A study of detection of *Neisseria gonorrhoeae* among HIV positive and HIV negative patients by conventional and molecular methods

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Abstract

Introduction: Gonorrhea infection is caused by a Sexually Transmitted Infection (STI) that infects both men and women. Based on the clinical manifestations it is difficult to diagnosis *Neisseria gonorrhoeae*. Conventional diagnosis techniques like gram stain and culture for the diagnosis of gonorrhea are inexpensive but have poor sensitivity especially in asymptomatic patients. Rapid diagnosis is important for the control of the disease, PCR method is one such rapid method.

Materials and Method: A total of 611 specimens were collected from different anatomical sites in men and women of reproductive age group who attend the STD and non-STD clinic at Khaja Bandanawaz Institute of Medical Sciences, Gulbarga, Karnataka, during the period of June 2013 to June 2016. Direct smears for gram staining, Culture, Biochemical tests and molecular method (PCR) were performed.

Conclusion: in the present study, the comparison between conventional and Molecular methods was done; gram staining was less sensitive than culture and PCR. Culture on chocolate agar [CA] was not showing 100% results compare to the Modified Thayer-Martin Media [MTM] on which all 55 specimens were grown, but PCR showed 94.5% result even though there was less specimen in the swabs. PCR technique for the detection of orf1 gene of *Neisseria gonorrhoeae* directly from clinical samples is a promising investigative method. However, culture on selective media (MTM) is the method of choice for the identification of *Neisseria gonorrhoeae* and susceptibility testing, particularly in developing countries like India.

Keywords: ONPG, PCR, MTM, *Neisseria gonorrhoeae,* Sexually Transmitted Infection

Introduction

Gonorrhea infection is caused by a Sexually Transmitted Infection (STI) that infects both men and women. Based on the clinical manifestations it is difficult to diagnosis *Neisseria gonorrhoeae*. This is due to the fact that *Neisseria gonorrhoeae* can cause both symptomatic and asymptomatic genital as well as extra-genital tract infection with various symptoms, many of which are similar to those of other STIs. Due to the less specific clinical presentation and inadequate facilities, materials, or personnel for laboratory-based diagnosis gonorrhea and other non-ulcerative STIs is difficult to determine. Moreover, there is a lack of reporting mechanism and reluctance to report STIs to the public health authorities. Since gonococcal infections are asymptomatic or have nonspecific symptoms, it is difficult to diagnosis *Neisseria gonorrhoeae* in the early stage of infection (Korenromp et al., 2002). It is important to control the disease because of the high incidence of acute asymptomatic cases, complications and its role in the transmission of HIV infection.

A number of advanced laboratory methods are developed to diagnosis genital infections caused by *Neisseria gonorrhoeae*. Conventional diagnosis techniques like gram stain and culture for the diagnosis of gonorrhea are inexpensive but have poor sensitivity especially in asymptomatic patients. Moreover, delay in availability of the results makes these methods unsuitable for both screening and treatment, as a number of patients do not return to collect their results. Despite the low viability of the *Neisseria gonorrhoeae* in vitro, the current gold standard for diagnosis of infections caused by this organism is cultured on selective media, the success of culture methods requires good quality collection and transportation of clinical specimens. Culture provides the opportunity to determine antimicrobial susceptibility testing, and continuous checking of the antibiotic resistance profile which is essential for appropriate management of cases as resistance could vary in different regions and over different time periods.

Rapid diagnosis is important for the control of the disease. PCR method is one such rapid method applied in the reference laboratories. The advantage of this method is that the identification is possible in a day and has high sensitivity of 100% with a specificity of 88.9% (Ho et al., 1992). The present study compares the sensitivity of the conventional and molecular methods for identifying *Neisseria gonorrhoeae* from various specimens.

