Evolution of *Neisseria gonorrhoeae* is a continuing challenge for molecular detection of gonorrhoea: false negative gonococcal *porA* mutants are spreading internationally

Catherine A Ison,1 Daniel Golparian,2 Pamela Saunders,1 Stephanie Chisholm,1 Magnus Unemo2

**ABSTRACT**

Objectives Identification of genetic targets specific to *Neisseria gonorrhoeae* for use in molecular detection methods has been a challenge. The *porA* pseudogene in *N gonorrhoeae* has been commonly used but recently gonococcal isolates giving a negative result in these PCRs have been reported. Here we describe the characterisation of two such gonococcal isolates received by the reference service at the Health Protection Agency, London, England.

Methods Phenotypic characterisation was achieved using conventional biochemical and immunological tests, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), antimicrobial susceptibility testing, serovar determination and detection of meningococcal *PorA* using monoclonal antibody 4BG4-E7. Genetic species confirmation was determined using commercial and in house PCRs and 16S rRNA gene sequencing. Molecular typing using the *N gonorrhoeae* multi-antigen sequence typing (NG-MAST) and multilocus sequence typing (MLST) was performed. The DNA sequence of the full-length gonococcal *porA* pseudogene was determined and compared with published sequences.

Results Both isolates were confirmed, biochemically and immunologically as *N gonorrhoeae*, but repeatedly gave negative results with two in house real-time PCR assays for the *porA* pseudogene. Further characterisation of these isolates identified the presence of a meningococcal *porA* sequence and showed these isolates belong to serovar Broptyp, and to NG-MAST sequence type (ST) 5967 and MLST ST1901.

Conclusions Gonococcal isolates that give false negative results with *porA* pseudogene PCR assays have now been identified in four countries, three of which are in Europe, and do not appear clonal. This report highlights the genetic diversity of *N gonorrhoeae*, which remains a challenge for the molecular detection methods.

**INTRODUCTION**

Molecular detection methods for the diagnosis of gonorrhoea have been hampered by the challenge of identifying genetic targets specific to the infecting organism, *Neisseria gonorrhoeae*. This is due primarily to the close relatedness with the genomes of other *Neisseria* species, that is, *Neisseria meningitidis* and several of the commensal *Neisseria* spp., and the high propensity of *N gonorrhoeae* to acquire DNA from other organisms.1 Considerable advances have been made in recent years and both commercial and in house molecular assays have become more robust and are in routine use in many laboratories, exhibiting increased sensitivity over conventional diagnostic methods such as culture.2 3 However, cross-reactivity with non-gonococcal *Neisseria* species, which has historically been a problem for many nucleic acid amplification tests (NAATs), particularly for samples from extragenital sites such as the pharynx, can still occur resulting in potentially false positive results.4 5 Supplementary testing using alternative targets for confirmation has been recommended both for positive results from extra-genital samples and from low prevalence populations, and often uses in house assays.6 7 8

A number of targets have been evaluated for in house assays and the *porA* pseudogene in *N gonorrhoeae* has proved to be a specific and popular target. The *porA* pseudogene, originally thought to be found as an expressed *porA*, has inactivating deletions in both the promoter and the hypothetical *PorA* coding region that prevent gene expression in *N gonorrhoeae*.9 The highly conserved *porA* pseudogene has been considered to be present in all gonococcal strains giving a high sensitivity.9 10 It is lacking in commensal *Neisseria* species and also is sufficiently different from the *porA* gene in *N meningitidis* to give a high specificity and is therefore useful for diagnostic purposes, both as a supplementary assay or in situations where commercial diagnostic NAATs are not appropriate or affordable. A number of in house assays detecting the *porA* pseudogene have been described and widely used.11 12 However, rare false negative results in *porA* pseudogene PCRs have recently been reported, firstly from Australia,13 and subsequently from Scotland14 and Sweden.15

In this report, we describe the first occurrence of false negative results with a *porA* pseudogene PCR from England.

