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Evaluation of an *opa* gene-based nucleic acid amplification test for detection of *Neisseria gonorrhoeae* in urogenital samples in North India

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SUMMARY

Due to the poor positive predictive value of nucleic acid amplification tests (NAATs) for gonorrhoea when applied to a low-prevalence setting, current guidelines recommend the use of supplementary polymerase chain reaction (PCR) targeting a different gene for confirmation of true positives in urogenital specimens. This study sought to standardize and evaluate performance of an in-house *opa* gene-based PCR assay for gonorrhoea compared to assays targeting the *porA* pseudogene and 16S rRNA gene. Four hundred samples (300 endocervical, 100 urethral swabs) from patients attending STD clinics in New Delhi, India were used. The sensitivity, specificity, positive predictive value and negative predictive value of the *opa*-based PCR were 100%, 97·9%, 89·5% and 100%, respectively. In females, the use of NAATs provided enhanced diagnosis of gonorrhoea.

Key words: Diagnosis, gonorrhoea, *Neisseria gonorrhoeae*, PCR.

INTRODUCTION

Genitourinary tract infections due to *Neisseria gonorrhoeae* is a major cause of morbidity in sexually active individuals [1, 2]. In an effort to prevent spread of this disease, increased attention is being focused on early diagnosis and treatment of symptomatic as well as asymptomatic individuals, which are not being adequately addressed by the conventional diagnostic methods, i.e. microscopy and culture [3]. Hence, various nucleic acid amplification tests (NAATs), predominantly polymerase chain reactions (PCRs) targeting different genes are being developed and evaluated by laboratories worldwide and include *opa*, 16S rRNA, *porA* pseudogene, *cppB* gene, CMT gene and the *pil* gene. A huge variation in sensitivity and specificity of these gene targets has been reported mainly depending on the primers and probes used and the population where they were evaluated [4]. Therefore, any *N. gonorrhoeae* NAAT must be validated in the prospective patient population prior to being introduced as a routine diagnostic test. Another major drawback of NAATs with respect to detection of *N. gonorrhoeae* is the cross-reactivity seen with non-*N. gonorrhoeae Neisseria* species (non-NgNS) [5, 6]. Therefore, a single PCR targeting one gene cannot be relied upon for definitive diagnosis of gonorrhoea in any patient population.

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Network (PHLN) and the UK's Health Protection Agency (HPA) guidelines, recommend the use of two PCRs targeting different genes known to have a discriminatory capacity. The PCR screening assay should be highly sensitive while the supplementary assay should be highly specific and have a sensitivity comparable to the screening assay [4, 7, 8]. Indeed, based on current US recommendations for *Chlamydia* and gonorrhoea testing and as reinforced by the PHLN, the acceptable performance criteria for test combinations requires that the reported result should have a positive predictive value (PPV) of at least 90% in the population being tested [7, 8]. Further, as recommended by the PHLN guidelines an on-going assessment of various gene assays is required [4]. The objective of the present study was to develop and evaluate the performance of an in-house PCR assay based on the *opa* gene compared to the *porA* pseudogene and 16S rRNA gene-based assays. In accordance with our earlier report, the 16S rRNA assay with its high sensitivity has been used as the screening assay and the highly specific *porA* pseudogene-based assay as the supplementary assay for detection of positives [9].

METHODS

Bacterial isolates and clinical samples

The panel of strains used for evaluation comprised *N. gonorrhoeae* (ATCC 49226, WHO B, WHO C, WHO F, WHO G, WHO K, WHO L, WHO M, WHO N, WHO O, WHO P; quality control strains received since 2005 as a part of the External Quality Assurance Scheme conducted by the Regional Reference Laboratory, Safdarjang Hospital, New Delhi ($n=26$); patient isolates ($n=14$); *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Mycoplasma hominis* (NCTC 10111), *Ureaplasma urealyticum* (NCTC 10177), *Streptococcus pyogenes* (ATCC 19615), group B streptococci (clinical isolate), *Candida* spp., *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 51299), *N. meningitidis* [ATCC 13077 serogroup A ($n=1$), clinical isolate ($n=3$)] and *N. sicca* (ATCC 29193 ($n=1$), clinical isolates ($n=8$)].

