Chapter 1
Evaluation and Development of In-house PCR for co-detection of C. trachomatis and N. gonorrhoeae
Introduction
1.1 INTRODUCTION

*Neisseria gonorrhoea* and *Chlamydia trachomatis* are two major pathogens of STDs. Of 448 million new cases of curable STIs (Chlamydia infection, gonorrhoea, syphilis and trichominiasis) occurring annually, 75% to 80% occur in developing countries (World Health Organization, 2011). These infections directly do not account for high rate of mortality but are responsible for high STI’s morbidity leading to acquisition of HIV and HPV infection and induced neoplasia (Smith *et al.*, 2002(2); White *et al.*, 2008). Clinical symptoms include conjunctivitis, urethritis, epididymitis, proctitis, and salpingitis. Chronic infection may lead to infertility, low birth weight, premature delivery and pelvic inflammatory disorder (Gaydos *et al.*, 2004; Mullick *et al.*, 2005a). Approximately 70% to 80% women and 50% men remain asymptomatic (Gaydos *et al.*, 2004). In developing countries, 66.7% - 100% and 31.2% to 80% of selected female population were found to be asymptomatic for *Neisseria* and *Chlamydia* respectively (Detels *et al.*, 2011). Asymptomatic and under diagnosed infections affect public health as these patients continue to infect their partners resulting in increase in disease burden.

Several reports have shown co-infection of gonococci and *Chlamydia* with much higher prevalence found in women and adolescents (Miller *et al.*, 2004; Mullick *et al.*, 2005a; van Bergen *et al.*, 2006b). Creighton and co-workers (2003) reported that 24.2% of men and 38.5% of women infected with *Neisseria* were also infected with *Chlamydia* whereas 18.8% of men and 13% of women infected with *Chlamydia* were co-infected with *Neisseria*. A prevalence study among school students showed overall rate of 0.3% co-infection. *Chlamydia* infection amongst gonorrhoea infected patients was found to be as high as 69.7% whereas 7.29% infected with *Chlamydia* were also found to be *Neisseria* positive (Miller *et al.*, 2004).

A high proportion of laboratory confirmed symptomatic and asymptomatic STI has been reported by several studies (Detels *et al.*, 2011; Patel *et al.*, 2010; Ray *et al.*, 2006; Sachdeva *et al.*, 2009). In developing nations like India, culture is used as gold standard because of its low cost, (Becker *et al.*, 2010) but requirement of technical expertise and long duration of culturing, makes them less popular in most of healthcare centres (Aledort *et al.*, 2006(11); Madhivanan *et al.*, 2009; Mullick *et al.*, 2005). Inability of patients to return and collect the reports, especially in case of pregnant women and adolescents, leads to further delay or lack of treatment which may lead to neonatal conjunctivitis (Kakar *et al.*, 2010).
Thus, to avoid delay in treatment, WHO issued guidelines on the basis of “vaginal discharge syndrome”. However, such guidelines are neither specific nor sensitive for *Chlamydia* and *Neisseria* especially in antenatal clinics (Mullick *et al.*, 2005; Vickerman *et al.*, 2006; Vishwanath *et al.*, 2000a). Vaginal discharge and pain in lower abdomen are common during pregnancy thus, they become less relevant for identification of infections among pregnant women (Romoren *et al.*, 2007). Therefore, syndromic management not only misses out on asymptomatic patients but could also lead to over treatment. Hence, there is an urgent need for intervention to control transmission of common STI from asymptomatic patients for effective disease management, especially in developing countries. This in turn is critically dependent on the development of an inexpensive, sensitive and point of care test that could be used to improve diagnosis of both *N. gonorrhoeae* and *C. trachomatis*.

An attempt in this direction was made by our laboratory and using in-house PCR based assay, we reported high prevalence of infection by *Neisseria* and *Chlamydia*, in symptomatic patients (Chaudhry & Saluja, 2002; Patel *et al.*, 2010; Sachdeva *et al.*, 2009). However, prevalence of co-infection in India is poorly understood. Although commercial kits are available for simultaneous detection of *Chlamydia* and *Neisseria*, but their high cost and/or time taken for diagnosis, as well as the requirement of trained technicians to conduct the assays, discourage their routine use in India. In the present study, duplex PCR (dPCR) assay has been developed for simultaneous detection of *N. gonorrhoeae* and *C. trachomatis*. The performance of in-house dPCR method was evaluated against uniplex (uPCR) and compared with existing commercial PCR assay (AMPLICOR MWP, *N. gonorrhoeae* detection Test; Roche Diagnostics Systems, USA). The dPCR assay developed in the present study is highly cost effective, specific and sensitive. The performance of the test for its specificity, sensitivity, positive and negative predictive values against currently used diagnostic methods suggest that in-house PCR assay was highly comparable in its performance to that of commercial kits.

The uPCR has been also evaluated using wet and dry endocervical swabs to test the feasibility of sample collection and transportation at room temperature. Further, use of molecular beacon as detection method has been used to further improve the sensitivity and specificity of the test. Availability of the test result within few hours and stability of premix reagents at 4°C could allow treatment at the initial visits even peripheral clinics and help in preventing future transmission of disease to occur.
Review of Literature
1.2 REVIEW OF LITERATURE

1.2.1 GONORRHOEA and its Causative Organism

*Neisseria* gonorrhoeae is one of the oldest diseases known in human history having reports in Chinese and Greek history (Edwards & Apicella, 2004). Gonorrhoea, also known as “The Clap”, remains a frequently reported STD and an important cause of pelvic inflammatory disease (PID) and subsequent infertility (Sherrard & Bingham, 1995). *Neisseria gonorrhoeae* was discovered by Albert Ludwig Sigesmund Neisser in 1879 by microscopy of stained smears from vaginal, urethral and conjunctival exudates. It is a member of *Neisseriaceae* family of the β subclass of the class Proteobacteria, consisting of thirty genera including *Neisseria, eikenella and Kingella* (Enright et al., 1994). *Neisseria* genus, which includes at least 21 members i.e., *N. gonorrhoeae, N. meningitidis, N. animalis, N. bacilliformis, N. canis, N. cinerea, N. denitrificans, N. dentiae, N. elongate, N. flava, N. flavescens, N. iguanae, N. lactamica, N. macace, N. mucosa, N. perflava, N. pharyngis, N. polysacchara, N. sicca, N. subflava, N. weaver* (Knapp, 1988). *N. gonorrhoeae* and *N. meningitidis* are the human pathogenic members whereas others are non-pathogenic commensal species although some of them can cause opportunistic infections in human (i.e., *N. lactamica*). Commensal *Neisseria* and *N. meningitidis* are frequent part of the normal flora of human especially the oro and nasopharynx.

![Figure 1.1: Gram stained smear showing gram negative diplococcus Neisseria gonorrhoeae.](image)

*Neisseria gonorrhoeae* is diplococcus, gram negative, aerobic, capnophilic, non-spore forming bacteria which infects mucosal sites of warm blooded animals (Figure 1.1). The bacterium has special nutrient requirement of sulphur in the form of cysteine and
iron in the form of Heme. It can also utilize iron bound to host iron-binding proteins within the host. It utilizes only glucose as carbon source. Laboratory cultures of *Neisseria* are grown on special Thayer martin medium with hemoglobin at 35°C under humid conditions with 5-10% of CO$_2$ (Kellogg et al 1963). These are usually seen in pairs under microscope. In clinical specimens, the intracellular pathogen is found in polymorphonuclear leukocytes. Most gonococci taken up by PMNs are killed, but up to 2% can survive as they avoid oxidative killing by various mechanisms. Tissue damage is caused by the host inflammatory response to two structural components of the organism: lipo-oligosaccharide (LOS) and peptidoglycan. Recent studies indicate that different molecular mechanisms are used to initiate infection in different anatomical sites. In the male urethra and in the upper female genital tract, gonococcal interaction with epithelial cells triggers cytokine release and promotes the influx of PMNs (Edwards & Apicella, 2004; Simons *et al.*, 2005). By contrast, gonococcal engagement of complement receptor-3 on epithelial cells of the cervix does not promote inflammation. Understanding of the pathogenesis of gonococcal infection has been hindered by the lack of a suitable animal model. The pili and opacity (Opa) proteins have been shown to be important for the success of infection. Piliated strains and strains that express Opa proteins adhere better to human columnar epithelial cells, and adherence can be blocked by monoclonal antibodies to these proteins. Pili also play an important role in adherence of *N. gonorrhoeae* to human PMNs and thus increasing resistance to phagocytosis (Heckels, 1989).

Antibiotic therapy remains the primary line of treatment since no effective vaccine is currently available for *N. gonorrhoeae*. Antibiotics are recommended at the initial clinical visit, prior to any knowledge of the organism’s susceptibility pattern or local sensitivity pattern. This has led to an alarming increase in the number of isolates of *N. gonorrhoeae* to commonly used drugs (Bala *et al.*, 2003; Bala *et al.*, 2007; Bala *et al.*, 2008; Bhalla *et al.*, 2002; Chaudhry *et al.*, 2002; Edwards & Apicella, 2004; Jabeen *et al.*, 2011). The major reason for increasing resistance across the world is due to injudicious use of antibiotics and travel of people around the globe (Hawkey & Jones, 2009) which increases the selective pressure on bacterium and transfer of resistance genes via Horizontal gene transfer (Hamilton & Dillard, 2006; Spratt *et al.*, 1992).
1.2.2 Prevalence and Incidence of Infection by Neisseria

*N. gonorrhoeae* is recognised as one of the major STI causing bacteria. Approximately 62 million cases of gonorrhoeae occur worldwide annually (World Health Organization, 2001a) which have now increased to 82 million cases (World Health Organization, 2011) with two-third cases occurring in developing countries. Sub-Saharan Africa, Southern and Southeast Asia have particularly high burden of disease, with an estimated 17 million new cases occurring in Africa and 27 million new cases in Southern Asia every year (Figure 1.2). Although, a 10% decline is observed in the infection rate, it still remains second most commonly reported infectious disease in the U.S. (CDC, 2012). In some parts of the developing World over 90% of the population is infected. The economic burden of STIs is so huge, especially for developing countries where they account for 17% of economic losses caused by ill health (Mayaud *et al.*, 2004). Although, a population survey in various Asian countries had shown that incidence of *Neisseria* was highest in China (0.8%) and lowest in India (0.3%) (Detels *et al.*, 2011), an increase in incidence of *Neisseria* from 13.7% to 15.4% over the period of fourteen years (1990-2004) among the male and female patients attending a regional STD centre at Safdarjung Hospital had been observed (Ray *et al.*, 2006).

