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Identification of dengue type 2 virus in febrile travellers returning from Burkina Faso to France, related to an ongoing outbreak, October to November 2016

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Dengue fever is rarely reported in travellers returning from Africa. We report two cases of dengue fever in travellers returning from Burkina Faso to France. One of them presented a severe dengue fever with ALT>1,000 IU/L and pericarditis. Serotype 2 was identified. The cases reflect a large ongoing outbreak with over 1,000 reported cases between August and November in the capital city. Clinicians should consider dengue fever in malaria-negative febrile travellers returning from Africa.

Dengue fever in returning travellers to non-endemic areas has been mainly reported after visits to South-east Asia, Central Asia, or South America [1]. In Africa, its epidemiology is poorly described even if the disease has long been known to exist [2]. We report two cases of dengue fever identified in travellers returning from Ouagadougou, Burkina Faso, to Marseille, France, in late autumn 2016, reflecting a large ongoing local outbreak.

Case descriptions

The first case was a woman in her mid-twenties, who travelled to Burkina Faso as a logistician for a medical non-governmental organisation (NGO). She spent 10 days in Ouagadougou from 23 October to 3 November 2016 and used atovaquone/proguanil for malaria prophylaxis. Three days after her arrival, she developed fever, headache, myalgia, nausea and diarrhoea. Two days after she came back to France, she presented at our centre with persisting diarrhoea on day 9 after symptom onset. At examination she had no fever, complained of weakness and had a painful abdomen. Dengue nonstructural protein 1 (NS1) antigen (Ag) and serology (IgM) was negative (SD BIOLINE Dengue Duo Combo Device, Standard Diagnostics Inc, Korea) and malaria rapid diagnostic tests (Palutop+4, All. Diag, France) were negative, as was serology with an in-house MAC ELISA for IgM and in-house indirect ELISA for IgG [3]. A serum sample was positive for dengue viral RNA [4] and typed as dengue 2 viral RNA [5]. Blood and stool cultures, parasitological examination of stools and RealStar Chikungunya RT-PCR kit 1.1 (Altona Diagnostics, Germany) were negative. Convalescent serum was not collected because the patient did not attend the follow-up visit (Table).

The second case was a woman in her 50s who travelled from 12 October to 10 November 2016 to Ouagadougou, where she worked with the organisation of a theatre festival. One week after arrival, she presented fever up to 40.5 °C, arthralgia and diarrhoea. She presented at a local medical centre and was treated by quinine for malaria without blood tests performed. Fever and diarrhoea persisted and on day 3 of illness dark urine appeared. She thus consulted the International Medical Center in Ouagadougou where blood sample analysis revealed severely elevated liver transaminases (Table). Malaria was ruled out by microscopic blood smear examination and Dengue NS1 Ag testing was performed and found positive. She came back to France and consulted a cardiologist because of chest pain. Chest computerised tomography (CT)-scan ruled out pulmonary embolism and pericarditis was diagnosed by echocardiography. She presented at our centre two weeks after resolution of symptoms because she had questions about the prevention of dengue fever.

Epidemiological situation in Burkina Faso and neighbouring countries

The first dengue fever outbreak in Burkina Faso was reported in 1925 [2]. In 1982, a second outbreak was described between September and December, with 30 cases reported (mainly European expatriate patients), and two strains of dengue virus serotype 2 were isolated for the first time in this country [6]. In 2013, another epidemic occurred between October and November, and serotype 3 was isolated [7]. On 18 November 2016, the World Health Organization (WHO) reported 1,061 suspected cases of dengue fever in

Table

Laboratory test results, two cases of dengue fever in travellers returning from Ouagadougou, Burkina Faso, to Marseille, France, October to November 2016[a]

Laboratory results (norm)	Case 1	Case 2
Leukocyte count/µl (4,000-10,000)	1,650	2,700
Platelet count/µl (150,000-400,000)	296,000	142,000
ALT/AST IU/L (10-40)	62/53	1,800/NA
Dengue NS1 Ag (NA)	Negative	Positive
Dengue serology (IgM and IgG)[b] (NA)	Negative	Negative
Real-time RT-PCR detecting all dengue virus RNA (NA)	Positive	Negative
Type specific real-time RT-PCR (NA)	Positive serotype 2	Negative

Ag: antigen; ALT: alanine transaminase; AST: aspartate aminotransferase; NA: not available NS 1: non-structural protein RT: reverse transcription.

[a] Only values deviating strongly from the norm are presented.

[b] MAC ELISA for IgM and in-house indirect ELISA for IgG [3].

Burkina Faso between August and November 2016, including 15 fatal cases [8]. Serotype 2 has been identified in the current outbreak, but further investigations are in progress.

Concerning other West African countries, data are scarce. The last epidemic in Senegal was reported in 2009, caused by dengue type 3 virus [9]. In Sierra Leone, Mali and the Ivory Coast, seroprevalence studies among febrile patients reflect the circulation of the virus [10-12]. Cases from Togo and Benin have been reported only in travellers [2,13].

Discussion

We report two cases of dengue fever in travellers returning from Burkina Faso to France in late autumn 2016. The first patient had a non-complicated dengue fever according to the WHO dengue fever classification criteria [14]. The second one fulfilled the criteria for severe dengue fever, with ALT›1,000 IU/L, and a pericarditis was diagnosed when she came back to France. Pericarditis has been rarely reported after dengue fever, possibly because of a lack of detection in endemic areas. Some ten cases have been reported from Malaysia, Sri Lanka, Singapore, Brazil in total and one case in a French traveller returning from Guadeloupe [15-18].

The two cases here should remind us that dengue screening should be performed in malaria-negative travellers with history of fever returning from Africa [1,19]. For systematic screening of returning travellers for dengue fever, rapid diagnostic tests (as commonly done for malaria) are available and should be used. Rapid and early detection of cases could allow implementing measures to prevent further spread i.e. mosquito control around the residence of the returning travellers in areas where competent vectors are present and adapting the prevention message for travellers who wish to visit Africa.

It is a well-known fact that travellers may serve as sentinels to local risks and this has been proven in numerous instances. In countries with scarce public health reporting, they may inform the international community on the onset of epidemics. Data from the African continent on dengue fever illustrates this phenomenon: dengue infections have been detected in 34 African countries, and for 12 of them the only available information was from travellers [2]. At the time we diagnosed our first case, the outbreak in Burkina Faso had not yet been notified by the WHO and the serotype involved was not known.

An international festival of theatre named 'Les récréâtrales' took place in Ouagadougou, from 29 October to 5 November. This festival occurs every two years in the capital between October and November, the months in which all previous dengue outbreaks were described. Our second patient mentioned that two other members of the festival staff were diagnosed with dengue fever during her stay. This festival takes place in a popular district of Ouagadougou (Bougsemtenga) and some presentations are organised in familial yards surrounding houses. A recent entomological survey in Ouagadougou identified that these yards were major places of vectors' breeding sites [20]. The most frequent breeding sites identified were water storage containers, garbage such as food tins, and tyres [20]. In this survey, *Aedes aegypti* specimens were captured from breeding sites but no *Ae. albopictus* was identified [20]. *Aedes* mosquitoes bite during daytime. Hence, clinicians should remind travellers to endemic areas, including those in Africa, of the importance to protect themselves against mosquito bites during the day.

Conflict of interest

None declared.

Authors' contributions

Carole Eldin and Philippe Gautret wrote the manuscript. Philippe Parola and Philippe Brouqui critically revised the manuscript Antoine Nougairede, Laetitia Ninove and Remi Charrel performed the virological anaysis. Melanie Sentis, Matthieu Million, and Nadia Saidani made the clinical diagnosis and description of cases.

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Evaluation of molecular typing of foodborne pathogens in European reference laboratories from 2012 to 2013

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In 2012, the European Centre for Disease Prevention and Control (ECDC) initiated external quality assessment (EQA) schemes for molecular typing including the National Public Health Reference Laboratories in Europe. The overall aim for these EQA schemes was to enhance the European surveillance of food-borne pathogens by evaluating and improving the quality and comparability of molecular typing. The EQAs were organised by Statens Serum Institut (SSI) and included *Salmonella enterica* subsp. enterica, verocytotoxin-producing *Escherichia coli* (VTEC) and *Listeria monocytogenes*. Inter-laboratory comparable pulsed-field gel electrophoresis (PFGE) images were obtained from 10 of 17 of the participating laboratories for *Listeria*, 15 of 25 for *Salmonella*, but only nine of 20 for VTEC. Most problems were related to PFGE running conditions and/or incorrect use of image acquisition. Analysis of the gels was done in good accordance with the provided guidelines. Furthermore, we assessed the multilocus variable-number tandem repeat analysis (MLVA) scheme for *S.* Typhimurium. Of 15 laboratories, nine submitted correct results for all analysed strains, and four had difficulties with one strain only. In conclusion, both PFGE and MLVA are prone to variation in quality, and there is therefore a continuous need for standardisation and validation of laboratory performance for molecular typing methods of food-borne pathogens in the human public health sector.

Introduction

Salmonellosis, verocytotoxin-producing *Escherichia coli* (VTEC) infections and listeriosis are some of the most commonly reported zoonotic diseases within the European Union (EU) [1]. Since 2006, the European Centre for Disease Prevention and Control's (ECDC) Food- and Waterborne Diseases and Zoonoses (FWD) Programme has been responsible for the EU-wide surveillance of salmonellosis, VTEC infections and listeriosis including the facilitation of the detection and investigation of food-borne outbreaks. Phenotypic parameters of the isolated pathogens are reported by the EU Member States to The European Surveillance System (TESSy) and molecular typing data are reported to the molecular surveillance service within TESSy [2].

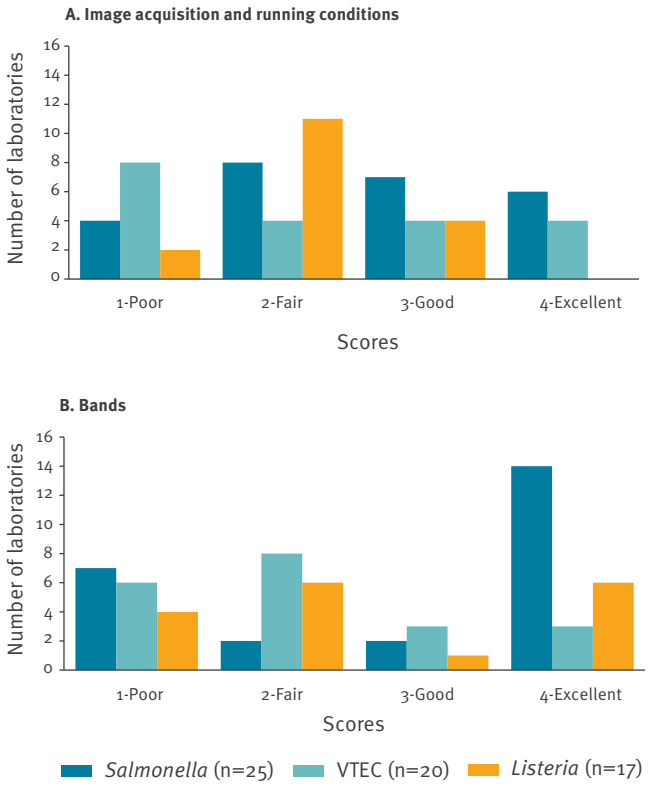
In view of the surveillance objectives, ECDC has developed a set of specific principles and prerequisites for the systematic incorporation of molecular typing data into routine EU-level surveillance [3,4]. One of the principles includes that the use of internationally agreed molecular typing methods is supported by external quality assessment (EQA) schemes to enhance data quality and comparability. For three food-borne pathogens, namely *Salmonella*, VTEC and *Listeria,* globally agreed standard molecular typing methods, namely pulsed-field gel electrophoresis (PFGE) and multilocus variable-number tandem repeat analysis (MLVA) [5] enable a comparison with isolates from food/feed and animals.

PFGE is used widely for surveillance [6-8] and outbreak investigations of all three pathogens [9-11]. It is the only generic method for typing of all *Salmonella* serovars and *Listeria* serotypes and global protocols have been developed and standardised by the United States (US) Centers for Disease Control and Prevention (CDC) [12,13].

MLVA is serotype specific, and has been developed for *S.* Typhimurium [14,15] with standardisation by the use of reference strains [16]. The method has a higher discrimination power compared with PFGE for *S.* Typhimurium and is widely used for surveillance [17,18] and outbreak investigations [19,20].

Figure 1

Number of laboratories according to their pulsed-field gel electrophoresis (PFGE) gel quality scores for the parameters a) 'image acquisition and running conditions' and b) 'bands', European Union/European Economic Area, 2012–2013



VTEC: verocytotoxin-producing *Escherichia coli*.

Each parameter is evaluated and presented separately.

PFGE and MLVA methods have been standardised in order to allow comparable results across laboratories [12,13,21,22], thus the FWD network decided to use those for developing molecular surveillance at EU level. This study presents the results from the first round (2012–2013) of the EQAs for molecular typing of *Salmonella*, VTEC and *Listeria monocytogenes* in National Public Health Reference Laboratories (NPHR-Ls) in EU/European Economic Area (EEA) countries and EU candidate countries. The objectives of the EQAs were to assess the quality and comparability of PFGE and MLVA results from participating laboratories.

Methods

Organisation

The EQAs were funded by ECDC and organised by Statens Serum Institut (SSI), Denmark. One NPHR-L from each of the 31 EU/EEA countries and four EU candidate countries in 2012–2013 were invited to participate in each of the three EQA schemes (one scheme for each bacterial species). Some countries have different NPHR-L for each species and some countries have one NPHR-L responsible for all three species. The EQA schemes and their different components were optional and laboratories could chose to only participate in selected parts (e.g. only submitting a PFGE gel without performing the analysis of the gel). The *Salmonella* EQA included PFGE, MLVA and phage typing, the VTEC EQA included PFGE, serotyping, genotyping (including subtyping and virulence genes) and phenotypic tests and the *Listeria* EQA included PFGE and serotyping. All details of the EQAs are published as technical reports by ECDC [23-25]. Only the molecular typing results are presented here.

Strains

For the PFGE parts of the EQAs, bacterial strains (10 *Salmonella*, 10 *Listeria* and 11 VTEC) were selected based on their relevance for the epidemiological situation in Europe, including in recent outbreaks. The serotypes included for PFGE were for *Salmonella*: Aberdeen, Dublin, Enteritidis, Infantis, Mbandaka, Poona, Saintpaul, Strathcona, and Typhimurium (2 strains), for VTEC: O26:H11, O41:H26, O103:H2, O104:H4, O111:H8/H-, O121:H9, O146:H21, O177:H25, O157:H7 (2 strains), O166:H15, for *Listeria*: 1/2a (2 strains), 1/2b (1 strain), 1/2c (3 strains), 4a/4c (1 strain) and 4b (3 strains). For the MLVA part of the *Salmonella* EQA, a total of 10 different representative *S.* Typhimurium strains were selected.

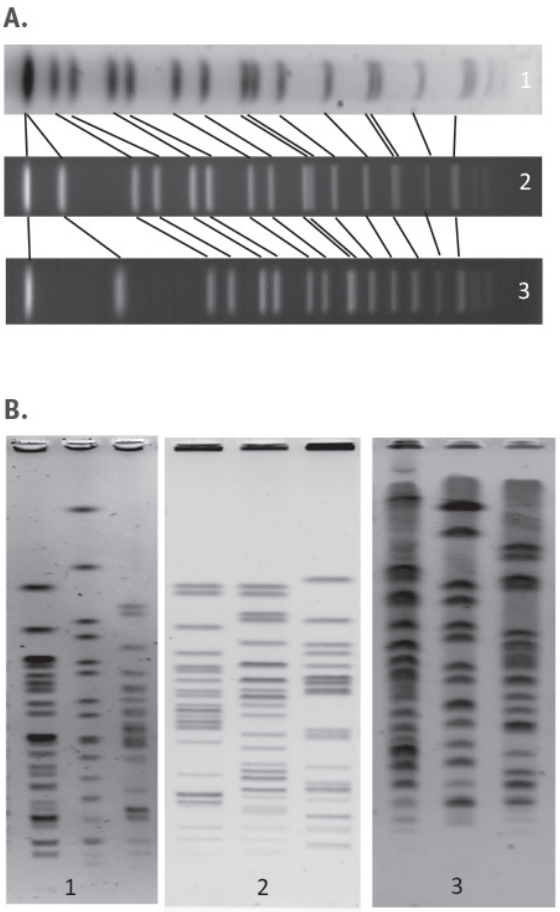
All the strains included in either MLVA or PFGE were stability tested, blinded and packed for distribution according to the International Standard ISO/IEC 17043:2010 (appendix B.5) [26]. In addition to these strains, reference strains for the different assays were delivered to participants upon request. These included the PFGE reference strain *S.* Brandrup and/or 33 MLVA reference strains, consisting of an original set of 31 previously described MLVA reference strains [16] as well as the recently added STm-SSI32: (3,17,21,18,311) and STm-SSI33: (2,13,9,11,112) strains. The participants were also provided a detailed study protocol specifying all suggested standardised methods for each of the specific species. Moreover, a pre-configured BioNumerics (BN) database with experiment settings and a guide for creating a new database was also made available to them if their BN software was older than version 5. Furthermore, guidance on how to export the BN analysis of PFGE data was provided as well as an Excel sheet converting the obtained MLVA fragment sizes to true allele numbers based on the results obtained when analysing the 33 MLVA reference strains.

Testing

The participants were instructed to use the Standard PulseNet PFGE protocol for *Salmonella*, VTEC O157 [27] and *Listeria monocytogenes* [28]. For the *S.* Typhimurium MLVA, the *S.* Typhimurium MLVA Standard protocol was suggested [29].

Figure 2

Examples of gel selections with a) incorrect running conditions and b) fuzzy/thick bands



The middle examples (2) scored 'excellent' (i.e. score = 4) in all parameters.

Data analysis

For PFGE, the data were evaluated as two separate parts (i) the quality of gels and (ii) the quality of the further gel analysis. (i) The gel quality was evaluated according to the ECDC FWD MolSurv Pilot - SOPs 1.0 - Annex 5 - PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines) [23-25], by scoring the gel with respect to seven parameters (image acquisition and running conditions, cell suspension, bands, lanes, restriction, gel background, and DNA degradation). (ii) The participant's ability to perform gel analysis was evaluated separately from the evaluation of the gel quality. However, the gel analysis (part ii) was based on the gels produced in the respective laboratories and therefore the outcome of a participating laboratory's band assignment is to some degree influenced by its gel quality (part i). The gel analysis (ii) was evaluated by scoring five parameters (position of gel, strips, curves, normalisation, and band assignment) using the BN gel analysis quality guidelines, developed by SSI. All parameters were scored between 1 and 4: 1 (poor), 2 (fair), 3 (good), and 4 (excellent). The evaluation of the participating laboratories' gel analysis was independently carried out by two experts in PFGE, who subsequently discussed and agreed upon the scores.