Materials and Method

Patient enrollment, A total of 611 specimens were collected from different anatomical sites in men and women of reproductive age group who attend the STD and non-STD clinic at Khaja Bandanawaz Institute of Medical Sciences, Gulbarga, Karnataka, during the period of June 2013 to June 2016. An informed consent form (questionnaire in English language) was provided; the procedure was informed to all the higher authorities of all department HODs (especially STD and non-STD

clinic departments). Ethical consideration was taken from the same institution and confidentiality was maintained where it was found necessary. Biosafety measures were taken for sample processing.

Specimens for the laboratory diagnosis of *Neisseria gonorrhoeae* were collected before starting antibiotics to the patients. Specimen collection was done with a sterile Dacron swab and bleeding that can render the specimen unsatisfactory was avoided. For the diagnosis of *Neisseria gonorrhoeae* urethral, cervical, vaginal, rectal, urine, oropharyngeal, conjunctiva, sterile body fluids and blood samples were collected. Two swab samples were taken, one for gram stain preparation and culture, if delayed samples were kept in Stuart transport media, which preserves the bacterial morphology was used for the transportation of clinical specimens to the research laboratory. The specimens were then used for direct microscopy and inoculation into the selective and non-selective culture medium. The second swab was kept in phosphate buffer saline (PBS) for molecular method. The samples were transported on the same day to the microbiology laboratory in an ice pack and were stored at -20˚C temperature till further use.

Direct Microscopy: Direct smears for gram staining were performed as soon as the swab specimen was collected. The swab was rolled gently onto the slide to preserve the cellular morphology and over an area less than 1 cm. Under oil immersion (1000 × magnification) for the presence of intracellular and extracellular gram negative kidney shaped diplococci and polymorphonuclear leukocytes were seen.

Culture diagnostics: Specimens were streaked on a selective (Modified Thayer-Martin) medium and non-selective (chocolate agar) medium. Isolation of *Neisseria gonorrhoeae* on culture media includes a blood agar base medium supplemented with bovine blood to enhance the growth of the gonococcus. Selective media are different from routine culture media in that they contain antimicrobial agents (vancomycin, colistin, trimethoprim and nystatin) which inhibit the growth of commensal and other pathogenic bacteria and fungi. Selective media might improve isolation if the anatomic source of the specimen normally contains other bacterial species. The culture plates were incubated at 35°C to 36°C in a moist atmosphere enriched with CO_2 (5 to 10%)

Biochemical tests: Bacterial Isolates recovered from a specimen on a selective and non-selective medium which are gram-negative diplococcic. A presumptive positive identification test like gram stain, catalase and oxidase test indicates the presence of gram-negative diplococcus in smears. For a confirmed laboratory diagnosis, additional biochemical tests were done. However, the presumptive investigation results are sufficient to initiate antimicrobial therapy.

O-Nitrophenyle-β-D-Galactopyranoside test (ONPG): This test distinguishes late lactose microbial ferments. A loop full of bacterial culture was suspended in 0.85% of saline in the test tube and ONPG disc was placed in the tube and was incubated for 4 hours at 37 ^{o} C in ambient air, after incubation, the tubes were examined for colour change (indicates the absences of β-galactosidase) and differentiates *Neisseria gonorrhoeae* from other *Neisseria sp*. Quality control was set up by using known organism *Escherichia coli* as positive control and *Proteus vulgaris* as a negative control.

Molecular Methods

Deoxyribonucleic acid (DNA) extraction and selection of primers: DNA was extracted as described by (Uma Chaudhry et al., 2002) The swabs preserved for PCR identification were brought to room temperature and then vortexed for one minute to release the material contained in the swab. The swabs were then discarded and the suspension was centrifuged for 30 minutes at 14,000 revolutions per minute to pellet the cells. The cell pellet was lysed with 50 mm TRIS-HCl (pH 8.0) 1% Triton X-100, 1mM EDTA, 10 µl (2.5 µg) of proteinase K per ml at 37°C for 1 hour, boiled for 10 minutes, and centrifuged. Then, the ten microlitres of this solution were subsequently added to 90 ml of PCR mixture and was stored at -20^{o}C for subsequent PCR amplification.