Another report suggests 5.4% incidence in Men attending STI clinic (Lindan *et al.*, 2005). High rate of infection has been reported among adolescents and pregnant women in various published and unpublished medical data. In a study among pregnant women in Africa, gonococcal infection rate was found to 0.02% in Gabon to
7.8% in South Africa (Gewirtzman et al.). The incidence of gonococcal infection in female sex workers ranged from 4.8% in China (Zhu et al., 2012) to 9.7% in Pakistan (Khan et al., 2011). High risk populations are screened and studied in various parts of the country. 5-7% of FSW in Goa were either infected with Neisseria or Trichomonas (Wayal et al., 2011).

An increasing number of studies have shown high probability of coexistence of gonococcal infections with Chlamydia. A recent study by Vonck and co-workers has shown that infection by gonococci increases in CT infected mice (2011). Creighton and co-workers (2003) reported 24.2% of men and 38.5% of women infected with Neisseria were also infected with Chlamydia whereas 18.8% of men and 13% of women infected with Chlamydia were co-infected with Neisseria. Higher rate of infection by Neisseria and Chlamydia infection has been found in women and adolescents with young girls between 15-19 years disproportionately affected (Dicker et al., 2003; Miller et al., 2004; Mullick et al., 2005b; Van Bergen et al., 2006a).

Approximately half of the STI reported in US and other parts of world occurs in men and women among age group of 15 to 24 years (CDC, 2010; Berggren & Patchen, CDC 2009). Co-infection rate of 0.3% to 0.8% is readily reported among school based study screening programmes in US. Much higher Chlamydia co-infection in Neisseria positive individuals (42.7%- 69.7%) in comparison to gonococcal co-infection among Chlamydia (7.29%-11.1%) infected adolescents is reported (Miller et al., 2004). Co-infection rate is much higher in females as compared to male patients visiting STI clinics (David et al., 1997; Dragovic et al., 2002; Hijazi et al., 2002; Lyss et al., 2003 Aug) and in adolescents as compared to older population (Dicker et al., 2003; Dragovic et al., 2002; Hijazi et al., 2002). A higher rate of infection with either one or both the pathogens is found during the pregnancy (Berggren & Patchen, 2011; Meade & Ickovics, 2005). Berggren has also reported that 31% of pregnant women get infected with one or more STI during pregnancy. 11% of them showed re-infection whereas 7% showed infection on retesting. It is important to screen pregnant women for Neisseria and Chlamydia especially in areas with high incidence, since in the newborn these are major cause of purulent conjunctival infection (ophthalmia neonatorum), which constitutes a medical emergency as blindness may rapidly ensue.
1.2.3  Clinical Manifestations and Complications of Gonorrhea

*Neisseria* and *Chlamydia* are transmitted during vaginal, anal or oral sex and therefore, in addition to heterosexuals, men who have sex with men are also at risk for infection. The primary infection sites of *N. gonorrhoeae* and *C. trachomatis* are the columnar and transitional epithelium of the urogenital tract (the urethra in males and the uterine cervix in females), the rectal mucosa, the conjunctiva, and pharynx (Hook I & Handsfield, 1990; Sparling *et al.*, 1990). The highest infection rate in both men and women occurs between 15 and 29 years of age. Host-related factors such as the number of sexual partners, contraceptive practices, sexual preference, and population mobility contribute to the incidence of infection. The incubation period is typically 1-7 days for *Neisseria* whereas 1-3 weeks for *C. trachomatis*. Over 50% of women and 10% of men who have gonorrhea are asymptomatic during the entire infection process. This gives rise to misdiagnosis and delay of treatment, facilitating spread of the pathogen. Asymptomatic infected men rarely suffer from long-term health problems but serve as carriers of the disease whereas women and infants are at high risk of developing severe complications of infection. Babies born to mothers with infection of their genital tract frequently present eye infection within a week of birth 'ophthalmia neonatorum' which if untreated, results in blindness due to corneal ulceration and perforation. Neonates may also develop disseminated gonococcal infection (DGI) and pneumonia (Kakar *et al.*, 2010; Rours *et al.*, 2008). Figure 1.3 represents pictorial depiction of clinical symptoms of gonococcal infection. Blindness due to neonatal gonococcal infection is still a serious problem in developing countries whereas it is now uncommon in the United States and in other countries where neonatal conjunctival prophylaxis with antimicrobial therapy is routine. In adults also, *Neisseria* and *Chlamydia* infects the inner upper eyelid and cornea making it one of the common infectious causes of blindness. The disease starts as an inflammatory infection of the eyelid and evolves to trachomatous trichiasis and blindness due to corneal opacity.

In men, primary urethral infection results in acute urethritis, manifested by purulent discharge from the tip of the penis and symptoms of urethral itch and pain or burning micturition. Ascending infection of *N. gonorrhoeae* and *C. trachomatis* causes epididymitis, a painful condition of the testicles that can lead to infertility if left untreated. Additionally, the prostate gland is also affected which may cause scarring in the urine canal.
The simplest gonococcal infections in women involve mucosal surfaces of the endocervix, urethra, anus, or pharynx with endocervix being the most common site. Most of infections are either silent or generate only mild symptoms, including discharge and mild irritation, that may or may not be appreciated until the infection spreads to the upper genital tract. Pharyngeal infections are nearly always asymptomatic. When an etiologic organism is isolated in the presence of cervicitis, it is typically *Chlamydia trachomatis* or *N. gonorrhoeae*. The essential criteria developed for presumptive diagnosis of mucopurulent cervicitis (MPC) include the presence of mucopus (discoloration) on a cervical swab and bleeding induced by swabbing the endocervical mucosa (Brunham *et al.*, 1984). There may be a strong smelling vaginal discharge that may be thin and watery or thick and yellow/green. Pain or a burning micturition may also be present. Women with either infection in the lower genital tract may develop an ascending infection to fallopian tubes leading to acute salpingitis with or without endometritis, also known as PID, (Murray *et al.*, 2003) whose long-term consequences include chronic pain, ectopic pregnancy and infertility (Westrom *et al.*, 1992). Figure 1.3 shows pictorial depictions of few symptoms. Severe cases may lead to abdominal pain, fever and result in tubo-ovarian abscesses and systemic infection.

Figure 1.3: Clinical manifestations of Neisseria.

Approximately 10%- 20% of infected women develop PID if left untreated (Edwards & Apicella, 2004). Other clinical presentations include abnormal vaginal bleeding and low abdominal pain or tenderness. Rectal infections may cause irritation or discharge of the anus in both men and women, but are often asymptomatic (Hook & Handsfield, 1990; Sparling *et al.*, 1990). Acute perihepatitis (Fitz-Hugh-Curtis syndrome) occurs primarily through direct extension of *N. gonorrhoeae* or *Chlamydia trachomatis* from the fallopian tube to the liver capsule and overlying peritoneum (Gonorrhoeae clinical manifestation, medscape).
In addition, infection can spread to adjacent sites of the urogenital tract or through the bloodstream to other parts of the body, causing damage and serious complications. Disseminated gonococcal infection (DGI) is a rare complication among only 0.5-3% (Kerle et al., 1992) of infected individuals. A systemic spread of the bacteria occurs in the bloodstream along with combination of certain strains of *N. gonorrhoeae*. Individual’s deficient in, complement factors C7, C8 and C9 appear to be predisposed to DGI (Petersen et al., 1979). The classic presentation of DGI is an arthritis dermatitis syndrome which is characterized by Joint or tendon pain leading to migratory polyarthritis, especially of the knees (gonococcal arthritis), elbows, and more distal joints in extreme cases. Lesions, usually number 5-40, are peripherally located, and may be painful before they are visible. Fever is common but rarely exceeds 38°C. The second stage of DGI is characterized by septic arthritis. Rare complications of DGI include gonococcal meningitis and endocarditis. Gonococcal endocarditis is more common in men than in women, with the aortic valve affected most commonly. A subacute onset of fever, chills, sweats, and malaise may indicate the presence of gonococcal endocarditis. Patients with endocarditis may develop atypical chest pain, cough, and dyspnea and may also develop the arthralgias and rash typical of DGI (Brown et al., Oct.,(1999)).

Infection with *N. gonorrhoeae* has been associated with increased infectiousness of and susceptibility to the sexually transmitted human immunodeficiency virus (HIV) (Shannon & Cohen, 2004). Comprehensive reviews have summarized the findings of clinical trials and epidemiological studies on the interrelationships between HIV and STIs including gonorrhea (Fleming & Wasserheit, 1999; Galvin & Cohen, 2004; Wasserheit, 1992). It has been shown that both genital ulcerative diseases (syphilis and human simplex virus infections) and non-ulcerative STIs (gonorrhea, urogenital chlamydia infections and urogenital trichomoniasis) increase the risk of HIV transmission approximately 3 to 5 fold (Wasserheit, 1992). Gonorrhea enhances the infectiousness of HIV due to several pathophysiological factors. These factors include increased HIV viral load in the urethra, semen, and cervical fluid due to high degree of inflammation, as well as increased HIV replication due to the influx of PMNs during gonococcal infections (Shannon & Cohen, 2004). Gonorrhea makes a person
more susceptible to HIV infection due to damage of the columnar epithelial barriers and increased infiltration of the HIV co-receptor containing cells (Shannon & Cohen, 2004; Wasserheit, 1992). Studies on prevalence of co-infection of Neisseria and/or Chlamydia amongst HIV infected patients are not available in India. One of the major reasons for this is the high cost of diagnostic assays for these and other STD causing pathogens. We consider that efforts are required to develop in-house diagnostic assays so that cohort studies could be carried.