The MLVA typing results were scored as correct or incorrect for each strain and the percentage of correct answers was used as the score for each participant.

Results

Participation

In total, 35 countries were invited to participate in each EQA. The highest number of laboratories participating was in the PFGE EQA for *Salmonella* and VTEC with 25 and 20 NPHR-L, respectively, compared with 17 for *Listeria*. The number of participants that submitted a PFGE gel (without analysis) were 11/25 for *Salmonella*, 8/20 for VTEC and 4/17 for *Listeria*. The number of laboratories analysing their PFGE gels and submitting export files according to the instructions were 14/25 for *Salmonella*, 12/20 for VTEC and 13/17 for *Listeria* (Table 1). Fifteen laboratories participated in the MLVA part of the *Salmonella* EQA.

Pulsed-field gel electrophoresis (PFGE)

Gel quality

The majority (61/62) of the submitted results were profiles recognisable as the profile for the relevant EQA strain, i.e. indicating that the laboratory had not by mistake interchanged strains. One laboratory seemed to have exchanged one PFGE VTEC strain with one VTEC strain for the phenotypic tests.

The average scores of all laboratories by parameter and pathogen are listed in Table 2, along with the conditions for being graded an excellent score. For all three pathogens and for four of the seven parameters, the gel quality was good, scoring on average 3.0 or above (Table 2). For VTEC gels, the parameter 'gel background' was only 2.9 on average as 3/20 gels were scored 'poor', mostly due to large amount of debris in the gels – which can be easily prevented. None of the *Salmonella* or *Listeria* gels obtained the lowest score for this parameter.

For all pathogens, with respect to the two important parameters 'image acquisition and running conditions' and 'bands' the average gel quality was only fair (between 2.1 and 2.9). Very diverse individual scores were obtained for these parameters (Figure 1a and 1b). Critical scores (1 or 2 ~ poor or fair) for the parameter 'image acquisition and running conditions' were given to 12/25, 12/20 and 13/17 of the gels of *Salmonella*, VTEC and *Listeria* (Figure 1a), respectively. Correct running conditions and thereby the correct spacing of the global standard is crucial for the possibility of inter-laboratory comparison. Incorrect spacing of the standard was more frequently observed on the VTEC gels than

Table 1

Number of national public health reference laboratories (NPHR-L) submitting external quality assessment (EQA) results by pathogen and method, European Union/European Economic Area, 2012–2013

Pathogen	Number of NPHR-L participating to the MLVA EQA	Number of NPHR-L participating to the PFGE EQA			TOTAL
		PFGE gel only[a]	PFGE gel + analysis[b]	Total	
Salmonella	15	11	14	25	27[c]
VTEC	NA	8	12	20	20
Listeria	NA	4	13	17	17

MLVA: multilocus variable-number tandem repeat analysis; NA: not applicable; NPHR-L: national public health reference laboratories; PFGE: pulsed-field gel electrophoresis; VTEC: verocytotoxin-producing *Escherichia coli*.

[a] Submitting a TIFF file of the PFGE profile.

[b] Analysing the gel profile and submitting export files.

[c] Two NPHR-L did not participate in the PFGE part of the external quality assessment, but only in MLVA.

the *Salmonella* and *Listeria* gels. For the parameter 'bands', clear and distinct bands were seen on 14/25 of the *Salmonella* gels, 6/17 of the *Listeria* gels and only 3/20 of the VTEC gels scored 'excellent' (Figure 1b). In addition, 7/25 of the *Salmonella*, 6/20 of the VTEC and 4/17 of the *Listeria* gels obtained the score 'poor' (Figure 1b), which indicates that further analysis of the gel was impossible and generally it would be difficult or impossible to compare with profiles on other gels. Examples of submitted gels of poor quality are shown in Figure 2.

Since a low quality score in just one parameter has a high impact on the ability to further analyse the image, the overall across-parameter results showed that inter-laboratory comparable PFGE images could only be obtained from 10 of 17 of the participating laboratories for *Listeria*, 15 of 25 for *Salmonella*, but only nine of 20 for VTEC.

Gel analysis

In the PFGE part of the EQAs, involving *Listeria*, *Salmonella* and VTEC, 17 to 25 laboratories per pathogen participated (Table 1) by submitting raw gel images (TIFF files). Depending on the pathogen, between 12 and 15 laboratories also analysed their gels and submitted the results in the form of export files (Table 1). However, one laboratory's submission was excluded in the *Salmonella* EQA due to incompatibility between the BN versions 6.0 and 7.0, i.e. 14 datasets were included in the gel analysis. Gel analysis was graded on five parameters. The average gel analysis quality scores of each parameter and EQA are listed in Table 3.

Laboratories received high scores for all three pathogens on the parameters 'strips' and 'curves' (Table 3). For both *Salmonella* and VTEC, high scores were also obtained on the parameter 'position of gel' but the score was a bit lower for *Listeria*. Two laboratories failed to place the frame below the wells and this had critical influence when the gel was normalised. With regard to the parameter 'normalisation', the participants in the VTEC EQA were graded lower than *Salmonella* and *Listeria* with an average of 2.8 because of incorrect band assignment of the reference lanes or failure to include the reference strains in the export files. The average scores of the parameter 'band assignment' were equal for all three pathogens (Table 3).

Multilocus variable-number tandem repeat analysis (MLVA) of *Salmonella* Typhimurium

Of the 15 laboratories that participated in the MLVA part of the EQA, nine laboratories were able to correctly MLVA type all ten EQA strains. Four laboratories reported the correct MLVA profile for nine of the strains, one laboratory had correct results for seven strains, and one for five strains. The typical error accounting for the vast majority of incorrect profiles by these six laboratories was to either replace an absent (NA) locus with a repeat number or vice versa. One of the laboratories seemed to have analysed/reported the MLVA profile for one EQA strain under two strain numbers, thereby obtaining an incorrect profile for one strain. One laboratory had multiple allele errors in several MLVA profiles and these were probably caused by incorrect or lack of calibration of the measured fragment sizes. Table 4 shows the number of laboratories able to submit the correct MLVA profile per strain.

In less stable loci: STTR5, STTR6 or STTR10 [18], the reporting of one repeat change was evaluated as an acceptable result. For one of the EQA-strains, strain ID 19, the STTR6 locus seemed to have changed immediately before shipment resulting in the presence of two alleles in some of the culture vials. This is clear from the variability in results obtained for this locus of strain 19 (data not shown). Both alleles were evaluated as correct.

Discussion

The EQA schemes for typing of *Salmonella*, VTEC and *Listeria* organised for the NPHR-Ls in the EU/EEA were the first ones specifically including globally agreed molecular typing methods.

Table 2

Average pulsed-field gel electrophoresis (PFGE) gel quality scores of laboratories participating in a typing external quality assessment (EQA), by parameters and pathogen, European Union/European Economic Area, 2012–2013

Parameters	Conditions for excellent score	*Salmonella* (n = 25)	VTEC (n = 20)	*Listeria* (n = 17)
Image acquisition and running conditions	Wells included, bottom band 1.5 cm from edge Spacing of standard match global standards	2.6	2.2	2.1
Cell suspension	Even distribution of DNA	3.9	3.5	3.8
Bands	Clear and distinct bands	2.9	2.2	2.5
Lanes	Straight lanes	3.7	3.6	3.8
Restriction	Complete restriction in all lanes	3.6	3.2	3.5
Gel background	Clear background	3.3	2.9	3.2
DNA degradation	No degradation	3.3	3.1	3.2

VTEC: verocytotoxin-producing *Escherichia coli*.
The scores 1 (poor), 2 (fair), 3 (good), and 4 (excellent) were given according to the TIFF Quality Grading Guidelines [23-25].

Evaluation of the PFGE gel quality showed that the laboratories generally obtained acceptable scores ('fair' or above) for the parameters 'cell suspension', 'lanes', 'restriction', 'gel background', and 'DNA degradation'. These parameters were therefore not the most problematic, but it is still desirable to improve the laboratories' capacity in these areas. However, many laboratories had problems with the critical parameter 'image acquisition and running conditions' as well as the parameter 'bands'. Incorrect running conditions will make it impossible to compare the PFGE profiles with profiles from others gels. It is important to ensure that the running conditions (switch time, buffer temperature, gel material etc.) are as described for the relevant organism, as these vary significantly between species. Generally, the *Salmonella* and *Listeria* gels had a higher quality than the VTEC gels. This is probably due to the fact that PFGE is a less used method in laboratories specialised in VTEC.

Many laboratories seemed to increase the contrast at image acquisition in order to enhance weak bands. Unfortunately, that resulted in thicker bands and made it hard to distinguish double bands. This, together with overloading plugs with DNA, mostly contributed to the low scores for the parameter 'bands'. In general, it is highly recommendable to take the time to get familiar with the image acquisition equipment and ensure its maintenance as well as the maintenance of the electrophoresis equipment. Several laboratories probably produced a high quality gel, but failed to document this due to poor image capturing.

The grading guidelines indicate that the score 'fair' can be obtained for the parameter 'image acquisition and running conditions' even when the band spacing of the standard does not match the global standard. In such cases, the score depends on other criteria included in the evaluation of this parameter. This is, however, inappropriate as it gives the impression that a gel that cannot be normalised correctly is still acceptable. In this EQA, some of the gels that obtained the score 'fair' for all parameters were not suitable for inter-laboratory comparison. Therefore, in the coming EQAs the scoring system will be modified to ensure that a gel with such severe quality deficiencies, that it is impossible to reliably compare with gels obtained in other laboratories, is scored ´poor´ in the relevant parameters. In general, an acceptable quality should be obtained for each parameter since a low quality score in just one parameter can have a high impact on the ability to further analyse the image and compare it with other profiles.

On average, 65% (40/62) of laboratories that performed PFGE on the different pathogens conducted also the subsequent gel analysis, i.e. the normalisation and band assignment that provides the actual PFGE profiles for comparison. This analysis requires the use of a specialised software, BN, which some laboratories might not have access to or limited experience with for PFGE analysis. However, to be able to carry out national surveillance and submit profiles to TESSy, it is important to have the capacity to analyse and interpret the PFGE gels, as submission of raw TIFF images to TESSy is not possible. Correct normalisation of the gel is critical for inter-laboratory comparability. The ability to normalise a gel according to an international standard depends on the use of standard running conditions and reference strains (as evaluated by the scoring parameter 'image acquisition and running conditions' from the TIFF Quality Grading Guidelines) as well as the correct use of the reference lanes for normalisation in the further analysis of the gel using the BN software. Standardisation of band assignment is difficult since the ability to recognise and distinguish bands (e.g. the presence of double bands, weak bands, etc.) is highly dependent on gel quality. In these EQAs, focus was on increasing the laboratories' ability to produce high quality PFGE gels that can be normalised and compared when submitted to a shared database. The participants' ability to assign bands on their produced gels was also evaluated; however, the large variability

Table 3

Average gel analysis quality scores of laboratories participating in a typing external quality assessment (EQA), by parameter and pathogen, European Union/European Economic Area, 2012–2013

Parameters	Conditions for excellent score	*Salmonella* (n = 14)[a]	VTEC (n = 12)	*Listeria* (n = 13)
Position of gel	Placement of gel in the frame, inverted	3.5	3.5	3.1
Strips	All lanes correctly defined	4.0	3.8	3.5
Curves	1/3 of the lanes is used for averaging of curve thickness	3.6	3.4	3.5
Normalisation	All bands (incl. below 33kb) assigned correctly in all reference lanes	3.4	2.8	3.2
Band assignment	Bands assigned correctly according to gel quality	3.3	3.3	3.3

The average score of the participating laboratories is presented for each pathogen, and for each of the five parameters. The scores 1 (poor), 2 (fair), 3 (good), and 4 (excellent) were given according to the BioNumerics gel analysis Quality Guidelines [23-25].

[a] For *Salmonella* 15 laboratories analysed their gels, however one laboratory's submission was excluded due to incompatibility between the BN versions 6.0 and 7.0.

in gel quality across laboratories made it difficult to classify profiles into definitive types. Therefore, we did not evaluate the performance in relation to participants' ability to assign a standard nomenclature. The main goal was to obtain a sufficient gel quality and normalisation for comparison in a centralised and curated database, where the nomenclature is centrally assigned by the curators of the database. In future EQAs, the ability to perform standardised band assignment could be evaluated by providing images of high quality PFGE gels to the participants. One of the challenges for standardisation within EU is that standard protocols can only be recommended. In order to include as many laboratories as possible, ECDC decided that it was not possible to make protocols mandatory for this EQA. In the public health sector within the EU there are no obligatory methods when uploading to TESSy, in contrast to other networks such as PulseNet in the US where the use of standardised methods are mandatory.

Fifteen laboratories participated in the MLVA part of the EQA, which consisted of typing ten strains of *S.* Typhimurium including monophasic variants of this serovar. Of the 15 laboratories, nine typed all MLVA strains correctly and an additional four reported correct MLVA profiles for nine strains. One laboratory had major problems with the correct allele calling. Except for this one laboratory that seemed to have general problems with the calibration of fragment sizes, most other errors were related to overlooking the presence of a locus (reporting as absent allele where a fragment should have been detected) or reporting an allele number for an absent locus. This can be due to the use of an unbalanced primer mix resulting in variability in peak heights and thereby either missing a peak or misidentifying background noise for a signal. Another explanation can be that the samples for capillary electrophoresis were overloaded, which can cause large peaks to pick up other primer dyes used in the mix and thereby be mistaken for a peak representing another locus.

One of the EQA test strains had a mix of alleles in the cultures sent to at least some of the laboratories. Three laboratories were impressively able to find both alleles and submit the results. For a highly discriminatory method like MLVA, there is always a risk of changes occurring in the strains during the transport and culturing before testing. In general, changes only occur in the fast changing loci STTR5, STTR6 and STTR10 and changes in these loci were therefore accepted when evaluating the results of this EQA. To our knowledge, several of the laboratories participating in the EQA are not performing the MLVA method on a routine basis and we therefore expect that the performance could be even higher with more experience.

This first comprehensive EQA scheme on molecular typing for NPHR-Ls in the EU/EEA provides invaluable information for the development of molecular typing-based surveillance of food-borne infections and gradual implementation of molecular typing in the routine surveillance at the EU level.

The results showed high variation of the typing capabilities between the laboratories, but the results also varied depending on the pathogens and methods. The MLVA results were reassuring with more than half of the laboratories providing correct results for all strains and most of the problems reported were errors in single loci. Mistakes in MLVA profiles submitted to TESSy will have a direct impact on the possibility of detecting clusters as MLVA results are not curated, but used directly for cluster detection and case definition. More MLVA profiles than PFGE profiles of *S.* Typhimurum are submitted to TESSy. The majority of laboratories participating in the *Salmonella* and *Listeria* EQAs were able to produce PFGE profiles that could be compared with profiles from other laboratories. Less than half of the laboratories participating in the VTEC EQA produced images with acceptable quality for comparison and need further improvements before submitting to

Table 4

MLVA profiles of 10 *Salmonella* Typhimurium strains used in a typing external quality assessment (EQA) and number of laboratories assigning an incorrect, accepted and correct MLVA profile to each strain, European Union/European Economic Area, 2012–2013 (n = 15 participating laboratories)

Strain number	MLVA profile					Result categories of the MLVA analysis with number of laboratories per result category			
	STTR9	STTR5	STTR6	STTR10	STTR3	Incorrect[a]	Accepted[b]	Correct	**Total correct**
11	2	9	15	5	212	2	0	13	**13**
12	3	12	9	NA	211	0	1	14	**15**
13	3	13	NA	NA	211	2	1	12	**13**
14	4	14	12	8	211	0	0	15	**15**
15	3	16	15	23	311	2	1	12	**13**
16	4	18	NA	10	212	1	2	12	**14**
17	3	16	NA	NA	311	1	0	14	**14**
18	3	13	16	14	311	1	0	14	**14**
19	4	8	19 (18)	10	211	2	10	6	**16c**
20	2	17	NA	15	212	1	0	14	**14**

MLVA: multiple-locus variable-number tandem repeat analysis; NA: not applicable (locus not present) [16].

[a] Incorrect profiles have repeat change in one or more loci, the exception being 'accepted profiles'.

[b] Accepted profiles have one repeat change in one of the highly variable loci STTR5, STTR6 or STTR10.

[c] The total number of laboratories for this strain is higher than the number of participating laboratories (n=15) because three laboratories correctly identified two MLVA profiles for strain 19, STTR6: 18 (accepted) /19 (correct).

TESSy. The most common problems were related to the running conditions and use of the image acquisition equipment, which in some cases are easily overcome and related to thick bands. There are no formal requirements regarding the proficiency in PFGE for a NPHR-L to be allowed to submit profiles to TESSy. However, all laboratories submitting to TESSy except one have so far participated in the relevant EQA. The PFGE data in TESSy are curated and poor quality profiles will be marked as 'rejected' and not used in the cluster detection unless linked to an ongoing cross-border outbreak. The data submitter will be notified of rejected profiles, but as new improved data are not always submitted, the EU-wide surveillance is influenced by the sub-optimal performance in PFGE. In general, there is a correlation between poor performance in the EQAs and lower quality of gels submitted to TESSy. Therefore, it is important for the European surveillance of food-borne infections that the laboratories use the feedback from the EQA to improve the quality of the molecular typing used for the national surveillance and for submission to TESSy.

The fact that PFGE is laborious, personnel sensitive, and prone to quality variations warrants the need for identifying more robust and reproducible methodologies for the molecular typing-based surveillance in the future. ECDC supports the standardisation of methods that fulfil the criteria for integration into EU level surveillance and follows closely the rapid development of whole genome sequencing techniques in the international scientific community [30]. However, at this point, PFGE and MLVA are still the most widely used methods for food-borne bacterial pathogens in NPHR-Ls in EU. The continued use of PFGE and MLVA in some countries and the parallel introduction of new sequence based methods in other countries pose a challenge for the EU level surveillance. The support of quality improvement in the laboratory procedures and interpretation of results, e.g. sustaining EQA schemes and training courses, will also be important for the inter-laboratory comparability of typing results in the future.

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Conflict of interest

None declared.

Authors' contributions

Susanne Schjørring has evaluated the data and drafted the initial manuscript. Taina Niskanen has contributed to writing of the manuscript. Mia Torpdahl has assisted in the evaluation of data and has contributed to writing of the manuscript. Jonas Björkman has assisted in the evaluation of data and has contributed to writing of the manuscript. Eva Møller Nielsen has contributed to writing of the manuscript.

