y is the number of confirmed laboratory reports of rotavirus infection, X represents the covariates (number of genotyped rotavirus specimens and month of specimen), α is the intercept term and ε represents the error term.

Table 1

Number of rotavirus specimens collected in season and out of season, by country, 12 European Union countries, September 2007–August 2013 (n = 39,786)

Country	Total specimens	In season		Out of season		In-season weeks (calendar weeks)
	Number	Number	%	Number	%	Number
Bulgaria	2,627	2,296	87	331	13	31–17
Denmark	1,392	1,192	86	200	14	1–26
France	5,044	4,584	91	460	9	48–21
Greece[a]	2,115	1,447	68	668	32	50–21
Hungary	2,263	1,835	81	428	19	1–23
Italy	6,955	5,685	82	1,270	18	48–22
Lithuania	2,990	2,582	86	408	14	49–23
The Netherlands	2,508	2,346	94	162	6	48–22
Slovenia	2,779	2,272	82	507	18	1–22
Spain	4,609	4,227	92	382	8	47–21
Sweden	1,232	1,030	84	202	16	1–20
United Kingdom	5,272	5,014	95	258	5	1–25
Total	**39,786**	**34,510**	**87**	**5,276**	**13**	**NA**

NA: not applicable.

[a] Data between September 2008 and August 2013.

All data handling and statistical analyses were performed using R Version 3.1.2. and Stata Version 14 [17,18]. The R packages 'Vegan' and 'boot' were used for analysis of genotype biodiversity [19,20]. Data tables are available through the EuroRotaNet website or available on request from the authors [10].

Results

Descriptive statistics

A total of 39,786 rotavirus-positive specimens from 12 countries were typed between September 2007 and August 2013. Rotavirus seasonality for genotyped rotavirus-positive specimens was variable across the countries studied (Figure 1). In the UK, the peak of the rotavirus season was well defined every year, typically occurring between weeks 10 and 12. The representativeness test for the UK confirmed that the seasonal pattern of the typed rotavirus specimens was representative of laboratory-confirmed rotavirus cases in the UK (adjusted $R^2 = 0.75$). Table 1 shows the total number of typed specimens for each country, the number in season and out of season, and the number of weeks per year classified as in season. The proportion of specimens referred for typing that were collected in season ranged from 68% in Greece to 95% in the UK.

The predominant genotype overall was G1P[8], representing 48% of the specimens included in the analysis (range: 24% in Bulgaria to 63% in France). G1P[8] predominated in all countries except Greece and Bulgaria where the predominant genotypes were G4P[8] and G2P[4], respectively (Table 2). Children younger than five years contributed 93% of the specimens (range: 77% in Denmark to 97% in Bulgaria, France and Italy).

It is difficult to distinguish aberrant events due to the data's stochastic nature (Figure 1). However, some can been explained by outbreaks of particular genotypes. For instance in Spain, the increased incidence during the 2011/12 surveillance year was due to an outbreak of G12P[8] in the north-eastern province of Gipuzkoa.

Genotypes in season and between rotavirus seasons

Across all countries studied, when adjusting for country, surveillance year and age group, the adjusted multinomial odds ratio (aM-OR) of infection caused by strains with DS-1-like genotype-constellation (aM-OR = 1.25; 95% CI: 1.13–1.37; $p < 0.001$), mixed or untypable genotypes (aM-OR = 1.55; 95% CI: 1.40–1.72; $p < 0.001$) and less common genotypes (group: 'other'; aM-OR = 2.11; 95% CI: 1.78–2.51; $p < 0.001$) increased out of season relative to G1P[8], while infection caused by strains with Wa-like genotype constellation declined (aM-OR = 0.93; 95% CI: 0.86–1.00; $p = 0.04$) (model 1).