**MATERIALS AND METHODS**

**Gonococcal isolates and conventional species verification**

Clinical isolates of *N gonorrhoeae* are referred to the Sexually Transmitted Bacteria Reference Unit at the Health Protection Agency, Colindale, UK from diagnostic laboratories across England for confirmation of anomalous results. In December 2011 two such
is isolates were received on chocolate agar slopes and were retrieved by inoculation on both gonococcal (GC) non-selective agar supplemented with 1% IsoVitaleX, and on gonococcal selective agar additionally supplemented with 5% laked blood and the anti-microbial agents; vancomycin, colistin, amphotericin B and trimethoprim, and then incubated at 36°C for 24 h in 5% carbon dioxide. Resulting colonies of oxidase positive, Gram negative diplococci were biochemically verified to species level using carbohydrate utilisation tests and preformed enzyme detection by API-NH (bioMérieux, Marcy l’Etoile, France) and Microscan HNID (Siemens Healthcare Diagnostics (UK), Camberley, UK), immunologically with the Phadebact GC Monoclonal Test (Bactus AB, Uppsala, Sweden) and by molecular confirmation using a porA pseudogene real-time PCR (RT-PCR)11 and any negative results further tested using a qps gene RT-PCR.16

Further phenotypic characterisation
These isolates were confirmed as N gonorrhoeae using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) with a MicroFlex LT (Bruker Daltonics, Bremen, Germany).

The susceptibility (mg/l) to cefixime, ceftriaxone, ciprofloxacin, azithromycin and spectinomycin was determined using the Etest methodology (bioMérieux, Solna, Sweden), according to the instructions from the manufacturer. The breakpoints used for determining antimicrobial resistance were according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org). β-lactamase production was detected using nitrocefin discs.

Serovar determination using the Phadebact GC Monoclonal Serovar Test (Bactus AB, Uppsala, Sweden) was performed in accordance with the instructions from the manufacturer. The monoclonal antibody 4BG4-E7 against N meningitidis PorA was used to confirm or rule out the expression of meningococcal PorA (http://www.nibsc.ac.uk).

Genetic characterisation
For additional species verification, the APTIMA Combo 2 and APTIMA GC (Gen-Probe, San Diego, California, USA) in the PANTHER system (Gen-Probe, San Diego, California, USA), GeneXpert CT/NG assay (Cepheid, Sunnyvale, California, USA) as well as 16S rDNA sequencing were applied according to the manufacturer’s instructions and as previously described, respectively.17 A dual-target PCR (targeting porA pseudogene and qps genes),18 and one additional porA pseudogene PCR12 were also performed.

Molecular epidemiological characterisation was performed using the N gonorrhoeae multi-antigen sequence typing (NG-MAST),19 and multilocus sequence typing (MLST),20 as described elsewhere.

Sequencing of the full-length gonococcal porA pseudogene was performed as previously described,9 however, including also meningococcal primers described elsewhere.21 in the sequencing. The porA gene sequence was used to acquire the meningococcal porA genosubtype and for GenBank BLAST analysis. Multiple-sequence alignments of the porA nucleotide sequences and the deduced corresponding amino acid sequences of PorA were performed in the software BioEdit Sequence Alignment Editor V7.0.9.0 with manual adjustment.

RESULTS
Species verification of porA pseudogene PCR negative N gonorrhoeae isolates from England (n=2)
In December 2011 two urethral isolates, 356 and 088, were received at the Health Protection Agency from male individuals who were 26 years old and had attended a genitourinary medicine clinic and were 50 years old and had attended general practice, respectively. These patients were not known to be linked and their sexual orientation was unknown. The isolates were positive for N gonorrhoeae in all phenotypic assays but gave negative results in both the porA pseudogene PCRs.11 12 All the conventional phenotypic tests, MALDI-TOF-MS analysis, APTIMA Combo 2 test, APTIMA GC test, GeneXpert CT/NG assay, the dual-target gonococcal PCR (only positive for the qps genes), qps gene PCR and 16S rDNA sequencing unequivocally identified the isolates as N gonorrhoeae (table 1).

Phenotypic characterisation of porA pseudogene PCR negative N gonorrhoeae isolates from England (n=2)
The two isolates displayed similar antimicrobial sensitivity pattern, and both isolates were resistant to ciprofloxacin but susceptible to cefixime, ceftriaxone, azithromycin and spectinomycin (table 1). Neither of the isolates produced β-lactamase. Furthermore, serovar determination assigned both isolates as gonococcal serovar Bropyst (table 1), and both reacted strongly with the monoclonal antibody 4BG4-E7 against N meningitidis PorA.