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Research article

Serodiagnosis of Zika virus (ZIKV) infections by a novel NS1-based ELISA devoid of cross-reactivity with dengue virus antibodies: a multicohort study of assay performance, 2015 to 2016

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Serological diagnosis of Zika virus (ZIKV) infections is challenging due to high cross-reactivity between flaviviruses. We evaluated the diagnostic performance of a novel anti-ZIKV ELISA based on recombinant ZIKV non-structural protein 1 (NS1). Assay sensitivity was examined using sera from 27 patients with reverse transcription (RT)-PCR-confirmed and 85 with suspected ZIKV infection. Specificity was analysed using sera from 1,015 healthy individuals. Samples from 252 patients with dengue virus (n = 93), West Nile virus (n = 34), Japanese encephalitis virus (n = 25), chikungunya virus (n = 19) or *Plasmodium* spp. (n = 69) infections and from 12 yellow fever-vaccinated individuals were also examined. In confirmed ZIKV specimens collected ≥ 6 days after symptom onset, ELISA sensitivity was 58.8% (95% confidence interval (CI): 36.0–78.4) for IgM, 88.2% (95% CI: 64.4–98.0) for IgG, and 100% (95% CI: 78.4–100) for IgM/IgG, at 99.8% (95% CI: 99.2–100) specificity. Cross-reactivity with high-level dengue virus antibodies was not detected. Among patients with potentially cross-reactive antibodies anti-ZIKV positive rates were 0.8% (95% CI: 0–3.0) and 0.4% (95% CI: 0–2.4) for IgM and IgG, respectively. Providing high specificity and low cross-reactivity, the NS1-based ELISA has the potential to aid in counselling patients, pregnant women and travellers after returning from ZIKV-endemic areas.

Introduction

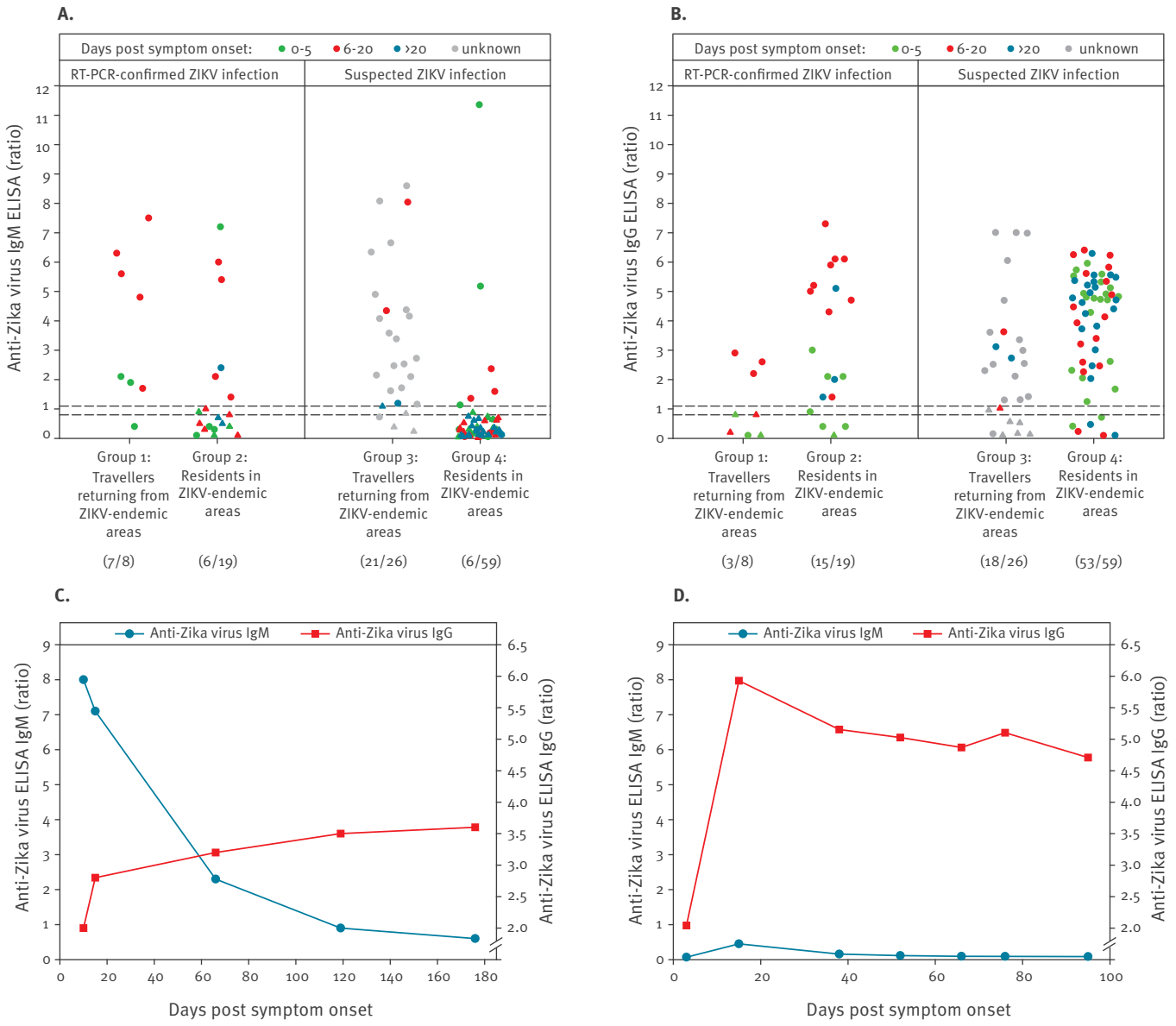
Zika virus (ZIKV) is an emerging mosquito-transmitted flavivirus currently causing large epidemics in South and Central America as well as in the Caribbean, presenting a global public health emergency [1]. It is closely related to other human pathogenic members of the flavivirus family such as dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and yellow fever virus (YFV). Besides their structural resemblance, most of these viruses share a partially overlapping geographical distribution, with tropical and subtropical regions representing the favourable environment of the main vector, mosquitos of the genus *Aedes* [2].

The diagnosis of ZIKV infections is increasingly relevant for European countries where, up to now, only a small number of travellers returning from endemic areas have contracted the virus [3]. However, there are concerns that ZIKV might be imported by infected individuals and spread through sexual transmission and via *Aedes* mosquitos that are endemic in parts of southern Europe.

The clinical symptoms associated with ZIKV infection include fever, rash, arthralgia, myalgia and conjunctivitis, and are normally self-limiting. The proportion of asymptomatic ZIKV infections is still unknown, but there is evidence that infection may go unrecognised

Figure 1

Anti-ZIKV reactivity in patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) ZIKV infection as determined by ELISA for (A) IgM and (B) IgG[a]; time course analysis of anti-ZIKV antibody levels in follow-up samples from (C) a German patient returning from Colombia (probable primary ZIKV infection)[b] and (D) a Colombian patient with RT-PCR-confirmed ZIKV infection (probable secondary flavivirus infection)[c]



RT-PCR: reverse transcription-PCR; US: United States; WHO: World Health Organization; ZIKV: Zika virus.

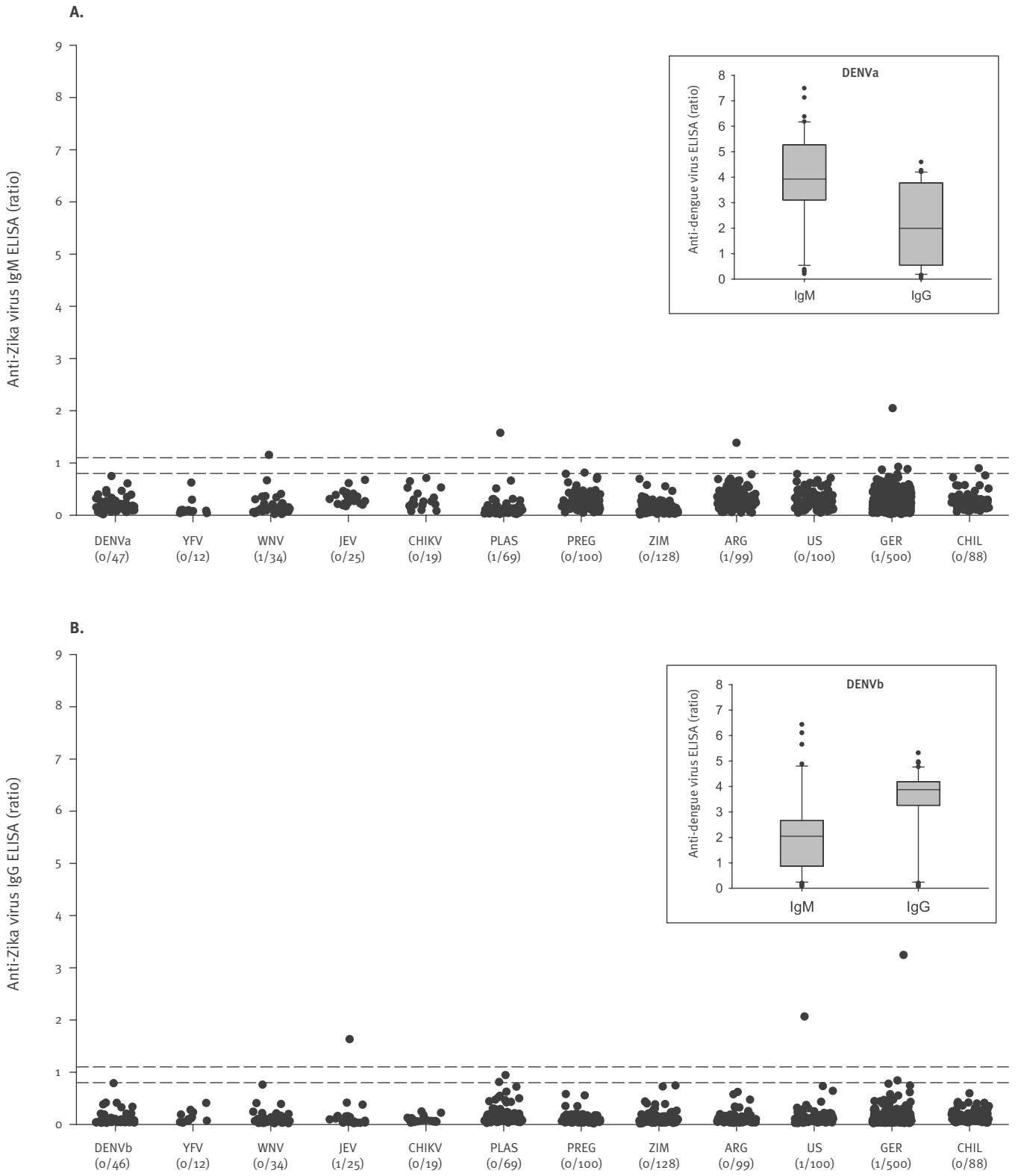
[a] Per patient, one sample was examined for anti-ZIKV IgM and IgG antibodies. Plotted data points represent ratio values (extinctionsample/extinctioncalibrator). Cut-off values for borderline results (≥ 0.8 to < 1.1) and positive results (≥ 1.1) are indicated by horizontal dashed lines. Positive and total cases are indicated in parentheses. Triangles indicate samples with a ratio for anti-ZIKV IgM or IgG below the cut-off (< 1.1), but a corresponding positive result in IgG or IgM testing, respectively.

[b] Samples were provided by the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Hamburg, Germany. Cut-off ratio: ≥ 1.1.

[c] Samples were provided by Biomex US LLC, Coconut Creek, Florida, US. Cut-off ratio: ≥ 1.1.

Figure 2

Anti-ZIKV reactivity in potentially cross-reactive samples (n = 252) and healthy controls (n = 1,015) as determined by ELISA for (A) IgM and (B) IgG[a,b], study evaluating a novel NS1-based ELISA, Germany 2016*



ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; GER: Germany; JEV: Japanese encephalitis virus; NS: non-structural protein; PLAS: Plasmodium; PREG: pregnant women; US: United States; WNV: West Nile virus; YFV: Yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[a] Plotted data points represent ratio values (extinctionsample/extinctioncalibrator); one data point per patient. Cut-off values for borderline results (≥ 0.8 to < 1.1) and positive results (≥ 1.1) are indicated by horizontal dashed lines. Positive and total cases are indicated in parentheses.

[b] To provide high levels of potentially cross-reactive anti-DENV IgM and IgG antibodies, the DENV-infected patients were divided into two groups: DENVa, high median ratio (3.9) anti-DENV IgM, anti-DENV IgM ratio ≥ 3.0 in 79% of cases (inset Panel A); DENVb, high median ratio (3.9) anti-DENV IgG, anti-DENV IgG ratio ≥ 3.0 in 80% of cases (inset Panel B). Cut-off ratio (anti-DENV ELISA, EUROIMMUN): ≥ 1.1.

in a considerable number of cases [1,4]. In the acute phase, fever due to ZIKV infection is difficult to differentiate clinically from that due to DENV infections [5]. Chikungunya virus (CHIKV), belonging to the *Togaviridae* family, should also be considered in differential diagnostics, as it is transmitted by the same mosquito vector and circulates in the same regions [2]. The common distribution and similar clinical presentation, in combination with high variation in disease outcome of ZIKV-, DENV- and CHIKV-infected patients, highlight the need for specific and reliable diagnostic methods. Knowing the infecting virus can be of clinical relevance, for example, when ZIKV infection is suspected in women during pregnancy, which could result in fetal malformations, or in men who could transmit the virus sexually, or, in cases of CHIKV infection with prolonged arthralgias, where correct diagnosis can help avoid unnecessary rheumatological analysis.

The current ZIKV epidemic, particularly in Brazil, has revealed two potential complications in ZIKV infections, which were initially suspected during the 2007 outbreak in Micronesia [6]. Firstly, a large rise in the number of cases of Guillain–Barré syndrome (GBS), an autoimmune disease resulting from damage of peripheral-nerve myelin, was triggered by ZIKV infections [1,7]. Secondly, a strong causative link was suggested between fetal abnormalities and ZIKV infection during early pregnancy, based on a 20-fold increase in newborn microcephaly in highly endemic regions in Brazil, followed by the first reports of ZIKV genome detection in amniotic fluid and fetal brain after intrauterine diagnosis of microcephaly [1,8-10].

At present, diagnosis of ZIKV infections is challenging because the only specific tool is direct virus detection using nucleic acid-based testing (NAT), with ZIKV RNA detectable in serum up to 7 days after symptom onset and even longer in saliva, urine (about 20 days) and semen (>20 days) [6,11-13]. Plaque-reduction neutralisation tests (PRNTs) can measure virus-specific neutralising antibodies, a fact that is relevant in regions where two or more flaviviruses co-occur. However, PRNTs do not discriminate between antibody classes and, especially in secondary flavivirus infections, cross-reactive neutralising antibodies may contribute to virus neutralisation [6,14,15], thus questioning the suitability of PRNTs for the confirmation of active infection. In addition, PRNTs are time-consuming, difficult to perform, not suitable for testing large panels, and therefore restricted to highly specialised laboratories. In contrast, ELISA-based measurement is a rapid, scalable and technically mature approach. IgM antibodies against flavivirus antigens are first produced 4 to 7 days after infection, and IgG antibodies appear a few days later. However, a major limitation of current ELISAs for diagnosing flaviviral infections is their extensive cross-reactivity within the *Flavivirus* genus [6].

The molecular organisation of flaviviruses is conserved. Virions consist of single-stranded positive RNA surrounded by an icosahedral capsid and envelope. The RNA encodes for a single polyprotein, which is processed into structural (C, prM, and E) and non-structural (NS1 to NS5) proteins [16]. Knowledge about NS1 is mainly derived from the well-studied flaviviruses (DENV, WNF, YFV), whereas little is known about NS1 from ZIKV. NS1 proteins (molecular mass 46–55 kDa) are present in two distinct variants [17]. Membrane-associated NS1 is mainly found as a dimer that interacts with intracellular membranes, such as the endoplasmic reticulum and vesicle packets, and with the cell surface [18,19]. Secreted NS1 assembles into barrel-shaped hexamers consisting of three dimers [20,21]. The NS1 function remains elusive, although roles in RNA replication [18], intracellular protein transport, virion release [22] and immunomodulatory activities [17] have been proposed. As reported for DENV and WNV, NS1 is secreted by infected cells into the bloodstream [23,24], stimulating the immune system to produce high NS1 antibody titres. However, acute-phase release of ZIKV-NS1 into patient's serum has not yet been verified, and a ZIKV-NS1 antigen assay is currently not available [25]. Recombinant NS1 proteins were used in a multiplex serological protein microarray for the detection of anti-DENV, -WNV, and -JEV IgM and IgG, demonstrating high sensitivity and limited cross-reactivity, suggesting NS1 may represent an efficient antigenic substrate [26].

Recently, an ELISA based on recombinant ZIKV-NS1 has been developed [27]. Here, the diagnostic performance of this assay was examined using sera from returning travellers and patients from ZIKV-endemic areas with laboratory-confirmed ZIKV infection, potentially cross-reactive samples from patients with flaviviral and other infections, as well as control panels from blood donors of different ages and geographical origin.

Methods

Human sera

The study included serum samples from 27 patients who had tested positive for ZIKV RNA by reverse transcription PCR (RT-PCR); Group 1: travellers returning from endemic areas (n = 8); Group 2: residents in ZIKV-endemic areas (n = 19). On the basis of direct detection of the pathogen's genome, these cases were referred to as having RT-PCR-confirmed ZIKV infection. Samples from a further 85 patients had been pre-characterised by anti-ZIKV indirect immunofluorescence assay (IIFA; EUROIMMUN, Lübeck, Germany) based on whole virus antigen, showing reactivity for anti-ZIKV IgM and/or IgG; Group 3: travellers returning from endemic areas (n = 26); Group 4: residents in ZIKV-endemic areas (n = 59). Since false-positive results due to cross-reactivity of this IIFA cannot be excluded, these cases were referred to as having suspected ZIKV infection (Table 1).

