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ORIGINAL ARTICLE

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

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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 Bropyst, 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 extra-genital 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.⁶⁻⁸

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* gene only in *N meningitidis*, 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

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 antimicrobial 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 *opa* 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 *opa* 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 V.7.0.9.0 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 30 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 *opa* genes), *opa* 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,¹³ 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 (53 nucleotides),¹⁵ and GC3 in Scotland (62 nucleotides)¹⁴ (figure 1). Both the primer and probe binding sites used in previously published *porA* pseudogene PCRs^{11 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

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.

	<i>porA</i> mutant gonococcal isolates (country)					
	356 (England)	088 (England)	Variant (Sweden)*	GC1 (Scotland)*	GC3 (Scotland)*	Variant (Australia)*
Screening and confirmatory tests						
<i>porA</i> pseudogene PCR†	Negative	Negative	Negative	Negative	Negative	Negative
Culture‡	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>
Gen-Probe APTIMA	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	§
16s rDNA (100% ID)	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	§	§	§
MALDI-TOF-MS	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	<i>N. gonorrhoeae</i>	§	§	<i>N. gonorrhoeae</i>
<i>porA</i> gene sequencing						
<i>porA</i> GenBank BLAST	98% identity to MC 278	98% identity to MC 278	92% identity to MC 278¶	99% identity to MC 278¶	99% identity to MC 278¶	99% identity to MC 278¶
Meningococcal <i>porA</i> genosubtype	P1.22-New,14-5,36-2	P1.22-New,14-5,36-2	P1.21-6,2-48,35-1	P1.¶,14-5,36-2	P1.¶,23-13,36-2	P1.¶,14-5,36-2
Molecular epidemiological typing						
NG-MAST	5967	5967	2382	5967	3149	5377
MLST	1901	1901	7367	§	§	1901
Serovar	Bropyst	Bropyst	Byust	WII/III	WII/III	Bropyst
Antimicrobial susceptibility, MIC (mg/l)						
Cefixime	<0.016	<0.016	<0.016	S**	S**	§
Ceftriaxone	0.016	0.032	0.002	S**	S**	0.03
Ciprofloxacin	>32	>32	0.002	R**	R**	16
Azithromycin	0.125	0.25	6	S**	S**	S**
Spectinomycin	8	12	8	S**	S**	S**
Case						
Anatomical site	Urethral	Urethral	Pharyngeal	Rectal	Urethral	Rectal, pharyngeal
Year	2011	2011	2011	2011	2010	2011
Sexual orientation	Not known	Not known	Heterosexual	MSM	MSM	MSM

*Previously published isolates.^{13–15}†*porA* pseudogene PCR,^{11, 12} and/or dual-target PCR (*porA* and *opa* genes).¹⁸

‡Including 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).

§Analysis not performed.

¶Performed 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}

**MIC data not available.

MALDI-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.

indistinguishable from one of the two previously described gonococcal *porA* mutants in Scotland¹⁴ and similar to the first reported *porA* mutant in Australia,¹³ but clearly different from the second *porA* mutant found in Scotland,¹⁴ 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 clone has acquired a meningococcal *porA* sequence, probably through horizontal gene transfer and subsequent recombination. Pharyngeal gonorrhoea is predominantly asymptomatic and more difficult to treat than urogenital gonorrhoea. The pharynx is an environment where gonococci and *N. meningitidis* strains can coexist and this may provide the means for this gene transfer. The pharynx is also a frequent site of gonococcal infection in men who have sex with men (MSM). Worryingly, most of the gonococcal *porA* mutant isolates previously reported have been identified among MSM (table 1), and accordingly these strains are already circulating in a high-frequency transmitting risk group.

In conclusion, gonococcal isolates that give false negative results with *porA* pseudogene PCRs have now been identified in

four countries, three of which are in Europe, and the identified strains 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 by standard methods and can cause asymptomatic infection can have a significant effect on the epidemiology of the infection. This was highlighted by the emergence of the new variant of *Chlamydia trachomatis*, which had a deletion covering the target sites of two commercial NAAT assays in frequent use 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.

