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Specific serology for emerging human coronaviruses by protein microarray

C Reusken (chantal.reusken@rivm.nl)[1,2], H Mou[1,3], G J Godeke[1,2], L van der Hoek[4], B Meyer[5], M A Müller[5], B Haagmans[6], R de Sousa[2], N Schuurman[3], U Dittmer[7], P Rottier[3], A Osterhaus[6], C Drosten[5], B J Bosch[3], M Koopmans[2,6]

1. These authors contributed equally to this work
2. Centre for Infectious Disease Control, Division Virology, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
3. Department of Infectious Diseases and Immunology, Utrecht University, Faculty of Veterinary Medicine, Utrecht, the Netherlands
4. Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
5. Institute of Virology, University of Bonn Medical Centre, Bonn, Germany
6. Department of Viroscience, Erasmus Medical Centre, Rotterdam, the Netherlands
7. Institute for Virology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

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We present a serological assay for the specific detection of IgM and IgG antibodies against the emerging human coronavirus hCoV-EMC and the SARS-CoV based on protein microarray technology. The assay uses the S1 receptor-binding subunit of the spike protein of hCoV-EMC and SARS-CoV as antigens. The assay has been validated extensively using putative cross-reacting sera of patient cohorts exposed to the four common hCoVs and sera from convalescent patients infected with hCoV-EMC or SARS-CoV.

Background

In 2012, a novel human betacoronavirus (hCoV-EMC) emerged in the Middle East [1]. At the end of March 2013, 17 confirmed cases of hCoV-EMC infection had been reported to the World Health Organization (WHO) [2,3]. Person-to-person transmission had been reported twice by the United Kingdom (UK) and may also have occurred in two family clusters in Saudi Arabia (SA) and a hospital cluster in Jordan [2-4]. Fifteen confirmed cases have presented with severe acute respiratory infection (SARI), in some cases accompanied by acute renal failure [5-7]. Eleven patients have died [3]. One confirmed contact case in the UK and one confirmed case in SA presented with mild illness, and the clinical manifestations also appeared milder in unconfirmed but probable cases in the hospital cluster in Jordan [2-4,8]. It is important to understand the full spectrum of illness associated with this new human infection, and to determine how that relates to infectivity and the ability to transmit the virus, as well as to outcomes of diagnostic tests.

The emergence of this novel hCoV lead to an international collaborative laboratory response resulting in the rapid availability of diagnostic real-time reverse transcription polymerase chain reaction (RT-PCR) assays [9-11]. Successful use of PCR-based diagnostics relies on timing and technique of sampling, with knowledge about kinetics of viraemia and shedding of virus during the course of infection. Investigations into epidemiologically linked clinical cases in SA and Jordan demonstrated that not in all symptomatic patients within a cluster viral RNA could be detected by RT-PCR, similar to what has been described for SARS and other infectious diseases [2,11]. For diagnosis of hCoV-EMC infection, virus detection by RT-PCR during the acute phase may be less sensitive, as samples from the lower respiratory tracts (tracheal aspirates, bronchoalveolar lavage) are necessary for optimal detection, and these are not as readily available as upper respiratory tract samples [1,6,12]. Therefore, serological testing is imperative to complement RT-PCR findings for adequate diagnosis. In addition serology is essential for the monitoring of the evolution of an outbreak, including (retrospective) studies of asymptomatic and mild cases and identification of animal reservoirs. [13-16].

Currently an immunofluorescence assay (IFA) using hCoV-EMC-infected cells is available [10]. However, as the authors caution, this assay may generate false-positive results due to the global co-circulation of four hCoVs namely hCoV-NL63, hCoV-OC43, hCoV-229E and hCoV-HKU1. Cross-reactivity to conserved viral proteins limits the use of such whole virus-based IFAs, especially as antibodies against coronaviruses within a genus are generally known to cross-react [2,17]. Therefore, the European Centre for Disease Prevention and Control (ECDC) advised not to screen patients by whole virus IFA unless second stage serology is conducted [2]. For confirmation, virus neutralisation assays are the gold standard, but these are difficult to

implement and not widely available. Therefore, there is a need for alternative methods.

Here, we describe the use of antigen-microarrays to measure antibodies directed against the receptor-binding spike domain S1 of hCoV- EMC and SARS-CoV. The most variable immunogenic CoV antigen is the amino-terminal S1 subunit of the spike protein, which exhibits at most some 30% amino acid identity between human CoV isolates (data not shown). We describe a specific serological tool, distinguishing cross-reactivity with the four common hCoVs belonging to the same genus as hCoV-EMC and SARS-CoV (genus *Betacoronavirus*, hCoV-OC43, hCoV-HKU1), and to the genus *Alphacoronavirus* (hCoV-NL63 and hCoV-229E).

Methods

Protein expression

Plasmids encoding the amino-terminal receptor-binding spike domain S1 of hCoV-EMC and SARS-CoV, fused to the Fc part of human IgG, were expressed in HEK-293T cells, and S1-Fc proteins were purified from the culture supernatant by protein A chromatography as described [18]. Purified S1-Fc was cleaved by thrombin at the S1-Fc junction. Soluble S1 was subsequently purified by gel-filtration chromatography and concentrated using Amicon Ultra-0.5 filter (Merck, Darmstadt, Germany).

Preparation and testing of microarrays

Purified hCoV-EMC S1 and SARS-CoV S1 were spotted in quadruplicate in two drops of 333 pL each in a two-fold dilution series ranging from 1:2 to 1:8 (starting at 200 µg/mL for undiluted antigen) on 16-pad nitrocellulose-coated slides (Fast Slides, Maine Manufacturing, Grand Blanc, US) using a non-contact Piezorray spotter (PerkinElmer, Waltham, US) as described earlier [19]. Slides were pre-treated with Blotto blocking buffer to avoid non-specific binding as described [19]. Dilutions of serum in Blotto containing 0.1 % Surfact-Amps 20 (Thermo Fisher Scientific Inc. Breda, the Netherlands) were transferred in a volume of 90 µL to the slides and incubated for 1 h at 37 ºC in a moist chamber. Sera tested for the presence of IgM were treated with GullSORB (Meridian Bioscience Inc., Cincinnati, US) to eliminate rheumatoid factor and immune IgG, which can interfere with IgM assays. Upon washing, goat anti-human IgG (Fc-fragment specific) or IgM (Fc5µ-fragment specific) conjugated with DyLight649 fluorescent dye (Jackson Immuno Research, West Grove, US) was incubated for 1 h at 37 ºC in a moist chamber. After washing with buffer and water, the slides were dried. Fluorescence signals were quantified by a ScanArray Gx Plus microarray scanner (PerkinElmer) using an adaptive circle (diameter 80–200 µm) with a saturated signal at 65,535. Median spot fluorescence foreground intensity (background subtracted) was determined using ScanArray Express vs 4.0 software.

Sera

For validation experiments the following serum samples were used. All sera were stored at -20 ºC or 80 ºC prior to testing.

- Anonymised serum samples from 72 persons ranging in age from 0 year to 95 years sampled during 2008. These sera had been sent to the Dutch National Institute for Public Health and the Environment (RIVM) for routine *Bordetella pertussis* serology and thus represented a cohort biased towards patients with non-influenza-like respiratory symptoms. Anonymised use of serum from RIVM was covered by the rules of the code of conduct for proper use of human tissue of the Dutch Federation of Medical Scientific Associations.
- Anonymised serum samples of 10 children, ages ranging from 9 to 14 months, known to be positive for antibodies to one of the four common hCoVs, as determined by comparative ELISA using N antigen at a dilution factor of 1:200 [20,21]. Samples were obtained in 2001, were stored at -80°C and were chosen from this age group because antibodies at this age most likely result from single exposures [21]. Two hCoV-HKU1, two hCoV-OC43, three hCoV-229E and three hCoV-NL63 IgG positive sera were used.
- Three anonymised hCoV-OC43-positive sera (including one paired sample) from patients with virologically (PCR) and serologically (IgG IFA) confirmed infection, and one hCoV-OC43 IgG positive serum as described in [22].
- Serum samples from two cynomolgus macaques infected with hCoV-EMC (virus stock obtained as described [23]) taken at 28 days post infection, including a pre-infection serum.
- A serum sample from a rabbit immunised with hCoV-EMC S1 taken 28 days post immunisation, including a pre-immunisation serum.
- One serum sample from an hCoV-EMC infected patient who was treated for SARI in a hospital in Essen, Germany taken at day 20 after onset of illness. This serum had an IgG titre of 1:10,000 and an IgM titre of 1:1,000 as determined by IFA on cells infected with hCoV-EMC and an IgM and IgG titre of ›1:320 as determined by IFA on cells expressing recombinant S protein [10,22,23].
- Convalescent serum samples from two SARS-CoV infected patients. Serum SARS-1 was taken 3.5 years after disease. It had an IgG titre of 1:160 and no IgM titre as determined by IFA on cells expressing recombinant S protein [22]. Serum SARS-2 was taken 36 days after onset of illness with an IgG titre of 1:1,000 in IFA and 1:1,600 in ELISA. No IgM titre was found by IFA (personal communication, M. Niedrig, March 2013).
- Convalescent serum samples of three patients with severe respiratory complaints who had travelled to SA, Dubai and Dubai/Qatar within 10 days before onset of illness, and therefore had been tested to exclude hCoV-EMC by RT-PCR, as recommended by WHO.