1.2.4 Diagnosis of Neisseria

Antigenic variability of Neisseria has helped the organism in overcoming host defence mechanism and development of effective vaccine. In the absence of vaccine, it is important to have an effective, cheap, and rapid diagnostic test for rapid diagnosis and therefore treatment of the infection. The methodology for the detection and/or identification of N. gonorrhoeae varies among countries and among locates within a country. There are a variety of physical and temporal conditions which affect a specimen from its acquisition to its receipt in the laboratory. In the sequence of their application, they are specimen collection, specimen management, detection procedure, identification procedure, and supplementary procedures. Specimens are collected for diagnostic purpose from a variety of sites. Cervix and rectum are two important sites of sample collection (Dans & Judson, 1975 Mar). In developing countries, microscopy and culture methods are most commonly used techniques for the detection of Neisseria.

1.2.4.1 Microscopy

Gram-stained smear of infected discharge or a culture for N. gonorrhoeae are most commonly used methods to diagnose gonococcal infection in developing countries. A direct smear for Gram staining may be performed as soon as the swab specimen is collected from the urethra, cervix, vagina or rectum. Sample is interpreted positive if more than one polymorphonuclear cell is detected with diplococcic. It reaches sensitivity and specificity of 90% and 95% in case of symptomatic males whereas it 50% to 80% sensitive for females and asymptomatic males (LCDC guidelines). Although microscopy is a fast, cheap and reliable diagnostic method but it requires experienced microscopist.
1.2.4.2 Culture Method

For decades culture method has been used as gold standard because of its high specificity and sensitivity. Numerous selective media has been developed for isolation of \textit{N. gonorrhoeae} (Carlson et al., 1980). It meets sensitivity of 95% of male urethral infections and 85% of female cervical infections when a single swab specimen is collected (Carlson et al., 1980; Dans & Judson, 1975 Mar). An advantage of culture is that it preserves the organisms for further studies such as genotyping or antimicrobial susceptibility testing. It is inoculated and grown on nonselective chocolate agar and selective agar containing antimicrobial agents like vancomycin, colistin, trimethoprim lactate and the antifungal agents like nystatin which inhibit the growth of commensal bacteria and fungi. Although highly specific, limitations like 2-3 days for generation of results, loss of viability of the sample during transportation under suboptimal conditions or on antibiotic treatment and failure to culture vancomycin-sensitive strains or other strains with fastidious growth characteristics, and time requirement up to 2 to 3 days before results are available has lead to use of alternative technologies have been developed for the laboratory diagnosis of gonococcal infections (Carlson et al., 1980; Dans & Judson, 1975 Mar).

Various tests based on biochemical assays, serological methods, colourimetric assays and detection of nucleic acid are available for confirmation of \textit{Neisseria}. Oxidase test is one of the biochemical assay used to confirm \textit{Neisseria}. This test requires heavy inoculums. Further tests based on the presence of preformed chromogenic enzymes like beta galactosidase, gamma glutamylaminopeptidase and prolylhydroxyprolyl aminopeptidase with in media are also available commercially (Gonochek II, EY Laboratories, USA; Gonochek II, TCS biosciences Ltd, UK; Identicult-Neisseria, Adams Scientific, USA; BactiCard Neisseria, Remel Inc, USA None of these tests were 100% sensitive or 100% specific. Thus, detection tests based on other techniques including detection of antigen or genetic material were developed and introduced. (Chapin-Robertson et al., 1992; Granato & Franz, 1990; Hale et al., 1993; Hosein et al., 1992; Limberger et al., 1992; Mahto & Mallinson, 2012; Miller et al., 1992; Panke et al., 1991; Stary et al., 1993; Vlaspolder et al., 1993). Probes using nucleotide sequences of the pilin gene (Meyer et al., 1982), the IgA1 protease gene (Roomey & Falkow, 1984), ribosomal RNA gene (Rossau et al., 1989) (Rossau et al., 1990) and the cryptic plasmid (Totten et al., 1983) have been reported.
1.2.4.4 DNA based Methods for the Laboratory Diagnosis of Gonococcal Infections

The advent of tests based on Nucleic Acid Amplification Technology (NAAT) has been one of the most important advances in the field of diagnosis. Since the detection limit of NAAT is low, it has major advantage in case of Neisseria since most of the patients are asymptomatic. It further facilitates the use of non-invasive sampling technique. NAAT based tests use specific primer sequences and enzymes to amplify targeted Nucleic acid region both RNA and DNA (plasmid or chromosomal). Although mRNA and rRNA are present in multiple copies, short half life, fragility and requirement of special buffer conditions for storage of RNA makes them less preferable than DNA based tests. Plasmid DNA which is also present in multiple copies could also be a good option but plasmid can be easily lost and exchanged or unevenly distributed during cell division. Few multi-copy genomic DNA target genes are also available but such genes are often preserved between species and hence, prone to false-positive test results. The pilin genes of the gonococci have more than one copy, but they are highly homologous with those of the meningococci. The use of cryptic plasmid derived probes for DNA hybridization was thought to have limited application for the detection of gonococci in clinical isolates. The polypeptide product of the cppB gene of this cryptic plasmid, pJD1, is also produced by the plasmid-free strains and it was shown that a copy of this gene was present in the gonococcal chromosome of all strains tested (Hagblom et al., 1986) Torres and co workers reported the evaluation of a cryptic plasmid-derived DNA probe in a dot-blot hybridization assay (1991). However, in symptomatic populations, these methods have similar performance as that of culture method (80% to 95%), but in asymptomatic population and settings with lower prevalence might be unsatisfactory (70% to 80%).

The direct detection of N. gonorrhoeae in specimens requires either an extremely sensitive and specific assay or a process by which a diagnostically useful gonococcal component can be amplified to a detectable level. The most widely known of the DNA amplification technology is PCR which provides the high sensitivity and specificity needed to detect organisms directly in clinical specimens. Several amplification procedures have been described for the detection of N. gonorrhoeae which includes polymerase chain reaction (PCR), strand displacement amplification (SDA) and ligase chain reaction (LCR).
Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) utilizes a thermostable DNA polymerase enzyme to extend specific primers and thus amplify specific parts of DNA. Running an agarose gel electrophoresis and staining with ethidium bromide verifies the specific amplified products. This helps in development of most specific, sensitive and fast method for detection of Neisseria. Since all NAATs detect nucleic acid targets, they do not depend on the viable organism, which would not ordinarily be detected by routine culture. Thus, NAATs are more sensitive than routine culture. However, a high sensitivity may be at risk by carryover contamination due to poor testing practices, thus generating false-positive results (Farrell, 1999). If the sequence, to which primers anneal, is unique, the NAATs have a high specificity. The presence of inhibitors in the sample may inhibit the amplification step resulting in false-negative results and thus reducing the specificity of assay (Akane et al., 1994; Vandenvelde et al., 1993; Verkooyen et al., 1996).

Ho and co-workers (1992) developed first PCR based test for Neisseria using cppB gene of the cryptic plasmid pJD1. Subsequent to the report by Ho and co workers numerous other PCR based assays with different target sequence and varying specificities and sensitivities have been described for the rapid detection of N. gonorrhoeae. Since there is considerable homology between two pathogenic species of Neisseria, N. gonorrhoeae and N. meningitides designing of primer becomes important point of consideration. It is particularly important since N. meningitidis can be occasionally isolated from the urogenital specimens (Conde-Glez & Calderon, 1991). Many single-copy genes encoding the outer membrane protein III, rmp gene, opa gene and the por gene were the used as targets in the development of the PCR assays to detect N. gonorrhoeae (Cooke et al., 1997; Liebling et al., 1994; O'Rourke et al., 1995). Since co-infection of Neisseria and Chlamydia common, attempts have been made to further extend the methodology for co-detection of two pathogens among the same clinical specimens (Crotchfelt et al., 1997; Mahony et al., 1995; Sowmya et al., 2001; Wong et al., 1995). The last decade has seen major improvements in our ability to detect these two STDs simultaneously using the multiplex-PCR assay.

SDA, Ligase chain reaction (LCR), TMA and Real-time PCR are two other NAAT based tests for detection of Neisseria. Real-time PCR is the predominant NAAT used worldwide for microbiological diagnostics, either as in-house methods or commercially
available kits. For *N. gonorrhoeae*, real-time PCR is used as in-house diagnostic assays (Hjelmevoll *et al.*, 2006; Hjelmevoll *et al.*, 2008; Whiley *et al.*, 2005; Whiley & Sloots, 2005), verification assays (Whiley *et al.*, 2004) and in commercial diagnostic assays such as, VERSANT CT/GC DNA 1.0 Assay (Siemens, Deerfield, IL, USA), COBAS TaqMan48 and 4800 (CT/NG) Test (Roche Molecular systems inc., San Diego, Calif) and RealTime CT/NG test (Abbott Laboratories, Abbot Park, Illinois, USA). Real-time PCR accounts for improved sensitivity as uses fluorescently labeled probe complementary to the amplified products for its detection. A number of different probing systems are used, such as; TaqMan probes, Molecular beacons and Fret Probes.