Sample sizes of the populations to be screened were determined on the basis of prevalence data. Considering the positivity rate in males as 40%, to estimate this prevalence with absolute precision of ± 10 with 95% confidence level, required 100 males and similarly for females with a positivity rate of 7%, to estimate this prevalence with absolute precision of ± 3 with 95% confidence level, the required number was calculated as 300. Four hundred patients (100 males, 300 females) aged 15–45 years with symptoms suggestive of gonococcal infections visiting the STD clinics, Dermatology Outpatient Department of the All India Institute of Medical Sciences (AIIMS) and the Regional STD, Teaching Training and Research Centre, Safdarjung Hospital, New Delhi, India were included in the study. The inclusion criteria for patients were urethral, cervical/vaginal discharge or lower abdominal pain. In addition, history of symptomatic partner or positive risk factors or partner with risk factors was taken into account for inclusion criteria. Urethral/endocervical swabs were collected in triplicate by standard protocol by a trained physician [10]: one swab was used to prepare a smear for microscopic examination, a second for culture and the third swab for DNA extraction for PCR.

The study was approved by the Ethics Committee, AIIMS, New Delhi, India.

Sample processing

The smear was Gram stained by standard method and swabs were cultured on saponin-lysed blood agar plus VCNT(A) inhibitors and chocolate agar. Suspected colonies were confirmed by oxidase test, superoxol and rapid carbohydrate utilization tests [10]. DNA was extracted from bacterial cultures and the third swab sample using QIAamp DNA mini kit (Qiagen Sciences Inc., USA) according to the manufacturer's instructions.

opa gene-based PCR

For the *opa* gene-based PCR, primers were designed using the available software ClustalX and Primer3 from the gene sequences available in the GenBank database. The selected oligonucleotide sequence was then analysed for non-specific binding using BLAST. The forward primer was 5′-CGG TGC TTC ATC ACC TTA G-3′ and the reverse primer was 5′-GGA TTC ATT TTC GGC TCC TT-3′. Sequences were validated by GenBank (GenBank accession no. PUID 9716120 SNUM 2706 Ng_opa). The optimum annealing temperature for the PCR was determined from a gradient range of 50–60 °C and the optimal $MgCl_2$ concentration from a range of $MgCl_2$ (1·5–2·5 mM). PCR was also performed with different

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Table 1. *Comparison of* opa *PCR with* porA *and 16S ribosomal assay*

opa-based PCR assay	Culture			*porA* and 16S-based PCR assay		
	Positive	Negative	Total	Positive	Negative	Total
Positive						
Males	42	2	44	44	0	44
Females	2	21	23	16	7	23
Total	44	23	67	60	7*	67
Negative						
Males	0	56	56	0	56	56
Females	0	277	277	0	277	277
Total	0	333	333	0	333	333
Total	**44**	**346**	**400**	**60**	**340**	**400**

* These seven patients were also positive by the 16S rRNA PCR.

concentrations of *Taq* polymerase (0·75–2·5 U) and forward and reverse primers (4–6 pmol). A 25-μl reaction contained a final concentration of reaction buffer (10 mM KCl, 10 mM HCl), 1·5 mM $MgCl_2$, *Taq* polymerase (1·5 U), 200 μM dNTP mix and 5 pmol primers. PCR conditions were denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s for a total of 30 cycles yielding a 188-bp product from positive samples.

porA pseudogene PCR

For *porA* pseudogene PCR primers 5′-CCG GAA CTG GTT TCA TCT GAT T-3′ and 5′-GTT TCA GCG GCA GCA TTC A-3′ were used [11]. The reaction mix (25 μl) was as above for the *opa* gene PCR and cycling conditions were denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 45 s for a total of 30 cycles yielding a 102-bp product from positive samples.

16S rRNA PCR

The forward and reverse primers were as described previously [12] utilizing the same reaction mix with denaturation at 95 °C for 45 s, annealing at 68 °C for 1 min and extension at 72 °C for 1 min for a total of 40 cycles to yield a 413-bp product.

All PCR assays were controlled for sample inhibition by the amplification of the β-globin gene as previously described [9].

Sensitivity and specificity

Tenfold dilutions were prepared from DNA extracted from a standard strain of *N. gonorrhoeae* to determine the detection limit of each of the PCR assays. Specificity was determined by reactivity of each PCR with non-NgNS and other control species in the strain panel.

Statistical analysis

True positives were defined as samples positive by culture and/or both the 16S rRNA and *porA* pseudogene-based assays [9]. The 95% confidence interval was calculated according to the efficient score method (corrected for continuity) using the online tool available at http://faculty.vassar.edu/lowry/VassarStats.html.