Classification into three stages of ZIKV infection was according to the Pan American Health Organization

Table 1A

Characteristics of patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) Zika virus infection, study evaluating a novel NS1-based ELISA, Germany 2016

Case ID	Age groups in years	Sex	Country of infection	Current/former residence	Sampling Dpso	Phase of infection[a]	Clinical symptoms[b]	Diagnostic centre/ provider of samples	ZIKV-RT-PCR assay/ performed at	ZIKV-RT-PCR result[c]	Virus neutralisation assay titre	IIFA IgM titre[d]	IIFA IgG titre[d]
Group 1: RT-PCR-confirmed ZIKV infection, travellers returning from ZIKV-endemic areas (n = 8)													
1	20–29	M	NA	Non-endemic	7	Active	Yes	WHOCC, Hamburg, Germany	RealStar Zika Virus RT-PCR (Altona Diagnostics, Hamburg, Germany)/ WHOCC	Pos	NA	1:3,200	1:3,200
2	30–39	F	Haiti	Non-endemic	≥ 4	Active	Yes			Pos	NA	1:320	1:32,000
3	50–59	M	NA	Non-endemic	3	Initial	No			Pos	NA	NA	NA
4	50–59	F	NA	Non-endemic	< 4	Initial	NA			Pos	NA	1:100	1:1,000
5	20–29	F	NA	Non-endemic	17	Active	NA	ITM, Antwerp, Belgium	RealStar Zika Virus RT-PCR (Altona Diagnostics, Hamburg, Germany)/ ITM	Pos	> 1:640	NA	NA
6	40–49	M	NA	Non-endemic	11	Active	NA			Pos	1:243	NA	NA
7	0–9	M	NA	Non-endemic	3	Initial	NA			Pos	NA	NA	NA
8	20–29	F	NA	Non-endemic	11	Active	NA			Pos	1:788	NA	NA
Group 2: RT-PCR-confirmed ZIKV infection, residents in ZIKV-endemic areas (n = 19)													
1	60–69	F	Suriname	The Netherlands/Suriname[e]	3	Initial	NA	AMC, Amsterdam, the Netherlands	In-house Zika RT-PCR/ AMC	Pos	NA	NA	NA
2	50–59	M	Suriname	The Netherlands/Suriname[e]	5	Initial	NA			Pos	NA	NA	NA
3	40–49	F	Suriname	The Netherlands/Suriname[e]	11	Active	NA			Pos	NA	NA	NA
4	40–49	M	Suriname	The Netherlands/Suriname[e]	9	Active	NA			Pos	NA	NA	NA
5	50–59	F	Suriname	The Netherlands/Suriname[e]	6	Active	NA			Pos	NA	NA	NA
6	50–59	M	Suriname	The Netherlands/Suriname[e]	6	Active	NA			Pos	NA	NA	NA
7	50–59	F	Suriname	The Netherlands/Suriname[e]	53	Late	NA			Pos	NA	NA	NA
8	50–59	F	Suriname	The Netherlands/Suriname[e]	17	Active	NA			Pos	NA	NA	NA
9	60–69	F	Suriname	The Netherlands/Suriname[e]	24	Late	NA			Pos	NA	NA	NA
10	70–79	M	Suriname	The Netherlands/Suriname[e]	6	Active	NA			Pos	NA	NA	NA
11	0–9	M	Dominican Republic	The Netherlands	1	Initial	NA			Pos	NA	NA	NA
12	50–59	F	Dominican Republic	Dominican Republic	20	Active	Yes	Boca Biolistics, Coconut Creek, Florida, US	Trioplex real-time RT-PCR (CDC, Atlanta, Georgia, US)/CDC	Pos	NA	0	1:32,000
13	50–59	F	Dominican Republic	Dominican Republic	31	Late	Yes			Pos	NA	1:100	1:32,000
14	20–29	M	Colombia	Colombia	3	Initial	Yes	Allied Research Society, Miami Lakes, Florida, US	Trioplex real-time RT-PCR (CDC, Atlanta, Georgia, US)/CDC	Pos	NA	0	1:1,000
15	40–49	F	Colombia	Colombia	5	Initial	Yes			Pos	NA	0	1:1,000
16	50–59	F	Colombia	Colombia	4	Initial	Yes			Pos	NA	1:10	1:3,200
17	10–19	M	Colombia	Colombia	3	Initial	Yes			Pos	NA	0	1:3,200

AMC: Academic Medical Center; CDC: Centers for Disease Control and Prevention; dpso: days post symptom onset; F: female; IIFA: indirect immunofluorescence assay; ITM: Institute of Tropical Medicine, M: male; NA: not available; NS: non-structural protein; Pos: positive; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); ZIKV: Zika virus.

[a] Phase of infection at the time of sample collection: initial phase: ≤ 5 dpso; active phase: 6 to 20 dpso; late phase: > 20 dpso [28].

[b] Fever, skin rash, joint pain, myalgia, headache, conjunctivitis, eye pain, diarrhoea and malaise.

[c] ZIKV-RT-PCR results can also refer to serum or urine samples taken at an earlier date than the samples used for anti-ZIKV serological testing.

[d] IIFA was performed at EUROIMMUN, Lübeck, Germany, using the Anti-Zika Virus IIFA test kit (EUROIMMUN). Cut-off IgM: ≥ 1:10; IgG: ≥ 1:100.

[e] Sera from Dutch residents who were born and raised in Suriname and/or had visited their country of origin occasionally.

Table 1B

Characteristics of patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) Zika virus infection, study evaluating a novel NS1-based ELISA, Germany 2016

Case ID	Age groups in years	Sex	Country of infection	Current/former residence	Sampling Dpso	Phase of infection[a]	Clinical symptoms[b]	Diagnostic centre/ provider of samples	ZIKV-RT-PCR assay/ performed at	ZIKV-RT-PCR result[c]	Virus neutralisation assay titre	IIFA IgM titre[d]	IIFA IgG titre[d]
18	20–29	F	Colombia	Colombia	6	Active	Yes	Biomex GmbH, Heidelberg, Germany	RealStar Zika Virus RT-PCR (Altona Diagnostics, Hamburg, Germany)/ Altona Diagnostics	**Pos**	NA	**1:3,200**	**1:32,000**
19	10–19	M	Colombia	Colombia/US	15	Active	**Yes**		Trioplex real-time RT-PCR (CDC, Atlanta, Georgia, US)/CDC	**Pos**	NA	**1:10**	**1:32,000**
Group 3: Suspected ZIKV infection, travellers returning from ZIKV-endemic areas (n = 26)													
1	NA	NA	NA	Non-endemic	NA	NA	NA	WHOCC, Hamburg, Germany	NA	NA	NA	**1:3,200**	**1:10,000**
2	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:10,000**
3	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:3,200**	**1:10,000**
4	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:32,000**
5	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:3,200**
6	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:3,200**	**1:10,000**
7	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:100**	**<1:100**
8	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:100**
9	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:10,000**
10	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:32,000**
11	NA	NA	Brazil	Non-endemic	19	Active	**Yes**			NA	NA	**1:320**	**1:10,000**
12	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:100**	**1:100,000**
13	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:320**
14	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:3,200**
15	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:1,000**
16	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:10,000**
17	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:10,000**
18	NA	NA	NA	Non-endemic	32	Late	**NA**			NA	NA	**1:100**	**1:32,000**
19	NA	NA	Colombia	Non-endemic	45	Late	**NA**			NA	NA	**1:100**	**1:3,200**
20	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:1,000**	**1:10,000**
21	NA	NA	Denmark	Non-endemic	NA	NA	**NA**			NA	NA	**1:100**	**1:32,000**
22	NA	NA	NA	Non-endemic	NA	NA	**NA**			NA	NA	**1:3,200**	**1:32,000**
23	NA	NA	Colombia	Non-endemic	NA	NA	**NA**			NA	NA	**1:100**	**1:10,000**
24	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:32,000**
25	NA	NA	Brazil	Non-endemic	NA	NA	**NA**			NA	NA	**1:320**	**1:32,000**
26	NA	NA	Colombia	Non-endemic	15	Active	**NA**			NA	NA	**1:3,200**	**1:10,000**

AMC: Academic Medical Center; CDC: Centers for Disease Control and Prevention; dpso: days post symptom onset; F: female; IIFA: indirect immunofluorescence assay; ITM: Institute of Tropical Medicine, M: male; NA: not available; NS: non-structural protein; Pos: positive; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); ZIKV: Zika virus.

[a] Phase of infection at the time of sample collection: initial phase: ≤ 5 dpso; active phase: 6 to 20 dpso; late phase: > 20 dpso [28].

[b] Fever, skin rash, joint pain, myalgia, headache, conjunctivitis, eye pain, diarrhoea and malaise.

[c] ZIKV-RT-PCR results can also refer to serum or urine samples taken at an earlier date than the samples used for anti-ZIKV serological testing.

[d] IIFA was performed at EUROIMMUN, Lübeck, Germany, using the Anti-Zika Virus IIFA test kit (EUROIMMUN). Cut-off IgM: ≥ 1:10; IgG: ≥ 1:100.

[e] Sera from Dutch residents who were born and raised in Suriname and/or had visited their country of origin occasionally.

Table 1c

Characteristics of patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) Zika virus infection, study evaluating a novel NS1-based ELISA, Germany 2016

Case ID	Age groups in years	Sex	Country of infection	Current/former residence	Sampling Dpso	Phase of infection[a]	Clinical symptoms[b]	Diagnostic centre/ provider of samples	ZIKV-RT-PCR assay/ performed at	ZIKV-RT-PCR result[c]	Virus neutralisation assay titre	IIFA IgM titre[d]	IIFA IgG titre[d]
Group 4: Suspected ZIKV infection, residents in ZIKV-endemic areas (n = 59)													
1	30–39	F	Colombia	Colombia	6	Active	Yes	Allied Research Society, Miami Lakes, Florida, US	NA	NA	NA	1:1,000	1:320,000
2	20–29	M	Colombia	Colombia	8	Active	Yes			NA	NA	1:100	1:1,000
3	30–39	F	Colombia	Colombia	11	Active	Yes			NA	NA	0	1:1,000
4	40–49	M	Colombia	Colombia	14	Active	Yes			NA	NA	1:3,200	1:320,000
5	30–39	F	Colombia	Colombia	17	Active	Yes			NA	NA	1:3,200	1:320,000
6	80–89	M	Colombia	Colombia	20	Active	Yes			NA	NA	1:320	1:320,000
7	50–59	F	Colombia	Colombia	23	Late	Yes			NA	NA	1:320	1:10,000
8	30–39	M	Colombia	Colombia	30	Late	Yes			NA	NA	1:3,200	1:320,000
9	40–49	F	Colombia	Colombia	49	Late	Yes			NA	NA	1:100	1:10,000
10	10–19	F	Colombia	Colombia	54	Late	Yes			NA	NA	1:10	1:1,000
11	50–59	F	Colombia	Colombia	6	Active	Yes			NA	NA	0	1:3,200
12	40–49	F	Colombia	Colombia	4	Initial	Yes			NA	NA	0	1:1,000
13	10–19	M	Colombia	Colombia	66	Late	Yes			NA	NA	0	1:32,000
14	40–49	F	Colombia	Colombia	68	Late	Yes			NA	NA	1:10	1:32,000
15	50–59	F	NA	Colombia	70	Late	Yes			NA	NA	0	1:32,000
16	40–49	F	NA	Colombia	2	Initial	Yes			NA	NA	0	1:10,000
17	20–29	F	Colombia	Colombia	7	Active	Yes			NA	NA	1:100	1:320,000
18	50–59	F	NA	Colombia	4	Initial	Yes			NA	NA	1:100	1:100,000
19	40–49	M	Colombia	Colombia	3	Initial	Yes			NA	NA	1:10,000	1:32,000
20	40–49	F	Colombia	Colombia	4	Initial	Yes			NA	NA	1:32	1:32,000
21	30–39	M	Colombia	Colombia	4	Initial	Yes			NA	NA	1:32	1:32,000
22	40–49	F	Colombia	Colombia	4	Initial	Yes			NA	NA	0	1:100,000
23	30–39	M	Colombia	Colombia	4	Initial	Yes			NA	NA	0	1:32,000
24	20–29	F	Colombia	Colombia	5	Initial	Yes			NA	NA	1:10	1:10,000
25	40–49	F	Colombia	Colombia	5	Initial	Yes			NA	NA	1:1,000	1:100,000
26	30–39	F	Colombia	Colombia	3	Initial	Yes			NA	NA	0	1:3,200
27	40–49	F	Colombia	Colombia	4	Initial	Yes			NA	NA	0	1:32,000
28	20–29	F	Colombia	Colombia	3	Initial	Yes			NA	NA	0	1:320
29	50–59	F	Colombia	Colombia	4	Initial	Yes			NA	NA	0	1:10,000
30	20–29	F	Colombia	Colombia	3	Initial	Yes			NA	NA	1:32	1:10,000

AMC: Academic Medical Center; CDC: Centers for Disease Control and Prevention; dpso: days post symptom onset; F: female; IIFA: indirect immunofluorescence assay; ITM: Institute of Tropical Medicine, M: male; NA: not available; NS: non-structural protein; Pos: positive; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); ZIKV: Zika virus.

[a] Phase of infection at the time of sample collection: initial phase: ≤ 5 dpso; active phase: 6 to 20 dpso; late phase: > 20 dpso [28].

[b] Fever, skin rash, joint pain, myalgia, headache, conjunctivitis, eye pain, diarrhoea and malaise.

[c] ZIKV-RT-PCR results can also refer to serum or urine samples taken at an earlier date than the samples used for anti-ZIKV serological testing.

[d] IIFA was performed at EUROIMMUN, Lübeck, Germany, using the Anti-Zika Virus IIFA test kit (EUROIMMUN). Cut-off IgM: ≥ 1:10; IgG: ≥ 1:100.

[e] Sera from Dutch residents who were born and raised in Suriname and/or had visited their country of origin occasionally.

Table 1d

Characteristics of patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) Zika virus infection, study evaluating a novel NS1-based ELISA, Germany 2016

Case ID	Age groups in years	Sex	Country of infection	Current/former residence	Sampling Dpso	Phase of infection[a]	Clinical symptoms[b]	Diagnostic centre/ provider of samples	ZIKV-RT-PCR assay/ performed at	ZIKV-RT-PCR result[c]	Virus neutralisation assay titre	IIFA IgM titre[d]	IIFA IgG titre[d]
31	30–39	F	Colombia	Colombia	3	Initial	Yes	Biomex GmbH, Heidelberg, Germany	NA	NA	NA	0	1:32,000
32	20–29	F	Colombia	Colombia	4	Initial	Yes			NA	NA	1:100	1:32,000
33	10–19	F	Colombia	Colombia	9	Active	Yes			NA	NA	1:100	1:32,000
34	20–29	F	Colombia	Colombia	12	Active	Yes			NA	NA	0	1:32,000
35	10–19	F	Colombia	Colombia	20	Active	Yes			NA	NA	1:100	1:10,000
36	20–29	F	Colombia	Colombia	27	Late	Yes			NA	NA	1:320	1:10,000
37	30–39	F	Colombia	Colombia	36	Late	Yes			NA	NA	1:10	1:32,000
38	10–19	F	Colombia	Colombia	56	Late	Yes			NA	NA	1:100	1:10,000
39	30–39	F	Colombia	Colombia	67	Late	Yes			NA	NA	1:10	1:32,000
40	10–19	F	Colombia	Colombia	2	Initial	Yes	Allied Research Society, Miami Lakes, Florida, US	NA	NA	NA	0	1:10,000
41	30–39	F	Colombia	Colombia	5	Initial	Yes			NA	NA	1:320	1:10,000
42	20–29	F	Colombia	Colombia	6	Active	Yes			NA	NA	1:100	1:10,000
43	20–29	F	Colombia	Colombia	8	Active	Yes			NA	NA	1:100	1:32,000
44	30–39	F	Colombia	Colombia	15	Active	Yes			NA	NA	0	1:10,000
45	20–29	F	Colombia	Colombia	21	Late	Yes			NA	NA	1:10	1:100,000
46	20–29	F	Colombia	Colombia	29	Late	Yes			NA	NA	1:320	1:32,000
47	20–29	F	Colombia	Colombia	38	Late	Yes			NA	NA	1:1,000	1:320,000
48	10–19	F	Colombia	Colombia	50	Late	Yes			NA	NA	1:10	1:10,000
49	20–29	F	Colombia	Colombia	88	Late	Yes			NA	NA	0	1:1,000
50	40–49	F	Colombia	Colombia	2	Initial	Yes			NA	NA	0	1:3,200
51	20–29	M	Colombia	Colombia	5	Initial	Yes			NA	NA	1:1,000	1:32,000
52	30–39	F	Colombia	Colombia	6	Active	Yes			NA	NA	0	1:1,000
53	20–29	M	Colombia	Colombia	8	Active	Yes			NA	NA	0	1:10,000
54	30–39	F	Colombia	Colombia	15	Active	Yes			NA	NA	1:320	1:320,000
55	30–39	M	Colombia	Colombia	21	Late	Yes			NA	NA	1:100	1:32,000
56	40–49	M	Colombia	Colombia	29	Late	Yes			NA	NA	1:32,000	1:32,000
57	40–49	F	Colombia	Colombia	38	Late	Yes			NA	NA	0	1:320
58	50–59	F	Colombia	Colombia	50	Late	Yes			NA	NA	0	1:100,000
59	50–59	M	Colombia	Colombia	85	Late	Yes			NA	NA	0	1:32,000

AMC: Academic Medical Center; CDC: Centers for Disease Control and Prevention; dpso: days post symptom onset; F: female; IIFA: indirect immunofluorescence assay; ITM: Institute of Tropical Medicine, M: male; NA: not available; NS: non-structural protein; Pos: positive; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); ZIKV: Zika virus.

[a] Phase of infection at the time of sample collection: initial phase: ≤ 5 dpso; active phase: 6 to 20 dpso; late phase: > 20 dpso [28].

[b] Fever, skin rash, joint pain, myalgia, headache, conjunctivitis, eye pain, diarrhoea and malaise.

[c] ZIKV-RT-PCR results can also refer to serum or urine samples taken at an earlier date than the samples used for anti-ZIKV serological testing.

[d] IIFA was performed at EUROIMMUN, Lübeck, Germany, using the Anti-Zika Virus IIFA test kit (EUROIMMUN). Cut-off IgM: ≥ 1:10; IgG: ≥ 1:100.

[e] Sera from Dutch residents who were born and raised in Suriname and/or had visited their country of origin occasionally.

(PAHO) /World Health Organization (WHO) recommendations on ZIKV surveillance in the Americas [28]: ≤5 days post symptom onset, initial stage; 6–20 days post symptom onset, active stage; >20 days post symptom onset, late stage. Samples from travellers returning from endemic areas were provided by the diagnostic institutes (listed in Table 1) to which they had been sent for routine diagnostic testing. Samples from patients residing in Latin America (i.e. Dominican Republic and Colombia) were purchased from Boca Biolistics (Coconut Creek, Florida, United States (United States (US)), Allied Research Society (Miami Lakes, Florida, US) and Biomex GmbH (Heidelberg, Germany). As confirmed by these institutes and companies, written informed consent had been obtained from all patients, and there were no legal or ethical restrictions to using the samples.

To evaluate cross-reactivity, samples were used from 252 patients with either a post-YFV vaccination status (n = 12), or with other flaviviral (DENV = 93; WNV = 34, JEV = 25), non-flaviviral (CHIKV = 19) and *Plasmodium* spp. (PLAS: n = 69) infections. In samples from DENV-infected patients, the confirmation of DENV as the infectious agent was based on NS1 antigen detection [28]. Sera from 1,015 healthy individuals (pregnant women, blood donors and children) living in flavivirus-endemic and non-endemic areas served as negative controls. Pre-characterisation data for all control cohorts are reported in Table 2. To the best of the authors' knowledge, none of these samples were analysed in previous studies.

Specimens, anamnestic/clinical information and pre-characterisation data were provided anonymised to the Institute for Experimental Immunology (affiliated to EUROIMMUN). All sera were stored at -20 °C until assayed. The study was performed according to the recommendations of the Central Ethical Committee of Germany [29].