Selection of primers: Primers were designed using PRIMERSELECT software. DNA sequence of about 39 genes of *Neisseria gonorrhoeae* was fetched from GenBank TM, maintained in a 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 *Neisseria 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-1) was selected as it is specific to *Neisseria gonorrhoeae*. The primer sequences were tested for homology, dimerization and other parameters. It was observed that the *orf1* gene primer pair was unique to *Neisseria gonorrhoeae* and did not show any sequence homology with the sequences available for other *Neisseria sp*, the oligonucleotides were custom synthesized from Bangalore Genei, India.

The sequence data on the cppB gene carried on a chromosome as well as on 4.2 KB cryptic plasmid of *Neisseria gonorrhoeae* was used to select two 20-mer oligonucleotide primers designing for orf1 and cppB gene based on the previous study with minor changes. (Alamet et al., 2002; Uma Chaudhry et al., 2002). Sense and Antisense primers of each gene of *Neisseria gonorrhoeae* and PCR conditions with temperature as mentioned in Table 1.

PCR amplification: For PCR amplification, a positive control (*Neisseria gonorrhoeae* ATCC strain 49226), and DNA ladder was used. The conditions and selection of primers used in PCR are mentioned in table 1. Amplification was performed in 100 ml reaction volumes containing 50 pmol of each oligonucleotide primer, 200 mM each of dATP, dCTP, dTTP, Dgtp, reaction buffer (50 mMKCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl2, 0.1% gelatin), 1.5 U of Taq DNA polymerase and 50 ng of genomic DNA. All the reagents were taken in a PCR tube and mixed by gentle vortexing before overlaying with a drop of mineral oil. Thirty-five cycles of amplification were performed in a gradient thermal cycler.

The amplified PCR product (10 ml) was analyzed by electrophoresis on 2% agarose gel and staining with ethidium bromide verifies the specific amplified products using a UV transilluminator, and the results were documented. An amplified product of 260 base pairs (bp) of orf1 and 390 bp of the cppB genes were observed in all *Neisseria gonorrhoeae* isolates.

Agarose gel electrophoresis: Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA and proteins. Here DNA molecules were separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Agarose gel electrophoresis was carried out in a horizontal matrix of 2% agarose with 1X TAE buffer in 100 ml and was melted in an oven for about 2-3 min. Agarose gel was allowed to cool at 50°C. Ethidium Bromide (EtBr) is an intercalating agent which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel and were added to get a final concentration of 0.5 µg/mL and poured into the gel-casting tray fitted with a comb. The agarose was allowed to polymerize for 30 minutes. The comb was then removed and the gel was immersed in 1X TAE buffer in horizontal electrophoresis tank. The amplified product was mixed with 1/6 volume of 6X Gel loading buffer 6ml and electrophoresed at 5 V/cm. A 1kbp DNA marker was run in parallel as size standard. The bands were visualized by short wave (300 nm) UV transilluminator. Fig. 1

Table 1: Sense and Antisense primers of each gene of *Neisseria gonorrhoeae* and PCR conditions with temperature

Name of the gene	Primers sequence (5' to 3')	Amplicon size	PCR conditions
orf 1	Sense primer CAA CTA TTC CCG ATT GCGA Antisense primer GTT ATA CAG CTT CGC CTGAA	260 bp	Initial denaturation: 94°C for 5 min Cycling: 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min Final extension: 72°C for 10 min.
CppB	Sense primer: GCT ACG CAT ACC CGC GTT GC Antisense primer: CGA AGC CCT TCG AGC AGA CA	390bp	Initial denaturation:94°C for 4 min Cycling: 94°C for 60 sec, 48°C for 60 sec, 72°C for 60 sec Final extension: 72°C for 10 min.

Table 2: Comparison of Gram stain smears and Culture on selective & Nonselective media in diagnosis of *Neisseria gonorrhoeae*

Test (n=55)	Gram stain		Culture			
			Growth on non-selective Chocolate agar (CA)		Growth on selective (MTM)	
	No.	%	No.	%	No.	%
Positive	49	89.1	52	94.5	55	100
Negative	6	10.9	3	5.5	0	0
Total	55		55		55	

Table 2 is showing 100% culture sensitivity in Modified Thayer Martian media and 94.5% on non-selective Chocolate agar.