RESULTS

Microscopy and culture

Out of 400 patients (100 males, 300 females) 46 (44 males, 2 females) were positive by microscopy and 44 (42 males, 2 females) by culture.

Performance characteristics of PCR assays

Specificity tests confirmed that no species other than *N. gonorrhoeae* reacted in the *opa* and *porA* pseudogene PCR assays but cross-reactivity with *N. sicca* was found with the 16S rRNA assay. The limit of detection of the *opa* and 16S rRNA assays was determined as 0·4 pg *N. gonorrhoeae* DNA but the *porA* pseudogene-based PCR was tenfold less sensitive (4 pg DNA).

Performance of PCR assays in patient samples

The 16S rRNA assay was positive in 82, *opa* in 67 and *porA* assay in 60 patients (Table 1). Sixty patient

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samples were classified as 'true positives' on the basis of their positivity in both the *porA* and 16S rRNA assays.

Of the 67 patients positive by *opa* assay, 60 were positive by both 16S and *porA* and seven only by 16S rRNA. The sensitivity, specificity, PPV and negative predictive value (NPV) of the assay based on the 60 confirmed positives was 100% (95% CI 92·5–100), 97·9% (95% CI 95·6–99·1), 89·5% (95% CI 79·1–95·3) and 100% (95% CI 98·6–100), respectively. Of the seven discrepant samples positive by *opa* assay which were positive in both the *opa* assay and the 16S rRNA assay but discrepant in the *porA* assay, *opa* amplicons for four samples could be sequenced; the other three had faint bands and hence could not be sequenced. The sequences gave a perfect match with the *N. gonorrhoeae* opacity gene sequence available in GenBank (three with gonococcal protein II opacity gene and one with opacity gene variant V28), confirming that the DNA being amplified in the patient samples was from *N. gonorrhoeae* (Fig. 1*a*, *b*).

DISCUSSION

There has been a significant change in the approach to the diagnosis of gonorrhoea in recent years and NAATs are now routinely used for detection of *N. gonorrhoeae* DNA in clinical samples. However, to date no single gonococcal NAAT has proved to be both sensitive and specific across a broad range of patient populations perhaps due to the well developed capacity of the gonococcus for genetic variation and recombination [6, 13]. These characteristics coupled with the fastidious nature of the organism have created considerable difficulties for the laboratory diagnosis of gonorrhoea using conventional as well as molecular approaches.

In this study an in-house conventional PCR targeting the *opa* gene was standardized and its analytical and diagnostic performance was determined and compared against two published PCR assays [11, 12]. The *porA* pseudogene and the *opa*-based PCR assays proved to be highly specific for gonococcal DNA whereas cross-reaction with *N. sicca* was evident with the 16S rRNA assay. The high specificity of the *porA* pseudogene-based assay was in accordance with other studies which have shown high conservation of this gene across *N. gonorrhoeae* subtypes [13, 14]. Similarly, cross-reactivity of 16S genes with commensal *Neisseria* spp. has been observed by others [15] and it is possible that different primer sequences to amplify this gene may improve specificity of the assay. The sensitivity of the *porA* pseudogene assay was tenfold less than the other assays perhaps owing to its presence as a single copy in the gonococcal genome. Additionally, the published primers used in this study were designed for real-time PCR in conjunction with probes which might account for the lower sensitivity also reported by other studies [16, 17]. Increasing the number of cycles, modification of reaction conditions may improve the sensitivity of this PCR.

The sequence homology of the seven discrepant results (16S rRNA+, *opa*+, *porA*−) with *N. gonorrhoeae* standard sequence suggests that these patient samples were indeed 'true positives' implying that the specificity and PPV of the assay was perhaps better than the projected figures. The discrepancy in the result may be due to the lower sensitivity of the *porA* pseudogene. The recent report of sequence variation in *porA* pseudogene further raises concern over the impact of sequence variation on the performance of gonococcal NAATs [18].

The 16S rRNA PCR revealed 15 positive samples negative by both other assays. As the sensitivity of the *opa* assay is equivalent to the 16S rRNA assay it was considered unlikely that these samples were false negative by the *opa* PCR and hence they were not processed further. In fact the *opa* gene-based assay is reported to be five times more sensitive than the 16S rRNA assay presumably due to the higher copy number ($n=11$) of the former compared to the latter ($n=4$) in the *N. gonorrhoeae* genome [19]. It is noteworthy that all of the discrepant 16S rRNA results were from female patients and this might have been due to cross-contamination of the assay with the diverse microbial flora of the female genital tract and the contribution of the high rate of horizontal genetic exchange within the *Neisseria* genus. Further, *N. gonorrhoeae* species comprises a broad range of subtypes which exhibit considerable genetic variation. The continually changing prevalence of these subtypes in a patient population can have a significant impact on the success of any *N. gonorrhoeae* NAAT [13]. Therefore, assays need to be routinely monitored in order to identify any new variation in the target sequence used.