Enzyme-linked immunosorbent assays

Anti-Zika Virus IgM and IgG ELISA (EUROIMMUN) were used as recommended by the manufacturer. These kit assays are based on standardised reagents and microtitre plates coated with recombinant ZIKV-NS1. Briefly, sera diluted 1:101 in sample buffer were added to the wells and allowed to react for 60 min at 37 °C. Before IgM detection, sera were pre-incubated with sample buffer containing IgG/rheumatoid factor (RF) absorbent (EUROIMMUN) to remove class IgG antibodies and class IgM RF from the sample. This step prevents specific IgG from displacing IgM from the antigen (leading to false IgM-negative results) and RF-IgM from reacting with specifically bound IgG (leading to false IgM-positive results). Bound antibodies were detected by applying goat anti-human IgM peroxidase conjugate or rabbit anti-human IgG peroxidase conjugate for 30 min at room temperature, followed by staining with tetramethylbenzidine for 15 min. The enzymatic reaction was stopped by addition of one volume 0.5 mol/L sulphuric acid. A calibrator (chicken–human chimeric ZIKV antibody with a concentration adjusted to give an extinction value defining the upper limit of the reference range of non-infected persons) as well as positive and negative controls were provided with the test kit and assayed with each test run. Colour intensity of the enzymatic reactions was determined photometrically at 450 nm (reference 620 nm), resulting in extinction values. A signal-to-cut-off ratio (extinctionsample/extinctioncalibrator) was calculated for each sample.

Receiver-operating characteristics (ROC) analysis based on the initial validation dataset of positive and negative samples was done by the manufacturer to evaluate assay performance at each possible cut-off, demonstrating optimal sensitivity and specificity at ratio values of 0.8 (IgM) and 0.6 (IgG). To ensure high specificity, the borderline range (≥0.8 to<1.1) was established between the highest negative and the lowest positive validation sample, resulting in a positivity cut-off of ≥1.1.

Anti-dengue Virus IgM and IgG ELISA (EUROIMMUN) were used.

Statistics

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, California, US) and SigmaPlot 13.0 (SSI, San Jose, California, US). Sensitivity was calculated as the proportion of ZIKV patients (referring to groups 1 to 4 as indicated) identified as positive by the assay. Specificity was calculated as the proportion of negative test results obtained among healthy controls. We calculated 95% confidence intervals (CIs) according to the modified Wald method. The study was performed in compliance with the Standards for Reporting of Diagnostic accuracy (STARD) statement [30].

Results

Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of the novel NS1-based anti-ZIKV ELISA was evaluated in sera from 27 patients with RT-PCR-confirmed ZIKV infection that had been sub-grouped into travellers returning from ZIKV-endemic areas and endemic-area residents. Among eight infected travellers returning from ZIKV-endemic areas (group 1), positive anti-ZIKV IgM and IgG reactivity was found in seven (87.5%) and three (37.5%) cases, respectively. Of 19 infected residents in endemic-areas (group 2), six (31.6%) were positive for anti-ZIKV IgM and 15 (79.0%) for IgG. In addition, sera from 85 patients with suspected ZIKV infection were examined. Here, of 26 infected travellers returning from ZIKV-endemic areas (group 3) 21 (80.8%) were positive for anti-ZIKV IgM and 18 (69.2%) for IgG, while among 59 infected residents in endemic-areas (group 4), six (10.2%) showed positive reactivity for anti-ZIKV IgM and 53 (89.9%) for IgG. For the total of RT-PCR-confirmed and suspected

Table 2a

Characteristics of control cohorts, study evaluating a novel NS1-based ELISA, Germany 2016

Cohort	n	Origin of sample donors	Type	Diagnostic centre (provider of samples)	Sample receipt	Precharacterisation
Flavivirus infection or vaccination						
DENVa (high IgM)	47	Germany, Italy	Returning travellers from endemic areas with DENV infection (contracted e.g. in Brazil, Bali, Thailand, Laos, Philippines, India, Cambodia, Taiwan)	MVZ Diamedes GmbH Bielefeld, Germany; University of Bologna, Bologna, Italy; WHOCC, Hamburg, Germany	2011–2014	• Panbio or BIO-RAD DENV-NS1 ELISA[a,b]: 47/47 (100%) DENV-NS1 positive • DENV-RT-PCR (only 8/47 tested)[b]: n = 4 subtype DENV-1, n = 2 subtype DENV-2, n = 2 subtype DENV-3 • EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 40/47 (85%) anti-DENV IgM positive, 30/47 (64%) anti-DENV IgG positive, 37/47 (79%) anti-DENV IgM ratio ≥ 3.0, 10/47 (21%) anti-DENV IgM ratio < 3.0, anti-DENV IgM median ratio = 3.9
DENVb (high IgG)	46	Germany, Italy	Returning travellers from endemic areas with DENV infection (contracted e.g. in Brazil, Bali, Thailand, Laos, Philippines, India, Cambodia, Taiwan)	MVZ Diamedes GmbH Bielefeld, Germany; University of Bologna, Bologna, Italy	2011–2014	• DENV-NS1 ELISA[a,b]: 46/46 (100%) DENV-NS1 positive • DENV-RT-PCR (only 1/46 tested)[b]: n = 1 subtype DENV-4 • EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 35/46 (76%) anti-DENV IgM positive, 40/46 (87%) anti-DENV IgG positive, 37/46 (80%) anti-DENV IgG ratio ≥ 3.0, 9/46 (20%) anti-DENV IgG ratio < 3.0, • anti-DENV IgG median ratio = 3.9
YFV	12	France	Individuals vaccinated against YFV	Cerba Specimen Services, Saint-Ouen l'Aumône, France	2015	• YFV seroneutralisation test[d]: 12/12 (100%) anti-YFV positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 0/12 (0%) anti-WNV IgM positive, 0/12 (0%) anti-WNV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/12 (0%) anti-CHIKV IgM positive, 1/12 (8%) anti-CHIKV IgG positive
WNV	34	US	Patients from endemic areas with WNV infection	MAYO Clinic, Scottsdale, Arizona, US	2014	• WNV PRNT[e]: 34/34 (100%) anti-WNV positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 23/34 (68%) anti-WNV IgM positive, 26/34 (76%) anti-WNV IgG positive
JEV	25	Vietnam	Patients from endemic areas with JEV infection	National Hospital of Tropical Disease, Hanoi, Vietnam	2016	• DRG JE IgM capture ELISA[f]: 25/25 (100%) anti-JEV IgM positive • EUROIMMUN Anti-JEV ELISA (IgM, IgG)[c]: 25/25 (100%) anti-JEV IgM positive, 19/25 (76%) anti-JEV IgG positive
Non-flavivirus infection						
CHIKV	19	Réunion	Patients from endemic areas with CHIKV infection	Cerba Specimen Services, Saint-Ouen l'Aumône, France	2015	• CHIKV VRP neutralisation test[g]: 19/19 (100%) anti-CHIKV positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/19 (0%) anti-CHIKV IgM positive, 19/19 (100%) anti-CHIKV IgG positive
Parasite infection						
PLAS	69	France (including overseas department and region Mayotte), French Guiana, Tunisia, Madagascar, Switzerland	Blood donors living in and travellers returning from *Plasmodium*-endemic areas, acute or past *Plasmodium* infection	TheBindingSite, Schwetzingen, Germany Cerba Specimen Services, Saint-Ouen l'Aumône, France Swiss Red Cross, Bern, Switzerland	2016	• BioMérieux Plasmodium IFA (IgM, IgG)[d,h]: 1/15 (7%) anti-*Plasmodium* IgM positive, 15/15 (100%) anti-*Plasmodium* IgG positive • BIO-RAD Malaria ELISA (IgG)[i]: 54/54 (100%) anti-*Plasmodium* positive

ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; IFA: immunofluorescence assay; GER: Germany; JEV: Japanese encephalitis virus; IIFA: indirect immunofluorescence assay; NA: not available; PLAS: *Plasmodium*; PREG: pregnant women; PRNT: plaque reduction neutralisation test; RT-PCR: reverse transcription-PCR; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[a] Performed at MVZ Diamedis GmbH, Bielefeld, Germany.

[b] Performed at the University of Bologna, Italy.

[c] Performed at EUROIMMUN, Lübeck, Germany.

[d] Performed at Cerba Specimen Services, Saint-Ouen l'Aumône, France.

[e] Performed at the University of Leipzig, Germany.

[f] Performed at the National Hospital of Tropical Disease, Hanoi, Vietnam.

[g] Performed at the University of Bonn, Germany.

[h] Performed at TheBindingSite, Schwetzingen, Germany.

[i] Performed at the Swiss Red Cross, Bern, Switzerland.

Table 2b

Characteristics of control cohorts, study evaluating a novel NS1-based ELISA, Germany 2016

Cohort	n	Origin of sample donors	Type	Diagnostic centre (provider of samples)	Sample receipt	Precharacterisation
Healthy controls: pregnant women, blood donors and children						
PREG	100	Germany	Pregnant women from non-flavivirus endemic areas without clinical symptoms	Laboratory Schottdorf, Augsburg, Germany	2007	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 2/100 (2%) anti-DENV IgM positive, 7/100 (7%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c] 3/100 (3%) anti-WNV IgM positive, 4/100 (4%) anti-WNV IgG positive • EUROIMMUN Anti-JEV ELISA (IgM, IgG)[c]: 2/100 (2%) anti-JEV IgM positive, 14/100 (14%) anti-JEV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-CHIKV IgM positive, 0/100 (0%) anti-CHIKV IgG positive
ZIM	128	Zimbabwe	Blood donors from flavivirus and parasite endemic areas without clinical symptoms	National Blood Transfusion Service, Zimbabwe, Africa	2003	• EUROIMMUN Anti-DENV ELISA (IgG)[c]: 4/128 (3%) anti-DENV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgG)[c]: 3/128 (2%) anti-CHIKV IgG positive • EUROIMMUN Anti-Plasmodium ELISA (IgG)[c]: 36/128 (28%) anti-*Plasmodium* IgG positive
ARG	99	Argentina	Blood donors from flavivirus endemic areas without signs of viral infection (routine samples for parasitology)	IACA Laboratory, Buenos Aires, Argentina	2014	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 2/99 (2%) anti-DENV IgM positive, 4/99 (4%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 2/99 (2%) anti-WNV IgM positive, 3/99 (3%) anti-WNV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 3/99 (3%) anti-CHIKV IgM positive, 1/99 (1%) anti-CHIKV IgG positive • EUROIMMUN Anti-Trypanosoma ELISA (IgM, IgG)[c]: 2/99 (2%) anti-Trypanosoma IgM positive, 1/99 (1%) anti-Trypanosoma IgG positive
US	100	US	Blood donors without clinical symptoms (n): Hispanic (25), African American (30), Caucasian (43), Asian (1), Colombian (1)	Serologix, New Hope, Pasadena, US	2014	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 1/100 (1%) anti-DENV IgM positive, 6/100 (6%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-WNV IgM positive, 4/100 (4%) anti-WNV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-CHIKV IgM positive, 4/100 (4%) anti-CHIKV IgG positive
GER	500	Germany	Blood donors from non-flavivirus endemic areas without clinical symptoms	University Medical Center Schleswig-Holstein, Campus Lübeck, Lübeck, Germany	2012	NA
CHIL	88	Germany	Children (≤10 years) form non-flavivirus endemic areas without clinical symptoms	Praxis Dr Fischer-Wassels, Dortmund, Germany	2007–2008	• EUROIMMUN Anti-DENV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-DENV IgM positive, 0/100 (0%) anti-DENV IgG positive • EUROIMMUN Anti-WNV ELISA (IgM, IgG)[c]: 1/100 (1%) anti-WNV IgM positive, 0/100 (0%) anti-WNV IgG positive • EUROIMMUN Anti-JEV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-JEV IgM positive, 0/100 (0%) anti-JEV IgG positive • EUROIMMUN Anti-CHIKV ELISA (IgM, IgG)[c]: 0/100 (0%) anti-CHIKV IgM positive, 0/100 (0%) anti-CHIKV IgG positive

ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; IFA: immunofluorescence assay; GER: Germany; JEV: Japanese encephalitis virus; IIFA: indirect immunofluorescence assay; NA: not available; PLAS: *Plasmodium*; PREG: pregnant women; PRNT: plaque reduction neutralisation test; RT-PCR: reverse transcription-PCR; US: United States; WHOCC: World Health Organization Collaborating Centre (for Arbovirus and Haemorrhagic Fever Reference and Research); WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[c] Performed at EUROIMMUN, Lübeck, Germany.

cases, the combined ELISA sensitivity (IgM and/or IgG) amounted to 23/27 (85.2%) and 78/85 (91.8%), respectively.

Confining the time point of serological evaluation to the active and late phase of ZIKV infection, i.e. ≥ 6 days after symptom onset, anti-ZIKV IgM reactivity was observed in 10/17 (58.8%) patients with positive ZIKV-RT-PCR and 3/38 (7.9%) patients with suspected ZIKV infection, while anti-ZIKV IgG was detectable in 15/17 (88.2%) and 34/38 (89.5%) cases, respectively. Thus, the combined sensitivity (IgM and/or IgG) reached 17/17 (100%) among RT-PCR-confirmed cases and 34/38 (89.5%) among suspected cases (Table 3).

Comparing ZIKV-infected travellers returning from endemic areas (groups 1 and 3) with infected residents in these areas (groups 2 and 4), a tendency of distinct ZIKV antibody kinetics could be observed: in most returning travellers, high IgM ratio values (median 5.6; interquartile range (IQR): 4.6–6.9,) and moderate IgG ratios (median 2.2; IQR 0.9–2.8,) were detectable in the active phase of infection (cut-off ratio: 1.1). By contrast, the majority of endemic-area residents had infections with very high IgG ratios (median 4.8; IQR 3.3–5.9) during the active phase, while IgM ratios were variable, but predominantly negative or low (median 0.5; IQR 0.2–1.3) (Figure 1A and 1B).

Time course analysis of a German patient who showed clinical symptoms after returning from a stay in Colombia revealed very high anti-ZIKV IgM ratios on first testing (day 10 after symptom onset), while IgG ratios increased to moderate levels during the acute phase of infection and thereafter (Figure 1C). On the other hand, follow-up samples taken from a Colombian resident with RT-PCR-confirmed ZIKV infection indicated a significant rise in the ZIKV-specific IgG response between days 3 and 15 after symptom onset, followed by a slow decrease, while anti-ZIKV IgM was negative 3 days after symptom onset and remained below detection threshold for 14 weeks (Figure 1D).

Cross-reactivity of the enzyme-linked immunosorbent assay

Cross-reactivity was analysed first in sera from 93 DENV-infected patients whose diagnosis had been secured by positive DENV-NS1 detection. This cohort was divided into one group (DENVa) with high anti-DENV IgM (median ratio 3.9) and another group (DENVb) with high anti-DENV IgG (median ratio 3.9), ensuring the presence of high levels of potentially cross-reactive antibodies. In both groups, anti-ZIKV reactivity was below the threshold, indicating absence of cross-reactivity in these specimens. Further testing, on a supplementary basis, included 159 sera from patients positive for IgM and/or IgG against YFV, WNV, JEV, CHIKV or PLAS. Anti-ZIKV IgM was positive in 1/34 (2.9%) patients infected with WNV and 1/69 (1.4%) patients infected with PLAS. Anti-ZIKV IgG was found in 1/25 (4.0%) patients infected with JEV (Figure 2). For the total of 252 potentially cross-reactive samples, the overall positivity rate amounted to 2/252 (0.8%) for IgM and 1/252 (0.4%) for IgG (Table 4).

Specificity of the enzyme-linked immunosorbent assay

Assay specificity was assessed by testing 1,015 sera from healthy controls. Only 1/99 (1.0%) Argentinian and 1/500 (0.2%) German blood donors were found anti-ZIKV IgM positive, while all 128 Zimbabwean and 100 US American blood donors as well as 100 German pregnant women and 88 children in Germany were negative. Anti-ZIKV IgG was present in 1/100 (1.0%) US American and 1/500 (0.2%) German blood donors, but absent in the cohorts of Zimbabwean and Argentinian blood donors, pregnant women and children. Thus, overall specificity amounted to 99.8% for either Ig class (Table 4, Figure 2).

Discussion

The serological diagnosis of ZIKV infections has been challenging due to cross-reactions with other flaviviruses, secondary infections and previous vaccinations, which complicate interpretation, sometimes leading to unreliable or false-positive results [6,31,32]. Here, we evaluated a newly-developed ELISA with recombinant ZIKV-NS1 protein as solid-phase antigen. Huzly et al. recently provided evidence that this assay is highly specific, as demonstrated on a limited number of European patients with DENV, YFV, tick-borne encephalitis virus (TBEV) or hepatitis C virus infection [27]. In the present study, testing on specimens collected ≥ 6 days after onset of symptoms (i.e. after the viraemic phase) revealed a combined sensitivity (IgM/IgG) of 100% for RT-PCR-confirmed cases of ZIKV infection at 99.8% specificity. Among suspected ZIKV cases, the combined sensitivity amounted to 89.5%. Notably, we included only one serum sample for each of the studied patients in our analysis, except for the time course analysis. For the serological diagnosis of patients, however, the evaluation of follow-up samples is important and recommended to demonstrate seroconversion or a 4-fold increase in antibody titre [28]. In four of 27 RT-PCR-confirmed ZIKV cases, samples were negative for both IgM and IgG against ZIKV-NS1, presumably because all of them were taken only ≤ 4 days after symptom onset, i.e. when antibodies had not yet reached detectable levels. Among 85 suspected ZIKV patients, too early sampling may account for two cases with negative IgM and IgG, while the remaining five double-negative cases could be due to the absence of ZIKV infection (deficits in pre-characterisation) or to false-negative results.