Table 3: Detection of *Neisseria gonorrhoeae* isolates as per Biochemical tests

Test (n = 55)	Glucose With acid production	Superoxal test	Oxidase test	ONPG	Nitrate reduction
Positive	55	55	55	0	0
Negative	0	0	0	55	55
ONPG= O-Nitrophenyle-β-D-Galactopyranoside					

Table 4: Comparison of gram stain, culture and PCR method for detection of *Neisseria gonorrhoeae* in HIV-positive and HIV-negative specimens

Disease (n= 55)	Gram stain (Direct microscopy)		Culture		PCR			
					Orf 1		cppB	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
NG + HIV	12	1	13	0	13	0	13	0
NG + Non-HIV	37	5	39	3	40	2	39	3
Total	49	6	52	3	53	2	52	3

Orf 1 and cppB gene was found in all HIV co-infected strains where as two strains of Orf 1 and 3 strains of cppB gene was missing in HIV negative co-infected strains.

Fig. 1: Detection of *Neisseria gonorrhoeae* genes (orf 1 and cppB genes) The amplified fragments were separated by electrophoresis by agarose gel stained with ethidium bromide

Discussion and Conclusion of conventional methods

Development in the laboratory techniques for the diagnosis of common Sexually Transmitted Diseases (STD) gives an opportunity to exactly measure sexual risk behaviours of young people. For isolation of the fastidious bacteria like *Neisseria gonorrhoeae*, ideal sampling, transport media, time, and conditions are necessary for performing culture diagnosis with high sensitivity and specificity.

Specimens collected from urethral, vaginal, cervical secretions and other samples were stained by modified gram-staining technique and samples that demonstrate the polymorphonuclear leukocytes and intracellular or extracellular gram-negative diplococci in symptomatic and asymptomatic patients were considered the causative agent of gonorrhea. In the present study out of 611 samples, 49 were found positive for *Neisseria gonorrhoeae* by direct microscopy. However the gram stain was not sufficient to rule out the infection in men, microscopy of urethral smears is more sensitive in symptomatic (90-95%) than in asymptomatic (50-75%) patients (Sherrard et al., 1996). In women, microscopy of gram-stained endocervical smears is more sensitive than urethral smears (37-50% vs 20%) (Barlow et al., 1978). Microscopy is not appropriate for pharyngeal and rectal specimens, as they are insufficient and is not recommended for the confirmation of infection by direct microscopy.

Conventional diagnosis of gonorrhea relies mainly on the demonstration of intracellular gram-negative diplococci in smears of genital discharge or swabs (Mukenge et al., 2002). The diagnosis of *Neisseria gonorrhoeae* can be established readily in the acute stage as urethral discharge contains a large number of gonococci. However, chronic cases often present great difficulties.

In the present study, a culture of *Neisseria gonorrhoeae* on non-selective chocolate agar were 52/611 and selective MTM media were 55/611 (Table 2). This shows that selective media is better than non-selective media for isolation of pathogenic bacteria from different specimens. Definitive diagnosis of gonorrhea involves culture of a clinical specimen on Modified Thayer-Martin medium in an atmosphere of 5-10% CO_2 which was provided by candle extension jar. Growth requires a minimum of 24 to 48 hours of incubation and the organism was identified by gram staining and enzymatic methods. As culture is subject to several variable factors, some gonococci fail to grow in culture or are misidentified, making the assay less reliable. Several strains of *Neisseria gonorrhoeae* are also susceptible to the concentrations of vancomycin used in the selective medium and hence the recovery rate is less than 100% (Wong et al., 1995). Colonies

of *Neisseria gonorrhoeae* was identified by colony morphology, gram stain and other biochemical tests.

The advantages of culture are high sensitivity and specificity, low cost, suitability for use with different types of specimens, and the ability to retain the isolate for additional testing. The major disadvantage of culture for *Neisseria gonorrhoeae* is that specimens must be transported under conditions, adequate to maintain the viability of organisms. Another disadvantage is that a minimum of 24–72 hours is required from specimen collection to the report of a presumptive culture result.