Table

Summary results of the validation of the hCoV-EMC and SARS-CoV S1 protein microarray (n=94)

		hCoV-EMC Ag[a]		SARS-CoV Ag[a]	
Sera	Number	IgG	IgM	IgG	IgM
Human					
Population sera human	72	Negative	Negative	Negative	Negative
hCoV-OC43 human	6	Negative	Negative	Negative	Negative
hCoV-229E human	3	Negative	Negative	Negative	Negative
hCoV-NL63 human	3	Negative	Negative	Negative	Negative
hCoV-HKU1 human	2	Negative	Negative	Negative	Negative
hCoV-EMC human	1	Positive	Positive	Negative	Negative
SARS-CoV human	2	Negative	Negative	Positive	Negative
Animal					
Pre-immunisation rabbit	1	Negative	Not tested	Negative	Not tested
hCoV-EMC post-immunisation rabbit	1	Positive	Not tested	Negative	Not tested
Pre-infection macaque	1	Negative	Not tested	Negative	Not tested
hCoV-EMC post-infection macaque	2	Positive	Not tested	Negative	Not tested

Ag: S1 antigen.

[a] Reactivity was scored based on the arbitrary set cut-off.

All human sera were collected in accordance with the ethical principles set out in the declaration of Helsinki; Macaque and rabbit sera were collected in compliance with Dutch laws on animal handling and welfare.

Results

Testing antigen quality

The amino-terminal receptor-binding spike domains S1 of hCoV-EMC and SARS-CoV were spotted in serial dilutions (1:2–1:8) on nitrocellulose slides and incubated with two-fold serial dilutions (1:20–1:640) of sera from hCoV-EMC-infected macaques, a rabbit immunised with hCoV-EMC S1, or a SARS-CoV-infected patient. All sera showed high-level IgG reactivity with their homologous S1 antigen, while only background reactivity was observed with the heterologous antigen. Pre-immune serum of macaque and rabbit were non-reactive (Table). Based on these observations it was concluded that the antigens as printed on the array slides were intact and in the proper conformation for immuno-reactivity with homologous antibodies.

Validation of protein array

To analyse the specificity of the microarray for detection of hCoV-EMC and SARS-CoV IgM and IgG antibodies, the reactivity of a cohort of human sera submitted to the RIVM for whooping cough diagnostics was tested. The cohort consisted of 72 sera of non-exposed patients, ranging from 0–95 years of age. This cohort represents the putative cross-reacting potential in the Dutch population, where previous studies have shown high seroprevalences for one or more of the four common hCoVs [20,21]. The sera were tested for IgM and IgG reactivity with the hCoV-EMC and SARS-CoV antigens at dilutions 1:20 and 1:40 (Table, Figure 1). The observed reactivity was low. Based on these results an arbitrary cut-off was set at 5,000 for IgM and at 10,000 for IgG measurements.

The specificity of the microarray was confirmed using serum samples from children with known recent exposure and antibody responses to one of the four common hCoVs, including the betacoronaviruses OC43 and HKU1. Sera were tested at dilutions 1:20 and 1:160, with one serum for each hCoV tested in a two-fold dilution series of 1:20 to 1:640. None of the 14 sera showed reactivity above background, for either IgG or IgM, with the hCoV-EMC and SARS-CoV antigens (Table, Figures 1 and 2).

Subsequently, the array was tested with a single serum sample taken in the third week of illness of a patient infected with hCoV-EMC [22], and convalescent serum samples of two patients taken during the SARS-CoV epidemic. The serum of the hCoV-EMC patient showed a clear positive reactivity for IgG with EMC S1 in the dilution range from 1:20 to 1: 20,480, declining only at dilutions 1:5,120 and higher. The IgM reactivity of the hCoV-EMC serum with EMC antigen was saturated in the dilution range from 1:20 to 1:80, with declining, but clearly positive, levels of reactivity at higher dilutions. No reactivity was observed with SARS antigen for either IgG or IgM.

Figure 1

IgM and IgG reactivity of two-step serially diluted sera with hCoV-EMC S1- and SARS-CoV S1-spotted microarrays (n=89)

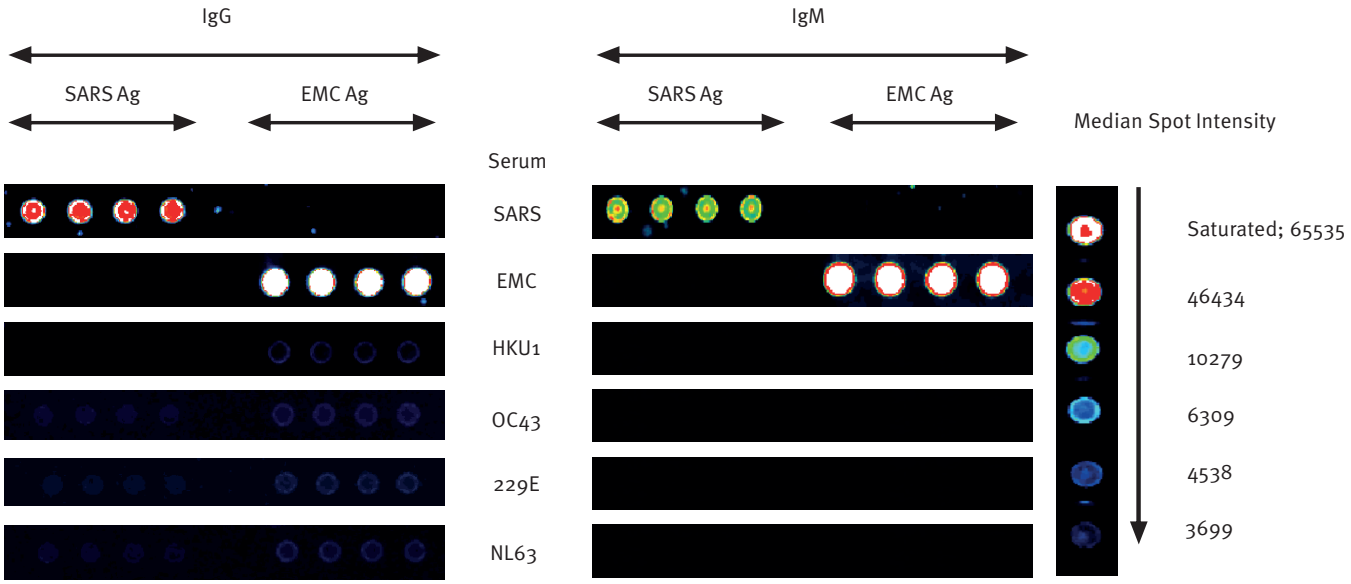
Sera: 72 population sera 1:20 diluted (panel A (IgM) and E (IgG)), hCoV-EMC (panel B (IgM) and F (IgG)), SARS-CoV serum SARS-1 (panel C (IgM) and G (IgG)) and hCoV-OC43 (panel D (IgM) and H (IgG)). Panels C and G are representative for all SARS-CoV sera tested (n=2). Panels D and H are representative for all common hCoV sera tested (n=14).

X-axes denote serum numbers (panel A and E) or serum dilutions: two-step serial dilutions, staring dilution 1:20.

Y-axes denote the measured median spot foreground fluorescence intensities.

Figure 2

Representative pictures of the protein microarray analysis of convalescent sera from patients infected with the six known hCoVs (n=17)



Ag: antigen.

Vertically from top to bottom: Incubation with sera containing antibodies to SARS-CoV, hCoV-EMC, hCoV-HKU1, hCoV-OC43, hCoV-229E or hCoV-NL63.

IgG (left panel) and IgM (right panel) reactivity of the six sera to SARS-CoV and hCoV-EMC S1 protein (SARS Ag and EMC Ag respectively).

Colours reflect median spot intensity as shown in the legend on the right.

Antigens spotted in quadruplicate with dilution factor 1:2; sera dilution factor 1:20.

The two SARS-CoV sera SARS-1 and SARS-2 gave a clear positive reaction with the SARS antigen for IgG at dilutions from 1:20 to 1:80 and from 1:20 to 1:160, respectively, with no reactivity for IgM using the chosen cut-off. No reactivity was observed with the EMC antigen (Table, Figures 1 and 2).

Serological diagnosis

Convalescent sera from three patients with severe respiratory symptoms and a travel history to the Middle East were tested using the newly developed microarray. None of the patients showed positive reactivity for IgM or IgG with EMC-S1.

Discussion

We present a protein microarray-based serological test for the confirmation of hCoV-EMC and SARS-CoV infections. A major obstacle in the development of detection tools for novel, emerging viruses is the availability of sufficient, well-defined negative and positive sera for the assessment of the specificity and sensitivity of the assays. Nevertheless, results so far suggest that our microarray is highly specific for the detection of IgM and IgG antibodies against these emerging hCoVs, with no false-positive reactivity in 72 population sera and 14 sera known to be positive for one of the four widely circulating hCoVs -OC43, -HKU1, -229E and -NL63. Samples with a high titre were preferred for assay validation, but the exact titres of the antibodies against the common hCoVs in the latter validation cohort were not known.

However, previous studies from the Netherlands have found that by the age of 30 months, more than 50% of children seroconverted to one or more of the alpha- (hCoV-NL63, hCoV-229E) or betacoronaviruses (hCoV-OC43, hCoV-HKU1), and seropositivity reached 100% by 10 years of age for alphacoronaviruses [20,21]. The seroprevalence for betacoronaviruses was not specifically tested in the Netherlands, but found to be 91% in adults in the United States [24]. Therefore, the absence of false-positives in our population samples is strong evidence for the specificity of the method. IgG and IgM antibodies to hCoV-EMC and IgG to SARS-CoV were clearly detectable in positive patient sera. However, due to the small number of available positive patient sera, determination of the sensitivity of the assay in relation to viral load, clinical manifestation and phase

of infection requires further investigation. For this essential clinical validation, international sharing of positive sera by (national) laboratories in possession of such sera is a prerequisite.