### 1.2.4.4 Other Diagnostics

Other diagnostic methods like direct fluorescent antibody (DFA) test, enzyme immunoassay (EIA), nucleic acid hybridization and syndromic diagnosis are also available, but these have low sensitivity and specificity. The Digene Hybrid Capture II (Digene Corp., Beltsville MD, USA) is the most common nucleic hybridization test for detecting *N. gonorrhoeae* and *C. trachomatis* and this method uses signal amplification to increase sensitivity. The sensitivity and specificity of Hybrid Capture II is lower for optimized culture diagnostics (Darwin *et al.*, 2002), and do not provide AMR profiles. Various POC are also available for diagnosis of *Neisseria* and *Chlamydia*. POC tests are often limited by their low sensitivity and should be used only for populations unlikely to return for follow-up. Alarmingly poor performance of three commercially available POC (between 12% and 27% ) for *Chlamydia – Handilab C* (enzymatic assay), Biorapid and Quick vue (antigen based) make them less popular to be used specially in low resource settings. Another recent antigen based *Chlamydia* rapid test also has low sensitivity of 42.0% and 39.4% in low risk and high risk women in Suriname as compared to commercial NAAT (Aptima, Gen-Probe). The gram stain is one of the important POC test available for *Neisseria*. A recently introduced POC test, PATH GC check rapid test (program for Appropriate technology Seattle, USA) is an immunochromatographic strip test has moderate sensitivity ranging from 81% to 31.2 under different clinical setting as compare to Roche Amplicor CT/NG PCR assay and 16s rRNA PCR assay (Alary *et al.*, 2006). In these settings, rapid POC diagnostics, followed by immediate treatment of positive patients, with lower sensitivity than reference test are shown in several studies to lead to treatment of more patients where patients have to return for results and treatment.
1.2.5 Development of Molecular Beacon

Molecular beacon is a hairpin shaped oligonucleotide which fluoresces on the binding with specific complementary target DNA. These are commonly used in real time settings or detecting RNA in living cells. These also help in detection single base pair mismatch. Molecular beacons consist of complementary target probe flanked with GC rich arms. It is equipped with a fluorophore - quencher pair attached covalently at two ends of the beacon. Further fluorophore quencher pair is chosen to ensure that when two moieties are close together, the energy received by fluorophore is transferred to quencher and is dissipated as heat rather than being emitted as light thus probe is unable to fluoresce. In the absence of specific target, the arms keep the two moieties close together, but when the probe binds to its target, the greater stability of the probe-target helix forces the stem to unwind, resulting in a separation of the fluorophore from the quencher, resulting restoration of fluorescence. Multiple targets can be detected in same sample using Molecular beacon with different fluophores. DABCYL a non-fluorescent chromophore, serves as the universal quencher for any fluorophore in molecular beacons.

PCR based detection is often associated with some degree of false positivity, and needs agarose gel electrophorosis to be carried out in order to visualize the gel. This is both cumbersome, time consuming and as well as uses ethidium bromide as detector dye which is carcinogenic thus handling requires precautions and technical expertise. It also involves risk of cross contamination as well as carryover contamination. To overcome this hurdles, we had used molecular beacon probe which both enhances the specificity and abrogates the use of agarose gel (Haldar et al., 2007). However, they have been mostly used in real-time formats for detection of Neisseria gonorrhoeae and other infectious diseases (Gullsby et al., 2008; Tsourkas & Bao, 2003; Tyagi & Kramer, 1996; Xia et al., 2007) which is not a feasible method for diagnosis in developing countries like India. In previous studies by our laboratory, we had developed PCR based diagnosis for Chlamydia (Patel et al., 2010) and Neisseria (Chaudhry & Saluja, 2002). Against this background, we developed an asymmetric PCR assay and using 6-carboxyfluorescein (FAM)-labeled and Cy3 labeled molecular beacons for end-point detection of Neisseria and Chlamydia respectively. The molecular-beacon based detection assay was compared with the PCR described previously.
Figure 1.4: Operation of Molecular Beacons. On their own, these molecules are non-fluorescent, because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence.

1.2.6 Why we Needs New Diagnostic Methods

*Chlamydia* and *Neisseria* are two most common STD causing pathogens leading to a huge burden of morbidity and mortality that is borne disproportionately by women and infants. WHO estimated that 150 million cases occur annually with approximately 2/3rd of cases occur in developing countries with limited diagnostic facilities cause. Genital *Chlamydia* is a main cause of pelvic inflammatory disease, tubal infertility and ectopic pregnancy in women in developing countries. In Africa, the prevalence rates for these infections are upto 66% in high-risk population (for example, commercial sex workers) and up to 40% even in low-risk population and about 3% of babies born to infected mothers suffer with ophthalmia. These also increase the shedding of HIV in genital secretions, thereby increasing the infectivity of the virus. Although easily curable by a single dose of antibiotic, large population goes undiagnosed due to absence of rapid, sensitive, specific and cost effective diagnostic methods. NAAT based commercial kits are available for diagnostics of these pathogens but their high cost makes them less popular in developing countries. Also, in developing countries, especially among the low income group people women may be at risk of violence from their sexual partners if they are asked to refer their partners for STI treatment when they do not have an STI. Thus, WHO has recommended syndromic management on the basis of Vaginal Discharge syndrome in women and urethral discharge in men to administer treatment regimen. Although syndromic management works well for men with urethral discharge and genital
ulcers, but several studies showed it lacks both sensitivity (30% - 80%) and specificity (40% - 80%) for the identification of women with vaginal discharge for Chlamydia trachomatis and Neisseria (Alary et al., 2001; Mayaud et al., 2004; Mukenge et al., 2002). Treatment on the basis of syndromic management not only misses the asymptomatic patients (Choudhry et al., 2010b; Vishwanath et al., 2000b) also leads to overtreatment which may leads to increased drug costs, change in pattern of endogenous flora, side effects of multiple drugs and emergence of antibiotic resistant strains by lateral gene transfer.

New diagnostics with 85% sensitivity and 90% specificity for both Neisseria and Chlamydia that requires minimal laboratory infrastructure could save ~3 million victims from suffering Disability Adjusted Life Years (DALYs). More than 12 million incidences of gonorrhoea and Chlamydia infections can be averted. About 161,000 HIV infections can be preventable among female commercial sex workers in sub-Saharan Africa, China and Southeast Asia. A test that requires no laboratory infrastructure could save ~4 million DALYs, avert >16.5 million incident gonorrhoea and Chlamydial infections and prevent >212,000 HIV infections (Urdea et al., 2006(11)). Available data indicates better performing and more-accessible diagnostic tools are required to prevent STIs in the developing world (Aledort et al., 2006). The introduction of new diagnostic tools that are rapid, sensitive and cost-effective can help to reduce the large burden of disease especially in the developing world. Burden of mortality and morbidity will be greatly reduced by introduction of new tests which can accurately discriminate between patients who do and do not need treatment. Although high-performance tests are available, are usually expensive and require greater levels of infrastructure and are therefore accessible to fewer people (Federico et al., 2006; Herring et al., 2006, TDR (WHO) 2006). A critical need to address these issues will be a rapid, sensitive and cost-effective diagnostic test that can be used for global screening, treatment and test-of-cure of infected individuals instead of empirical therapy that not only drives drug resistance but is not cost effective. This type of diagnostic would allow clinicians and researchers to evaluate the true incidence and prevalence of gonococcal infections in both developed and developing countries.
Objectives
1.3 OBJECTIVES

- Evaluation of clinical performance of the in-house PCR and comparison with the Roche Amplicor MWP kit.
- Development and evaluation of dPCR for co detection of *N. gonorrhoeae* and *C. trachomatis*.
- Evaluation of using dry swabs for isolation of gDNA from clinical samples.
- Stabilization of PCR reagents at 4°C.
- Development of a visualized assay for detection of *N. gonorrhoeae*. 
Materials and Methods
1.4 MATERIALS AND METHODS

1.4.1 Chemicals

All chemicals and biochemicals were purchased from the following standard commercial sources such as: Sigma Chemical Co., St. louis, USA; Bio-Rad, Richmond, USA; New England Biolabs Inc, Beverly, MA, USA; Himedia India Ltd, Bangalore Genei India Pvt. Ltd., Bangalore, India.

Tris Base, Agarose, DithioThreitol, β-Mercaptoethanol, proteinase K, glycogen, sodium acetate, glycine, Immidazole, IPTG and EDTA were purchased from Sigma Chemicals. Potassium dihydrogen phosphate, disodium hydrogen phosphate, monosodium dihydrogen phosphate from Qualigens. Propanol, Sodium Chloride, Potassium Chloride, Glacial Acetic Acid, Methanol from Merck, Taq Polymerase from Bangalore Genei. Cotton swabs for sample collection were purchased from Himedia India Ltd.

Kits Used

Gel extraction Kit (Qiagen), Roche MWP Amplicor Kit CT/NG kit was from Roche, USA

Enzymes

Taq DNA Polymerase was purchased from Bangalore Genei India Pvt. Ltd., Bangalore, India; T4 DNA from New England Biolabs Inc, Beverly, MA, USA., Proteinase K from Sigma Aldrich.

Primers

All the primers used in the study were got synthesized from Sigma Chemical Co., St. louis, USA. Molecular beacon probes used were synthesized from MWG Biotech (Euorofins MWG Operon, Germany).

1.4.2 Methods

1. Evaluation and Development of In-house PCR for co detection of C. Trachomatis and N. Gonorrhoeae.
1.4.2.1 Enrollment of Patients

A total of 810 non-pregnant symptomatic women (18 years - 55 years of age with 90% patients in the age group of 18-31 years) seeking diagnosis and treatment of vaginal discharge syndrome (VDS) or PID, from the Gynaecology Outpatient Clinic of Safdarjung Hospital and Hindu Rao Hospital, New Delhi, India, during the period October 2006 to September 2010 were enrolled in this study as per the guidelines of institutional ethical committee (committee of Dr. B.R. Ambedkar Centre for Biomedical Research, University of Delhi, No.F50-2/ Eth.Com/ACBR/11/2107) and guidelines of Indian Council of Medical Research, India. The results of this study were not to influence the treatment of the patients. Written informed consent was taken from all patients.

1.4.2.2 Specimen Collection

A thorough speculum examination of vulva was performed for the presence of ulcers, wrats, ectopy and vaginal or cervical discharge by the clinician. Exocervix was cleaned using cotton swab (Hi Media, Mumbai, India) and two endocervical swabs were taken from the individual patient and tested for the presence of infection by PCR and Roche MWP CT/NG Kit. In group 1, for PCR, the swab was immersed in 1ml transport medium (Chaudhry et al., 2002) (130 mM NaCl, 2.6 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4), while another swab was immersed in Buffer provided in Roche MWP kit. In group 2, first swab was immersed in above mentioned transport medium whereas second swab was collected in dry vial. All specimens were transported to laboratory at Dr. B. R. Ambedkar Center for Biomedical Research, on same day in mini cooler. Dry swabs were transported at room temperature.