In country-stratified analyses (model 2), the proportional distribution of rotavirus genotypes varied by country (Figure 2). There were significant differences in the proportional representation of genotypes from specimens collected in season and out of season in 10 of the 12 countries studied. In these 10 counties, out-of-season specimens were more likely to belong to a less common genotype (group: 'other') than specimens collected in season (Figure 3). However, this was only significant in eight countries, with the highest aM-OR observed in Spain (aM-OR = 8.18; 95% CI: 4.57–14.64) and Slovenia (aM-OR = 4.49; 95% CI: 1.56–12.88). DS-1-like genotypes were significantly more likely to occur out of season in

Table 2

Distribution of rotavirus genotypes and age of infection in 12 European Union countries, September 2007–August 2013 (n = 39,786)

	Bulgaria n = 2,627		Denmark n = 1,392		France n = 5,044		Greece[a] n = 2,115		Hungary n = 2,263		Italy n = 6,955		Lithuania n = 2,990		The Netherlands n = 2,508		Slovenia n = 2,779		Spain n = 4,609		Sweden n = 1,232		United Kingdom n = 5,272		Total n = 39,786	
Genotype	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
G1P[8]	636	24	614	44	3,182	63	566	27	934	41	3,636	52	1,140	38	1,247	50	1,195	43	2,120	46	747	61	2,958	56	18,975	48
G2P[4]	682	26	87	6	339	7	310	15	273	12	471	7	276	9	186	7	525	19	461	10	119	10	314	6	4,043	10
G4P[8]	605	23	198	14	154	3	768	36	414	18	632	9	742	25	242	10	729	26	133	3	110	9	403	8	5,130	13
G9P[8]	267	10	159	11	686	14	37	2	288	13	878	13	138	5	189	8	181	7	564	12	132	11	565	11	4,084	10
G3P[8]	12	0	115	8	392	8	47	2	13	1	274	4	516	17	339	14	23	1	239	5	83	7	511	10	2564	6
G12P[8]	4	0	3	0	79	2	51	2	14	1	36	1	30	1	44	2	27	1	589	13	8	1	145	3	1030	3
G8P[4]	1	0	2	0	1	0	0	0	0	0	1	0	3	0	4	0	1	0	4	0	4	0	122	2	143	0
Mixed and untypable	325	12	144	10	110	2	299	14	251	11	887	13	82	3	223	9	82	3	429	9	15	1	186	4	3,033	8
Other	95	4	70	5	101	2	37	2	76	3	140	2	63	2	34	1	16	1	70	2	14	1	68	1	784	2
Age group[b]	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
0–11 months	754	29	352	25	2,616	53	837	40	440	20	2,208	32	569	19	1,145	46	554	20	1,841	42	413	34	1,698	35	13,427	34
12–23 months	879	34	520	37	1,402	28	511	24	552	25	2,124	31	893	30	752	30	1,089	39	1,579	36	443	37	1,818	37	12,562	32
2–4 years	892	34	195	14	760	15	665	31	731	33	2,421	35	1,205	40	457	18	893	32	787	18	192	16	918	19	10,116	26
5–14 years	76	3	39	3	113	2	99	5	365	16	164	2	309	10	46	2	122	4	135	3	15	1	164	3	1,647	4
15–64 years	8	0	143	10	28	1	1	0	91	4	12	0	13	0	33	1	82	3	60	1	62	5	107	2	640	2
≥ 65 years	1	0	143	10	26	1	0	0	38	2	3	0	0	0	62	2	35	1	22	1	90	7	195	4	615	2

[a] Data between September 2008 and August 2013.

[b] There were 779 specimens with unknown case age. Percentages are calculated from total specimens with known case age.