Currently, WHO and ECDC recommend the collection of paired serum samples, preferably from the acute and convalescent phase, of all cases under investigation, as serological testing might be necessary to confirm infection when clinical presentation and epidemiology suggest an infection with hCoV-EMC despite negative PCR results [2,12]. In addition, serology is needed for contact investigations and source tracking. A two-staged serological approach is recommended, which proved effective in a contact investigation of an hCoV-EMC infection treated in Germany. It uses IFA with virus-infected cells for screening, and as second-stage recombinant spike- and nucleocapsid-transfected cells and virus neutralisation tests [22]. Our protein microarray enables specific, one-stage, high-throughput testing, with the benefit of minimal sample requirement. This technique can use dried blood spots for testing, which greatly facilitates shipping of samples.

The serological assay presented here is available and of great value for human and animal population screening, both of which are necessary to gain insight in the epidemiology of the novel hCoV. The array format can be modified to identify primary and intermediate animal reservoirs by simple adaptation of the conjugate used to visualise reactivity on the array (data not shown). Our assay is available to aid diagnosis in individual patients, for confirmatory testing of positive tests and for (large-scale) contact studies.

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Conflict of interest

None declared.

Authors' contributions

CR: assisted in designing the study, coordinated the study, analysed data, wrote manuscript. HM: performed laboratory testing and serum production. GJG: assisted in designing the study, performed laboratory testing, analysed data, read and revised manuscript. LvdH: performed laboratory testing, provided sera, read and revised manuscript. BM: performed laboratory testing, read and revised manuscript. MM: provided sera, performed laboratory testing, read and revised manuscript. BH: serum production, read and revised manuscript. RdS: assisted in study design, read and revised the manuscript.NS: performed laboratory testing and serum production. UD: provided sera, read and revised manuscript. PR: read and revised the manuscript. AO: provided sera, read and revised the manuscript. CD: provided sera, read and revised the manuscript. BJB: produced design antigen, provided antigens and serum, read and revised the manuscript. MK: assisted in designing the study, analysed data, read and revised the manuscript.

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Increased incidence of invasive group A streptococcal infections in Sweden, January 2012–February 2013

J Darenberg (Jessica.Darenberg@smi.se)[1], B Henriques-Normark[1,2,3], T Lepp[1], K Tegmark-Wisell[1], A Tegnell[1], K Widgren[1]
1. Swedish Institute for Communicable Disease Control (SMI), Solna, Sweden
2. Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden
3. Department of Laboratory Medicine, Division of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden

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The incidence of invasive group A streptococcal infections in Sweden was 6.1 per 100,000 population in 2012, the highest since the disease became notifiable in 2004. Furthermore, January and February 2013 marked a dramatic increase of cases notified, partly explainable by an increase of *emm*1/T1 isolates, a type previously shown to cause severe invasive disease more often than other types. Healthcare providers in Sweden and health authorities in neighbouring countries have been informed about this increase.

Background

Group A streptococci (GAS) can cause a variety of diseases, ranging from mild or moderate infections, such as tonsillitis and erysipelas, to more severe and sometimes life threatening conditions with septicaemia, shock and/or necrotizing fasciitis [1]. Furthermore, many individuals are asymptomatic carriers of GAS, especially children. Analyses have shown that 12% of children older than five years are carriers, as compared to 4% among younger children [2].

The number of invasive group A streptococcal (iGAS) infections often has a seasonal pattern with peaks during the winter and spring months. Furthermore, variations in the yearly number of reported cases are not uncommon. The cause of these variations is not fully known, but is likely dependent on factors such as underlying co-existing medical conditions, seasonal influenza infections, the GAS types that are circulating in the community, and the immunity against these types in the population. The prevalence of specific GAS types in a community shows high variability depending on geographic area and time period studied [3-6].

Invasive infection with GAS is notifiable in Sweden, according to the Communicable Diseases Act, since 1 July 2004. Both the physician at the diagnosing laboratory and the treating physician are obliged by this law to report iGAS cases to the county medical officer and to the Swedish Institute for Communicable Disease Control (SMI). Invasive isolates are normally collected in Sweden at national level at least every fifth year. Due to the noted increase of reported cases in 2012, we started collecting isolates ahead of time.

Here, we report an increased number of notified cases of severe GAS infection in Sweden, with a predominance of type *emm*1/T1 during 2012, and a further increase during the first two months of 2013.

Clinical data collection

Demographic information on iGAS cases were analysed using data from notification forms collected in the national surveillance database SmiNet. In order to establish the date of death, if applicable, the notification forms were linked to the national death register, using the personal identification code of each individual. Additionally, we calculated case-fatality rate for deaths within seven days from bacterial sampling.

Epidemiological typing

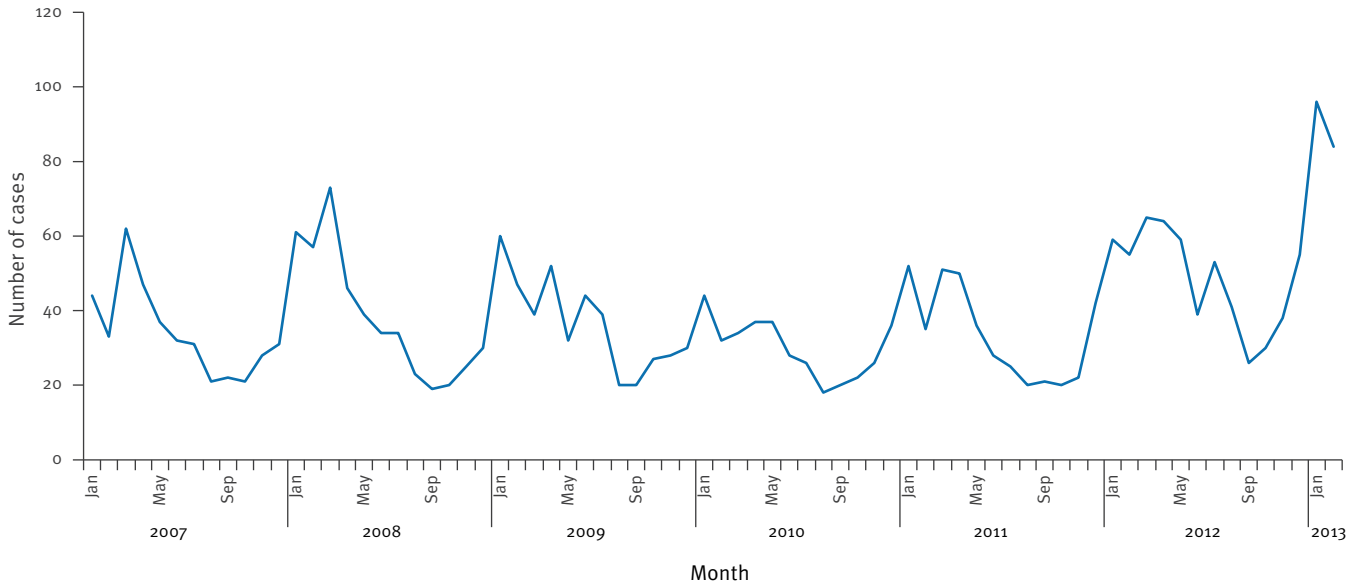
Available iGAS isolates (85% of all reported cases) from the clinical microbiological laboratories in Sweden were collected during 2012 and characterised by serological T-typing (n=400) and sequence-based *emm* typing (n=494 in 2012, and n=113 in 2013). The T-typing was performed using poly- and monospecific T antisera (Sevapharma). Genomic DNA was prepared from the isolates, and the *emm*-types determined by direct sequencing of PCR-products covering the N-terminal hypervariable portion of the *emm* gene [7].

Statistics

Incidence was calculated using population data as of 1 November each year from Statistics Sweden. The incidence in 2012 was compared to the incidence of each of the preceding years between 2006 and 2011, and we calculated the mean of the differences in incidence and the mean of the incidence rate ratios. A one-sample two-sided t-test was then applied to test the null hypothesis that the incidence was the same in 2012 as in previous years. Data from 2004 and 2005 were excluded as there could have been suboptimal completeness in the first few years of the notification system. Differences in type distribution between 2012

Figure 1

Reported cases of invasive group A streptococcal infections per month, Sweden, January 2007–February 2013



Table

Number of cases of invasive group A streptococcal infections and incidence per 100,000 inhabitants, Sweden, 2006–2012

Age group (years)	2006	2007	2008	2009	2010	2011	2012	Mean incidence 2006-2011	Difference in incidence (95% CI)[a]	Incidence rate ratio (95% CI)[a]
	Number of cases (incidence)									
0-19	25 (1.2)	40 (1.9)	31 (1.4)	30 (1.4)	17 (0.8)	33 (1.5)	45 (2.1)	1.4	0.73 (0.35-1.11)	1.65 (1.09-2.22)
20-39	42 (1.8)	58 (2.5)	60 (2.6)	60 (2.5)	38 (1.6)	58 (2.4)	69 (2.8)	2.2	0.57 (0.12-1.01)	1.30 (1.00-1.60)
40-59	79 (3.2)	85 (3.5)	95 (3.9)	91 (3.7)	78 (3.2)	77 (3.1)	116 (4.7)	3.4	1.23 (0.90-1.56)	1.37 (1.24-1.49)
60-79	110 (6.5)	137 (7.9)	157 (8.8)	156 (8.5)	129 (6.9)	118 (6.2)	218 (11.4)	7.5	3.89 (2.76-5.02)	1.55 (1.31-1.78)
≥80	67 (13.4)	89 (17.8)	118 (23.5)	101 (20.1)	98 (19.3)	116 (22.9)	136 (26.8)	19.5	7.30 (3.45-11.15)	1.42 (1.09-1.75)
Total	**323 (3.5)**	**409 (4.5)**	**461 (5.0)**	**438 (4.7)**	**360 (3.8)**	**402 (4.2)**	**584 (6.1)**			

CI: confidence interval.

[a] Comparisons of the incidence in 2012 with the mean incidence 2006–2011.

and the first months of 2013 were tested using the chi-square test. The alpha error was set to 0.05.