1.4.2.3 Primer and Probe (Molecular Beacon) Designing A Primers

The primers used in our previous studies (Chaudhry et al., 2002; Patel et al., 2010) were modified to improve the performance of in-house PCR assay as well as to develop duplex PCR. Modified stretches of nucleotides were aligned using the BLAST program from NCBI to find matching sequence with other organisms if any. The sequences that were highly unique to C. trachomatis (gyrA gene (CT189)) and N.
gonorrhoeae (orf1 gene (NGO0364)) (see Table 1.1, Primers 3 and 4) producing an amplicon of size 462-bp and 269-bp respectively. Primers were designed using software Gene Runner 3.05 and their sequences are given in the Table 1.1.

Table 1.1: Showing the Sequence of Primers designed for in-house PCR for detection of *N. gonorrhoeae* and *C. trachomatis*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1F</td>
<td>5’-GATCCAACTATTCCCGATTGC-3’</td>
</tr>
<tr>
<td>Orf1R</td>
<td>5’-GCAAAGTTATACAGCTTCGCCTGA-3’</td>
</tr>
<tr>
<td>C2</td>
<td>5’-TGATGCTAGGGACGGATTTAAAAACC-3’</td>
</tr>
<tr>
<td>C5</td>
<td>5’-TTCCCTAAATTATGCGGTGGAA-3’</td>
</tr>
</tbody>
</table>

### B Molecular Beacon

Molecular beacon is a fluorescent probe which generates fluorigenic response on the binding with specific complementary target DNA. It is a stem loop forming structure of synthesized nucleotides with fluorophore- quencher pair (Figure 3). The loop is an anti-sense target-binding domain flanked by two GC rich complementary short arm sequences is labeled at one end with a reporter dye and at the opposite end with a quencher. The stem sequence was designed to ensure that these two moieties remain in close proximity at the optimal annealing temperature of the PCR when no target amplicons are present to avoid nonspecific fluorescence emission. This is ensured by choosing a stem that melts 7-10˚C higher than the annealing temperature of the PCR. Usually the stems are 5-7 base pairs long and have a very high GC content (75 to 100 percent). Further, fluorophore quencher pair is chosen to ensure that when two moieties are close together, the energy received by fluorophore is transferred to quencher and is dissipated as heat rather than being emitted as light. This reduces noise to signal ratio. When probe finds the target molecule, it forms longer and more stable hybrid opening the hairpin structure, and separating fluorophore and quencher resulting in restoration of fluorescence. Fluorescence could be easily visualized using a simple dark reader fitted with LED same as excitation wavelength of fluorophore in molecular beacon. The probes were designed using molecular beacon designer (www.premierbiosoft.com/molecular_beacons). Fluorophore-quencher combination
used was Cy3-BHQ1 and Fam – Dabcyl for *C. trachomatis* and *N. gonorrhoeae* respectively. The 6bp stem sequence is highlighted.

NG B: 5’ fam CCATGCCTGACTGCCAACAAGAAAAAAGCCATCC CGCATGG-Dabcyl 3’

CT B: 5’ cy3 GCGAGG GCGGTAGGGATGGCAACAAATA CCTCGC- BHQ2

### 1.4.2.4 Processing of Samples for PCR

**I. Lysis Method**

400 µl sample of specimen was centrifuged for 30 minutes at 14,000 x g at 4°C for 30 minutes and the pellet was treated with 40 µl of lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM EDTA, 250 µg of proteinase K per ml). After incubation at 37°C for 2 hour, 1mM of DTT was added and lysates were boiled for 10 min and centrifuged briefly. 5 µl of the supernatant was used for PCR.

**II. Extraction of Purified gDNA**

Crude lysate obtained above was treated with equal volume of phenol:chloroform. gDNA was precipitated using ehanol and sodium acetate.

### 1.4.2.5 PCR Amplification

Supernatant (5µl) of processed sample or crude lysate was used for PCR in a reaction volume of 25 µl containing 1X Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2), 200 µM each of the four dNTPs (New England Biolabs Inc, Beverly, MA, USA), 10 pmoles each of forward and reverse primers, 1U of Taq DNA polymerase (Bangalore Genei India Pvt. Ltd., Bangalore, India). Negative and positive controls were included in each PCR run. Amplification for *orf1* and *gyrA* alone was performed in thermal cycler (I cycler, Bio-Rad, Richmond, USA) for 35 cycles with following parameters: 95°C for 5 min for initial denaturation, cycling of 95°C for 30 sec, 60°C for 30sec, 72°C for 30 sec, final extension at 72°C for 10 min. The amplicons were analyzed by agarose gel (1%) electrophoresis containing ethidium bromide and were visualized by UV transilluminator. The amplicons from positive samples (10% of the total) were eluted using DNA isolation kit (Geneaid, USA) according to the manufacturer’s instructions and were custom
sequenced. DNA sequence of the amplified product was compared to known orf1 nucleotide sequences (1991) in the GenBank databases using BLAST program to determine the percent identity. Duplex PCR for Orf1/GyrA was carried out using 1.6X Taq DNA polymerase buffer (80 mM KCl, 16 mM Tris-HCl pH 8.3), 2.8 mM MgCl₂, 200 µM each of the four dNTPs (New England Biolabs Inc, Beverly, MA, USA), 10 pmoles each of forward and reverse primers, 1.2U of Taq DNA polymerase (Bangalore Genei India Pvt. Ltd., Bangalore, India). Negative and positive controls were included in each PCR run. Amplification for duplex PCR was performed in thermal cycler (I cycler, Bio-Rad, Richmond, USA) for 35 cycles with following parameters: 95°C for 5 min for initial denaturation, cycling of 95°C for 30 sec, 58°C for 45 sec, 72°C for 30 sec, final extension at 72°C for 10 min.

Further, to make the above test an easy assay to be carried out in peripheral laboratories with minimum resources, stability of reagents was checked for different time intervals. 2X PCR master mixes containing all the requisite reagents excepting DNA template was prepared for orf1 and stored at 4°C. At regular intervals of time (15 days, 1 month, 2 months, 3 months, 4 months, 6 months and 8 months) from the date of preparation of master mix, an aliquot was taken and checked for the stability and integrity of the PCR mastermix. The respective DNA templates were added to the pre-prepared mastermix and subjected to PCR at the conditions standardized for orf1 amplification.

1.4.2.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in horizontal matrix of agarose with 1X TAE buffer as described by Sambrook et al., (2001). Generally 1.5% agarose was melted in 1X TAE buffer in microwave for 2-3 min. Agarose gel was allowed to cool to ~50°C. An aliquot of ethidium was added to get a final concentration of 0.5μg/ml and poured onto gel casting tray fitted with comb. The agarose was allowed to polymerize for 30 min. The comb was then removed and the gel was immersed in 1X TAE buffer in horizontal electrophoresis tank. The DNA samples were mixed with 1/6 volume of 6X loading buffer and electrophoresed at 5 V/cm. A 100 bp DNA marker was run in parallel as size standard. The bands were visualized by short wave (300 nm) UV transilluminator and photographed using gel documentation system.
1.4.2.7 Elution of PCR Products from Agarose Gel

After agarose gel electrophoresis the desired DNA band was excised from the gel using a scalpel blade and DNA was eluted from the gel slice using gel extraction kit (geneaid) according to the instructions supplied by the manufacturer. Briefly the gel piece containing the DNA fragment of interest were chopped into fine pieces and transferred to a 2 ml microfuge tube. The agarose was melted by addition of 3 gel volumes of DF Buffer and incubated at 55°C to get a homogenous solution. The sample was loaded onto a column and centrifuged at 8000 rpm for 30sec. The DNA bound to the matrix was washed once with W1 followed by second wash buffer with ethanol and centrifuged twice for 2 minutes each to remove the traces of the buffer. The bound DNA was eluted by addition of 30 µl of distilled water and centrifugation at 14000 rpm for 5 minutes.

1.4.2.8 Visualization of Amplicons using Molecular Beacons

Molecular beacons (5 pmoles) were added either during PCR or after the PCR is completed. An additional of 1.0mM MgCl₂ was added before ramping. Subsequent to the amplification, the PCR Product was heat denatured for 5min at 95°C and then allowed to cool to room temperature (20°C) at a ramping rate of 0.1°/sec. PCR products were stored at -20°C unless checked by i) Phosphorimager, ii) fluorescent ELISA Reader iii) Dark Reader or iv) agarose gel electrophoresis. To visualize in ELISA reader, the reaction contents (50 µl) were transferred into wells of a 96 well plate. To each well added 150 µl 20 mM Tris/Cl (pH 8.0) and 1 mM MgCl₂ and the fluorescence was measured using appropriate excitation/emission wavelength pair using a spectrofluorimeter (M200 infinite, TECAN,) at 37°C. In method 3, the tubes were placed over a blue light source (Dark Reader; Clare Chemical Research) or in the slot of in house dark reader manufactured by DSS for detection of fluorescence by visual inspection. In the beginning, 100 samples were also analysed using Phosphorimagery (GE Healthacre). Samples were placed in 96 well plates and scanned under fluorescence mode. The excitation and emission wavelength of Cy3 is 554 nm and 568 nm respectively whereas excitation wavelength of fam is 560 nm and emission wavelength 515 nm.
1.4.2.9 Characterization of PCR Products by Sequencing

All the sequencing experiments were carried out using automated DNA sequencer (ABI Prism 377, PE Applied Biosystems) using Big Dye terminator kit (Perkin Elmer, USA) at TCGA commercial sequencing facility. About 100 ng of gel purified PCR products was subjected to sequencing using gene specific primers.