The conservation and stability of target DNA also has a major impact on the sensitivity of the assay. Other authors have also reported the *opa* gene-based assay to be sensitive, specific and reliable for detection of *N. gonorrhoeae* in clinical samples and/or for confirmation of less specific tests [13, 20]. However, a

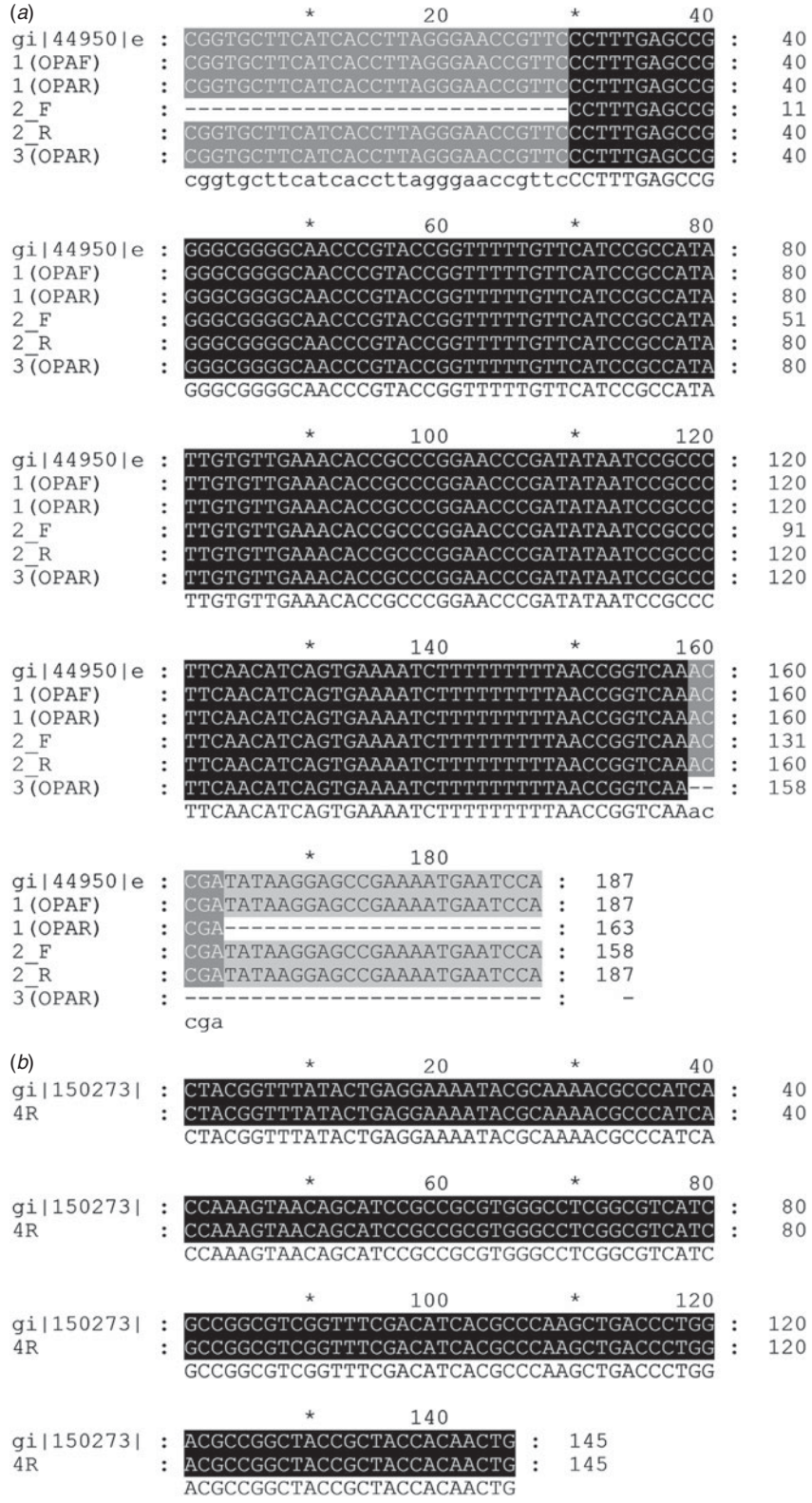

Fig. 1. (*a*) Sequence alignment of *opa* amplicons of three patients with protein II opacity gene of *N. gonorrhoeae*. (*b*) Sequence alignment of *opa* amplicon of one patient with opacity gene variant V28 of *N. gonorrhoeae*.