Results

In 2012, 584 cases of iGAS infections were notified in Sweden which corresponds to an annual incidence of 6.1 cases per 100,000 inhabitants. This is the highest yearly number and incidence of reported cases since these infections became notifiable in 2004 (Figure 1). Furthermore, in January and February 2013, an additional 96 and 84 cases respectively were reported, which indicates a continuing increase in incidence rate also during 2013 (Figure 1).

The median age of the cases in 2012 was 65 years and the highest incidence was seen among people above 80 years of age. Men and women were equally affected, except for the age group 20–39 years where women were overrepresented. Of 43 women in this age group, 30% had confirmed puerperal sepsis. In total, 60% of these women had gynaecological infections.

The incidence rate ratio was significantly higher in 2012 compared to previous years, in all age groups except for a borderline difference for the 20–39 years age group (Table). However, the difference in incidence was the highest in the age group 80 years and above (an increase of 7.3 cases per 100,000 inhabitants per year).

In 2012, 46 of the 584 cases (8%) died within seven days from bacterial culture sampling. This is comparable with Swedish data from 2010–2011 (data not shown). The median age for deceased patients was 77 years.

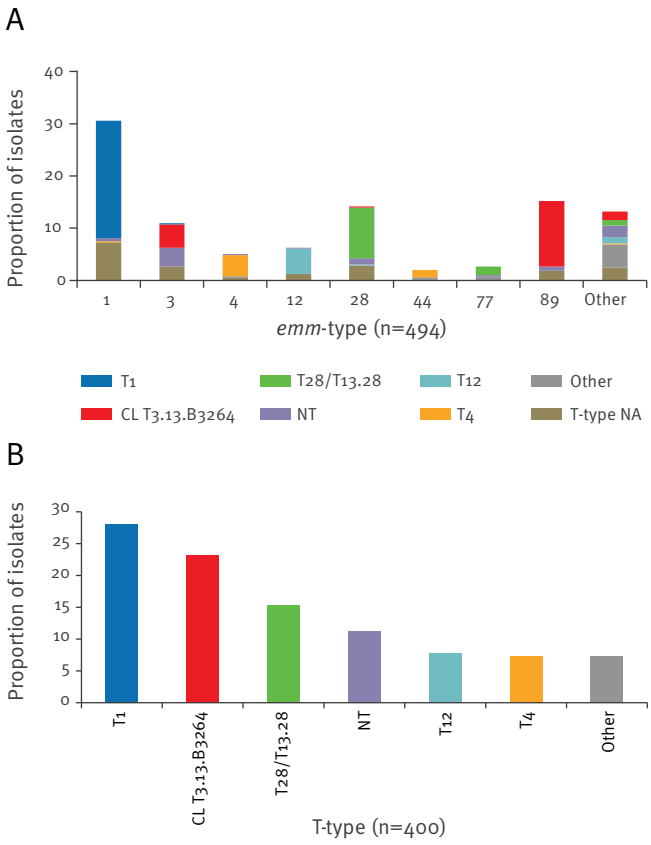
So far 400 (68%) isolates from the reported cases in 2012 have been received for T- and *emm*-typing. In addition, 94 isolates have been *emm*-typed only. Overall, 30% of the typed 494 isolates were *emm*1/T1 in 2012, followed by *emm*-types 89 (15%) 28 (14%) and 3 (11%) (Figure 2). However, preliminary data from isolates sampled during the first two months 2013 shows a significant increase of *emm*1/T1 constituting about 55% of the 113 typed isolates ($p<0.001$) (Figure 3). Bacterial isolates from 39 of the 46 deceased patients in 2012 have been received for typing; 13 of the isolates were *emm*1, followed by 7 *emm*3 and 6 *emm*89. The type distribution for these patients cannot be compared to previous years, as isolates were not collected for typing then.

Discussion

In 2012 we observed the highest yearly number of notified cases of invasive GAS infections since it became notifiable in Sweden 2004. January 2013 marked the highest number of cases reported in a single month so far, continuing also during February. Importantly, we noticed an emergence of *emm*1/T1 among these invasive cases. The relative increase in incidence in 2012 compared to previous years was similar for all age groups. However, as the incidence was highest among the elderly (80 years and above) the largest increase in absolute numbers was seen within this group. A similar increase in the number of cases has not been seen in our neighbouring countries during 2012. However, Finland noted a somewhat increased incidence in 2012 as compared to 2011 (4.4 cases per 100,000 population in 2012, compared to 3.13 cases per 100,000 population in 2011). Furthermore, an increase has been noted in Norway during the first months of 2013 as compared to the same time period last year, with twice as many reported cases. So far, 60 cases have been reported in Norway in 2013 and 56 have been typed; 24% of the isolates typed are *emm*1 (personal communications, S Hoffmann (SSI, Denmark), J Voupio-Varkila (THL, Finland), M Steinbakk (FHI, Norway), March–April 2013). Several reasons can be suggested to explain this increase in incidence in Sweden. Raised awareness in local authorities and laboratories, who had been informed in spring 2012, may partly explain this increase. Also an increased transmission of respiratory

Figure 2

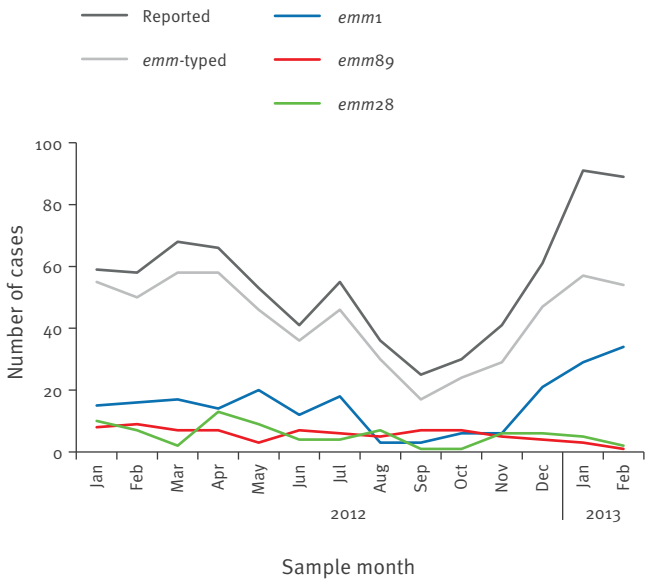
Type distribution of invasive group A streptococcal isolates sampled in 2012, Sweden



CL: cluster (consists of different combinations of T3.13.B3264).

Figure 3

Reported cases of invasive group A streptococcal infection per month and proportion of *emm* sequenced isolates, including the three most prevalent *emm*-types, Sweden, 2012–2013



viral diseases could potentially be a factor, since GAS infections can be transmitted secondary to these infections, especially influenza virus infection [8,9]. An increase of viral infections, not only the seasonal influenza, has been noted in Sweden during the present season. The increase of iGAS infection was first noted during the spring months of 2012, with the prevalence remaining high in autumn 2012, before the influenza season started.

A correlation has previously been found between certain *emm*-types and disease. The *emm*1/T1 type has been shown to cause more severe infections, often with higher fatality rates than other serotypes and was, together with type *emm*3 isolates, the predominant cause of outbreaks of severe iGAS disease during the 1980s, also in Sweden [10-12]. Interestingly, the type distribution among the isolates in our surveillance study from 2002 to 2004 showed a completely different type distribution as compared to the present study. During that time period, *emm*-types 89 and 81 accounted for 30% of the isolates and *emm*1 for 12% only [13]. Furthermore, *emm*3 isolates were very scarce, as compared to 11% of the isolates typed in 2012. In 2009 we performed another survey of iGAS infection where 83% of the reported isolates were T-typed. The type distribution was more similar to the present study, as the major types in descending order then were T1 (27%), T28 (21%), those in the cluster T3.13. B3264 (20%), NT (11%) and 12 (7%) (unpublished data). The present study, with the collection of bacterial isolates, makes it possible to characterise and study specific groups of isolates, such as the *emm*1/T1, more in-depth in the future, and also to compare these with isolates collected during previous study periods.

The epidemiology of GAS disease changes over time, but also between countries. In a European Union-funded collaboration, with 13 participating countries, differences in incidence and fatality rates were found between the countries which could be explained partly by differences in circulating types [4,14]. Only in a few European countries iGAS is a notifiable disease. However, we would recommend countries to monitor the national incidence of the disease, and that each country has alert systems to detect and analyse epidemiological changes.

Due to the observed increase of iGAS incidence in Sweden, SMI now informs healthcare providers through different channels about the emergence of these infections. The main purpose has been to create increased awareness of the disease to improve management and thereby possibly reduce mortality. Neighbouring countries have been contacted and informed of the situation in Sweden in order to increase awareness among healthcare providers in these countries.

SMI also encourages the laboratories to send clinical isolates for typing and will improve the collection of clinical data within the surveillance system in order to gain a better understanding of the epidemiology and transmission patterns of iGAS disease.

Acknowledgements

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Authors' contribution

Jessica Darenberg, Tiia Lepp and Katarina Widgren performed the analyses and drafted the report. All authors supported the analysis, wrote the manuscript and approved the final report submitted. Anders Tegnell has an overall responsibility for the Swedish surveillance system that found the signal. Birgitta Henriques-Normark has the overall responsibility for the epidemiological typing.

Conflict of interest

None declared.

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More reasons to dread rain on vacation? Dengue fever in 42 German and United Kingdom Madeira tourists during autumn 2012

C Frank (FrankC@rki.de)[1], M Höhle[1], K Stark[1], J Lawrence[2]
1. Robert Koch Institute, Dept. for Infectious Disease Epidemiology, Berlin, Germany
2. Travel and Migrant Health Section, Centre for Infectious Disease Surveillance and Control, Public Health England, London, United Kingdom

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In October and November 2012 residents and tourists in the Autonomous Region of Madeira, Portugal, were affected by dengue fever. The outbreak waned during the unusually dry winter. Using a Monte Carlo test we investigated the hypothesis that rainy weather conveyed increased risk of dengue virus infection among tourists. Results confirmed the hypothesis. As it is unclear whether the outbreak is over, upkeep and emphasis on mosquito avoidance on rainy days may help residents and tourists reduce infection risk.