1.4.2.10 Roche AMPLICOR MWP Neisseria gonorrhoeae Detection Assay

412 cervical specimens were tested by Roche AMPLICOR NG detection kit (Roche Diagnostic Systems) according to the manufacturer’s instructions. Briefly, 1 ml of dilutent was added to endocervical samples and mixed well by vortexing followed by 10 minutes incubation at room temperature. Samples were stored overnight at 4°C. 50µl of the clinical sample was added to each PCR tube containing 50 µl of the PCR Master Mix. The PCR master mix contains primers for internal control as well. The assay was developed as per instructions given by manufacturers. To resolve the discrepant samples genomic DNA was isolated from the aliquots of frozen specimens. Samples were centrifuged at 15,000X g for 30 minute. Pellet was resuspended in 500 µl of lysis buffer (Tris EDTA 50mM pH 8.0, Proteinase K 400 µg/ml) and incubated at 55°C for two hours and then boiled at 100°C for 10 minute with 1mM, DTT. Thereafter DNA was extracted with phenol:chloroform and centrifuged at 12,000X g for 10 minute. To the supernatant, isopropanol was added with 1/10 volume of 3M sodium acetate and incubated overnight at -20°C. Pellet was collected by centrifugation at 12,000X g for 10 minute, washed with 70% ethanol, air-dried and dissolved in water before performing PCR (Sachdeva et al., 2009 ).

1.4.2.11 Evaluation of Assays

All assays on clinical specimens were performed blinded to the result of one another. Discrepant results (n=18) were also tested using rmp a house keeping gene of Neisseria. A specimen was considered true when 1) in-house PCR and Roche PCR positive 2) Roche PCR positive 3) in-house PCR positive and positive using rmp amplification. Roche PCR was taken as gold standard.
1.4.2.12 Evaluation of Specificity and Sensitivity

To evaluate the specificity of the primer pair, DNA extracted from various stains of N. gonorrhoeae: *Neisseria gonorrhoeae* [24], *Neisseria meningitidis* (genital isolates) [3], *Neisseria lactamica* 94D4 [1], *Neisseria sicca* 94C1 [1], *Neisseria subflava* 86G7 [1] (kind gift from Prof. J. W. Tapsall, WHO Collaborating Centre for STD and HIV, Department of Microbiology, The Prince of Wales Hospital, Randwick, New South Wales, Australia) other sexually transmitted disease causing pathogens including *C. trachomatis* serovar L2 (kind gift from Dr. Peter Braun, Department of Molecular Biology, Max Plank Institute for Infection Biology, Berlin, Germany), other sexually transmitted disease (STD) causing and related microorganisms such as *Mycoplasma* sp. [8] *Chlamydia pneumoniae* [5], *Candida* species [10], *Ureaplasma* [11], *Trichomonas* [8], *Pseudomonas aeruginosa* [2], *Klebsiella pneumoniae* [2], *Acinetobacter baumannii* [2], *Staphylococcus aureus* [2], *Herpes simplex* virus 1 (HSV-1) and *Herpes simplex* virus 2 (HSV-2) [2], *Cytomegalovirus* [2], *BK virus* [2] were kind gift from Department of Microbiology AIIMS, New Delhi, India, were used as templates for PCR.

Ten human genomic DNA samples were also used to evaluate the specificity of primer pair.

To determine the sensitivity of finally selected primer pairs and molecular beacons against *orf1* and *gyrA1* serial dilutions from 50 ng to 10 fg of purified genomic DNA of *Neisseria* and *C. trachomatis* and various dilutions of positive clinical samples were tested by PCR. For both assays PCR conditions were similar as described above with additional step of denaturation at 95°C followed by slow cooling (0.1°C/sec) was added in the tubes with molecular beacon. Sensitivity of molecular beacon was checked in fluorimeter as well as under dark reader.

1.4.2.13 Definition of a Positive Sample

410 clinical specimens were tested by Roche Amplicor MWP kit and by in house PCR assay. Amplification of known *N. gonorrhoeae* gene *rmp* (NGO1577) was carried out for discrepant samples. Samples were considered positive if they tested positive by at least two PCR methods: Roche Amplicor MWP kit/ inhouse PCR/ amplification of *rmp* gene.
Thereafter, duplex PCR (dPCR) assay was evaluated using 533 samples against uniplex PCR for each pathogen. Discrepant analyses as well as 67 blind samples were evaluated using Roche kit as gold standard. Samples were considered positive if they tested positive by at least two PCR methods: in-house uPCR/in-house dPCR /Roche Amplicor MWP kit.

Further, performance of our in house PCR using gDNA isolated from dry swabs samples (n=133). We found that performance of PCR assay using gDNA isolated from dry swabs was as good as that of wet swabs. Discrepant analysis was done using housekeeping genes (ompA for C. trachomatis and rmp for Neisseria gonorrhoeae). A sample was considered positive if it tested positive using two methods: in house PCR using gDNA isolated from dry swab/wet swab/PCR with housekeeping gene.

**Evaluation of Molecular beacon**

1.4.2.14 Performance of Molecular Beacon was Evaluated Against In-house PCR

100 samples were evaluated using molecular beacon of orf1 gene using Roche Amplicor MWP kit as gold standard. Agarose gel analysis was also performed simultaneously. Beacon was visualised under phosphor-imager, ELISA reader as well as under dark reader. A standard curve of concentration gradient v/s fluorescence was plotted to calculate the sensitivity of beacon against agarose gel electrophoresis. Similarly conditions for visualising molecular beacon were also standardised for detecting C. trachomatis using gyrA gene.

Once all the conditions were standardised for molecular beacons. 150 samples were further evaluated using dark reader for orf1 gene as well as gyrA gene against agarose gel electrophoresis.

1.4.2.15 Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Sensitivities, specificities, PPVs and NPVs were calculated with 95% confidence intervals to test the significance of the estimates.
Results
1.5 RESULTS

1.5.1 Standardization of In-house PCRs for the Detection of *N. gonorrhoeae*

In-house PCR conditions were standardised using purified gDNA isolated from culture plates of *N. gonorrhoeae* by varying annealing temperatures ranging from 54°C to 62°C (Figure 1.5A). Concentration of MgCl\(_2\) has a major role to confer specificity and the yield of amplification. Therefore, titration of Mg\(^{2+}\) concentration in the range of 1.5 to 3.5 mM was performed (Figure 1.5 B). Except for the reagent whose effect was being tested, all other conditions were kept constant. Based on these experiments, *orf1* was amplified at wide range of 54-62°C (Lane 1-5, Figure 1.5A) and 3mM MgCl\(_2\) (Lane 4, Figure 1.5B).

![Figure 1.5: Agarose gel (1%) showing standardization of PCR amplification of orf1 using orfF and orfR and purified genomic DNA of *N. gonorrhoeae* (FA19) as template. Panel A represents the temperature gradient PCR and panel b represents Mg\(^{2+}\) concentration gradient PCR. Lane M represents 100 bp DNA ladder. Panel A: PCR amplification was carried out at a range of annealing temperatures (62, 60, 59, 58, 56 and 54°C) shown in Lane 1-6 respectively. Panel B: orf1 amplification at different MgCl\(_2\) concentration of 1.5, 2.0, 2.5, 3.0 and 3.5mM was performed (Lane 1-5 respectively).](image)

1.5.2 Specificity and Sensitivity of Primers

To evaluate the specificity of the primer pair, amplification was performed using gDNA isolated from *N. gonorrhoeae*, various other species of *Neisseria*, other STD causing pathogens and using Human gDNA. Amplicons of desired size (~269 bp) were obtained when purified genomic DNA of *N. gonorrhoeae* was used as template for the in-house PCR, while no amplicon was detected when other STD causing and related microorganisms, as well as, human genomic DNA were tested (Figure 1.6).
The specificity was further confirmed by sequencing the amplicons obtained from 10% of positive clinical samples. The primer pair was highly sensitive as an amplicon could be seen when as low as 100 fg of purified Neisseria genomic DNA was used as template (Figure 1.7) The primer pair amplified the target sequence even when crude lysate of clinical samples were used as template.

Figure 1.6: Specificity of in-house primers using purified genomic DNA of various STD causing pathogens: PCR amplification of orf1 using purified genomic of various STD causing pathogens.

Figure 1.7: Sensitivity of in-house primers using purified Neisserial genomic DNA: PCR amplification of orf1 using purified genomic DNA (10ng, 1ng, 100pg, 10pg, 1pg, 100fg, 10fg, and 1fg in lane 2-9) of N. gonorrhoeae as template was carried out by using Ngu1/Ngu2 primers. Sensitivity of primers was found to be 100fg.
1.5.3 Clinical Performance of In-house PCR for Detection of Neisseria and its Comparison with Roche Amplicor MWP kit

To evaluate the efficacy of in house PCR, performance of uniplex PCR (uPCR) was evaluated against commercially available, Roche Amplicor MWP kit. uPCR for N. gonorrhoeae was established and evaluated using swabs samples collected from 412 symptomatic women patients with median age of 24 years. Two swabs were collected from each patient to avoid the swab sample variation that may occur when multiple swabs are taken (especially when infection load is low). 143 (34.70%) tested positive and 240, (58.2%) tested negative by both in-house PCR and commercial PCR assay and 29 samples showed discrepant results (Table 1.2). To resolve the discrepancy, gDNA was purified and tests were repeated. One major problem that we encountered during this study was occurrence of inhibition of the amplification reaction when the Roche MWP kit was used, as has also been observed by others (Niederhauser & Kaempf, 2003 Feb; Shattock et al., 1998). When gDNA was purified for all 29 discrepant samples, 14 turned to be positive both by in house PCR and commercial PCR. These samples also tested positive with rmp, a housekeeping gene. Three other samples which tested negative with commercial PCR assay, tested positive by rmp gene and were thus considered true positive. Five samples tested negative by both commercial PCR assay and rmp gene thus considered to be true negative. Seven samples which were negative by in house PCR but tested positive with commercial assay, tested positive with rmp and thus were considered as true positive (Table 1.3). Thus overall, our primer pair tested 95.8% (160/167) positive samples correctly whereas commercial kit was able to detect 98% of positive samples only when DNA was purified from samples. In this part of the study, compared with the Roche MWP PCR assay, the sensitivity and specificity of the in-house uPCR Ngu1/ Ngu2 were 95.3% and 91.6% respectively. After discrepant analysis, the sensitivity of the PCR method increased to 95.8% and the specificity increased to 97.9%. Thus, our in house PCR has 95.8% sensitivity and 97.9% specificity after discrepant analysis. The PPV of in-house PCR assay increased from 86.7% to 97.0% while the NPV remained unchanged at 97.2% (Table 1.5).
## Chapter 1: Results

Table 1.2: Comparison of in house PCR with Roche Amplicor MWP kit: before after discrepant analysis for *N. gonorrhoeae*.