Bulgaria (aM-OR = 1.98; 95% CI: 1.35–2.90), France (aM-OR = 1.67; 95% CI: 1.18–2.37), Italy (aM-OR = 1.94; 95% CI: 1.56–2.42), the Netherlands (aM-OR = 2.79; 95% CI: 1.65–4.71), Slovenia (aM-OR = 1.61; 95% CI: 1.18–2.18) and the UK (aM-OR = 1.90; 99CIs: 1.25–2.90), whereas they were less likely to occur out of season in Spain (aM-OR = 0.32; 95% CI: 0.19–0.57) and Greece (aM-OR = 0.41; 95% CI: 0.29–0.59). Untypable and mixed genotypes had significantly higher proportional representation out of season in Bulgaria (aM-OR = 2.47; 95% CI: 1.63–3.73), Italy (aM-OR = 1.32; 95% CI: 1.10–1.60), the Netherlands (aM-OR = 2.57; 95% CI: 1.53–4.29), Spain (aM-OR = 2.14; 95% CI: 1.55–2.98) and the UK (aM-OR = 4.13; 95% CI: 2.59–6.57). Only the UK (aM-OR = 1.38; 95% CI: 1.00–1.90) showed a significant change in the proportional representation of other genotype-constellation 1 (Wa-like) genotypes out of season compared with in season. Although Sweden and Denmark were the only two countries that did not show significant changes in genotype distribution out of season compared with in season, they had very different genotype distributions (Table 2).

Age of cases in season and out of season

Across all countries studied, when adjusting for country, surveillance year and genotype, the aM-OR of infection in two- to four-year-olds (aM-OR = 1.13; 95% CI: 1.04–1.22; p<0.01) and in those five years and older (aM-OR = 1.13; 95% CI: 1.00–1.27; p=0.04) increased out of season relative to the younger children 12–23 months of age, while declining in those younger than 12 months (aM-OR = 0.92; 95% CI: 0.85–0.99; p=0.03) (model 3). Country-stratified analyses (model 4) showed that when adjusting for genotype and surveillance year, half of the countries experienced significant variation in the age group of cases out of season as compared with in season (Figure 3). Cases five years and older constituted a higher proportion of the out-of-season than of the in-season cases in Greece, Italy, the Netherlands, Slovenia, Spain, Sweden and the UK. This difference was only significant in Spain (aM-OR = 1.76; 95% CI: 1.11–2.81) and the UK (aM-OR = 2.53; 95% CI: 1.67–3.82). In France (aM-OR = 1.51; 95% CI: 1.12–2.04) and the Netherlands (aM-OR = 1.79; 95% CI: 1.13–2.82), two- to four-year olds were significantly more commonly represented out of season compared with in season. Lithuania had significantly smaller proportions of cases 0–11 months of age (aM-OR = 0.56; 95% CI: 0.39–0.78) in season compared with out of season, whereas Greece (aM-OR = 1.36; 95% CI: 1.07–1.73) and the UK (aM-OR = 1.66; 95% CI: 1.20–2.30) had a significantly higher proportion of cases younger than 12 months out of season compared with in season.

Relationship between age of cases and genotype group

There was a significant association between increasing age and the genotypes causing disease regardless of season. Those five years and older were more likely to be infected with non-G1P[8] genotypes than those younger than five years (model 5). This was most pronounced in the DS-1-like genotype-constellation (aM-OR = 2.56; 95% CI: 2.27–2.90; p<0.001), but also significant for mixed or untypable genotypes (aM-OR = 1.92; 95% CI: 1.65–2.23; p<0.001), less common genotypes (group: 'other') (aM-OR 2.32; 95% CI: 1.79–3.02; p<0.001) and Wa-like genotype constellations (aM-OR = 1.15; 95% CI: 1.04–1.27; p<0.01). The 0–11-months-old infants were also more likely than the reference group (12–23-month-olds) to be infected with mixed or untypable genotypes (aM-OR = 1.23; 95% CI: 1.11–1.35; p<0.001) and less common genotypes (group: 'other') (aM-OR = 1.30; 95% CI: 1.08–1.56; p<0.01)