Background

Public health authorities in the Autonomous Region of Madeira (RAM), Portugal have been monitoring an outbreak of dengue fever beginning October 2012 [1]. RAM is an archipelago in the Atlantic, politically part of Portugal but closer to Africa and on the same latitude as the African north coast. At 32° northern latitude the islands have a maritime and subtropical climate. Rains fall predominantly in the months of October through March [2]. As of 3 February 2013, over 2,000 cases among residents of Madeira had been reported, most between October and November 2012 [3]. In addition, 78 cases from 13 European countries (including travellers from mainland Portugal) have been reported by the European Centre for Disease Prevention and Control (ECDC) in tourists to Madeira, including 19 from Germany and 23 from the United Kingdom (UK) [4]. The number of cases decreased in December 2012 [2] although a small number of cases in residents and tourists have continued to be reported throughout January and February 2013 [5-7].

A non-statistical comparison of German case data with rainfall information from an internet data source [8] led to the hypothesis, that visiting Madeira in autumn 2012 during rainy days conferred a higher dengue virus infection risk than visiting during drier periods. To test this hypothesis, a Monte Carlo simulation with information from 19 German and 23 UK cases together with Madeira weather data, was used. The results may help to interpret a relative dearth of new cases since December and offer risk reduction guidance for residents in and tourists visiting Madeira.

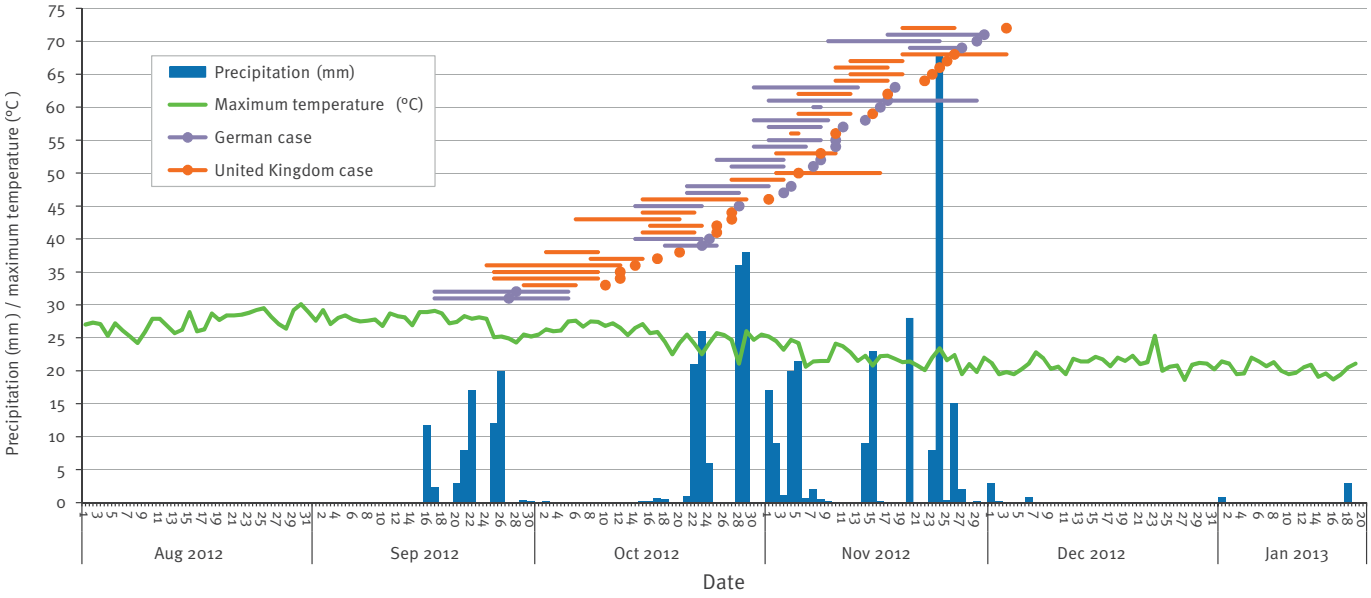
Methods

The German National Weather Service provided validated information on rainfall at Funchal, Madeira, weather station between 1 July 2012 and 20 January 2013 (6 am 24h reading projected on the preceding date) [9]. Laboratory-confirmed clinical dengue cases with known travel history to Madeira, as well as dates of travel to Madeira, sex, age and symptom onset dates, were extracted from the German database on notifiable infectious diseases [10] and the UK national dengue database [11]. Starting with the first reported cases which occurred in September 2012 this yielded a total of 42 cases. Based on a potential dengue fever incubation period of three to 14 days [12], likely risk periods on the island were calculated: date of arrival, or date of onset minus 14 days (whichever came later), to three days before symptom onset or date of departure (whichever came earlier).

A Monte Carlo test was performed as follows: in the absence of actual control travellers, the travels to Madeira of 42 German and UK hypothetical travellers were simulated 9,999 times. Data on German [13] and UK [14] traveller numbers per month were used as weights to account for any fluctuations in traveller numbers. For each simulation, 42 start dates (i.e. arrival dates to Madeira) between 15 September and 21 December 2012 were drawn at random from all available dates, each day's probability to be drawn proportional to the country-respective traveller numbers. In the two separate country groups, the cases' risk periods were randomly allotted with replacement to these starting dates, yielding 42 risk periods per set. These 9,999 control sets and the actual 42 cases were compared on the following criteria: Whether they had

Figure 1

Travel periods to Madeira and symptom onset dates of German and United Kingdom laboratory-confirmed dengue cases, relative to daily precipitation and maximum temperature in Funchal, Madeira, 1 August 2012–19 January 2013 (n=42 cases)



Travel periods of the cases to Madeira are indicated by horizontal bars. Dots represent cases at time of symptom onset.

experienced any rain at all (>0 mm), what proportion of days during their risk period had been rainy, and the mean daily mm of rain during their risk period. A one-sided Monte Carlo p-value was determined as the number of realisations among the 10,000, having a value equal to or more extreme than the actual observed value in the set of cases.

Results

Between September 2012 and March 2013, 42 cases of dengue fever were reported in Germany and the UK, including 19 from Germany and 23 from the UK. For all cases, complete data were available. Symptom onset dates ranged from 27 September to 3 December 2012. Sex distribution was even, median age was 57 (range: 20–73) years. Median duration of travel was eight days (range: 1–29).

Figure 1 shows cases' travel periods and onset dates relative to daily rainfall totals and maximum daily temperature between 1 August 2012 and 19 January 2013.

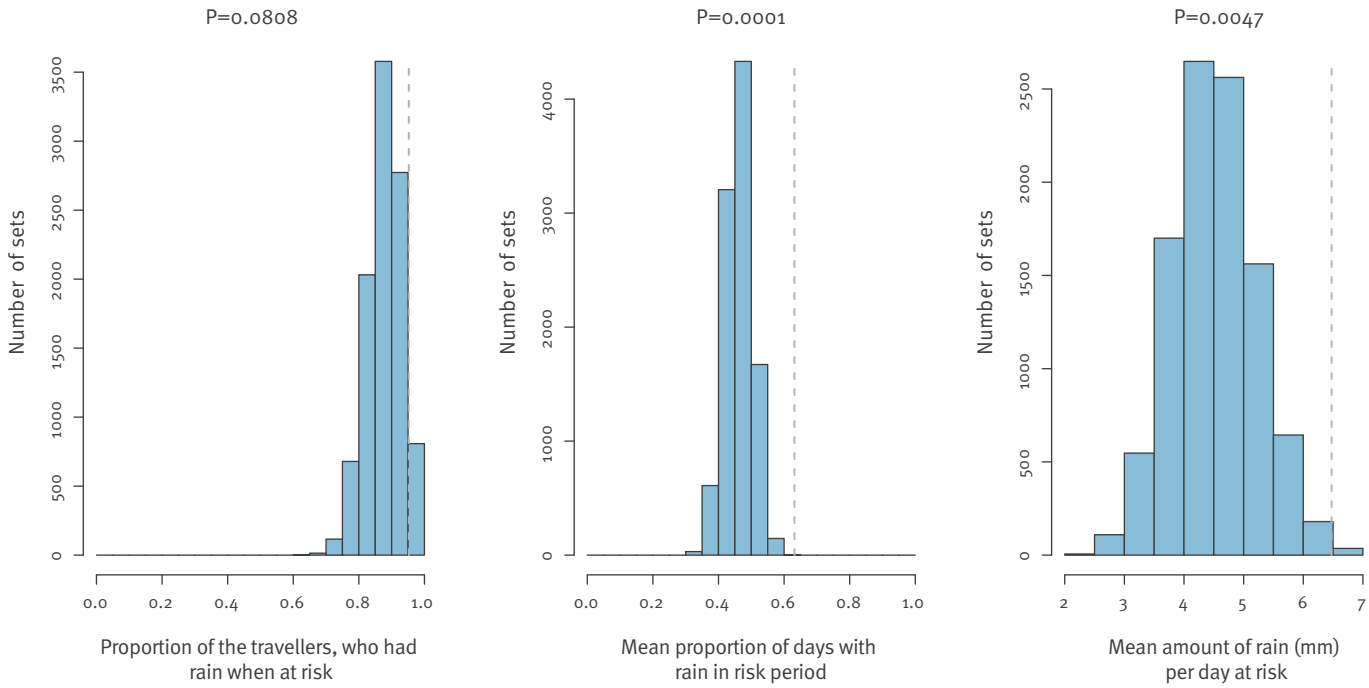
In the statistical comparison between the 42 cases and the simulated travellers (Figure 2), 40 of 42 cases (95.2%) experienced at least one day of rain during their risk period. Altogether, in the 10,000 realisations, a set with 92.5% or more* of the simulated travellers experiencing any rain during their risk period was very unlikely (808/10,000, p=0.0808). For the only two cases without rain in their risk period it rained on the day of their departure, two days before disease onset. For the cases, 63.2% of their risk period days were rainy – the highest value among all 10,000 realisations, i.e. p=1/10,000 or 0.0001. The cases also experienced statistically significant higher mean amounts of rain (6.5 mm per day on average during their risk period) than the simulated travellers (4.5 mm per day, p=0.0047). One case each from Germany and the UK were in Madeira during one-day cruise ship stops; on both days it rained.