<table>
<thead>
<tr>
<th>PCR results</th>
<th>Roche</th>
<th>After resolution of discordant results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>NgU1/Ngu2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>143</td>
<td>22</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>240</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>150</td>
<td>262</td>
</tr>
</tbody>
</table>

Table 1.3: Resolution of discrepant results of group I by PCR based detection of *rmp* for *Neisseria gonorrhoeae*. (N=29)

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Ngu1/Ngu2</th>
<th>Processed and developed with Roche</th>
<th>Purified DNA, developed with Roche *</th>
<th>rmp</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>14$^5$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>NR</td>
<td>true positive</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>false positive</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>true positive</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>NR</td>
<td>+</td>
<td>false negative</td>
</tr>
</tbody>
</table>

$^5$ The patient samples when processed and tested with Roche Amplicor MWP did not give positive results even for the internal control suggesting that the PCR reaction was inhibited due to the presence of inhibitors in the processed patient samples.

* Total DNA from patient sample was purified followed by NAAT based detection of *Neisseria gonorrhoeae* using Roche Amplicor MWP kit.
Standardization of Duplex PCR for Simultaneous Detection of *N. gonorrhoeae* and *C. trachomatis*, and its Evaluation against Roche Amplicor MWP kit

Although there are several commercial kits which allow simultaneous detection of these two pathogens, but their high cost and lack of trained technicians, makes them far from use in routine practice in India. The method developed in present study is not only cost effective but also highly specific and sensitive and allows simultaneous detection of the two pathogens in single test assay (Figure 1.8). For simultaneous detection of *N. gonorrhoeae* (present study) and *C. trachomatis*, (established in previous studies, Patel *et al.*, 2010), duplex PCR was standardized and evaluated using 533 samples. Each clinical sample was tested individually for *N. gonorrhoeae* and *C. trachomatis* as well as by duplex PCR using in-house primers. Discrepant samples were analyzed using Roche Amplicor MWP kit as gold standard. Out of the 533 symptomatic patients enrolled in the above study, 119 tested positive for *Chlamydia trachomatis*, 164 tested positive for *Neisseria gonorrhoeae* while 84 were found to be co-infected with both bacteria based on the results of uPCR. Using in-house dPCR, only 72 samples out of these 84 samples were found to be positive for both the pathogens. In addition, there were 12 samples which were found to be infected with single pathogen using uPCR (7 with *C. trachomatis* and 5 with *N. gonorrhoeae*), but were found to be co-infected using dPCR. All the discrepant samples were also checked with Roche Amplicor MWP kit.

Out of the 24 discrepant samples, 12 samples tested positive for both the pathogens (*Chlamydia* and *Neisseria*) using uPCR (in-house) but tested positive for only one of the two pathogens (8 tested positive for *Chlamydia* and 4 for *Neisseria*) when dPCR was done. Using Roche kit, 11 samples tested positive for both the pathogens and hence were considered to be co-infected (Table 1.4). The other 12 discrepant samples when tested by in-house uPCR or Roche kit were found to be infected either with *Chlamydia* (7 samples) or *Neisseria* (5 samples). However, in-house dPCR results suggested co-infection with *Chlamydia* and *Neisseria* (Table 1.4, lower 2 rows). Based on the concordance between uPCR and Roche assay, these samples were
scored positive for either *Chlamydia* (7 samples) or *Neisseria* (5 samples). As per these results the sensitivity of in house dPCR was found to be 85.7% (95 CI: 77.1%-92.8 %) with specificity of 97.3% (95 CI: 95.3%-98.5 %) (Table 1.4)

![Image of agarose gel detecting infection load](image-url)

**Figure 1.8: Agarose gel (1.5%) detecting the infection load in clinical samples using in house PCR.** Amplicon of 463 bp shows successful amplification of gyrA of *C. trachomatis* (Panel A), 269 bp of orf1 of *N. gonorrhoeae* (panel B) and their co-amplification (Panel C). Lane 1 is No template control, lane 2-8 are clinical isolates and Lane 9 is positive template control in each panel

Out of the total 822 symptomatic female subjects enrolled in the current study, 314 (38.19%) were infected either with *Neisseria* or *Chlamydia* or both. The infection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* was 27.8% (95% CI: 22.9% - 32.7%) and 26.3% (95% CI: 21.3% - 31.3%) respectively with 11.3% of co-infection. Further, we also studied that among 229 patients who tested positive for gonococcal infections, 93 also tested positive for *Chlamydia trachomatis* (40.6%), whereas out of 217 samples that tested positive for *Chlamydia*, 93 samples tested positive with *N. gonorrhoeae* (42.85%)
Although we did not observe any significant difference in our study, the community based studies carried out on symptomatic patients, in US (Miller et al., 2004) and Netherlands (van Bergen et al., 2006) had suggested 69.7% and 100% probability, respectively, of Chlamydia infections among gonococcal patients.

**Table 1.4: Resolution of discrepant samples:** Discrepancy of dPCR was resolved using Roche Amplicor MWP kit.

<table>
<thead>
<tr>
<th>Discrepant samples</th>
<th>uPCR</th>
<th>dPCR</th>
<th>Roche</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>NG</td>
<td>CT</td>
<td>NG</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 1.5: Performance characteristics of in-house PCR assays based on expanded spectrum of positivity after discrepant analysis.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>95% CI</th>
<th>Specificity</th>
<th>95% CI</th>
<th>PPV</th>
<th>95% CI</th>
<th>NPV</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1 PCR</td>
<td>95.8</td>
<td>91.2-98.8</td>
<td>97.9</td>
<td>95.2-99.0</td>
<td>97</td>
<td>93.1-98.7</td>
<td>97.2</td>
<td>94.3-98.6</td>
</tr>
<tr>
<td>Duplex PCR</td>
<td>85.7</td>
<td>77.1-92.8</td>
<td>97.3</td>
<td>95.3-98.5</td>
<td>86</td>
<td>76.1-92.3</td>
<td>97</td>
<td>95.3-98.2</td>
</tr>
</tbody>
</table>
Use of Dry Swabs for Clinical Evaluation

Lack of resources and technical expertise make diagnosis of STDs difficult in rural areas of India. Thus, to make our method as test of choice, reagents have been stabilized such that assay reaction mixture can be kept at 4°C for up to 6 months, (Figure 1.9) making it an easy assay to be carried out in peripheral laboratories where deep freezers are not available. The assay so designed will be of great use for easy, rapid diagnosis and surveillance programs in developing countries. Dry swabs could be transported at room temperature making the method of collection and transport of the samples easy from remote areas to the nearest laboratory (Feng et al., 2010; Krech et al., 2009). We had checked the performance of our in-house PCR using dry swabs samples (n=133) and found similar sensitivities of PCR assay to that of gDNA isolated from wet swabs (Table 1.6). Discrepant samples were analysed using housekeeping genes (ompA for C. trachomatis and rmp for Neisseria gonorrhoeae). Out of 133 samples, 16 were positive for C. trachomatis using both dry and wet swabs while 110 were negative for Chlamydia infection. For Neisseria, 33 samples out of 133 were found positive using both the swabs whereas discordance in PCR results using dry and wet swabs was observed for 4 samples. These samples were analysed for another gene (rmp for Neisseria and ompA for Chlamydia) as shown in Table 1.7. Although we found good concordance of results with dry and wet swabs, use of dry swabs as a preferred method of collection needs further evaluation.

Figure 1.9: Agarose gel showing the stability of PCR master mix at 4°C. Amplification of Orf1 was performed with PCR master mix stored at 4°C for 10, 8, 6, 5, 4, 3 and 1 month (Lanes 1, 2, 3, 4, 5, 6 and 7 of Panel a) Amplicon of expected size was observed on 1.5% Master mix was stable and functional for more than six months. The stability of reagents at 4°C for long periods is especially important in resource limited laboratories where deep freezers (-20°C and -80°C) are not available.
Table 1.6: Comparison of in house PCR using dry swabs with wet swabs as mode of sample collection, for *C. trachomatis* and *N. gonorrhoeae*.

<table>
<thead>
<tr>
<th>Wet swabs</th>
<th>PCR results for <em>gyrA</em> gene using GyrA1/GyrA2 primers</th>
<th>Dry swab</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td></td>
<td>16</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td></td>
<td>2</td>
<td>110</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>18</td>
<td>115</td>
<td>133</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wet swabs</th>
<th>PCR results for <em>orf1</em> gene using orf1F/ orf1R primers</th>
<th>Dry swabs</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td></td>
<td>33</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td></td>
<td>2</td>
<td>96</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>35</td>
<td>98</td>
<td>133</td>
</tr>
</tbody>
</table>

Table 1.7: Resolution of discrepant samples: Discrepancy of PCR using DNA isolated from wet and dry swabs was resolved using a house keeping gene *ompA* for C2/C5 and *rmp* for orf1F/orf1R primers.