Genetic characterisation of porA pseudogene PCR negative N gonorrhoeae isolates from England (n=2)
Both isolates were of the same NG-MAST sequence type (ST), that is, ST5967, and were assigned as MLST ST1901. The isolates displayed an identical porA gene sequence, which had a 98% sequence identity to the porA gene sequence of N meningitidis strain MC 278 (GenBank accession no. GQ173789) (figure 1), and was assigned to meningococcal genosubtype P1.22-New,14-5,36-2.

DISCUSSION
In this report, we describe the identification and detailed characteristics of two isolates of N gonorrhoeae from patients diagnosed in England, which lacked the traditional gonococcal porA pseudogene. Both isolates were confirmed, biochemically and immunologically as N gonorrhoeae, but repeatedly gave false negative results in two different in house real-time PCR assays for the porA pseudogene,11 12 which are commonly used in many laboratories globally. Further characterisation identified the presence of a meningococcal porA sequence instead of the conventional gonococcal porA pseudogene.

The isolates displayed an identical porA gene sequence, which was also identical to the partial porA gene sequence reported in the previously described isolate in Australia,15 and, with exception of a single nucleotide deletion, GC1 in Scotland.14 However, the porA gene sequence substantially differed from the porA gene in the previously described isolate in Sweden (55 nucleotides),15 and GC5 in Scotland (62 nucleotides)14 (figure 1). Both the primer and probe binding sites used in previously published porA pseudogene PCRs11 12 have multiple mismatches and deletions in all the described gonococcal porA mutants (figure 1), which explains the false negative reaction for these gonococcal strains. This evidence is confirmed further by the positive reaction of the isolates to the monoclonal 4BG4-E7 indicating the presence of the expressible meningococcal porA gene and not the porA pseudogene.

The isolates were assigned to gonococcal serovar Bropyst, NG-MAST ST5967, an ST which has not been identified in other studies performed at the Health Protection Agency, and MLST ST1901 indicating again that these isolates were
The second porA mutant found in Scotland, and the one reported in Sweden,13 but clearly different from the second porA mutant in Australia,13 and the one reported in Sweden.15

Accordingly, several different gonococcal strains that cannot be detected in porA pseudogene PCRs are spreading internationally (presently identified in three European countries and Australia). Thus it appears clear that more than one gonococcal strain do not appear clonal. This report highlights the genetic diversity and ongoing evolution of N. gonorrhoeae, which remains a major challenge for the molecular detection methods. Strains circulating in the population which can go undetected among MSM (table 1), and the target sites of two commercial NAAT assays used in Sweden and other countries worldwide.22 The porA pseudogene is mostly used as a diagnostic target of in house assays and these assays are unlikely to be in widespread use in England, and the target is not used in any of the commercial assays from the main manufacturers of gonococcal NAATs used in the European Union or the USA, so the potential for these isolates to spread widely in these regions is limited. However, enhanced awareness in laboratories and among clinicians of the spread of such strains is needed, and screening for them can be crucial. The opportunities to use combinations of different diagnostic methods (such as NAAT and culture) and multi-target (separately detected) NAATs in a laboratory remain exceedingly valuable.

Table 1 Detailed description of PCR false negative porA mutant Neisseria gonorrhoeae isolates identified in England, and comparison to previously reported porA mutant isolates from Sweden, Scotland and Australia.

