Evaluation of an *Opa* Gene-Based Real-Time PCR Assay for Detection of *Neisseria gonorrhoeae* in South African Population

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BACKGROUND

Gonorrhoea, the second most widespread sexually transmitted infection, affects 80 million people annually. Female infections are often asymptomatic, leading to silent transmission and severe complications like pelvic inflammatory disease. Untreated *Neisseria gonorrhoeae* (*N. gonorrhoeae*) infections during pregnancy result in various complications, emphasizing the need for effective identification.

While culture is the "gold standard," nucleic acid amplification tests (NAATs) offer >95% sensitivity and >99% specificity, with faster results. Opa proteins on *N. gonorrhoeae's* outer membrane play a crucial role, making them suitable targets for real-time PCR assays. Geraats-Peters *et al.*, 2005 reported that an *opa* gene-based PCR assay is suggested to be more sensitive than a 16s rRNA PCR assay. Other studies also reported that the *opa* gene assays have a higher sensitivity than *porA* pseudogene assays. The *cppB* and CMT genes exhibit broad sensitivity due to the absence of the genes in specific strains of *N. gonorrhoeae*. The *CMT* gene demonstrates reduced specificity as it can react with commensal *Neisseria* species. Consequently, the *cppB* and *CMT* genes are unsuitable for detecting *N. gonorrhoeae.*

Therefore, this study aimed to develop and assess the performance of an in-house *opa*-based real-time PCR assay, comparing it to a published primer (Verma *et al.*, 2012).

METHODS

Study Population

- Full ethics- Biomedical Research Committee (BREC) UKZN-(BREC/00005104/2022).
- The study included stored bacterial isolates DNA: *N. gonorrhoeae* reference strains (WHO O, N, X, Y, and Z) from the WHO, clinical isolate DNA from pregnant women (n=6), and non-gonococcal *Neisseria* control isolate DNA (*N. cinera*, *N. weaver, N. sicca*). Clinical DNA samples comprised 29 endocervical swabs, 20 self-collected vaginal swabs, and 11 urine samples.

Design of the in-house *opa* primers

- Primers were designed based on a conserved region of the *opa* gene using GenScript software (Piscataway, New Jersey, United States), referencing Roth *et al*., 2012's description (GenBank accession: KC503485.1). Primer details are in Figure 1 and Table 1.

Qualitative PCR of *opa* genes-Comparator *opa* primer

- The Verma *et al*., 2012 *opa* primer set was used in this study, Real-time SYBR Green assay conditions included 5µl PowerUp SYBR Green Master Mix, 0.2nM forward and reverse primers, 1.5µl template DNA in a 10µl final volume. Amplification was performed in a Quant Studio 5 PCR instrument. Cycling conditions: UDG activation (2 min at 50°C), initial denaturation (2 min at 95°C), 40 cycles of denaturation (15 sec at 95°C), annealing (15 sec at 52°C), and extension (1 min at 72°C). Negative controls were included for contamination and PCR efficiency assessment.

Qualitative PCR of *opa* genes- in-house *opa* primers

- The in-house assay utilized Table 1 primer sets, 5µl PowerUp SYBR Green Master Mix, 0.2nM primers, and 1.5µl template DNA brought up to a final volume of 10ul with nuclease-free water. The reactions were run in a Quant Studio 5 PCR instrument (ThermoFisher Scientific, United States). Cycling conditions: all three primer sets had very similar melting temperatures, an annealing temperature of 56°C, UDG activation stage for 2 minutes at 50° C, initial denaturation for 2 minutes at 95° C, denaturation for 15 seconds at 95° C, annealing 15 seconds at 56°C with extension for 1 minute at 72° C for 40 cycles.

Limit of detection

- Ten-fold serial dilutions of the genomic DNA of the clinical isolates and clinical samples were prepared. The dilution series ranged from 1: 10 - 1: 100 000. The comparator *opa* and in-house *opa* primers were performed on each dilution series. The DNA concentration of each dilution was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, South Africa). The limit of detection of the assays was determined by calculating the copy numbers yielding positive amplification for each gene investigated.

Statistical Analysis

- The data was analysed using IBM SPSS Statistics version 27. For this study, sensitivity was calculated as the probability that the in-house *opa* primers produced positive amplification for culture isolates, endocervical, vaginal, and urine DNA samples.

Figure 1: The template used for the design of the primers was based on a conserved region of the *opa* region which includes all 11 *opa* types as described by Roth *et al.*, (2012) (GenBank accession number: KC503485.1) (2). The positions of the primer pairs are on the *opa* gene. There is an overlap between the *opa* 2 reverse primer and *opa* 3 forward primer. The region where the overlap occurs is underlined.