Discussion

Our investigation supports a temporal association between tourists experiencing rain on Madeira and acquiring dengue fever in late 2012. While the lack of specific control persons may be considered a limitation, the Monte Carlo method is a common statistical technique to assess associations [15]. In our study the lack of specific control persons is compensated by generating hypothetical control individuals based on only few assumptions, e.g. that travel length in controls is similar to the cases regardless of time of arrival. Weighting the analysis with monthly traveller numbers buffers the results against fluctuations caused by other factors (e.g. school holiday periods). The dengue mosquito vector, *Aedes aegypti*, was first identified in

Figure 2

Rain exposure, comparison between the set of 42 laboratory-confirmed cases of dengue fever and 9,999 simulated sets of 42 travellers arriving on Madeira, 15 September–21 December 2012 *



The 42 laboratory-confirmed dengue cases are indicated by a vertical gray dashed line. The 9,999 simulated sets of 42 travellers are shown in the form of blue histograms based on the total 10,000 sets.

Madeira in the mid-2000s [16] but no dengue cases were reported in Madeira until October 2012. The lack of recognised cases during the dry and warm summer period before the outbreak and the fact that there were no cases among travellers from Germany and the UK in December, a month with little rain in Madeira, would support the importance of rainfall for dengue virus transmission in Madeira – the trickle of reported cases in travellers from other countries and among Madeirans in December or thereafter is hard to interpret without known onset dates (published data only relies on notification dates). A combination of decreasing temperatures (Figure 1) in December and vector control efforts by the Madeiran authorities [17] may have also contributed to the decrease in cases reported into December [17].

The exact role of rain in dengue virus transmission in Madeira is outside the scope of this paper, but it is important to identify rain as a marker for the temporarily increased risk to those exposed. Our data are in agreement with a tight temporal association – either the infective bites occur on the same days as the rain (possibly before, between or after showers rather than during actual rain), or with a short lag after the rain on following days. Aside from rain-increased mosquito activity another contributing factor could be that travellers may believe they do not need to use mosquito bite avoidance measures as rigorously on rainy days, or it is possible that indoor biting may play a role. Individual risk factors for bites constitute an area that requires further study.

December 2012 and January 2013 so far have been dryer than average (4 mm of rain in both of these months, compared with a median of 113 mm and 67 mm, for December and January, respectively between 1994 and 2011) [2]. In the coming months both increasing temperatures and rainy periods are to be expected. Until it is clear that the outbreak will not flare up again in 2013, tourists to Madeira (as well as residents) should take precautions as for any area endemic for dengue fever, regardless of the weather: Risk of infection can be reduced by using personal mosquito bite avoidance measures (e.g. cover-up clothing, repellents) during the day, especially around dawn and dusk when *Aedes* mosquitoes are most active [18].

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Conflict of interest

None declared.

Authors' contributions

Christina Frank devised the study, contributed analysis and wrote much of the manuscript. Michael Höhle conducted the Monte Carlo test and contributed to writing. Klaus Stark provided advice on methodology and contributed to writing. Joanne Lawrence supplied UK data for the analysis and contributed to the writing and critical review of the manuscript.

*Erratum:

The text and Figure 2 were updated on 05 April 2013.

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Polymerase chain reaction-based screening for the ceftriaxone-resistant *Neisseria gonorrhoeae* F89 strain

N Goire[1,2], M M Lahra[3], M Ohnishi[4], T Hogan[3], A E Liminios[3], M D Nissen[1,2,5], T P Sloots[1,2,5], D M Whiley (d.whiley@uq.edu.au)[1,2]

1. Queensland Paediatric Infectious Diseases Laboratory, Queensland Children's Medical Research Institute, The University of Queensland, Queensland, Australia
2. Sir Albert Sakzewski Virus Research Centre, Children's Health Service, Queensland, Australia
3. WHO Collaborating Centre for STD, Microbiology Department, South Eastern Area Laboratory Services, The Prince of Wales Hospital, Sydney, New South Wales, Australia
4. National Institute of Infectious Diseases, Tokyo, Japan
5. Microbiology Division, Pathology Queensland Central Laboratory, Herston, Queensland, Australia

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Emergence and spread of *Neisseria gonorrhoeae* resistant to extended spectrum cephalosporins is a major problem threatening treatment of gonorrhoea and is further highlighted by the recent report of a second ceftriaxone-resistant *N. gonorrhoeae* strain (F89) in Europe, initially observed in France and subsequently identified in Spain. *N. gonorrhoeae* antimicrobial resistance (AMR) surveillance has acquired new importance and molecular tools have the potential to enhance bacterial culture-based methods. In this study, we established a polymerase chain reaction (PCR) protocol for direct detection of the F89 strain. A key component of this screening protocol was the development of a hybridisation probe-based melting curve analysis assay (mosaic501-hybPCR) to detect the presence of an A501P substitution on the *N. gonorrhoeae* mosaic penicillin binding protein 2 (PBP2) sequence, an important characteristic of the F89 strain. The mosaic501-hybPCR was evaluated using plasmid-derived positive controls (n=3) and characterised gonococcal (n=33) and non-gonococcal (n=58) isolates. The protocol was then applied to 159 clinical specimens from Sydney, Australia, collected during the first half of the year 2012 that were *N. gonorrhoeae* PCR-positive. Overall, the results indicate that the PCR-based protocol is suitable for direct detection of the *N. gonorrhoeae* F89 strain in non-cultured clinical samples. It therefore provides an additional tool to aid investigations into the potential spread of F89 strain throughout Europe and elsewhere.

Introduction

With the emergence of the ceftriaxone-resistant and extensively-drug resistant (XDR) *Neisseria gonorrhoeae* strains H041 in Japan [1] and F89 in France and Spain [2,3], there exists a real threat that such strains may emerge and spread worldwide. Molecular characterisation of these strains has implicated mutations in the penicillin binding protein 2 (PBP2) in conferring resistance to ceftriaxone [1,2]. Notably, the 'mosaic' variant of PBP2 has long been associated with reduced susceptibility to ceftriaxone and other extended spectrum cephalosporins (ESCs), and both the Japanese H041 and European F89 strains harboured a mosaic PBP2. In each of these cases however, additional mutations on the mosaic PBP2 were responsible for expression of resistant phenotypes displaying a raised ceftriaxone minimum inhibitory concentration (MIC). The Japanese H041 (ceftriaxone MIC=2 to 4 mg/L) featured several novel substitutions on the mosaic PBP2, of which two (A311V and T316S) have been experimentally shown to contribute to ESC resistance [1]. Similarly the European F89 strain (ceftriaxone MIC=1 to 2 mg/L) harboured an amino acid substitution at position 501 (A501P) of the mosaic PBP2, resulting in ESC resistance [2,3]. The contribution of other substitutions at position 501 of PBP2, especially A501V but also A501T, towards reduced susceptibility to ESCs has also been shown [4-7].

N. gonorrhoeae antimicrobial resistance (AMR) surveillance is a pivotal component of public health efforts to control gonorrhoea. Molecular methods for tracking gonococci of major public health importance have considerable potential to enhance *N. gonorrhoeae* AMR surveillance and to this effect we have previously described a rapid real-time polymerase chain reaction (PCR) method for direct detection of the H041 strain [8]. In this study we describe a testing algorithm for direct detection of the *N. gonorrhoeae* F89 strain. Briefly, the algorithm involved two steps. The first step screened *N. gonorrhoeae* nucleic acid amplification test (NAAT)-positive specimens for the presence of mosaic PBP2 using a previously described real-time PCR assay (mosaic-PCR; [9,10]). Samples testing positive by the mosaic-PCR were then subjected to a hybridisation probe-based melting curve analysis assay, developed and validated as part of this study, to characterise the 501 amino acid on the mosaic PBP2 sequence (mosaic501-hybPCR).

Table 1

Primers and probes used to analyse *Neisseria gonorrhoeae* mosaic penicillin binding protein 2 sequences

Designation	Sequence (5' to 3')[a]
Mosaic-PCR	
Mosaic-F	GTTGGATGCCCGTACTGGG
Mosaic-R	ACCGATTTTGTAAGGCAGGG
Mosaic-Probe	FAM-CGGCAAAGTGGATGCAACCGA-BHQ
Mosaic501-hybPCR	
Mosaic501-F	GGCGCAAAAACCGGTACG
Mosaic501-R	ATCACACGCGGATTTTTAGCC
Mosaic501-Probe1	CGCAAAAACCGGTACGCCG-fluorescein
Mosaic501-Probe2	Quasar 670-GTAAGTTGGTTAACGGTCGTTACGTCGATTACAAACAC-phosphate
Plasmid control development	
Control-A501P-F	GGCGCAAAAACCGGTACGCCG
Control-A501T-F	GGCGCAAAAACCGGTACGACG
Control-A501V-F	GGCGCAAAAACCGGTACGGTG
PenA-R	GCCCAAGATGTTCAGGCTGC

BHQ: black hole quencher dye; F: forward primer; PCR: polymerase chain reaction; R: reverse primer.

[a] When present, dye labels or 3' phosphates are also indicated.