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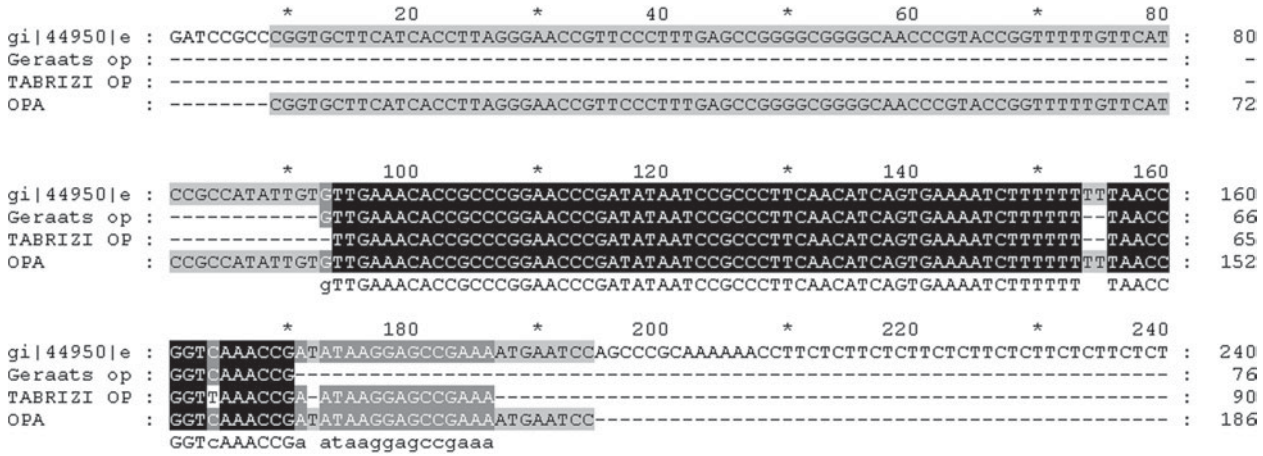

Fig. 2. Alignment of DNA sequence of *opa* amplicon with target sequences of Geraats-Peters *et al.* [19] and Tabrizi *et al.* [20] with respect to protein II (pFLOB1100) of *N. gonorrhoeae*.

recent study has described sequence variation in the multicopy *opa* gene leading to false-negative results [19]. Although, alignment of the sequence amplified by the in-house *opa* PCR with the target sequences of the earlier studies [19, 20] showed that the amplified product overlaps the target sequence in question (Fig. 2), the gene remains a highly stable target. By contrast with the earlier report by Geraats-Peters *et al.* [19], the present study picked up seven extra positives by the *opa*-based assay, four of which could be sequenced and were confirmed as true positives by DNA sequencing. Therefore, we have reason to believe that as suggested by others, variation observed in the *opa* gene of Dutch gonococci may not yet be as widespread [21]. Unlike the real-time PCR formats used by others [19, 20] our *opa* assay is a conventional PCR which after more extensive validation in different population groups would be suitable for laboratories which do not have access to real-time PCR machines. An investigation into the stability of the target sequence across a diverse range of gonococcal isolates from across the country may provide the necessary insight.

Although not surprising, an interesting finding was the low clinical sensitivity of conventional methods for the detection of *N. gonorrhoeae* particularly in female patients (21 false negatives). Even in male patients where the performance characteristic of culture is known to be good, there were two false negatives. Therefore, where possible diagnostic laboratories should adopt molecular techniques in conjunction with standard culture to optimize the detection and hence control this bacterial sexually transmitted infection. However, a close watch on the antimicrobial resistance of *N. gonorrhoeae* is mandatory but this at present cannot be met by NAATs.

To conclude we have shown an in-house *opa*-based PCR assay to be highly sensitive and specific for detection of *N. gonorrhoeae* in clinical swab samples. This assay may prove to be a suitable supplementary test in our population along with 16S rRNA as a screening assay. Moreover, the use of NAATs provided enhanced diagnosis of gonorrhoea in female patients.

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DECLARATION OF INTEREST

None.

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