Genotype diversity

Sweden and France had the lowest genotype diversity and Bulgaria the highest (Figure 4). Age group analysis showed that genotype diversity was highest in the age group five years and older in six of 12 countries based on Shannon's index and in eight of 12 countries based on Simpson's index of diversity. When cases five years and older were compared with the reference category of 12–23-month-olds, diversity was significantly higher in Shannon's index, Simpson's index of diversity or both indices in Denmark (H': p=0.021/D: p=0.585), Italy (H': p<0.001/D: p<0.001), the Netherlands (H': p=0.192/D: p=0.003), Sweden (H': p<0.001/D: p<0.001) and the UK (H': p<0.001/D: p<0.001). When comparing genotype diversity in season with out-of-season genotype diversity, only Italy and the UK showed significant differences in genotype diversity. Both Shannon's index and Simpson's index of diversity showed significantly higher genotype diversity out of season in Italy (H': p=0.012/D: p<0.001) whereas only Simpson's index of diversity indicated significantly higher genotype diversity out of season in the UK (H': p=0.098/D: p=0.003).

Survey

All countries responded to the survey. Only Hungary indicated that they had reporting laboratories which did not test for rotavirus all year round. There was little variation in the temporal definition of the peak rotavirus season between the questionnaire responses and the statistical coding specified in the Methods chapter. The exceptions were Bulgaria and Denmark. The questionnaire response for Bulgaria specified no seasonality, whereas we identified weeks 31 to 17 for this analysis. For Denmark, the questionnaire response specified peak rotavirus season as March to June, while for the analysis, we defined it as weeks 1 to 26 (i.e. beginning in January).

Diagnostic tests used included enzyme-linked immunosorbent assay (ELISA), dual adenovirus/rotavirus rapid immunochromatographic tests (RIT), real-time RT-PCR, single rotavirus RIT, and electron microscopy. Dual RIT (9/12 responses) and ELISA (8/12 responses) were the most common tests. During the time period studied, it was reported that one laboratory in France had changed testing from latex agglutination to Dual RIT,

and laboratories in four other countries had changed from ELISA to real-time RT-PCR or increased its use.

Age testing policies were variable across countries. Italy, Spain and the UK specified that they routinely test only children younger than five years, while other countries either included older children or tested all ages. Only one laboratory in France was identified as changing age group testing polices out of season. This laboratory specified that it changed from testing all ages to testing immunocompromised cases and children younger than five years only. In addition, a variety of factors were reported as influencing decision to test, but clinician request was selected in every survey response. Other common factors influencing decision to test included nosocomial outbreaks of acute gastroenteritis in a paediatric ward (10/12 responses) and diarrhoeal outbreaks in a nursery (8/12 responses). Apart from the aforementioned laboratory in France, respondents indicated that factors influencing testing for rotavirus were the same in season as out of season, and all countries stated that their decision to genotype did not vary in season and out of season.

Discussion

Significant differences in the circulating rotavirus genotypes in season compared with out of season were observed across the countries studied. Genotype G1P[8] was dominant in season but this dominance declined out of season in most countries, whereas the proportion of other less abundant genotypes increased out of season. Other than the dominance of G1P[8] in most countries, there was little consistency in genotype distribution across countries studied, highlighted by the country-to-country variation in genotype diversity and relative genotype dominance. For instance in Bulgaria, no genotype was identified as dominant, and the survey results further elucidated that Bulgaria does not appear to have a well-defined rotavirus season.

The analysis also showed that there were clear seasonal differences in the age distribution among rotavirus cases out of season vs in season. These differences were not consistent across all the countries studied. Generally, the proportion of cases five years and older increased out of season and in most countries, genotypes found in cases aged five years and older were more diverse than genotypes identified among younger age groups regardless of season. Relative to younger cases, cases aged at least five years were more likely to be infected with a non-G1P[8] genotype, in particular genotypes from the DS-1-like genotype constellation.

The relative decline of G1P[8] genotypes out of season is common in European countries and by definition coincides with a flattening of incidence, similar to countries with smoother incidence throughout the year, such as Bulgaria, where no single genotype is dominant. This pattern is also reflected in observations from countries which have introduced rotavirus vaccination, reinforcing the importance of understanding the pre-vaccine ecology of rotavirus infection across Europe for interpreting changes in rotavirus genotype distribution, seasonality and age of infection after vaccine introduction [21–24].