Table 1: Showing the *in-house opa* primer designed using the GenScript software.

Primer Name	Sequence	Amplicon size (bp)	Start position strand	Length	Primer Melting temperature (Tm)
***Opa* 1 RT Forward**	5' TAT GTG CAG GCG GAT TTA GC 3'	72bp	28	Forward 20	55.49
***Opa* 1 RT Reverse**	5' GTC TGT ACC GGT TGG TTT CG 3'		80	Reverse 20	56.00
***Opa* 2 RT Forward**	5' CTT CCA CGC CGT TTC TTC TC 3'	119bp	324	Forward 20	56.35
***Opa* 2 RT Reverse**	5' AAT CGA TGC TGT GTC TGA CG 3'		423	Reverse 20	55.50
***Opa* 3 RT Forward**	5' GAC ACG TCA GAC ACA GCA TC 3'	188bp	419	Forward 20	56.09
***Opa* 3 RT Reverse**	5' CTT GGG CGT GAT GTC GAA AC 3'		587	Reverse 20	56.98

RESULTS

Table 2: *N. gonorrhoeae* amplification and cross-reactivity of different *Opa* primer sets across the different sample types.

Primer Set	*N. gonorrhoeae* Amplification (Clinical Isolates)	Endocervical DNA Amplification	Vaginal DNA Amplification	Urine DNA Amplification	Cross Reactivity
Comparator *opa*	10/11 (90.9%)	8/29 (27.6%)	5/20 (25.0%)	4/11 (36.4%)	Yes (*N. sicca*)
In-house *opa* 1	11/11 (100%)	24/29 (82.8%)	19/20 (95.0%)	11/11 (100%)	No
In-house *opa* 2	11/11 (100%)	21/29 (72.4%)	19/20 (95.0%)	9/11 (81.8%)	Yes (*N. sicca*)
In-house *opa* 3	11/11 (100%)	17/29 (58.6%)	18/20 (90.0%)	8/11 (72.7%)	Yes (*N. cinera*, *N. sicca*)
Overall Performance	*opa* 1= *opa* 2= *opa* 3	*opa* 1 > *opa* 2 > *opa* 3	*opa* 1 = *opa* 2	*opa* 1 > *opa* 2 > *opa* 3	

Note: Percentages for amplification represent the proportion of positive results out of the total samples tested in each category. Cross-reactivity indicates whether the primer set showed positive amplification for non-gonococcal isolates.

Table 3: Limit of detection for different *opa* Primer Sets in various DNA sample.

Sample Type	Primer Set	Highest DNA Dilution	Copies Detected
Endocervical DNA	Opa 1	1:100 000	5.15 x 10^9
Endocervical DNA	Opa 2	1:100 000	3.11 x 10^9
Endocervical DNA	Opa 3	1:100 000	1.97 x 10^9
Vaginal DNA	Opa 1	1:100 000	5.15 x 10^9
Vaginal DNA	Opa 2	1:100 000	3.11 x 10^9
Vaginal DNA	Opa 3	1:100 000	1.97 x 10^9
Urine DNA	Opa 1	1:100 000	1.29 x 10^10
Urine DNA	Opa 2	1:100 000	7.79 x 10^9
Urine DNA	Opa 3	1:100 000	4.93 x 10^9
Overall Performance	Opa 1	1:100 000	Best Detection

DISCUSSION AND CONCLUSION

The study compared three in-house real-time PCR assays targeting the *opa* gene with the *opa* primer set by Verma *et al.*, 2012, for the detection of *N. gonorrhoeae*. The in-house *opa* 1 primer set performed better than *opa* 2 and *opa* 3, as well as the comparator *opa* primer set, which showed cross-reactivity with non-gonococcal isolates. The in-house *opa* 1 primer set detected the highest number of positives across different sample types and demonstrated superior sensitivity, especially at the highest DNA dilution (1:100000). The cost-effective and sensitive SYBR Green Assay used in this study holds promise for routine testing of STIs, as demonstrated by its reliability in *detecting N. gonorrhoeae* without cross-reactivity. While acknowledging the small sample size, the study's inclusion of various sample types, including self-collected vaginal swabs (less DNA material), adds to its significance. The in-house *opa* 1 assay stands out as a potential diagnostic tool for *N. gonorrhoeae*, offering specificity, sensitivity, and cost-effectiveness. In conclusion, the study contributes valuable insights into the development of a reliable and economical diagnostic assay for *N. gonorrhoeae*, with the in-house *opa* 1 primer set emerging as a promising candidate for further evaluation and potential clinical use.