<table>
<thead>
<tr>
<th>Screening and confirmatory tests</th>
<th>356 (England)</th>
<th>088 (England)</th>
<th>Variant (Sweden)*</th>
<th>GC1 (Scotland)*</th>
<th>GC3 (Scotland)*</th>
<th>Variant (Australia)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>porA pseudogene PCR1</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Culture4</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
</tr>
<tr>
<td>Gen-Probe APTIMA</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
</tr>
<tr>
<td>16s rDNA (100% ID)</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
</tr>
<tr>
<td>MALDI-TOF-MS</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
<td>N. gonorrhoeae</td>
</tr>
<tr>
<td>porA gene sequencing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GenBank BLAST</td>
<td>98% identity to MC 278</td>
<td>98% identity to MC 278</td>
<td>92% identity to MC 278f</td>
<td>99% identity to MC 278f</td>
<td>99% identity to MC 278f</td>
<td>99% identity to MC 278f</td>
</tr>
<tr>
<td>Meningococcal porA genosubtype</td>
<td>P1.22-New;14,5,36-2</td>
<td>P1.22-New;14,5,36-2</td>
<td>P1.21-6,2-48,35-1</td>
<td>P1.1,f;14-5,36-2</td>
<td>P1.1,f;23-13,36-2</td>
<td>P1.1,f;14-5,36-2</td>
</tr>
<tr>
<td>Molecular epidemiological typing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG-MAST</td>
<td>5967</td>
<td>5967</td>
<td>2382</td>
<td>5967</td>
<td>3149</td>
<td>5377</td>
</tr>
<tr>
<td>Serovar</td>
<td>Broplust</td>
<td>Broplust</td>
<td>Byust</td>
<td>Will/III</td>
<td>Will/III</td>
<td>Broplust</td>
</tr>
<tr>
<td>Antimicrobial susceptibility, MIC (mg/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefixime</td>
<td>&lt;0.016</td>
<td>&lt;0.016</td>
<td>&lt;0.016</td>
<td>S**</td>
<td>S**</td>
<td>S**</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.016</td>
<td>0.032</td>
<td>0.002</td>
<td>S**</td>
<td>S**</td>
<td>0.03</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.002</td>
<td>R**</td>
<td>R**</td>
<td>16</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.125</td>
<td>0.25</td>
<td>6</td>
<td>S**</td>
<td>S**</td>
<td>S**</td>
</tr>
<tr>
<td>Spectromycin</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>S**</td>
<td>S**</td>
<td>S**</td>
</tr>
<tr>
<td>Anatomical site</td>
<td>Urethral</td>
<td>Urethral</td>
<td>Pharyngeal</td>
<td>Rectal</td>
<td>Urethral</td>
<td>Rectal, pharyngeal</td>
</tr>
<tr>
<td>Year</td>
<td>2011</td>
<td>2011</td>
<td>2011</td>
<td>2011</td>
<td>2010</td>
<td>2011</td>
</tr>
<tr>
<td>Sexual orientation</td>
<td>Not known</td>
<td>Not known</td>
<td>Heterosexual</td>
<td>MSM</td>
<td>MSM</td>
<td>MSM</td>
</tr>
</tbody>
</table>

*Previously published isolates.14-15
1porA pseudogene PCR,11 12 and/or dual-target PCR (porA and ope genes).18
2Including species confirmation using selective culture, rapid oxidase production, microscopy after Gram staining, sugar utilisation test, API NH (bioMérieux, Marcy l'Etoile, France) and Phadebact GC Monoclonal Test (Bactus AB, Uppsala, Sweden).
3Analysis not performed.
4Performed in this study on available full-length or partial porA gene sequences. Variable region 1 in the meningococcal porA genotyping was not possible to determine due to the lack of full-length porA sequence in previously published papers from Scotland and Australia.13 14
5MIC data not available.
6MALDI-TOF-MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; MC 278, Neisseria meningitidis strain 278 (GenBank accession no. GQ173789); MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; MSM, men who have sex with men; NG-MAST, Neisseria gonorrhoeae multi-antigen sequence typing.
Key messages

- Molecular detection of gonorrhoea is dependent on choice of a nucleic acid target specific to Neisseria gonorrhoeae and presents an ongoing challenge.
- The porA pseudogene in N. gonorrhoeae is not expressed and is sufficiently different from the porA gene in Neisseria meningitidis to be useful for diagnostic purposes.
- This is the first report of two gonococcal isolates from England that lack the porA pseudogene.
- If these gonococcal isolates are undetected they have the potential to impact on the epidemiology of gonorrhoea.

Acknowledgements The authors would like to thank Professor Friedrich Muhlschlegel and the staff of the Department of Laboratory Medicine, East Kent Microbiology Service, Ashford, Kent for the primary isolation and referral of these isolates and Michael Dawson for critical review of the manuscript.

Contributors CI and MU designed the study, analysed and interpreted the data and wrote the initial draft of the manuscript. DG, PS and SC performed all the laboratory analysis. All authors were involved in the preparations of the final draft of the paper.

Funding This work was supported by the Örebro County Council Research Committee and the Foundation for Medical Research (09/029, 25/3 2009) at Örebro University Hospital, Sweden.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

3. Alexander S. The challenges of detecting gonorrhoea and chlamydia in rectal and pharyngeal sites: could we, should we, be doing more? Sex Transm Infect 2009;85:159–60.