Methods

Mosaic-PCR assay

The mosaic-PCR was performed using primers and TaqMan probe previously described by Ochiai et al. [10]. Briefly, the reaction mix consisted of 12.5 µl of QuantiTect Probe PCR Master Mix (QIAGEN, Doncaster, Australia), 10.0 pmoles each of forward and reverse primer (Mosaic-F and Mosaic-R; Table 1), 4.0 pmoles of probe (Mosaic-Probe; Table 1) and 2.5 µl of sample nucleic acid extract in a final reaction volume of 25.0 µl. Reactions were thermo-cycled on a Rotor-Gene 6000 real-time PCR instrument (QIAGEN, Doncaster, Australia) with the following parameters: initial hold at 95°C for 15 min followed by 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Results were analysed using the quantification analysis tool of the Rotor-Gene software and samples were considered positive if their amplification curves crossed above background fluorescence. We have previously shown this assay to be suitable for direct screening for the *N. gonorrhoeae* mosaic PBP2 sequence directly in non-cultured clinical samples [9].

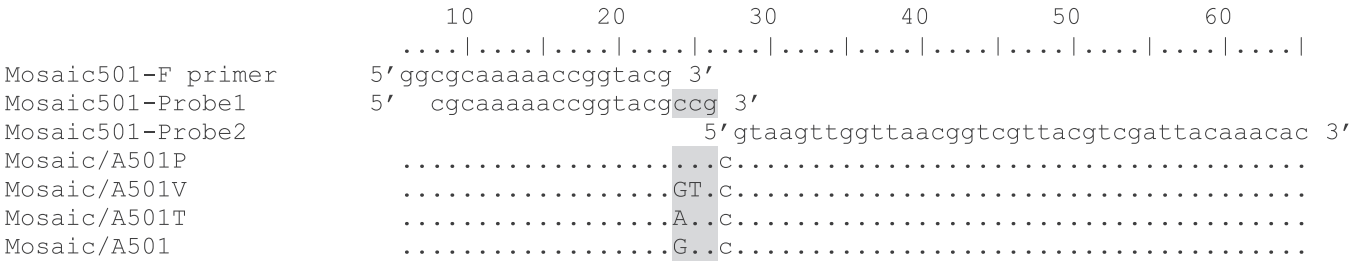
Mosaic501-hybPCR assay

The mosaic501-hybPCR was developed as per a modified hybridisation probe-based real-time PCR approach previously described by our laboratory [11]. Briefly, two primers (Mosaic501-F and Mosaic501-R; Table 1) were used for amplification of the target region and two probes (Mosaic501-Probe1 and Mosaic501-Probe2; Table 1) were used for detection, with Mosaic501-Probe1 being the sensor probe and Mosaic501-Probe2 being the anchor probe. In this modified hybridisation probe approach, the Mosaic501-F primer shares the same target sequence as the Mosaic501-Probe1 sensor probe except for the latter 3-base codon representing the 501 amino acid position (Figure). As previously described, this ensures the sensor probe has full homology with its target sequence with the exception of any variations, if present, in the 501 codon of interest [11].

The mosaic501-hybPCR assay was performed using the LightCycler FastStart DNA Master Hybridization Probes kit (Roche Diagnostics, Australia) on a LightCycler 2.0 real-time PCR instrument (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, each LightCycler capillary was loaded with 2 µl of 10 x kit Master reagent (Roche Diagnostics, Australia, reagent 1), 1.6 µl of $MgCl_2$ (25mM; Roche Diagnostics, Australia, reagent 2), 5.0 pmoles of Mosaic501-F primer (Table 1), 10.0 pmoles of Mosaic501-R primer (Table 1), 2.0 µl of nucleic acid extract or control and made up to a total reaction volume of 20 µl. Capillaries were then placed into LightCycler centrifuge adapters (Roche Molecular Biochemicals, Mannheim, Germany) and spun in a standard 24 x 1.5 ml microfuge at approximately 735 g for 10 seconds. A 2.0 µl suspension comprising a total of 4.0 pmoles of each probe (Table 1) was then added into the sample receptacle of each capillary. The capillaries were then capped but were not microfuged again at this point; this left the PCR reaction in the bottom of the capillary and separated from the probes in the upper receptacle. In this modified hybridisation probe format, the probes must remain separate from the reaction mix during PCR amplification otherwise amplification can be inhibited [11].

Figure

Alignment of the mosaic501-hybPCR forward primer and hybridisation probes with sequences of *Neisseria gonorrhoeae* strains harbouring mosaic penicillin binding protein 2 types differing at residue 501



PBP2: penicillin binding protein 2.

The nucleotide sequences of the mosaic PBP2 types are designated by 'mosaic' followed by details of the particular residue or mutation at position 501 of the protein sequence. Nucleotide sequence numbering is based on the first 60 nucleotides of the 107 base pair product obtained in the real-time polymerase chain reaction assay. The codon for the PBP2 '501' amino acid position is represented by nucleotides 19 to 21. Both probes match 100% with the mosaic/A501P sequence whereas Mosaic501-probe1 has mismatches with the other PBP2 types, enabling discrimination by melting curve analysis. Dots indicate sequence identity with the primer or probe sequences. Capitalised bases indicate mismatches with Mosaic501- Probe1.

Amplification was performed on the LightCycler with the following conditions: initial hold at 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 20 seconds. No data collection was conducted during the PCR as the probes were not present in the mix. Following PCR, the capillaries were spun upside down (without the LightCycler centrifuge adapters) in a 24 x 1.5 ml microfuge at approximately 735 g for 10 seconds, and then placed back into the LightCycler centrifuge adapters and respun right side up as above. This double spinning was used to wash the probes out of the top of the capillary and bring the whole contents (probes and PCR mix) into the bottom of the capillary. Capillaries were then placed back into the LightCycler and melting curve analysis conducted using the following parameters; 95°C for 5 seconds, then the reactions were continuously monitored as they were heated at a rate of 0.2°C/second, starting at 40°C for 30 seconds to a final temperature of 85°C.

Assay reference controls

A mosaic PBP2-harbouring *N. gonorrhoeae* isolate from a previous study (sequence pattern X; [5]) was used as the positive control for the mosaic-PCR assay. This isolate lacked further alterations at amino acid position 501 (mosaic/A501) and so was also used as the reference control for the mosaic501-hybPCR assay. Given that we did not have access to the *N. gonorrhoeae* F89 strain, which has the sequence encoding the mosaic PBP2 protein with the A501P mutation (mosaic/A501P), we had a mosaic/A501P plasmid control (i.e. a mosaic *penA* sequence with a A501P codon; CCG) synthesised by the University of Queensland Protein Expression Facility (University of Queensland, Australia). The mosaic/A501 clinical isolate was amplified using primers Control-A501P-F and PenA-R (Table 1). Use of the Control-A501P-F primer, with 'CCG' at the 3'end, enabled the A501P codon to be incorporated into the mosaic PBP2 sequence in the resulting PCR product. The PCR product was then cloned using the pGEM-T Easy Vector system and the plasmid purified. Plasmid controls were also created for the partial sequences of the mosaic PBP2 harbouring the A501T (mosaic/A501T) and A501V (mosaic/A501V) mutations as described above, using primers Control-A501T-F and Control-A501V-F respectively (Table 1).

Mosaic501-hybPCR assay evaluation

The specificity of the mosaic501-hybPCR assay was investigated using a panel of *Neisseria* species, comprising both gonococcal (n=33) and non-gonococcal strains (n=58). The 33 *N. gonorrhoeae* isolates were of mainly Australian and Asian origin (years of isolation: 1988 to 2009) from a previous study [5], comprising 29 different *N. gonorrhoeae* multi-antigen sequence types (NG-MAST) and 23 different PBP2 types. Four isolates comprised mosaic PBP2 sequences and included the *N. gonorrhoeae* H041 strain [1]. Of these four mosaic-harbouring isolates, one isolate also possessed an A501V alteration (mosaic/A501V) and was the previously described cefixime-resistant NG0304 strain [7], kindly provided by Dr. Makoto Ohnishi, institute of infectious diseases, Japan. The 58 non-gonococcal strains comprised *Moraxella catarrhalis* (n=6), *M. osloensis* (n=2), *N. cinerea* (n=4), *N. elongata* (n=1), *N. lactamica* (n=8), *N. meningitidis* (n=21), *N. polysaccharea* (n=4), *N. sicca* (n=1), *N. subflava* (n=10) and *N. weaveri* (n=1), and were predominantly isolated in New South Wales, Australia during the years 2007 to 2009.

Application to clinical samples

Following evaluation of the mosaic501-hybPCR assay, 159 clinical specimens were screened for the presence of mosaic PBP2 sequences and alterations at amino acid 501. The samples comprised DNA extracts from 62 urine specimens and 13 endocervical, eight vaginal, 74 rectal and two throat swabs that were PCR-positive for *N. gonorrhoeae* at the South Eastern Area Laboratory Services in Sydney, New South Wales during the first half of 2012. Samples were initially tested using the mosaic-PCR. Samples providing positive results in the mosaic-PCR were then characterised using the mosaic501-hybPCR assay.

Detection limit

A ten-fold dilution series of DNA from the *N. gonorrhoeae* NG0304 strain [7] in water (10E-1 to 10E-9) was prepared. The dilutions were tested in duplicate in the mosaic501-hybPCR and mosaic-PCR assays above, as well as an *N. gonorrhoeae* duplex real-time PCR assay (NG-duplex) routinely used by our laboratory for detection of gonorrhoea and targeting the gonococcal *porA* pseudogene and multicopy *opa* genes [12]. The detection limit of each assay was determined as the lowest concentration returning positive results in both duplicates.

Results

Table 2 provides the melting temperatures for the controls (samples 1 to 4; Table 2), all isolates (samples 5 to 30; Table 2) and all clinical specimens (samples 31 to 40; Table 2) for which melting curves were obtained in the mosaic501-hybPCR assay.

