DETECTION OF *NEISSERIA GONORRHOEAE* BY POLYMERASE CHAIN REACTION (PCR)

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ABSTRACT

Three different sets of primers were designed using FASTA homology search and PRIMERSELECT for the specific detection of *Neisseria gonorrhoeae* using polymerase chain reaction (PCR). These primers amplified the highly conserved regions of genes for Open Reading Frame (ORF), Outer Membrane Protein (OMP) and 23S rRNA sequences of *N.gonorrhoeae*. Each of the PCR primer set was evaluated using the DNA samples isolated from eight different positive isolates of *N.gonorrhoeae* cultured from urethral swabs of patients visiting Maulana Azad Medical College and Safdarjung Hospital. Amplification products were analyzed on agarose gel electrophoresis. Two sets of PCR primers, designated as Ngu1/Ngu2 and Ngu5/Ngu6, specific for ORF and OMP gene respectively, amplified four regions of the gene which may help to differentiate the various strains of *N.gonorrhoeae* infecting indigenous population. In contrast, a single, specific PCR product of 650bp was visualized on agarose gel with primers Ngu3/Ngu4, amplifying the 23S rRNA gene. Under optimum conditions, as low as 25ng of DNA isolated from eight different clinical strains of *N.gonorrhoeae* could be detected by PCR using Ngu3/Ngu4 set of primers. Our results suggested that Ngu3/Ngu4 could serve as good primers for the specific, reproducible and sensitive diagnosis of *Neisseria gonorrhoeae* from clinical samples.

KEY WORDS : Gonorrhoeae, Polymerase Chain Reaction, 23S rRNA

INTRODUCTION

The genus *Neisseria* includes human-specific pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitis*, which cause gonorrhoeae/ pelvic inflammatory disease and bacterial meningitis/sepsis respectively (1). Conventional diagnosis of gonorrhea relies mainly on the demonstration of intracellular Gram negative diplococci in smears of genital discharge or swabs (2). The diagnosis of *N.gonorrhoeae* can

be established readily in the acute stage as urethral discharge contains large number of gonococci. However chronic cases often present great difficulties. Definitive diagnosis of gonorrhoea involves culture of clinical specimen on modified Thayer Martin Medium in an atmosphere of 5% CO₂. Growth requires a minimum of 24 hours, and by 48 hours, the organism can be easily identified by Gram staining, enzymatic methods or immunofluorescence (3,4). As cultures are subject to several variable factors, some gonococci fail to grow in culture or are misidentified, making the

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assay less reliable. Several strains of *N. gonorrhoeae* are also susceptible to the concentrations of vancomycin used in the selective medium and hence the recovery rate is less than 100% (5). To eliminate some of these concerns associated with culture, alternative methods such as immunoblotting, ELISA, radioimmunoassay, and DNA hybridization have been developed (6,7). The specificity of these enzyme immunoassays is less than desired (94-100%). When compared with culture, the enzyme immunoassays are even less sensitive (87-98%) (4).

The use of DNA probes as a tool for detection of gonococci in urogenital specimens has been investigated extensively in recent years. Probes using nucleotide sequences of the pilin gene, the IgA gene, protease gene, ribosomal RNA and the cryptic plasmid have been reported (8). However, because of the low sensitivity of these methods, these are not useful as diagnostic tools. One of the reasons for low sensitivity is the low gene copy number. As the number of infectious organisms in clinical samples are usually too few, detection by culture or DNA probe assays or antigen assay does not provide desired sensitivity. Recently this problem has been greatly overcome by the development of the polymerase chain reaction (PCR) where, theoretically, even a single gene copy would be sufficient for amplification (5,9). The practical use of PCR methods developed in West would clearly be dependent on stability of the gene sequence during transmission over a period of time and geographical location.

In the present study, we have developed a polymerase chain reaction-based assay for the

detection of *N. gonorrhoeae* using primers specific for 23S rRNA gene. Though 23S rRNA gene is conserved among several bacteria, there are regions which are highly specific for *N. gonorrhoeae*. Use of rRNA gene provides a diagnostic advantage as rRNA gene is present in high copy number per cell. Presence of large number of target molecules increases the likelihood of initiating amplification even when the organism is present in low number. This will be particularly important in asymptomatic patients and for using urine samples instead of endocervical specimens.