Seasonal and age group differences in the distribution of rotavirus genotypes may be driven by differential virus fitness among susceptible and partially immune hosts. Younger children, who are more susceptible, may be preferentially infected by the G1P[8] genotype, which given its predominance in most countries may be better adapted to the host or to transmission. The out-of-season decline in G1P[8] dominance may then be driven by the accumulation of homotypic immunity to G1P[8] in the community during the rotavirus season, reducing the number of susceptible hosts out of season and enabling the potentially less fit non-G1P[8] genotypes to infect those who have homotypic immunity from previous exposure to G1P[8] (24–60-month-olds may only have partial protection due to limited number of exposures) and older individuals infected with other genotypes to which cross-protection may be incomplete [25,26]. Indeed, a Mexican study showed that natural rotavirus infection reduces host susceptibility after each infection and that secondary infections are more likely to be caused by a different genotype than the one causing the first infection [25]. Furthermore, this explanation may be consistent with previous findings in which birth cohort effects were identified as potential drivers for differences in seasonality across the United States (US) [27].

Such differences between heterotypic and homotypic protection conferred by the dominant G1P[8] genotype support results from vaccine efficacy and observational studies of the monovalent Rotarix vaccine, which show that although the vaccine does protect against completely heterologous genotypes (e.g. G2P[4]), it may do so to a lesser extent [28–31].

The analysis also showed that mixed and untypable genotypes proportionally increased in a number of countries out of season. The types available for partially typed rotaviruses (G or P type unobtainable) appear to be representative of the more commonly found types (typically G1 or P[8]). Insufficient sensitivity of the typing procedures is the most likely cause for the typing failures [32]. These samples may, therefore, contain lower viral loads, which are likely to be associated with infections in previously exposed individuals with partial protection and/or subclinical infections.

Therefore, a plausible explanation for the increase in the proportional representation of older children and adults and of mixed and untypable genotypes out of season might be the accidental detection of an (asymptomatic) rotavirus infection in previously exposed individuals protected from severe RVGE, coinciding with infection by another pathogen causing gastrointestinal symptoms that has peak incidence in the summer months, such as some gastrointestinal bacterial

pathogens. This could be supported by a study in the US that found that in adults admitted to hospital with diarrhoea, rotavirus was as commonly detected as bacterial gastrointestinal pathogens [33]. Furthermore, pre-vaccine studies suggest that there are high symptomatic and asymptomatic infection rates in adults regardless of epidemic season and that re-infection in adults persists across the year, which may suggest that older children and adults may be a reservoir from which the winter/spring paediatric epidemic emerges [34–36].

Our findings also suggest rapid genotype cycling from in-season to out-of-season periods and, as noted by Pitzer et al. [26], this could be caused by relatively stronger homotypic immunity than heterotypic immunity in the population, which renders the less common genotypes increased fitness, permitting them to persist in the population [26,37]. Moreover, age increases among RVGE cases as the predominant genotype declines, and the rapid cycling to less common genotypes out of season may explain the proportional increase in two- to four-year-olds and those five years and older seen in our analysis out of season [26,27]. However, an increase in those five years and older out of season may also be influenced by delayed transmission to this group because of mixing and contact patterns in younger children and infants. Additionally, the change to older age groups and less common genotypes out of season could potentially be related to importations associated with travel.

Interpretation of the proportional increase in specimens from those five years and older is, however, complicated by testing practices. The survey suggests that laboratories in some countries routinely test for rotavirus only in children younger than five years or, in some cases, those younger than 18 years, while limited testing occurs in older age groups. However, only one laboratory among the study countries reported changes in either age-specific testing procedures or clinician requests in season compared with out of season. Also, specifically in the UK, published guidance suggests a consistent testing algorithm all year, indicating that the reported variation in age of infection is representative [38].