Cross-reactivity with high-level DENV antibodies was not detectable and, according to preliminary analysis with a limited amount of samples, there was no indication for DENV serotype-dependent differences in cross-reactivity (data not shown). To better judge assay performance in endemic areas, samples from endemic residents who experienced multiple DENV (and other

Table 3

Anti-ZIKV reactivity in patients with RT-PCR-confirmed (n = 27) and suspected (n = 85) ZIKV infection as determined by ELISA for IgM and IgG, study evaluating a novel NS1-based ELISA, Germany 2016

Group	Characteristics		Anti-ZIKV ELISA reactivity (≥ 1 day post symptom onset)[c]				Anti-ZIKV ELISA reactivity (≥ 6 days post symptom onset)[d,e]			
			n	IgM	IgG	IgM/IgG	n	IgM	IgG	IgM/IgG
1	RT-PCR-confirmed ZIKV infection, travellers returning from ZIKV-endemic areas	Positive	8	7	3	7	5	5	3	5
		Sensitivity %[b] (95% CI)	–	87.5 (50.8–99.9)	37.5 (13.5–69.6)	87.5 (50.8–99.9)	–	100 (51.1–100)	60.0 (22.9–88.4)	100 (51.1–100)
2	RT-PCR-confirmed ZIKV infection, residents in ZIKV-endemic areas[a]	Positive	19	6	15	16	12	5	12	12
		Sensitivity %[b] (95% CI)	–	31.6 (15.2–54.2)	78.9 (56.1–92.1)	84.2 (61.6–95.3)	–	41.7 (19.3–68.1)	100 (71.8–100)	100 (71.8–100)
Total 1 + 2	RT-PCR-confirmed ZIKV infection	Positive	27	13	18	23	17	10	15	17
		Sensitivity %[b] (95% CI)	–	48.1 (30.7–66.0)	66.7 (47.7–81.5)	85.2 (66.9–94.7)	–	58.8 (36.0–78.4)	88.2 (64.4–98.0)	100 (78.4–100)
3	Suspected ZIKV infection, travellers returning from ZIKV-endemic areas	Positive	26	21	18	25	NA[e]			
		Sensitivity %[b] (95% CI)	–	80.8 (61.7–92.0)	69.2 (49.9–83.7)	96.2 (79.6–100)				
4	Suspected ZIKV infection, residents ZIKV-endemic areas	Positive	59	6	53	53	38	3	34	34
		Sensitivity %[b] (95% CI)	–	10.2 (4.4–20.8)	89.9 (79.2–95.6)	89.9 (79.2–95.6)	–	7.9 (2.0–21.5)	89.5 (75.3–96.4)	89.5 (75.3–96.4)
Total 3 + 4	Suspected ZIKV infection	Positive	85	27	71	78	38[e]	3	34	34
		Sensitivity %[b] (95% CI)	–	31.8 (22.8–42.3)	83.5 (74.1–90.1)	91.8 (83.7–96.2)	–	7.9 (2.0–21.5)	89.5 (75.3–96.4)	89.5 (75.3–96.4)

CI: confidence interval; NA: not available or not applicable; NS: non-structural protein; RT-PCR: reverse transcription-PCR; ZIKV: Zika virus.

[a] This group contains 10 sera from residents of the Netherlands who were born and raised in Suriname and/or had visited their country of origin occasionally.

[b] Referring to the total number of samples in the respective patient group during the indicated sampling period.

[c] Referring to the whole study population of ZIKV-infected patients, i.e. samples (one per patient) taken between day 1 and day 88 post symptom onset, representing the initial (day 1–5 post symptom onset), active (day 6–20) and late phase (> 20 days) of infection.

[d] Samples (one per patient) taken between day 6 and day 88 post symptom onset, representing the active (day 6 to 20 post symptom onset) and late phase (> 20 days post symptom onset) of infection [28].

[e] Group 3 is not represented in the sampling period ≥ 6 days post symptom onset, because the sampling date was available for only four out of a total of 26 samples in this group.

flavivirus) infections should be included in further assessments, as these samples have a potential for increased cross-reactivity. Future studies should also address a comparison of cross-reactivity with acute vs convalescent anti-DENV-positive samples, considering that the extent of cross-reactivity may be influenced by the level of circulating DENV-NS1 antigen-binding DENV-NS1 antibodies. Analysis of all potentially cross-reactive specimens resulted in positive rates of 0.8% (IgM) and 0.4% (IgG) caused by one case each with WNV and PLAS with low-level anti-ZIKV IgM and one JEV case with low-level anti-ZIKV IgG. In these cases, however, double infections cannot be excluded, so it remains unclear if ELISA positivity resulted from the presence of ZIKV antibodies due to co-infection with ZIKV (true-positive) or from cross-reactivity (false-positive). In case of PLAS infection, PLAS-induced polyclonal B-cell activation may cause the production of potentially cross-reactive antibodies [33]. Among patients with current PLAS infection, up to 30% false-positive or borderline reactions were reported using the presented NS1-based ELISA [34], which is in contrast to only 1.4% in the present study and probably explained by the fact that our cohort was comprised mainly of individuals with past PLAS infection status. Possible interferences should thus be considered when applying the assay.

In sera from travellers returning from ZIKV-endemic areas, we observed a tendency of ZIKV-specific IgM to appear at high ratios during the active phase of infection, paralleled by a moderate rise in IgG. In contrast, most residents in endemic areas had high anti-ZIKV IgG and low/negative IgM ratio values, irrespective of whether their samples were taken during the initial, active or late phase of infection. IgM responses in travellers returning from ZIKV-endemic areas tended to be higher compared with residents in such areas, whereas the IgG-positivity rate was higher in the latter subgroup. Such differences in ZIKV antibody kinetics were

Table 4

Anti-ZIKV reactivity in potentially cross-reactive specimens (n = 252) and healthy controls (n = 1,015) as determined by ELISA for IgM and IgG, study evaluating a novel NS1-based ELISA, Germany 2016

Cohort	Characteristics		Prevalence % (CI 95%)[c]		Specificity (CI 95%)[c]	
			IgM	IgG	IgM	IgG
DENVa	Dengue virus infection (high median anti-DENV IgM)[a]	47	0 (0–9.0)	0 (0–9.0)	100 (91.0–100)	100 (91.0–100)
DENVb	Dengue virus infection (high median anti-DENV IgG[b]	46	0 (0–9.2)	0 (0–9.2)	100 (90.8–100)	100 (90.8–100)
YFV	Yellow fever virus vaccination	12	0 (0–28.2)	0 (0–28.2)	100 (71.8–100)	100 (71.8–100)
WNV	West Nile virus infection	34	2.9 (0–16.2)	0 (0–12.1)	97.1 (83.8–100)	100 (87.9–100)
JEV	Japanese encephalitis virus infection	25	0 (0–15.8)	4.0 (0–21.1)	100 (84.2–100)	96.0 (78.9–100)
CHIKV	Chikungunya virus infection	19	0 (0–19.8)	0 (0–19.8)	100 (80.2–100)	100 (80.2–100)
PLAS	*Plasmodium* spp. Infection	69	1.4 (0–8.5)	0 (0–6.3)	98.6 (91.5–100)	100 (93.7–100)
Total	Potentially cross-reactive samples	252	0.8 (0–3.0)	0.4 (0–2.4)	99.2 (97.0–100)	99.6 (97.6–100)
PREG	German pregnant women	100	0 (0–4.4)	0 (0–4.4)	100 (95.6–100)	100 (95.6–100)
ZIM	Zimbabwean blood donors	128	0 (0–3.5)	0 (0–3.5)	100 (96.5–100)	100 (96.5–100)
ARG	Argentinian blood donors	99	1.0 (0–6.1)	0 (0–4.5)	99.0 (94.0–100)	100 (95.5–100)
US	US-American blood donors	100	0 (0–4.4)	1.0 (0–6.0)	100 (95.6–100)	99.0 (94.0–100)
GER	German blood donors	500	0.2 (0–1.2)	0.2 (0–1.2)	99.8 (98.8–100)	99.8 (98.8–100)
CHIL	German children	88	0 (0–5.0)	0 (0–5.0)	100 (95.0–100)	100 (95.0–100)
Total	Healthy control samples	1,015	0.2 (0–0.8)	0.2 (0–0.8)	99.8 (99.2–100)	99.8 (99.2–100)

ARG: Argentina; CHIKV: chikungunya virus; CHIL: children; DENV: dengue virus; GER: Germany; JEV: Japanese encephalitis virus; PLAS: *Plasmodium*; PREG: pregnant women; US: United States; WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus; ZIM: Zimbabwe.

[a] Median anti-DENV IgM ratio 3.9 (79% of samples with anti-DENV IgM ratio ≥ 3.0), as indicated in the inset of Figure 2A.

[b] Median anti-DENV IgG ratio 3.9 (80% of samples with anti-DENV IgG ratio ≥ 3.0), as indicated in the inset of Figure 2B.

[c] Referring to the total number of samples in the individual cohorts.

also illustrated by time course analysis of antibody levels in two representative patients, possibly reflecting that travellers returning from ZIKV-endemic countries predominantly had a primary flavivirus/ZIKV infection, while most residents probably contracted ZIKV as a secondary flavivirus infection. Similar kinetics have been described for primary and secondary infections in the Micronesian ZIKV epidemic [6] and for DENV-infected patients [35,36], suggesting that the detection of both specific IgM and IgG is diagnostically important and relevant for differentiating primary from secondary infections. Regarding our comparison of patients residing in endemic countries vs travellers, however, systematic differences in the background of these populations (e.g. genetic, ethnic) cannot be excluded.

Another limitation of our study is that it does not comprise side-by-side testing with additional assays, such as the Zika MAC-ELISA (Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, US) or PRNT, to provide comparative data on these current tests. In addition, the non-deliberate absence of a uniform serological reference standard for the pre-characterisation of all ZIKV samples resulted in a high number of suspected cases of ZIKV infection.

Although ZIKV usually causes rather mild infections, there has been convincing evidence of a causal link to neuronal impairment, such as newborn microcephaly and GBS [37]. Furthermore, there have been studies showing that DENV NS1 antibodies have the potential of inducing autoantibodies in secondary infections, probably mediated by cross-reactive binding of antigens on platelets and endothelial cells, followed by cellular damage and inflammatory activation [17]. Basic research is needed to fully elucidate the causal relations between neuronal disorders and ZIKV infection. Epidemiologic assessment of pregnant women and their babies, and of travellers returning from endemic areas, the surveillance of donated blood and the investigation of ZIKV prevalence in endemic and non-endemic areas may provide crucial information. These studies need reliable, fast, and easy-to-handle diagnostic tests that have low cross-reactivity and allow a definite diagnosis.

In conclusion, our study revealed that the NS1-based anti-ZIKV ELISA is a sensitive and highly specific tool for the serodiagnosis of ZIKV infections, eliminating cross-reactions with antibodies to DENV and other flaviviurses. The assay format is suitable for use in routine laboratories worldwide enabling high-throughput testing in epidemic settings. Serological identification of ZIKV infections is maximised by parallel testing for IgM and IgG. Further studies will be necessary to determine the accuracy of this and other current assays in a

larger set of well-defined samples, and to clarify how ZIKV infection triggers GBS, newborn microcephaly and other neurological manifestations.

*Erratum

The title of the y-axis in Figure 2B was corrected to read 'Anti-Zika virus IgG ELISA (ratio)' on 22 December 2016

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Conflict of interest

KS, CP, CR, JMW, EL, LK, AD, and SS are employees of EUROIMMUN AG. WSt and WSch are board members of EUROIMMUN AG. All other authors declare no competing interests.

Authors' contributions

This study was designed, supervised and coordinated by KS, WSt, and WSch. All authors provided important intellectual input at different stages of the work. KS, JSC, PE, MVE, JS, MPG, AG, JMW and EL contributed to the collection and, in part, pre-characterisation of patient samples. KS, CP, CR, LK, AD, WSt, and WSch contributed to assay development. KS and EL prepared and conducted all antibody determinations. The manuscript draft was written by JMW, AD and SS, including the analysis, statistical evaluation, and interpretation of all final data. All authors contributed to the writing of, and approved the final version of the manuscript.

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Research article

Rise of multidrug-resistant non-vaccine serotype 15A *Streptococcus pneumoniae* in the United Kingdom, 2001 to 2014

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Conjugate vaccines have reduced pneumococcal disease in vaccinated children and unvaccinated adults, but non-vaccine serotypes are of concern, particularly if antibiotic resistant. We reviewed *Streptococcus pneumoniae* collected via: (i) the British Society for Antimicrobial Chemotherapy (BSAC) surveillances from 2001–2014; (ii) Public Health England's (PHE) invasive isolate surveillance from 2005–2014 and (iii) referral to PHE for resistance investigation from 2005–2014. Serotype 15A increased in all series, with many representatives showing triple resistance to macrolides, tetracyclines and penicillin. 15A was consistently among the 10 most prevalent serotypes from 2011 in PHE and BSAC invasive isolate/bacteraemia surveillance but never previously; 26–33% of these invasive 15A isolates had triple resistance. BSAC respiratory isolates were only serotyped in 2013/14 and 2014/15 (October to September); 15A was most prevalent serotype in both periods, comprising 9–11% of isolates, 38–48% of them with triple resistance. Serotype 15A represented 0–4% of *S. pneumoniae* referred to PHE for reference investigation annually until 2008 but rose to 29% (2013) and 32% (2014). Almost all multidrug-resistant 15A isolates were sequence type (ST) 63 variants, whereas susceptible 15A isolates were clonally diverse. The rise of serotype 15A suggests that pneumococcal conjugate vaccines will need ongoing adaptation.

Introduction

Seven-valent pneumococcal conjugate vaccine (Prevenar 7, PCV7) first became available internationally in 2000, and protects against invasive *Streptococcus pneumoniae* infection by serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. Numerous countries have reported that deployment reduced the incidence of invasive (i.e. blood and cerebrospinal fluid (CSF)) *S. pneumoniae* disease both in children, who are vaccinated, and in elderly adults, who benefit through reduced carriage and transmission of virulent serotypes by children [1-4]. Antibiotic resistance was historically concentrated in five PCV7-targeted serotypes (6B, 9V, 14, 19F and 23F) [5] and several countries have reported reductions in the prevalence of resistance as these were displaced [6]. United Kingdom (UK) experience conforms to these general patterns [7], with the caveat that penicillin-non-susceptible *S. pneumoniae* were uncommon before the vaccine's introduction to the childhood schedule in 2006/07, meaning that little further fall occurred; macrolide resistance was reduced, reflecting displacement of a resistant serotype 14 lineage [8,9].

The success of PCV7 was partly offset by rises in other serotypes; notably 19A, where multidrug resistance to antibiotics became frequent [10,11]. This was countered by replacing PCV7 with a 13-valent conjugate vaccine (PCV13), additionally covering serotypes 1, 3, 5, 6A, 7F and 19A. PCV13 replaced PCV7 in the UK in April 2010 and this switch was followed by (i) reduced infant carriage of these additional serotypes [12], and (ii) a further 56% reduction in invasive disease incidence from a post-PCV7 baseline [13]. Again, however, rises are being seen in other, non-vaccine, serotypes, principally 8, 10A, 12F, 15A and 24F [13]. Serotype 15A is of particular interest since multidrug-resistant isolates belonging to this serotype have been reported as far apart as east Asia [14-16], North America [17,18], Norway [19], Italy [20] and Australia [21]. Here, we explore the rise

Table 1

Ten most-represented pneumococcal serotypes in the British Society for Antimicrobial Chemotherapy bacteraemia surveillance, United Kingdom and Republic of Ireland, 2001–2014 (n = 3,206 isolates)

	Serotype (bold); number and proportion of isolates										% for top 10[a]
Rank	1	2	3	4	5	6	7	8	9	10	
2001 (n = 227)	**14**	**8**	**9V**	**23F**	**3**	**4** **6B** **12F**	NA	NA	**1** 9F	NA	71.4%
	n = 36 15.9%	n = 23 10.1%	n = 20 8.8%	n = 17 7.5%	n = 15 6.6%	n = 11 4.8%	NA	NA	n = 9 4.0%	NA	
2002 (n = 220)	**14**	**9V**	**6B** **19F** **23F**	**NA**	**NA**	**1** **19A** **20** **22F**		**8**	**3**	**4** **7F** **6A**	69.5%
	n = 42 19.1%	n = 24 10.9%	n = 14 6.4%	NA	NA	n = 11 5.0%	n = 10 4.5%	n = 9 4.1%	n = 8 3.6%	n = 7 3.2%	
2003 (n = 239)	**14**	**9V**	**1**	**4**	**8**	**23F**	**3** **19F**	**NA**	**6B**	**18C**	72.0%
	n = 34 14.2%	n = 30 12.6%	n = 21 8.8%	n = 17 7.1%	n = 16 6.7%	n = 13 5.4%	n = 11 4.6%	NA	n = 10 4.2%	n = 6 3.8%	
2004 (n = 241)	**14**	**1**	**19F**	**4** **23F**	**NA**	**9V**	**8**	**3**	**7F** **19A** **22F**	**NA**	67.2%
	n = 37 15.4%	n = 24 10.0%	n = 16 6.6%	n = 14 5.8%	NA	n = 13 5.4%	n = 13 5.4%	n = 11 4.6%	n = 10 4.1%	NA	
2005 (n = 230)	**14**	**1**	**9V**	**23F**	**3** **4** **8**	**NA**	**NA**	**19F**	**7F**	**6B**	75.2%
	n = 35 15.2%	n = 30 13.0%	n = 21 9.1%	n = 18 7.8%	n = 14 6.1%	NA	NA	n = 10 4.3%	n = 9 3.9%	n = 8 3.5%	
2006 (n = 231)	**1**	**14**	**9V**	**23F**	**6A**	**4** **6B** **7F**	**NA**	**NA**	**8** **18C**	NA	75.8%
	n = 36 15.6%	n = 29 12.6%	n = 22 9.5%	n = 16 6.9%	n = 15 6.5%	n = 13 5.6%	NA	NA	n = 9 3.9%	NA	
2007 (n = 216)	**14**	**1**	**9V**	**8**	**7F**	**23F**	**3** **4** **6A**	**NA**	**NA**	**12F**	77.3%
	n = 30 13.9%	n = 26 12.0%	n = 20 9.3%	n = 19 8.8%	n = 14 6.5%	13 6.0%	n = 12 5.6%	NA	NA	n = 9 4.2%	
2008 (n = 201)	**1**	**14**	**8**	**7F** **22F**	**NA**	**9V**	**19A**	**3**	**20**	**4** **23F**	73.6%
	n = 32 15.9%	n = 20 10.0%	n = 17 8.5%	n = 15 7.5%	NA	n = 14 7.0%	n = 13 6.5%	n = 9 4.5%	n = 7 3.5%	n = 6 3.0%=	
2009 (n = 211)	**7F**	**3**	**19A**	**8**	**22F**	**1**	**6A**	**14**	**12F**	**4**	71.6%
	n = 26 12.3%	n = 23 10.9%	n = 18 8.5%	n = 17 8.1%	n = 14 6.6%	n = 13 6.2%	n = 12 5.7%	n = 11 5.2%	n = 9 4.3%	n = 8 3.8%	
2010 (n = 249)	**19A**	**7F**	**1**	**8**	**33F**	**22F**	**14**	**3**	**6A** **11A**	NA	68.7%
	n = 38 15.3%	n = 33 13.3%	n = 21 8.4%	n = 19 7.6%	n = 14 5.6%	n = 11 4.4%	n = 10 4.0%	n = 9 3.6%	n = 8 3.2%	NA	
2011 (n = 230)	**7F**	**19A**	**8**	**3**	**22F**	**1**	**23B**	**9N** **15A**	**NA**	**12F** **19F**	70.4%
	n = 29 12.6%	n = 28 12.2%	n = 22 9.6%	n = 19 8.3%	n = 18 7.8%	n = 14 6.1%	n = 9 3.9%	n = 8 3.5%	NA	n = 7 3.0%	
2012 (n = 229)	**7F**	**8**	**22F**	**19A**	**33F**	**12F**	**1** **3**	**NA**	**6C**	**15A**	69.4%
	n = 29 12.7%	n = 27 11.8%	n = 25 10.9%	n = 20 8.7%	n = 12 5.2%	n = 11 4.8%	n = 10 4.4%	NA	n = 8 3.5%	n = 7 3.1%	
2013 (n = 235)	**7F** **8**	**NA**	**22F**	**3** **19A**	**NA**	**23A**	**12F**	**15A** **33F**	**NA**	**1** **11A** **24F**	66.8%
	n = 36 15.3%	NA	n = 15 6.4%	n = 14 6.0%	NA	n = 10 4.3%	n = 9 3.8%	n = 8 3.4%	NA	n = 7 3.0%	
2014 (n = 247)	**8**	**22F**	**12F**	**15A**	**19A**	**3**	**7F**	**9N** **24F**	**NA**	**10A**	71.7%
	n = 43 17.4%	n = 22 8.9%	n = 20 8.1%	n = 19 7.7%	n = 16 6.5%	n = 14 5.7%	n = 13 5.3%	n = 11 4.5%	NA	n = 8 3.2%	

NA: not applicable.