MATERIALS AND METHODS

Bacterial Strains

The *Neisseria gonorrhoeae* strains used were all clinical isolates recovered from urethral or cervical swabs by inoculation onto modified Thayer Martin medium and subsequently identified. Clinical specimens were collected from men and women with acute gonococcal urethritis attending a sexually transmitted disease clinic in Safdarjung Hospital and Maulana Azad Medical College, Delhi. Two urethral swabs from the male and two cervical swabs from the female were taken with phosphate buffered saline pre wet swabs. One swab was inoculated directly onto modified Thayer Martin Medium, incubated in an atmosphere of 5% CO₂ for 48 hrs (3,4) and any suspected organisms were identified by Gram stain, colony morphology, positive oxidase, and rapid carbohydrate utilization tests following standard methods (2). The other swab was stored frozen at -20°C except during transport.

Isolation of genomic DNA

Bacterial DNA from eight *N. gonorrhoeae* strains (1a-1d and 2a-2d) was extracted essentially by the standard protocol (10). Briefly, bacterial suspension in Tris-EDTA (TE) was made from a 48 hour cultured plate to give about 10^8 cfu/ml. The following reagents were added sequentially to the suspension, sodium dodecyl sulfate, proteinase K and CTAB / NaCl at concentration described (10). The contents were incubated at 65°C for 20 min. The DNA was extracted once with phenol-chloroform and once with chloroform-isoamylalcohol followed by precipitation with ethanol. The extracted DNA was resuspended in 30 µl of TE and stored at -20°C. The concentration of DNA was estimated by $A_{260/280}$.

Selection of Primers

Primers were designed using PRIMER-SELECT software. DNA sequence of about 39 genes of *N. gonorrhoeae* was fetched from GenBank™, maintained at the National Center for Biotechnology Information (NCBI) branch of NIH using the Internet website <http://www.ncbi.nlm.nih.gov>. Using the FASTA Homology Search package, nucleotide sequences of genes which are highly conserved in *N. gonorrhoeae* and exhibit minimum homology to other bacterial pathogens and to known human gene sequences were screened. The gene for Open Reading Frame 1 (ORF, 1044 nucleotide (ntd) long), 23S rRNA gene and Outer Membrane Protein (OMP) gene were selected for the present study. From the nucleotide sequence, small regions (20-25ntd long) were chosen to design primers. The primer sequences were tested for homology, dimerization and other

parameters. The oligonucleotides were custom synthesized from Bangalore Genei, India.

PCR amplification

Amplification was performed in a 50 µl reaction volume containing 50 pmol of each oligonucleotide primer; 200 µM each of dATP, dCTP, dTTP and dGTP; 50 mM KCl, 10 mM Tris pH 8.3, 100 µg/ml bovine serum albumin, 1.5 mM $MgCl_2$ and 50 ng of DNA template. Forty cycles of amplifications were performed in a DNA thermal cycler (Perkin Elmer Cetus). Unless otherwise indicated, each cycle consisted of a denaturation step (94°C for 30 sec.), an annealing step (51°C for 30 sec.), extension step (74°C for 90 sec.) followed by a 10 min final extension at 74°C. An aliquot of the PCR product (10 µl) was size fractionated on Agarose gel (1.5%). Amplified product was visualized by ethidium bromide staining under UV light. Sigma PCR marker was used as DNA marker to estimate size of the PCR product.

RESULTS

Designing of PCR Primers

The key to any polymerase chain reaction is a pair of oligonucleotide primers of defined sequence. For *N. gonorrhoeae*, DNA sequences of three genes were selected for primer designing. These are Open Reading Frame (ORF), 23S rRNA gene sequence and an Outer Membrane Protein Sequence (OMP).

The Open Reading Frame (ORF) sequence was so chosen because this sequence is unique to *N. gonorrhoeae* species and can be used as a potential target to differentiate it from

its close relative *N. meningitis*. Similarly 23S rRNA gene sequence is also a good marker to differentiate phylogenitically these two species of *Neisseria*. Since more than one copy of the gene encoding the 23S rRNA is present in many bacterial species, this would further amplify the signal of PCR. The Outer Membrane Protein sequence (OMP) is a potential target to check for any inter-species divergence in *N. gonorrhoeae*. The exposed regions of this protein could show differences from one strain of *N. gonorrhoeae* to another.

Primers were designed using the PRIMERSELECT program for all the three sequences. On the basis of homology search (*data not presented*) and PRIMERSELECT program (*data not presented*), three pairs of primers were designed, (see Table 1 for their sequence) and ordered from Bangalore Genei, India Pvt. Ltd.