Unfortunately, there is no apparent explanation for increases in the proportion of rotavirus-positive infants younger than 12 months out of season in Greece and the UK and for the decline in Lithuania. Findings are unlikely to be explained by seasonal birth rates as birth rate seasonality is similar in all the countries studied, suggesting that other factors, such as low heterotypic immunity conferred by previous infection, may be responsible [39].

We have described a number of potential hypotheses that may contribute to the observed differences in genotype and age distribution in season and out of season. However, we recognise this is not exhaustive and there may be other plausible hypotheses.

Strengths and limitations

Our analysis benefited from using an established surveillance system that has achieved consistency over a number of years. We supplemented our understanding of these data with a network-wide survey of testing practices. Nevertheless, there are limitations. Firstly, the sample size of rotavirus-positive samples typed was calculated based on detecting genotypes with a prevalence of at least 1% and, depending upon the country population size and estimated rates of rotavirus infection, are therefore not representative of the incidence of RVGE [11]. Secondly, it is unknown how many samples are referred for rotavirus diagnosis or how many are positive in routine diagnostic laboratories given that rotavirus is not a notifiable disease in many of the countries studied. For this reason we were unable to provide the proportion of positive samples each country submits for genotyping. Consequently we could not quantify the effect of sampling bias on out-of-season increases in less common genotypes, and the smaller number of cases out of season means that we must be aware of random variation when considering the findings. However, the study design helped to increase precision by pooling data over a number of seasons. Thirdly, data completeness of sex in the EuroRotaNet database was inconsistent across the countries studied. Previous analysis of EuroRotaNet data has shown no differences in genotype distribution between the sexes [11]. For these reasons sex was excluded from our models. Fourthly, the survey has shown that diagnostic procedures can vary slightly between countries and that a small number of laboratories have changed testing practices during the study period, which may have influenced the number of detected cases. However, a study in the UK found no association between number of laboratory reports and proportion of cases diagnosed by each diagnostic method [40]. Fifthly, even though countries included in the study had either low vaccination coverage (< 35%) or total absence of routine rotavirus vaccination [13], we have been unable to account for the effect of low-level vaccination in countries in which vaccine is available in regions and/or in the private health care sector, or the effect of routine vaccination in neighbouring countries on our findings. Finally, it is important to acknowledge that EuroRotaNet data are likely to be representative only of moderate to severe cases because in many countries, rotavirus is not a notifiable disease and because symptoms often resolve without healthcare contact.

Conclusions

This study shows that rotavirus genotype distribution in Europe is variable and that most countries included in this study experience variation in genotypes typed from specimens collected during the peak rotavirus season compared with the out-of-season periods. Changes in age of infection between peak rotavirus season and out-of-peak season may be due to lower

cross-protection against heterotypic genotypes. These findings raise several questions about the genotype reservoirs and genotype persistence that may help direct future research to understand the temporal variability in the environment and in hosts. In addition, the true burden and epidemiology of rotavirus infections in adults and older children are not well understood due to age-exclusive testing policies, but the study further indicates that this could be critical to understanding re-infection and transmission that persists to re-ignite the epidemic season each year.

Finally, of the countries studied here, the UK has since introduced rotavirus vaccination into the childhood immunisation schedule. Critically, this work provides important pre-vaccine ecological data for the UK and other European countries introducing or expanding rotavirus vaccination programmes.

Members of the EuroRotaNet

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Conflict of interest

Rotarix is developed and licensed by GSK Biologicals. NC has recieved research grant support from GSK Biologicals and honoraria for participation in GSK Rotavirus Vaccine Advisory Board Meetings.

Authors' contributions

DH participated in study design, developed the survey, performed data management, conducted the analysis and wrote the manuscript. EuroRotaNet members contributed to study design and data collection. RV contributed to study design and survey design. JMR contributed to the analysis. VEP contributed to the analysis. NF participated in study design and contributed to the analysis. NC contributed to interpretation of data. MIG conceived of the study; contributed to survey design and data collection. All authors contributed to the interpretation of the data, drafting the article, and final approval of the version to be published. No person or persons other than the authors listed have contributed significantly to the study or manuscript preparation.