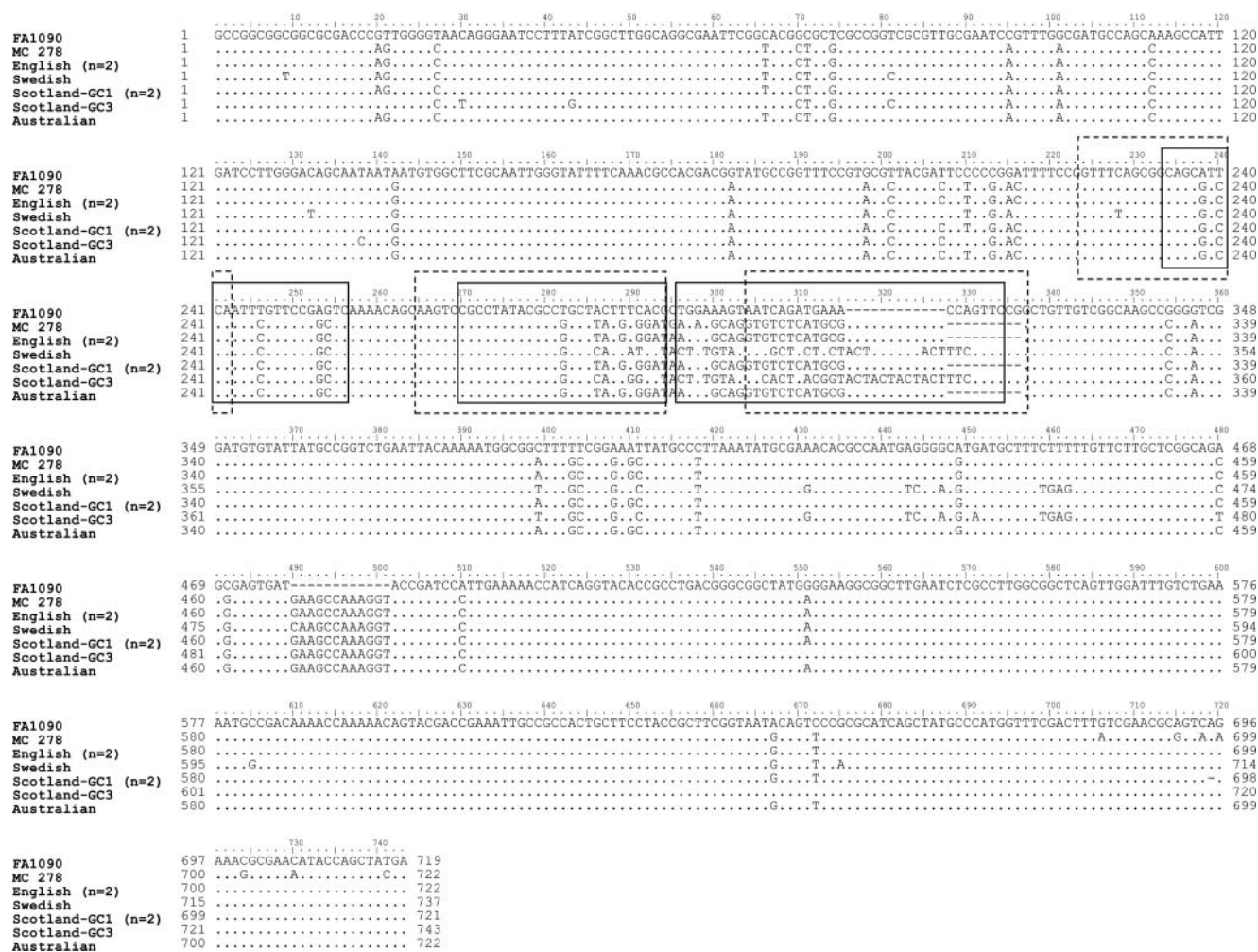


Figure 1 Nucleotide sequence alignment of *porA* gene sequences from the examined English *Neisseria gonorrhoeae* *porA* mutants, compared to the *porA* sequences of previously identified *porA* mutants in Sweden,¹⁵ Scotland,¹⁴ and Australia,¹³ as well as the wild-type *porA* pseudogene sequence of *N gonorrhoeae* reference strain FA1090 (GenBank accession no. AJ223447) and the *porA* gene sequence of *Neisseria meningitidis* strain 278 (GenBank accession no. GQ173789). Sequences in full boxes and in dashed boxes indicate PCR primer and probe binding sites for the *porA* pseudogene PCR described by Whiley *et al*¹¹ and Hjelmevoll *et al*¹² respectively.

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.

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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.

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