Table 1: Three pairs of Oligonucleotide Primers with the Tm values

Ngu1 : Sense primer from Orf1 of <i>N. gonorrhoeae</i> , Tm : 56°C
5'-CAA CTA TTC CCG ATT GCG A-3'
Ngu2 : Antisense primer from Orf1 of <i>N. gonorrhoeae</i> , Tm : 58°C
5'-GTT ATA CAG CTT CGC CTG AA-3'
Ngu3 : Sense primer from 23S rRNA gene of <i>N. gonorrhoeae</i> , Tm : 62°C
5'-CGT TCA TCG GCG TAG GGT AA-3'
Ngu4 : Antisense primer from 23S rRNA gene of <i>N. gonorrhoeae</i> , Tm : 56°C
5'-CAC TTC TCG GTG TTA AGA AA-3'

Ngu5 : Sense primer from omp gene of *N. gonorrhoeae*, Tm : 62°C

5'-GCC GCG TAT CTT GAG GCA TT-3'

Ngu 6 : Antisense primer from omp gene of *N. gonorrhoeae*, Tm : 60°C

5'-TGA AGC CTT TGG CAG AAC CT-3'

Isolation of the genomic DNA of *N. gonorrhoeae*

For clinical diagnosis and for culture of *N. gonorrhoeae*, two swabs were collected from patients suspected of suffering from gonorrhoea. One swab was used for Gram staining. The other swab was used for culture of *N. gonorrhoeae* on Thayer Martin Media in duplicate only if the first swab gave a positive reaction with Gram stain. The genomic DNA was isolated and estimated as described under methods from eight different clinical strains.

Specificity of primers for amplification of *N. gonorrhoeae*

The three sets of primers designed by us (Table 1) were evaluated in a PCR assay for the specificity. All the three sets of primers designed by us amplified *N. gonorrhoeae* DNA sequence isolated from all the eight strains (Fig. 1 and data not presented). However, the PCR primer pair Ngu3 and Ngu4 was found to be the most suitable candidate that could be used for PCR based detection of *N. gonorrhoeae* from the clinical samples. This set of PCR primers amplified the 23S rRNA gene sequence.

The Ngu1 and Ngu2 set of PCR primers amplified the open reading frame (ORF)

Fig. 1 : PCR with different *N. gonorrhoeae* isolates. Lane 1 : Sigma PCR Marker (50-2,000bp). Lanes 2-4 are strains of *N. gonorrhoeae* (1a,1b,2a resp) amplified using Ngu3/Ngu4 primers. Lanes 5 and 6 show PCR amplification of *N. gonorrhoeae* strains 2a and 1a resp. with primers Ngu1/Ngu2. Lanes 7 and 8, amplification of strain 1a with primers Ngu3/Ngu4 and Ngu5/Ngu6 resp.

sequence of the *N.gonorrhoeae*. The protein product of this gene and its putative function is not clear. This sequence is highly conserved in *Neisseria gonorrhoeae* and is used to differentiate *N.gonorrhoeae* from other *Neisseria* species. Using this set of PCR primers, four bands of 200bp, 500bp, 1000bp, and 2000bp were amplified, as shown in Fig. 2, lane2. Amplification products of four different sizes suggest that the primers annealed at more than one position in the genome. Similar results were obtained when DNA from other isolates of *N.gonorrhoeae* was used (as shown in Fig. 1. lanes 5, 6 and Fig. 2 lane 2).

Fig. 2 : Specificity of primers for amplification of genes of *N. gonorrhoeae*. PCR was carried out using DNA isolated from 2b and different sets of primers, under standard conditions. Lane 1: Sigma PCR marker (50-2,000bp). Lane 2 : Amplified product using Ngu1/Ngu2 primer pair. Lane 4 : Amplified product using Ngu3/Ngu4 primer pair. Lane 6 : Amplified using Ngu5/Ngu6 primer pair.

With Ngu5 and Ngu6 primer pair, amplifying the Outer Membrane Protein of *N.gonorrhoeae*, again four different bands of 150bp, 400bp, 1000bp and 2200bp were amplified (Fig. 1 lane 8). The regions in the protein which are membrane bound are highly conserved, whereas the DNA sequence for the protein contributing to the exposed regions are highly variant. The widespread occurrence of sequence variation of membrane proteins between different serovars and also within a serovar has potentially important implication. We envisage that this pair of PCR primer may serve as a suitable candidate to differentiate the

different strains of *N.gonorrhoeae*.