Green: covered by PCV7; yellow: additional types covered by PCV13; pink: not covered by any conjugate vaccine.

[a] When there is a tie for tenth rank, only one of the tied serotypes is counted into the percentage total for the top 10.

of serotype 15A *S. pneumoniae* in the UK and Ireland at epidemiological and molecular levels, using data from both the British Society for Antimicrobial Chemotherapy (BSAC) and Public Health England (PHE) surveillances.

Methods

British Society for Antimicrobial Chemotherapy surveillance

The BSAC Bacteraemia and Respiratory Surveillance Programmes have been described previously [22-24]. Both collect isolates from across the UK and Republic of Ireland. The Bacteraemia programme runs on the calendar year. Until 2009 we asked participating laboratories to send up to 10 consecutive bloodstream *S. pneumoniae* isolates per annum from each of 25 hospital laboratories; from 2010 we have similarly sought seven consecutive bloodstream isolates per annum from each of 40 hospital laboratories. Isolates have been serotyped throughout, and results were reviewed across the years 2001 to 2014, inclusive. The Respiratory Programme runs on an October–September year, designated e.g. 2013/14, so that isolates from each winter peak of respiratory disease are not split between calendar years. It examines consecutive isolates from lower respiratory tract infections (LRTIs) in non-hospitalised patients or those hospitalised for less than 48 hours. Until 2013/14 the BSAC Respiratory Surveillance Programme did not routinely serotype isolates, therefore only 2013/14 and 2014/15 data were reviewed. In both these years the surveillance sought 14 consecutive LRTI *S. pneumoniae* isolates from each of the same 40 laboratories contributing to the Bacteraemia surveillance. Actual numbers of isolates collected in both surveillances were somewhat below these targets (see Results) and, in most years, one or two recruited laboratories failed to collect, and were subsequently dropped and replaced by alternative sites. Hospital laboratory mergers, mostly in the past 5 years, have also meant that participating microbiology laboratories increasingly source isolates from multiple hospitals, augmenting representativeness.

Public Health England invasive isolate surveillance

PHE Colindale routinely seeks submission of all invasive (i.e. blood and CSF) *S. pneumoniae* from hospital laboratories in England, Wales and Northern Ireland, receiving ca 4,000–5,000 isolates each year), over 95% of them from blood. Results of this surveillance were reviewed from 2005 (i.e. 1 year before PCV7 was introduced) to 2014. Susceptibility testing is performed on a subset of these isolates, comprising all those from laboratories contributing to European Antimicrobial Resistance Surveillance (EARS)-net [25]; this total fluctuated between 1,159 and 2,066 organisms annually over the study period.

Public Health England reference laboratory submissions

Besides surveillance isolates from invasive infections, PHE receives variable numbers of *S. pneumoniae* as reference submissions from respiratory and other non-sterile sites, principally eye and ear infections. Most are sent for investigation because the sender perceives them to have unusual resistance patterns, although senders' definitions of unusual vary and may be contingent on the site of the infection. Over 95% of isolates are from laboratories in England, Wales and Northern Ireland, with the remaining ca 5% largely from Scotland and the Republic of Ireland. Data were reviewed across the period 2005 to 2014.

Identification, serotyping and susceptibility testing

All surveillance and referred isolates were confirmed as forming alpha-haemolytic colonies on horse blood agar and being inhibited by a 5 µg optochin (ethylhydrocupreine hydrochloride) disc (Oxoid-Thermofisher, Basingstoke, UK). Isolates with atypical colonial morphology, or which could not be serotyped (below), were confirmed as being lysed within 30 min by 2% sodium deoxycholate, and being catalase-negative when tested with 3% hydrogen peroxide. For serotyping, isolates were grown overnight in Todd Hewitt broth at 35 °C with 5% CO2, harvested by centrifugation at 453 *g* for 30 min, then re-suspended in a small residual volume of broth and subjected to slide agglutination tests with standard antisera (Statens Serum Institut, Copenhagen, Denmark) [26]. Agar dilution susceptibility tests were performed in accordance with BSAC guidelines [27], using IsoSensitest agar (Oxoid-Thermofisher) supplemented with 5% defibrinated horse blood and incubated at 35–37 °C in a 5% CO2 atmosphere. 'Triple resistance' was defined as resistant to erythromycin (minimum inhibitory concentration (MIC) › 0.5 mg/L) and tetracycline (MIC › 2 mg/L), and non-susceptible to penicillin (MIC › 0.06 mg/L), based on EUCAST breakpoints [28])

DNA extraction, sequencing and bioinformatic analysis

Isolates were grown on horse blood agar (PHE Media Services) and treated by the Qiagen-recommended method for lysis of Gram-negative bacteria (Qiagen, Manchester, UK), which is effective for *S. pneumoniae* and simpler than the Gram-positive protocol. DNA was extracted from the lysates using a QIAsymphony SP automated instrument (Qiagen) and a QIAsymphony DSP DNA Mini Kit, using a tissue extraction protocol. DNA concentrations were measured using the Quant-IT Broad Range DNA Kit (Life Technologies, Paisley, UK) and GloMax 96 Microplate Luminometer (Promega, Southampton, UK). After adjusting to a concentration of 10–30 ng/µl, DNA was sent for whole genome sequencing (WGS) by Illumina methodology. The resulting data were automatically analysed using a bespoke bioinformatic pipeline for *S. pneumoniae*, developed by PHE. Among other things, this (i) checks species

Table 2

Major serotypes and associations with resistance among *Streptococcus pneumoniae* from the British Society for Antimicrobial Chemotherapy Respiratory Surveillance, United Kingdom and Republic of Ireland, 2013/14 and 2014/15 (n=805)

Serotype	October 2013 to September 2014			October 2014 to September 2015		
	Count	% of total isolates	No (%) with triple resistance	Count	% of total isolates	No (%) with triple resistance
15A	34	9.1	13 (38.2%)	46	10.7	22 (47.8%)
23B	26	6.9	1 (3.8%)	21	4.9	0
3	22	5.9	0	26	6.0	0
11A	21	5.6	1 (4.8%)	34	7.9	1 (2.9%)
23A	21	5.6	0	30	7.0	4 (13.3%)
22F	19	5.1	0	17	4.0	0
6C	18	4.8	0	12	2.8	0
19A	17	4.5	5 (29.4%)	14	3.3	4 (28.6%)
24F	16	4.3	0	12	2.8	1 (8.3%)
35F	14	3.7	0	14	3.3	0
10A	14	3.7	0	12	2.8	0
31	14	3.7	0	16	3.7	0
16F	12	3.2	1 (8.3%)	19	4.4	0
15B	11	2.9	0	3	0.7	0
17F	11	2.9	0	16	3.7	0
19F	11	2.9	3 (27.3%)	14	3.3	5(35.7%)
35B	11	2.9	0	18	4.2	0
8	10	2.7	0	12	2.8	1 (8.3%)
Other serotypes, with<10 isolates in one or both years	73	19.4	3 (4.9%)[a]	85	(21.7)	10 (2.3%)[a]
PCV7 serotypes	17	4.5	NA	20	4.6	NA
PCV13 serotypes	63	16.8	NA	67	15.6	NA
Total	375	100	27 (7.2%)	430	100	49 (11.4%)

NA: not applicable; PCV: pneumococcal conjugate vaccine.

[a] In 2013/14, three 6B isolates had triple resistance; the 10 'Other serotype' isolates with triple resistance in 2014/15 comprised three non-typeable, two 12F and single representatives of 6B, 7F, 9N 9V and 23.

identification by a kmer method and (ii) automatically assigns MLST sequence types (STs), identified by mapping the reads against all *S. pneumoniae* allele variants held in the MLST database [29], using a modification of the short-read sequence typing (SRST) software [30]. Resistance genes affecting susceptibility for macrolides and tetracyclines were identified, and their sequences reviewed.

Results

Serotype trends, British Society for Antimicrobial Chemotherapy bacteraemia surveillance

Prior to widespread UK deployment of PCV7 in the 2006/07 season, *S. pneumoniae* belonging to its target serotypes accounted for around half (44.4–53.6% in each of the years 2001 to 2006 inclusive) of all the *S. pneumoniae* collected in the BSAC bacteraemia surveillance but these declined to 4.7% of isolates by 2013 and 2.0% in 2014. Serotype 14 was the most common type in 6 of the 7 years from 2001 to 2007, comprising 13–20% of all isolates (Table 1) and accounting for 61% of all erythromycin-resistant isolates. By 2013, however, serotype 14 had only a single representative (0.4%), and none in 2014. Other serotypes became relatively more frequent as the PCV7 types declined, notably 7F and 19A, whereas serotype 1 had been expanding since 2001. These three types are within the spectrum of PCV13 and have declined, with variable rapidity, following its replacement of PCV7 in 2010. A further PCV13 type, serotypes 3, shows much less evidence of decline, as also noted elsewhere [13].

Serotype 15A isolates were encountered in each year from 2010 and the serotype was in the top 10 from 2011 onwards, whereas previously the type was sporadic. Other types that had long been encountered at moderate to low prevalence also became more prominent,

Table 3

Predominant serotypes among *S. pneumoniae* serotyped by the Respiratory and Vaccine Preventable Bacteria Reference Unit, Public Health England from invasive infections, 2005–2014 (n = 45,645)

	Serotype (bold) number and proportion of isolates										Proportion for top 10
Rank	1	2	3	4	5	6	7	8	9	10	
2005 n = 4,662	**14**	**1**	**8**	**9V**	**4**	**23F**	**3**	**6B**	**7F**	**19F**	73.3%
	701	528	357	333	327	271	250	248	208	195	
	15.0%	11.3%	7.7%	7.1%	7.0%	5.8%	5.4%	5.3%	4.5%	4.2%	
2006 n = 4,857	**14**	**1**	**9V**	**8**	**23F**	**4**	**3**	**6B**	**7F**	**19F**	72.1%
	660	611	337	321	300	288	268	256	249	210	
	13.6%	12.6%	6.9%	6.6%	6.2%	5.9%	5.5%	5.3%	5.1%	4.3%	
2007 n = 4,673	**1**	**14**	**9V**	**8**	**7F**	**3**	**4**	**6A**	**23F**	**6B**	69.1%
	583	449	351	348	316	278	238	237	231	197	
	12.5%	9.6%	7.5%	7.4%	6.8%	5.9%	5.1%	5.1%	4.9%	4.2%	
2008 n = 4,978	**1**	**7F**	**8**	**3**	**22F**	**19A**	**6A**	**14**	**9V**	**23F**	66.4%
	592	474	372	359	328	307	239	238	206	189	
	11.9%	9.5%	7.5%	7.2%	6.6%	6.2%	4.8%	4.8%	4.1%	3.8%	
2009 n = 5,000	**7F**	**1**	**19A**	**3**	**22F**	**8**	**6A**	**12F**	**14**	**33F**	67.7%
	553	501	490	438	423	393	189	148	131	118	
	11.1%	10.0%	9.8%	8.8%	8.5%	7.9%	3.8%	3.0%	2.6%	2.4%	
2010 n = 4,881	**7F**	**19A**	**1**	**3**	**8**	**22F**	**33F**	**6C**	**12F**	**11A**	69.9%
	675	640	445	362	362	361	164	161	139	102	
	13.8%	13.1%	9.1%	7.4%	7.4%	7.4%	3.4%	3.3%	2.8%	2.1%	
2011 n = 4,549	**7F**	**19A**	**8**	**1**	**3**	**22F**	**12F**	**33F**	**6C**	**15A**	71.8%
	665	538	424	391	382	348	139	131	126	124	
	14.6%	11.8%	9.3%	8.6%	8.4%	7.7%	3.1%	2.9%	2.8%	2.7%	
2012 n = 4,092	**7F**	**8**	**19A**	**22F**	**3**	**1**	**15A**	**33F**	**6C**	**12F**	68.2%
	485	456	369	357	276	243	176	155	148	125	
	11.9%	11.1%	9.0%	8.7%	6.7%	5.9%	4.3%	3.8%	3.6%	3.1%	
2013 n = 3,995	**8**	**7F**	**22F**	**19A**	**3**	**15A**	**12F**	**1**	**24F**	**33F**	66.4%
	545	415	320	293	274	203	174	153	141	134	
	13.6%	10.4%	8.0%	7.3%	6.9%	5.1%	4.4%	3.8%	3.5%	3.4%	
2014 n = 3,959	**8**	**12F**	**22F**	**3**	**19A**	**15A**	**7F**	**9N**	**33F**	**24F**	67.9%
	599	336	334	243	229	224	219	170	168	167	
	15.1%	8.5%	8.4%	6.1%	5.8%	5.7%	5.5%	4.3%	4.2%	4.2%	

PCV: pneumococcal conjugate vaccine.

Green: covered by PCV7.

Yellow: additional types covered by PCV13.

Pink: not covered by any conjugate vaccine.

99% of isolates are from England, Wales and Northern Ireland, with the remaining few from Scotland, Crown Dependencies, Republic of Ireland and elsewhere.

including serotypes 8, and (albeit with considerable year-on-year variation) 22F.

Triple resistance was seen in just 60/3,206 isolates (1.97%) throughout the period reviewed and its prevalence exceeded 10% only among isolates of serotypes 37 (2/3 isolates), 6B (13/90 isolates, 14.4%) and, most strikingly, 15A (13/50, 26.0%). Triple-resistant serotype 15A *S. pneumoniae* were received in every year from 2011, although never previously. This observation, along with increasing numbers of 15A isolates among PHE reference submissions (below), prompted the present analysis.

Serotypes among British Society for Antimicrobial Chemotherapy respiratory isolates

Unlike those collected in the BSAC Bacteraemia Surveillance, *S. pneumoniae* from the BSAC Respiratory Surveillance were not routinely serotyped until 2013/14, when 15A proved to be the most frequent serotype (Table 2), comprising 34 9.1% of all 375 isolates collected, with a similar pattern in 2014/15, when 15A comprised 46/430 (10.7%) of isolates. What is more, 15A was one of only four serotypes (the others being 6B, 19A and 19F) where triple resistance was seen in over 10% of representatives. Overall, triple resistance was seen in 13/34 (38.2%) serotype 15A isolates vs

Table 4

Proportions of isolates with triple resistance to penicillin, erythromycin and tetracycline among frequent serotypes of *Streptococcus pneumoniae* from blood and cerebrospinal fluid infections, Public Health England surveillance, 2005–2014 (n = 13,551)

Serotype	Total	Triple resistance	% Triple resistance
15A	330	104	31.5
6B	420	51	12.1
19F	401	45	11.2
19A	987	83	8.4
23F	360	15	4.2
24F	124	5	4.0
9V	562	19	3.4
14	1,145	27	2.4
6A	366	3	0.8
8	1,197	3	0.3
6C	205	2	1.0
9N	261	1	0.4
3	777	2	0.3
33F	239	2	0.8
1	1,195	1	0.1
22F	761	1	0.1
12F	474	1	0.2
4	334	0	0
7F	1,155	0	0
All others	2,258	0	0
All isolates and serotypes	13,551	469	3.5

99% of isolates are from England, Wales and Northern Ireland, with the remaining few from Scotland, Crown Dependencies, Republic of Ireland and elsewhere.

Serotypes that reached a top-10 ranking in any surveillance year in Table 3 are line-listed.

14/341 (4.1%) of all other isolates in 2013/14 ($p < 0.001$, logistic regression adjusted for clustering by centre); there was an even sharper difference, 24/46 (52.2%) vs 25/384 (6.5%) ($p < 0.001$), in 2014/15.

Also notable was the fact that PCV7 serotypes accounted for only 17/375 (4.5%) of all the respiratory *S. pneumoniae* in 2013/14 and PCV13 types for just 63/375 (16.8%); corresponding figures in 2014/15 were 18/430 (4.3%) for PCV7 types and 68/430 (15.8%) for PCV13 types. The sole previous season when *S. pneumoniae* from the Respiratory Programme were typed was 2005/06, immediately before UK introduction of PCV7 [24]. Then, among 749 isolates, 312 (41.7%) belonged to PCV7 types and 450 (60.1%) to PCV13 types (assuming all serogroup 7 isolates belonged to serotype 7F) whereas 36 (4.8%) belonged to serogroup 15, which was not split to its component (15A/B/C/F) serotypes. The declines in PCV7 types, PCV13 types, and the rise in serotype 15A (compared with all serotype 15 in 2005/06) were all highly significant ($p < 0.001$, logistic regression adjusted for clustering by centre).

Serotype trends, Public Health England invasive isolate surveillance

PHE surveillance of invasive *S. pneumoniae* indicated dramatic changes in serotype prevalence over time, as reviewed previously [7,13], with these paralleling the shifts seen for BSAC bacteraemia isolates. Specifically, in each of the years up to and including 2007, five or six of the top 10 serotypes were PCV7 types, with serotype 14 the most abundant (Table 3), as in the BSAC series (Table 1). After 2007, PCV7 types declined, with none in the top 10 after 2009. Several of the additional types covered by PCV13, notably 3, 6A, 7F and 19A, became relatively more prominent from 2006 until 2011 while serotype 1, also a PCV13 additional type, was prominent even before 2011. Except for serotype 3, which showed little convincing trend, these additional PCV13 types declined in rank after 2010/11, with the peaking of serotypes 1 and 19A being especially marked.