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Three vole species and one (?) novel arvicolid hantavirus pathogen: Tula virus revisited

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To the editor: The recent confirmation by RT-PCR of a case of haemorrhagic fever with renal syndrome (HFRS), induced by Tula virus (TULV) in France [1], confirms the pathogenicity of this arvicolid hantavirus, a fact not generally acknowledged yet, or at least still contested [2]. The clinical presentation of the demonstrated TULV HFRS case was, however, unusual: besides the classic fever with thrombocytopenia and elevated transaminases, leukopenia instead of leukocytosis with left shift was found, and the renal function remained strictly within normal limits. However, renal involvement was nevertheless indicated by transient microscopic haematuria. Regrettably, transient but massive and unselective proteinuria, the renal hallmark in probably all hantavirus infections, was once more not discussed. Interestingly, a false-positive serological screening result for another arvicolid hantavirus, Puumala virus (PUUV), was obtained in three assays of two different formats (immunofluorescence assay (IFA) and ELISA), but could not be confirmed by routine RT-PCR, i.e. by using PUUV-specific primers [1]. As subsequently shown by Reynes et al., TULV and PUUV are two closely related, yet genetically distinct hantavirus species, both carried by distinct voles of the *Arvicolinae*, a subfamily of the *Cricetidae* rodent family [1].

Consequently, it is important to remember that the classic TULV rodent reservoir, the common vole (*Microtus arvalis*), is present throughout most of western Europe, except Fennoscandia and the British Isles, with however a presence on the Orkney Islands. The common vole is also present in northern and even central Spain. This means in serological practice that an HFRS-like infection in Fennoscandia and/or the British Isles, documented by standard IFA and/or ELISA to be IgM-positive for PUUV, could thus (until recently) readily be accepted as a true PUUV infection, given the complete absence of common voles in the area. However, the same conclusion is not so evident for a PUUV-positive HFRS case in the rest of north-western Europe, where even strong positive serological results for PUUV could in fact point to a TULV infection spread by common voles, as exemplified by this French case. This is valid also for northern and central Spain, northern Italy and the Balkan Peninsula.

Things become even more complex when the geographical spread of the field vole (*Microtus agrestis)* is also considered. Indeed, its habitat, much more extensive than that of its cousin *M. arvalis*, includes the whole of Europe except Ireland and Mediterranean countries. Several reports mention the presence of a TULV-like agent in field voles; the most recent example is Tatenale virus, the first biomolecularly proven arvicolid hantavirus in the United Kingdom, characterised in a field vole in north-western England. Like TULV, it provoked false-positive PUUV reactions in serology [3]. Finally, a TULV-like agent has also been documented in the Eurasian water vole (*Arvicola amphibius*, formerly *Arvicola terrestris*), which has the same extensive European spread as the field vole; infection with this virus therefore has the same potential of yielding PUUV-like serological cross-reactions [4]. Moreover, this novel TULV-like agent has already been found to infect asymptomatic forest workers, even in non-endemic areas of eastern Germany [5].

In summary, in western Europe, including Fennoscandia and mainland England, an ELISA-and/or IFA-positive result for PUUV does not automatically mean a true PUUV infection, as now convincingly shown [1]. Few isolated European HFRS case reports, and virtually no national European or Russian PUUV seroprevalence studies have so far excluded this possibility. Northern Ireland, where none of the above vole species are present, remains a noticeable arvicolid-free exception [6]. Admittedly, all this bears little practical clinical significance for physicians treating a suspected PUUV case, since TULV infections seem even milder than their

PUUV counterpart. In fact, it is even likely that a prior PUUV infection, subclinical or not, might confer at least partial, but probably life-long cross-immunoprotection against its cousin-pathogen TULV.

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