Of the three sets of primers, the Ngu3 and Ngu4 primer set, which amplifies 23S rRNA gene, gave a single PCR product of 650 bp and was used for further standardization process. It is important to mention here that each experiment was repeated at least three times. All assays were assembled at 4°C. Care was also taken to prevent any possible cross contamination by using gloves and disposable tips and tubes.

Optimization of PCR conditions

The optimum annealing temperature was found to be 51°C for primer sets Ngu1/Ngu2 and Ngu3/Ngu4 while for primer set Ngu5/Ngu6, the optimum annealing temperature was found to be 55°C. For all the three sets of primers, the optimum concentration of Mg²⁺ for maximum and specific amplification was found to be 2.5mM. The optimum concentration of primers was found to be 50pmoles/assay (Fig.3, lanes 5-7). High concentrations of primers (100pmoles and above), resulted in spurious PCR product, whereas at low concentrations (1–20pmoles), a decrease in the yield of the product was observed suggesting that primer concentration may not be sufficient (data not presented). These experiments also verified that all the four DNA bands observed on agarose gel that were being amplified with Ngu1/Ngu2 and Ngu5/Ngu6 primer pairs are specific to *N.gonorrhoeae*.

Sensitivity of PCR assay

In order to check the sensitivity of the PCR assay optimized by us, different concentrations of DNA (25ng to 100ng) were used. Using the serial

Fig 3 : Effect of DNA concentration and primer concentration on PCR. Lanes (1-3) : DNA concentrations used were 100ng, 40ng, and 25ng respectively. Lanes (5-7) : Primer concentrations used were 50 pmoles, 20pmoles and 10 pmoles respectively. Lane 8 : Sigma PCR marker (50-2,000bp).

dilution of DNA, the PCR was performed under the standard conditions using the Ngu3 and Ngu4 primer pair to amplify the 23S rRNA gene. The PCR product of 650 bp was detected from as little as 25 ng of DNA of *Neisseria gonorrhoeae* per assay (Fig. 3, lanes 1-3).

DISCUSSION

The ability to identify the pathogen reliably is essential for the management of infectious diseases such as Gonorrhea (or Sexually Transmitted Diseases). More recently, gonococcal infections have been shown to facilitate transmission of HIV (3-5 fold more likely). Culture method and Gram staining are

routinely used in clinical setup for routine identification of infectious diseases. For culture, the specimens should be inoculated immediately upon collection. Furthermore, in chronic cases, where mixed infection is common, it is essential to use a selective medium for growth. Methods other than culture such as enzyme immunoassays, DNA hybridization, though more sensitive and rapid than culture method, are not specific enough for routine diagnosis. This is because, the specificities of these methods rely on a single recognition step, such as antibody-antigen binding or hybridization of the probe to its target DNA. Furthermore, as most of these kits need to be imported, and require expertise and special instruments, they have not become a method of choice in India. Thus the effective control of *N.gonorrhoeae* in India and other developing countries is hampered due to the lack of cost effective yet rapid, effective, sensitive and specific method of detection.

We have developed a method for the rapid detection of *N.gonorrhoeae* by DNA amplification method using 23S rRNA gene as target. A single, specific band of 650bp is observed when DNA, isolated from eight different clinical samples was amplified with primers Ngu3/Ngu4. The presence of multiple copies of rRNA genes in each cell increases the probability of initiating amplification even when the pathogen is present in low number. This is evident from the fact that as little as 25ng DNA/assay was sufficient to give a positive band

on agarose gel electrophoresis. The other two sets of primers (Ngu1 / Ngu2 and Ngu5 / Ngu6) amplifying ORP and OMP genes respectively gave four specific bands. Experiments are in progress to check if PCR using these primers could be used to differentiate serovars of *N.gonorrhoea* infecting indigenous population.

Thus, our studies suggest that the use of 23S rRNA gene as a target molecule will provide a diagnostic advantage specially in asymptomatic patients. The method will also be useful for using urine samples instead of endocervical specimens. The DNA amplification method described here promises to be a sensitive, inexpensive and rapid method for the detection of *N.gonorrhoeae*. To offer this method as an alternate to culture in clinical set up, detection directly from the clinical samples without the isolation of DNA is under evaluation and standardization.

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