As in the BSAC series, serotype 15A first appeared in the top 10 in 2011. It then advanced to seventh rank by 2012 and sixth rank in both 2013 and 2014, accounting for 5.7% of isolates (224/3,959) in the latter year. Again, the proportion of resistance was striking: among the 330 tested, fully 104 (31.5%) of bloodstream 15A *S. pneumoniae* for all years pooled had triple resistance, whereas triple resistance rates for all other isolates that ever featured in the top 10 were under 12.5% (Table 4). Proportions of serotype 15A isolates, taking 2005–2014 pooled, rose with the patient's age, from 1.3% in the 0–5 year age group to 1.4% in the 6–35 year age group, 0.6% in the 36–45 year age group, 1.7% in the 46–55, 56–65 and 66–75 year age groups, reaching 2.4% in the 76–85 year age group and 3.1% among the over-85-year-olds ($p < 0.001$). Triple resistance was represented among serotype 15A *S. pneumoniae* throughout the surveillance period reviewed, with proportions as follows: 2005, 0/3 isolates with triple resistance; 2006, 1/4; 2007, 2/10; 2008, 4/13; 2009 7/13; 2010, 18/34; 2011, 10/33; 2012, 15/50; 2013, 19/63 and 2014, 33/114.

The isolates tested for antibiotic susceptibility and resistance (n = 13,551, annual range 1,159–2,966 p.a.) are a subset of those in Table 3 and comprise all isolates from hospitals that participate in the EARS-net surveillance along with those bloodstream isolates where the referring laboratory specifically sought susceptibility testing. Inclusion of the latter group may over-represent resistant organisms, although there is no reason why it should do so disproportionately within particular serotypes.

Serotype trends, isolates referred to Public Health England for investigation of resistance

Between 2005 and 2014, 1,536 *S. pneumoniae* from respiratory, ear and eye infections were referred to PHE (Table 5) for investigation of unusual resistance. These submissions constitute a heavily biased sample and lack a denominator, but do provide a rolling snapshot of *S. pneumoniae* isolates that sending laboratories

Table 5

Predominant serotypes among respiratory, ear and eye isolates of *Streptococcus pneumoniae* received by the Public Health England Colindale reference service, 2005–2014 (n=1,536)

	Number of isolates of indicated serotype in year:										Grand total	No with triple resistance	% with triple resistance
	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014			
Serotype 19F	15	31	41	50	55	35	23	14	12	14	290	232	80.0
Serotype 19A	2	10	15	27	45	44	28	17	8	17	213	169	79.3
Serotype 15A	0	4	3	8	23	22	17	26	31	31	165	137	83.0
Serotype 6B	5	18	39	35	25	13	4	7	3	2	151	104	68.9
Non-typeable rough	3	8	16	23	33	18	10	15	5	2	133	81	60.9
Serotype 9V	7	36	25	15	18	10	0	0	1	2	114	16	14.0
Serotype 14	9	18	21	6	11	11	4	2	3	1	86	30	34.9
Serotype 23F	7	9	6	16	16	9	3	2	1	3	72	35	48.6
Serotype 35B	1	3	4	9	7	6	9	4	7	4	54	9	16.7
No serotype data	1	2	0	0	0	6	1	1	5	26	42	22	52.4
Serotype 6A	0	3	5	5	3	6	1	2	0	1	26	7	26.9
Serotype 11A	0	0	1	1	4	3	3	5	2	4	23	12	52.2
Serotype 3	0	1	3	0	3	4	1	2	4	3	21	3	14.3
Serotype 1	0	0	1	2	3	5	1	1	1	0	14	1	7.1
Serotype 13	0	2	0	1	6	1	0	0	1	0	11	0	0.0
All other types[a]	8	5	5	9	16	17	15	10	13	23	121	38	31.4
Total	58	150	185	207	268	210	120	108	97	133	1,536	896	58.3
15A as % typed reference submissions[b]	0.0	2.7	1.6	3.9	8.6	10.8	14.3	24.3	33.7	29.0	11.0		

95% of isolates were from England, Wales and Northern Ireland with the remainder from Scotland, Crown Dependencies, Republic of Ireland or elsewhere.

[a] Not accounting for >10 isolates in total over the surveillance period.

[b] Excludes 'no data row' above from denominator.

(mostly in England and Wales) consider to be concerning. Overall, 896/1,536 (58.3%) had triple resistance. In the earlier years members of serotypes 19F, 9V, 6B and 14 dominated, collectively accounting for 82.8–92.4% of referrals from 2005 to 2007, before declining from the start of the 'PCV7 era'. Serotype 19A accounted for a growing proportion of referrals from 2005, peaking at 23.3% in 2011, while serotype 15A represented just 0–4% of submissions throughout the period 2005 to 2008 but thereafter increased progressively, becoming the most commonly referred serotype in 2012. In 2013, it accounted for 31/92 of all submissions where typing was undertaken, and for 31/107 in 2014. These proportions were greater than ever previously achieved by any other serotype. Fully 83.0% of serotype 15A isolates (137/165) had the triple resistance vs 68.9–80.0% among serotype 9V, 19A and 19F referrals, with lower proportions for other serotypes (Table 4).

Genomic sequencing and phenotypes of serotype 15A isolates

Genomic sequencing was performed on 156 serotype 15A *S. pneumoniae*. These represented a diversity of resistance patterns, and including 50 with triple resistance; a limitation was that all 156 sequenced isolates dated from 2013 and 2014. MLST types were deduced from the sequence data, and 78 (50%) of the isolates were identified as belonging to ST63 (n=61) or its single or double locus variants (n=17). All of these 78 ST63-related isolates were resistant to erythromycin (also clindamycin, not shown) and 49 (62.8%) had the triple resistance profile (Table 6). The macrolide and clindamycin resistance correlated with the consistent presence of *erm(B)* genes, as detected by WGS. All 78 ST63-related isolates were found also to carry the tetracycline-resistance determinant, *tet(M)*; those (n=65, 83.3%) that expressed tetracycline resistance had the intact gene, whereas those (n=13, 16.7%) that were tetracycline-susceptible (all of them classical ST63 isolates) had a deletion of two nucleotides at codon 339, generating a premature stop codon and thereby inactivating the gene. Most of the 49 isolates with triple resistance were susceptible to alternative agents: 37 remained susceptible to ampicillin, 47 to moxifloxacin, 48 to cefotaxime and all 49 to vancomycin, all based on EUCAST breakpoints. Sequence types (STs) 3811 (n=19), 58 and its single locus variants (SLVs) (n=21), and 73 and its SLVs (n=11) were all heavily represented among the 78 ST63-unrelated serotype 15A isolates and, among all these, just one isolate had triple resistance and three or fewer were non-susceptible to any one of erythromycin, tetracycline or penicillin.

WGS data were available for a further 141 non-15A *S. pneumoniae*, predominantly investigated owing to multidrug resistance. Six had ST63-related profiles and

Table 6

Sequence types in relation to resistance of serotype 15A *Streptococcus pneumoniae* subjected to genomic sequencing (n = 156)

		Number (%) non-susceptible (intermediate or resistant)			
	n	Erythromycin	Tetracycline	Penicillin	Triple resistance
ST63	61	61 (100%)	48 (78.7%)	46 (75.4%)	35 (57.4%)
ST63 SLV and DLV	17	17 (100%)	17 (100%)	14 (82.4%)	14 (82.4%)
Other 15A[a]	78	2 (2.6%)	3 (3.8%)	1 (1.3%)	1 (1.3%)

SLV: single locus variant; ST: sequence type.

[a] Includes 21 ST58 and SLVs, 19 ST3811, 11 ST73 and SLVs and 27 isolates belonging to sequence types with four representatives or fewer.

these all had triple resistance; three expressed serotype 19F, one serotype 21 and one 23F; the final isolate was typed using antisera as serotype 20 but was predicted to be serotype 11A based on WGS; review suggests that the original serotype determination was in error. The association with 19F (a PCV7 serotype) is notable (see Discussion), but members of this serotype were highly variable in terms of ST; among a total of 25 serotype 19F isolates sequenced, 22 with triple resistance, we recorded 12 different known STs, along with two new variants. No single ST had more than four representatives.

Discussion

Deployment of PCVs has had clear public health benefits. The incidence of invasive pneumococcal disease has been reduced not only in vaccinated children, but also in elderly adults, who benefit from herd immunity [31]. There is also evidence of impact on non-invasive disease: thus, PCV7 deployment in the UK in 2006 also was followed by a 19% reduction in hospital admissions for community-acquired pneumonia (CAP) among children aged<2 years, reversing a rising trend that had persisted during the preceding decade [32]. A similar reduction was reported in Italy [33]. Moreover, a Cochrane review concluded that PCV7 reduced the incidence of acute otitis media in healthy vaccinated children, although with less impact for those with a history of the illness or deemed to be 'high risk' [34]. Lastly, active PCV13 vaccination was recently shown to achieve a 50% reduction in the incidence of bacteraemia and non-invasive pneumonia in elderly adults, again reflecting displacement of vaccine serotypes [35].

A limitation to this pattern of successes is, however, that the PCV vaccines cover only the most prevalent pneumococcal serotypes, leaving scope for expansion of other types. Deployment of PCV7 was followed by increased prevalence of serotype 19A isolates, many of them multidrug-resistant, and, although serotype 19A is now covered by PCV13, a niche may be created for yet further types. Internationally, several groups have remarked on the increased prevalence of multidrug-resistant serotype 15A and 35B isolates [14-21] and a recent PHE analysis of invasive pneumococcal infections, using the data series of Table 3, noted 15A to be among several serotypes now increasing in numbers and proportion in the UK [13]. The present analysis extends these findings, confirming that serotype 15A *S. pneumoniae* are of growing importance, as also shown (i) in the BSAC bacteraemia series (Table 1), which overlaps the PHE series but also includes Scotland and Ireland, (ii) the BSAC series LRTI (Table 2), which is the sole UK surveillance to test *S. pneumoniae* from their predominant disease setting, and (iii) among PHE reference submissions, which provide a rolling snapshot of resistance phenotypes causing concern to microbiologists at sending laboratories, which are predominantly in England, Wales and Northern Ireland, although with a few isolates received from elsewhere (Table 5). By 2013 and 2014, serotype 15A was consistently (i) among the top 10 serotypes in both the PHE and BSAC surveillances of invasive *S. pneumoniae* (Tables 1 and 3), (ii) was the top serotype among respiratory isolates (Table 2) and (iii) accounted for almost one third of all the *S. pneumoniae* sent for reference investigation as 'unusually' resistant. Critically, and unlike other rising pneumococcal serotypes (8, 10A, 11A, 12F, and 24F – see Tables 1, 3 and ref [13]) serotype 15A isolates were commonly resistant or non-susceptible to multiple antibiotics, including macrolides, clindamycin, tetracycline and penicillin. While none of the surveillances captures clinical outcomes, the fact that serotype 15A is rising in invasive infections implies that these organisms are virulent.

Around one third of serotype 15A isolates had 'triple resistance' (i.e. to macrolides and tetracycline together with intermediate penicillin resistance), a higher proportion than for other serotypes (Table 4). This proportion did not change substantially over time (although assessment is complicated by small total numbers of isolates in the earlier years), indicating that the serotype was gaining prominence both generally and as a resistant type, again implying that the surface polysaccharides of serotype 15A support virulence.

Triple resistance among serotype 15A isolates was strongly associated ($p<0.0001$, Fisher's exact of chi-squared tests) with ST63 and its variants and extremely rare among serotype 15A isolates belonging to other

STs. This association between serotype 15A, ST63 and multidrug-resistance has been made by others too [18,36,37] and it was suggested by Frazao et al. [38] that multidrug-resistant ST63–15A organisms arose by type transformation of ST63 strains previously expressing the 19F capsular serotype. The present results provide very little support for this hypothesis. Although 3/22 multidrug-resistant serotype 19F *S. pneumoniae* examined were ST63 single- or double-locus variants, the remaining 19/22 belonged to diverse sequence types; moreover, ST63 alleles have been reported to be associated with other serotypes besides 19F and 15A, including serotype 8 in Spain [39], where it is suggested that they may have arisen via serotype switching of earlier Sweden 15A lineages [40].

In summary, the present findings imply that conjugate vaccines will face an ongoing game of 'catch-up', as new serotypes rise to prominence, and that expansion beyond a 13-valent formulation will be needed. They are pertinent also to the debate as to whether PCV13 should be adopted for prophylactic vaccination against pneumonia in the elderly, as is advocated based on recent positive trial results in the Netherlands [35]. Such positive findings must be set against the fact that PCV13 strains now account for less than 20% of community-onset pneumococcal pneumonias in the UK (Table 2).

While the rise of any new multidrug-resistant type is of concern for patient management, the ST63–15A *S. pneumoniae* had high level resistances only to macrolides, clindamycin and tetracyclines; MICs of penicillin mostly remained in the range 0.12 to 0.5 mg/L, and this level of 'non-susceptibility' is unlikely to compromise outcomes, except in meningitis. Susceptibility to moxifloxacin and cefotaxime remained near-universal, and ampicillin MICs were twofold below those of penicillin, remaining in the susceptible range and reversing the usual pattern for penicillin-non-susceptible *S. pneumoniae*, where ampicillin MICs mostly exceed those of penicillin. Treatment of infections therefore is unlikely to present especial problems, unless macrolides or tetracyclines are used alone, for example in beta-lactam allergic patients.

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Conflict of interest

DML has shares in Pfizer and GSK, who make pneumococcal conjugate vaccines, and occasionally lectures and does contract and consultancy work for both companies. Other authors declare no conflict of interest.

Authors' contributions

CS, MK: molecular characterisation of isolates; NF/TH: Public Health England reference surveillance and typing of S. pneumoniae, on which this analysis is predicated; RR/SM/RJ: British Society for Antimicrobial Chemotherapy surveillance of S. pneumoniae, on which this analysis is predicated; RP, RH, NW: reference investigation of resistant S. pneumoniae on which analysis is predicated; PS: extraction and consolidation of data series; MD: Bioinformatic analysis of sequence data; DML: primary observation of rise of 15A S. pneumoniae, wrote manuscript. All authors commented upon and contributed to improving the manuscript.

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Letter to the editor: New metrics to monitor progress towards global HIV targets: using the estimated number of undiagnosed HIV-infected individuals as denominator

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To the editor: Recently, Pharris et al. presented an important analysis of the current HIV situation in the European Union/European Economic Area (EU/EEA), focussing on the estimates produced by the European Centre for Disease Prevention and Control (ECDC) HIV Modelling Tool, and especially the number of people living with HIV who are not aware of their infection [1].

The ECDC HIV Modelling tool allows estimation of the number of undiagnosed HIV-infected individuals by country, by transmission route, or at European level [2,3]. This number is commonly compared with the total number of people living with HIV (PLHIV); the ratio of both numbers forms the undiagnosed fraction, providing information on the first of the Joint United Nations Programme on HIV/AIDS (UNAIDS) 90–90–90 global targets for 2020 '90% of persons living with HIV diagnosed' [4]. However, in countries where a large proportion of patients are in care, this ratio may be an insufficient indicator of testing and diagnosis success because PLHIV on treatment currently live longer with HIV and consequently, the proportion of undiagnosed persons with HIV will naturally become smaller in relation to the ever-increasing population of diagnosed PLHIV [1]. In a population with a large number of successfully treated patients, even a very high diagnosed fraction of 90% may hide a large and increasing number of undiagnosed infections that may sustain HIV transmission in the near future. Hence, the diagnosed fraction does not provide precise enough information to gauge testing services' effectiveness.

However, the estimated number of undiagnosed PLHIV can contribute to a more effective indicator of diagnosis timeliness and completeness. Used as a denominator, the number of undiagnosed PLHIV could be an appropriate indicator of quality of testing programmes when it is compared with the yearly number of new diagnoses. This comparison may be expressed as the ratio of yearly number of new diagnoses to the estimated number of undiagnosed individuals, or as a fraction, the yearly diagnosed fraction (YDF), expressed as follows: yearly number of new diagnoses / (yearly number of new diagnoses + estimated number of undiagnosed PLHIV). To monitor testing programmes, comparing the yearly number of diagnoses with the number of undiagnosed individuals is likely to provide more sensitive and dynamic information than the diagnosed fraction calculated among the total population of PLHIV.

Looking at today's epidemic in the EU/EEA, what would be the current value of the YDF?

Considering the HIV epidemic in EU/EEA, the estimated number of PLHIV who are not aware that they are infected is 122,000, or 15% of all PLHIV [1]. In 2015, 29,747 people were newly diagnosed with HIV [5]. The yearly number of new HIV diagnoses remains quite stable over time. The calculated YDF is 20% (29,747 / (29,747 + 122,000)). It means that only one in five of the estimated number of PLHIV susceptible to be diagnosed in 2015 was actually diagnosed in 2015. At constant number of diagnoses, four years at least would be necessary to diagnose the number of still undiagnosed PLHIV (not taking into account those expected to become infected during coming years), leading to many late diagnoses, and consequently fuelling HIV transmission. In fact, the diagnoses of the people who are currently unaware of their infection will likely take place over a longer period of time.

In order to reduce HIV transmission and improve individual and population health, the undiagnosed PLHIV should be diagnosed and offered treatment as soon as possible [6], ideally becoming the early diagnosed

of tomorrow rather than the late diagnoses of after-tomorrow. Increases in the number of diagnoses would be something positive if there was a concomitant increase in YDF. A considerable increase in the number of diagnoses, and therefore in YDF, would be necessary to cut the cycle of infection-transmission. So an ideal situation would be that all persons currently living with undiagnosed HIV receive a diagnosis next year (YDF = 100%).

The YDF is an indicator that relates 'work done' (persons diagnosed this year) to 'work remaining to be done' (persons undiagnosed). The value of YDF and its trend over time are indicators that can monitor the situation and progress on HIV testing and timely HIV diagnosis, thus helping guide efforts to reducing the number of undiagnosed PLHIV and curbing the epidemic.

Conflict of interest

None declared.

Authors' contributions

The author conceived the idea of the letter and wrote the manuscript.

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Authors' reply: New metrics to monitor progress towards global HIV targets: using the estimated number of undiagnosed HIV-infected individuals as denominator

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To the Editor: We thank Dr Sasse for his interest in the conclusions of our rapid communication, in which we used the European Centre for Disease Prevention and Control (ECDC) HIV Modelling tool to estimate for the number and proportion of persons living with HIV who remain undiagnosed in the European Union/European Economic Area [1]. In response to our discussion of the limitations of monitoring a proportion of those undiagnosed relative to persons diagnosed and living with HIV, because this will naturally decrease over time as the number of those undiagnosed becomes smaller in relation to the growing number of persons diagnosed and living longer with HIV, Dr Sasse proposes a new metric: the yearly diagnosed fraction (YDF), where diagnoses made in a given year are examined in relation to the number of persons undiagnosed [2]. We agree that the YDF is an interesting proposal which has the potential to inform monitoring of HIV testing programme efforts. However, it should be remembered that metrics based on estimates are not always straightforward to monitor over time. Estimates can change slightly from year to year, depending on data sources and assumptions used in the model, and changes in the YDF should be interpreted with this in mind, and including confidence intervals to understand the range of uncertainty in a given year.

Conflict of interest

None declared.

Authors' contributions

All authors drafted the reply.

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