Mosaic501-hybPCR assay evaluation

The results for the controls (samples 1 to 4; Table 2) showed that based on melting temperature, isolates with mosaic/A501P (68.0°C) or mosaic/A501V (56.5°C) could be distinguished from isolates with mosaic/A501 or mosaic/A501T (58.48°C and 58.50°C respectively). However, isolates with mosaic/A501 or mosaic/A501T could not be distinguished from each other given their similar melting temperatures. The latter was not considered a problem given that an isolate with a mosaic/A501T alteration has not been described to date.

The mosaic501-hybPCR assay results for the 33 *N. gonorrhoeae* clinical isolates were consistent with the results of DNA sequencing. The isolates with mosaic/A501 (n=3) and mosaic/A501V (n=1) were correctly characterised by the mosaic501-hybPCR assay (samples 5 to 8; Table 2). No melting curves were observed for the remaining 29 *N. gonorrhoeae* isolates and were consistent with all of these isolates lacking a mosaic PBP2 sequence.

Of the 58 non-gonococcal strains, 22 (samples 9 to 30; Table 2) provided melting curves in the mosaic501-hybPCR assay; *N. cinerea* (n=3), *N. elongata* (n=1), *N. lactamica* (n=7), *N. meningitidis* (n=3), *N. polysaccharea* (n=2) and *N. subflava* (n=6). Of these, 19 were characterised as mosaic/A501 whereas the remaining three isolates could not be characterised on the basis that their melting curves were not consistent with any of the reference controls.

Application to clinical samples

Of the 159 clinical specimens, 10 (6 rectal swabs and 4 urine samples) provided positive results in the mosaic-PCR assay. When tested by the mosaic501-hybPCR assay, all were characterised as mosaic/A501 strains (samples 31 to 40; Table 2).

Detection limit

The testing of ten-fold dilutions of DNA from the NG0304 strain showed that the mosaic501-hybPCR and mosaic-PCR assays had comparable detection limits, with both detecting to the 10E-7 dilution. However, both these methods were 10-fold less sensitive than the NG-duplex assay which detected to the 10E-8 dilution.

Discussion

The fact that the *N. gonorrhoeae* F89 strain has been found in both France and Spain suggests that this may indeed be the first high-level ceftriaxone-resistant gonococcal strain spreading internationally. Enhanced surveillance for this strain is therefore warranted, particularly in Europe. The main aim of this study was to develop a real-time PCR method that could distinguish *N. gonorrhoeae* strains of the mosaic/A501P PBP2 type so that it could be used for direct screening for the *N. gonorrhoeae* F89 strain. Overall, the approach appears suitable for this purpose, and in fact could potentially also be used to detect strains of the mosaic/A501V PBP2 type. When applied to 159 *N. gonorrhoeae* PCR-positive samples from our local Sydney (Australia) population, we found that 10/159 (6.3%) had a mosaic PBP2, but that none of these possessed the A501P or A501V alterations. These data are consistent with the fact that while isolates with ceftriaxone-reduced susceptibility are prevalent in the Australian population, no isolates exhibiting ceftriaxone MICs reflective of the H041 or F89 strains have yet been reported in the Australian population. Our ongoing concern is that with the speed with which *N. gonorrhoeae* isolates with the mosaic PBP2 have spread globally [2,13], importation of the F89 strain remains a very real threat.

One limitation of the approach was that there were high rates of cross-reaction observed with the commensal *Neisseria* species, with 22 of the 58 (38%) non-gonococcal species providing melting peaks in the mosaic501-hybPCR assay. While none of these strains were found to possess either the A501P or A501V alterations, the results nevertheless highlight the considerable sequence homology between gonococcal sequences associated with antimicrobial resistance and those of commensal *Neisseria* strains. In practical terms, this means that the method may not be suitable for use on pharyngeal specimens where commensal *Neisseria* species are prevalent, given that amplification of the *penA* of such species, together or instead

Table 2

Melting temperatures for controls, isolates and clinical samples obtained in the mosaic501-hybPCR assay, which was developed to detect nucleotide substitutions leading to mosaic penicillin binding protein 2 types differing at residue 501

Sample typeand number	Description	*penA*/PBP2 type[a]	Melting temperature (call)
Controls			
1.	*Neisseria gonorrhoeae* isolate	Mosaic/A501	58.48°C (mosaic/A501[b])
2.	Plasmid control	Mosaic/A501T	58.50°C
3.	Plasmid control	Mosaic/A501V	56.50°C
4.	Plasmid control	Mosaic/A501P	68.00°C
Neisseria isolate panel			
5.	*N. gonorrhoeae*	Mosaic/A501	58.51°C (mosaic/A501[b])
6.	*N. gonorrhoeae*	Mosaic/A501	58.54°C (mosaic/A501[b])
7.	*N. gonorrhoeae* (H041)	Mosaic/A501	58.63°C (mosaic/A501[b])
8.	*N. gonorrhoeae* (NG0304)	Mosaic/A501V	56.15°C (mosaic/A501V)
9.	*N. cinerea*	NA	58.49°C (mosaic/A501[b])
10.	*N. cinerea*	NA	58.67°C (mosaic/A501[b])
11.	*N. cinerea*	NA	58.02°C (mosaic/A501[b])
12.	*N. elongata*	NA	59.20°C (mosaic/A501[b])
13.	*N. lactamica*	NA	58.41°C (mosaic/A501[b])
14.	*N. lactamica*	NA	58.45°C (mosaic/A501[b])
15.	*N. lactamica*	NA	58.49°C (mosaic/A501[b])
16.	*N. lactamica*	NA	58.49°C (mosaic/A501[b])
17.	*N. lactamica*	NA	58.68°C (mosaic/A501[b])
18.	*N. lactamica*	NA	58.71°C (mosaic/A501[b])
19.	*N. lactamica*	NA	58.98°C (mosaic/A501[b])
20.	*N. meningitidis*	NA	58.96°C (mosaic/A501[b])
21.	*N. meningitidis*	NA	58.68°C (mosaic/A501[b])
22.	*N. meningitidis*	NA	53.23°C (NT)
23.	*N. polysaccharea*e	NA	58.38°C (mosaic/A501[b])
24.	*N. polysaccharea*e	NA	58.60°C (mosaic/A501[b])
25.	*N. subflava*	NA	45.75°C (NT)
26.	*N. subflava*	NA	46.98°C (NT)
27.	*N. subflava*	NA	58.40°C (mosaic/A501[b])
28.	*N. subflava*	NA	58.51°C (mosaic/A501[b])
29.	*N. subflava*	NA	58.55°C (mosaic/A501[b])
30.	*N. subflava*	NA	58.92°C (mosaic/A501[b])
PCR-positive clinical samples[c]			
31.	Rectal swab	NA	58.57°C (mosaic/A501[b])
32.	Rectal swab	NA	58.68°C (mosaic/A501[b])
33.	Rectal swab	NA	58.78°C (mosaic/A501[b])
34.	Rectal swab	NA	58.83°C (mosaic/A501[b])
35.	Rectal swab	NA	58.84°C (mosaic/A501[b])
36.	Rectal swab	NA	59.13°C (mosaic/A501[b])
37.	Urine sample	NA	58.57°C (mosaic/A501[b])
38.	Urine sample	NA	58.60°C (mosaic/A501[b])
39.	Urine sample	NA	58.61°C (mosaic/A501[b])
40.	Urine sample	NA	58.63°C (mosaic/A501[b])

NA: not available; NT: not typed, as melting temperature was not consistent with any of the reference controls; PBP2: penicillin binding protein 2; PCR: polymerase chain reaction.

[a] Based on DNA sequencing. The mosaic PBP2 types are designated by 'mosaic' followed by details of the particular residue or mutation at position 501 of the protein sequence.

[b] It should be noted that the assay cannot distinguish between mosaic/A501 and mosaic/A501T.

[c] These samples were positive by *Neisseria gonorrhoeae* PCR as well as the mosaic-PCR assay.

of *penA* of *N. gonorrhoeae*, could potentially interfere with characterisation by melting curve analysis. A second limitation of the mosaic501-hybPCR and mosaic-PCR assays, as shown by the detection limit studies, is that they are less sensitive than diagnostic *N. gonorrhoeae* NAAT methods, such as the NG-duplex method tested in this study. Hence, the approach will not be able to characterise all *N. gonorrhoeae* NAAT, particularly samples with very low *N. gonorrhoeae* DNA loads. It should also be noted that in addition to a 'CCG' codon, an A501P alteration may also arise via 'CCT', 'CCC' or 'CCA' and would therefore not be identified by the current method. We do not however consider this a major limitation in the context of screening for the F89 strain, which is known to possess the 'CCG' codon.

In summary, our results show that the mosaic501-hyb-PCR assay can be readily used to detect the presence of the mosaic/A501P PBP2 alteration, and therefore, when used in combination with mosaic-PCR, could be used for direct detection of the *N. gonorrhoeae* F89 strain in non-cultured clinical samples. Use of the assay could considerably strengthen bacterial culture-based investigations into the broader prevalence, if any, of the F89 strain in Europe. Likewise, for settings where AMR surveillance gaps exist because of increased use of NAAT for gonorrhoea diagnosis or where the scope for bacterial culture is limited, such methods also demonstrate considerable potential of molecular technology to enhance *N. gonorrhoeae* antimicrobial resistance surveillance.

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Novel influenza A(H7N9) virus linked to human disease in China, April 2013

Eurosurveillance editorial team (eurosurveillance@ecdc.europa.eu)[1]
1. European Centre for Disease Prevention and Control (ECDC) Stockholm, Sweden

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A novel influenza virus of avian origin, A(H7N9), has been isolated from patients with severe respiratory disease in China. Eleven human cases have now been confirmed, including five deaths. Monitoring of the cases' contacts suggests that the new virus currently does not spread easily from human to human.

As new information is collected and analysed, updates on the situation can be found, among others, on the websites of the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC).

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