The presumptive diagnosis based on the identification of suspected colonies with typical morphology, Confirmation of culture was done by biochemical tests. Rapid carbohydrate degradation test for *Neisseria gonorrhoeae* utilizes glucose only, but not sucrose, maltose, mannitol and lactose, whereas Superoxal & oxidase tests were found positive in all culture positive isolates, all 55 were ONPG negative and there was no nitrate reduction (Table 3). Improved diagnostics of *Neisseria gonorrhoeae* and STIs, in general, can help limit the spread of HIV as it has been shown in several studies that an underlying STI can increase HIV transmission 5-10 times (Rosebury et al., 2008). Adequate treatment is important to prevent the emergence of antimicrobial resistance (AMR), as the treatment options for gonococcal infection is getting worse. For the same reason, representative and timely AMR surveillance programs are important to ensure that the most effective drugs are recommended and used.

Discussion and conclusion of molecular methods

Diagnosis of *Neisseria gonorrhoeae* by molecular methods like Polymerase Chain Reaction (PCR) based assay for the detection of the Orf1 gene and a cppB gene using specific primers. The Open Reading Frame (ORF) sequence is unique to *Neisseria gonorrhoeae* and can be used as a potential target to differentiate from its closely related species. In the present study, 55 specimens that were found positive by the conventional method were tested by PCR assays using primers specific to the orf1 and a cppB gene. The orf1 gene was detected in HIV positive and HIV negative gonococcus cases, whereas the cppB gene was not found in three culture positive clinical specimens that were considered as gonococcal positive. With PCR, there was 96.4% detection of the orf1 gene, a cppB gene was found to be 94.5% (Table 4).

Due to the high incidence of acute asymptomatic patients, control of the disease is essential, rapid diagnosis is very important to control the disease and prevent transmission of HIV infection. Polymerase Chain Reaction method is one such rapid method, by this method identification of the causative agent of *Neisseria gonorrhoeae* is possible in a day and it has a high sensitivity and specificity. But there are several limitations to PCR method such as costly equipment's and reagents, experienced technicians and above all organisms cannot be isolated by this method for the further investigation like antibiotic susceptibility testing.

In the present study, the comparison between conventional and Molecular methods were done, gram staining was less sensitive than culture and PCR. Culture on chocolate agar [CA] was not showing 100% results compare to the Modified Thayer-Martin Media [MTM] on which all 55 specimens were grown, but PCR showed 94.5% result even though there was less specimen in the swabs. PCR technique for the detection of orf1 gene of *Neisseria gonorrhoeae* directly from clinical samples is a promising investigative method. However, culture on selective media (MTM) is the method of choice for the identification of *Neisseria gonorrhoeae* and susceptibility testing, particularly in developing countries like India.

Confirmatory tests using the *cppB* gene have been reported (HO B.S et al); however, the cryptic plasmid on which the *cppB* gene is located is suspected to be missing in 5.8% clinical isolates (Bruisten et al., 2004). Polymerase chain reaction utilizes a DNA polymerase enzyme to extend primers and thus amplify specific parts of DNA. The specificity and sensitivity of PCR reactions depend on the primers used (Farrell et al., 1999) and the presence of inhibitory substances in the specimens (Martin et al., 2000). Running an agarose gel electrophoresis and staining with ethidium bromide verifies the specific amplified products.

Cultivation of bacteria on the selective agar plate, colonies with morphology compatible with *Neisseria gonorrhoeae* must be recognized and investigated. Sensitivity in PCR can be reported as excellent compared to culture (Page-Shafer et al., 2002), but the possibility of false positive must be considered. Several Neisseria species are known to colonies the human pharynx and several commercial tests have been reported to produce false positives (Diemert et al., 2002). Conformational tests are recommended, the Roche test, for example has not been cleared by FDA for samples from the pharynx. (Johnson et al., 2002).

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