<table>
<thead>
<tr>
<th>Discrepant Samples</th>
<th>Wet swabs</th>
<th>Dry swabs</th>
<th>ompA/ rmp</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>True positive for <em>Chlamydia</em></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>True negative for <em>Chlamydia</em></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>True positive for <em>Chlamydia</em></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>True positive for <em>Neisseria</em></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>True negative for <em>Neisseria</em></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>True positive for <em>Neisseria</em></td>
</tr>
</tbody>
</table>
Development of Reagents for an Easy Visualization of PCR based Diagnostic Method

Our in-house PCR method has been proved to be highly sensitive and specific when evaluated against Roche Amplicor MWP kit, which uses ELISA-based detection methods. These methods are preferred because of their higher sensitivity and specificity. To increase the specificity of the Roche MWP detection method, a DNA probe against the target gene is hybridized to the MWP strips. This detection method requires technical expertise and is time-consuming (4-5 hrs). Thus to improve our detection assay, we introduced the fluorescent probe technique of molecular beacons, to detect *N. gonorrhoeae* and *C. trachomatis*. Molecular beacon probe specific to the sequence of the orf1 as well as for *gyrA* amplicon were designed as described under ‘Materials and Methods’. Asymmetric PCR was performed using orf1R primer in excess (20 pmoles in 50µl reaction) as compared to orf1F primer (10 pmoles) to preferentially amplify the strand to which the probe will hybridize. Molecular beacons to detect the amplified product was added (2.5 pmoles) either during PCR or after the PCR is completed. Subsequent to the amplification, the PCR product was heat denatured for 5 min at 95°C and then allowed to cool to room temperature (20°C) at a ramping rate of 0.1%/sec. PCR products were stored at -20°C or checked by i) Phosphorimager, (Figure 1.10 A) ii) ELISA reader (Figure 1.10B). In the standardization phase all the results were cross-checked by analyzing the PCR products by Agarose gel electrophoresis.

Figure 1.10: Detection of *N. gonorrhoeae* using Molecular Beacons (cy3 labeled). To the PCR product, beacon was allowed to hybridize as described before and fluorescence was measured either on phosphorimager (Figure 1.10A) or quantified by ELISA reader (Figure 1.10B). Panel A) Spots 1 & 2 represent no beacon & no template control respectively, spot 3 represents positive control. Spots 4-12 represent patient samples. Panel B represents corresponding values of the above samples on ELISA reader after dilution of the amplified PCR products with buffer (150 µl of 10 mM Tris/Cl (pH 8.0) and 1 mM MgCl₂). Fluorescence was measured using 554 nm excitation and 568 nm emission wavelength in an ELISA Reader (Tecan).
Evaluation of Beacon for orf1

For orf1 we had also performed a pilot study (176 samples) in which detection method using molecular beacon probe was evaluated using Roche Amplicor MWP kit as gold standard. 74 samples were detected positive by PCR method whereas 77 samples were detected positive by beacon. Discrepant samples (n=3) also tested positive using Roche amplicor MWP kit and thus considered true positive. Use of molecular beacon has improved the sensitivity of PCR method using agarose gel electrophoresis as detection method.

After standardization of molecular beacon for orf1 gene, beacon (Cy3) was also designed complementary to amplified region of gyrA gene for detecting chlamydial infections. We calculated the sensitivity of molecular beacon using serial dilutions of chlamydial gDNA. Sensitivity of beacon was found to be 10fg. (Figure 1.11)

Since our goal is to develop multiplex PCR for co-detection of Chlamydia and Neisseria, we used fam labeled beacon for detection of Neisseria and Cy3 labeled beacon for Chlamydia for all further detections. Sensitivity of Fam labeled beacon was also calculated using serially diluted gDNA. Fluorescence was measured using ELISA reader and line graph was drawn of DNA concentration vs Fluorescence intensity units. Sensitivity of gyrA beacon was found to be 40fg (Figure 1.11) whereas beacon for orf1 was sensitive till 100fg (Figure 1.13). Clinical samples (n=150) were tested for both orf1 and gyrA gene. 31 samples tested positive for Chlamydia and 22 for Neisseria using agarose gel electrophoresis. Amplified product probed with molecular beacon was also detected using phosphorimager (Figure1.12 panel A) Panel B represent corresponding values of the above sample on ELISA reader. Fluorescence was measured using 554 nm excitation and 568 nm emission in ELISA Reader (Tecan) as described above. Panel C represents direct visualization of PCR product on an indigenous Dark reader: Tube 1& 2 are clinical samples and tube 3 represents negative control. Similarly fluorescence was measured for detection of Neisseria gonorrhoeae using Fam labeled beacon for orf1 gene (Figure 1.14 A). Panel C represents direct visualization of PCR product on an indigenous Dark reader: Tube 1 & 2 are clinical samples and tube 3 represents negative control. Results using molecular beacon corresponded with agarose gel electrophoresis experiments for uPCR but use of molecular beacon for co-detection needs to be further standardized. Two samples which were considered to be at border line for Neisseria
infections, using agarose gel electrophoresis turned out to be true positive using molecular beacon suggesting high sensitivity of the method. It may also be emphasized that hybridization to internal regions of the amplicons also results in increasing the specificity of the assay.

Figure 1.11: Detection of *C. trachomatis* using Molecular Beacons. Panel A) Sensitivity of molecular beacon using purified chlamydial genomic DNA: PCR amplification of *gyrA* using purified genomic DNA (1ng, 100pg, 10pg, 1pg, 100fg and 10fg) of *C. trachomatis* as template was carried out by using C2/C5 primers probed using molecular beacon. Sensitivity of primers was found to be 40fg.

Figure 1.12: Detection of *C. trachomatis* using Molecular Beacons. Panel A) Spot 1 & 2 represent no beacon & no template control, Spot 3 represents positive control. Spots 4-7 represent patient samples Panel B) represent corresponding values of the above sample on ELISA reader. Fluorescence was measured using 554 nm excitation and 568 nm emission in a ELISA Reader (Tecan) as described above. Panel C) represents direct visualization of PCR product on an indigenous Dark reader: Tube 1 & 2 are clinical samples and Tube 3 represents negative control.
Figure 1.13: Detection of *N. gonorrhoeae* using Molecular Beacons (Fam labeled). Sensitivity of molecular beacon using purified Neisserial genomic DNA: PCR amplification of *orf1* gene using purified genomic DNA (10ng, 1ng, 100pg, 10pg, and 1pg) was carried out by using orf1F/orf1R primers and probed with fam labeled molecular beacon. Sensitivity of beacon was found to be less than 1pg.

Figure 1.14 Detection of *N. gonorrhoeae* using FAM labeled Molecular Beacons. A) The amplified PCR products were transferred into wells of a 96-well plate containing 150 µl of 10 mM Tris/Cl (pH 8.0) and 1 mM MgCl₂ and the fluorescence was measured using 489 nm excitation and 521 nm emission in a ELISA Reader. Column 1 and 2 are no template and positive template control respectively. Wells 3-6 represent fluorescence value on ELISA reader of the amplified products using DNA from patient samples. B) Represents direct visualization of PCR product on an indigenous Dark reader: Tube 1 represents negative control and Tube 2 & 3 are clinical samples. Our results strongly suggest that beacon can be used for detection of amplified product and this will help user in not only saving time as one can directly visualize on dark reader but also increases specificity as beacons hybridize to the internal region of the amplicons.
Discussion
1.6 DISCUSSION

Many studies have evaluated in-house PCR assays for detection of *Chlamydia* and *Neisseria* but high cost of PCR precluded their use in developing countries like India. In the present study we have developed and evaluated an in-house dPCR assay for the simultaneous detection of *Neisseria* and *Chlamydia*. Using uPCR, dPCR and commercial assays our study suggests high prevalence of *Neisseria* (27.8%) and *Chlamydia* (26.3%) amongst women seeking treatment. Most of the women were from age group of 18-30 years. Another study among the patients visiting STI clinics of Delhi has also reported 19.3% *Neisseria* and 16.1% *Chlamydia* infection using culture method and Bio-Rad Chlamydia Microplate EIA kit respectively (Choudhry *et al.*, 2010a).

High rates of chlamydial co-infection were observed with gonococcal infections in adolescents especially between the age group of 15-24 years (Dicker *et al.*, 2003; Forward, 2010; Nsuami *et al.*, 2009) and in pregnant women (Berggren & Patchen, 2011). In *Neisseria* infected individuals, 20% to 57% of women and 3.3% to 37% of men were infected with *Chlamydia*. Similarly 4.3% to 31% of women and 12% to 28% of men are co-infected with *Neisseria* in *Chlamydia* infected patients (Dicker *et al.*, 2003; Miller *et al.*, 2004; Nsuami *et al.*, 2009). Using a co-infection model of female BALB/c mice, Vonck and co-workers have shown that gonocccal infection increases if mice is pre-infected by *C. trachomatis* (2011). In the present study, we also found 11.3% of co-infection among symptomatic female patients. A similar report stating 4.4% of co-infection (95%CI 2.95% - 5.85%) was reported among male patients attending STD clinic in Brazil (Barbosa *et al.*, 2010). Varying rates of co-infection were reported among male (5% - 8%) and female (6% - 25%) patients depending upon age, in a population survey done in Nova Scotia (Forward, 2010).

Although screening programmes for *Chlamydia* and *Neisseria* are well established in developed countries, their high cost makes them difficult for routine use in developing countries. Moreover, only few health centres and hospitals are diagnosing *Chlamydia* and *Neisseria* before giving treatment to symptomatic patients. Since most of diagnostic centres use culture method as gold standard, results take long time to reach the clinician. Sensitivity of these methods in such laboratories is less than 45% (Aledort *et al.*, 2003).
Syndromic management is thus only rescue used in community screening programmes as well as in hospital settings. However, syndromic management misses out a good proportion of infection in asymptomatic patients (30% - 40% cases) and may detect false positives (Vishwanath et al., 2000). This further leads to spread of infection in the society and thus increases the cost of reproductive health (Kostick et al., 2010). Also co-infection of STI has also been observed with UTI whereas treatment regimen for UTI does not treat Chlamydia and Neisseria infection (Prentiss et al., 2011). Moreover, treatment regimen should be based on local antibiotic sensitivities but such pattern is rarely known in resource limited settings. This may lead to over treatment (90% in Chlamydia and 80% in Neisseria) which may contribute to emergence of antibiotic resistant strains. Another study carried out by Shilpee and co workers (2008) reported that the sensitivity of genital discharge syndrome (GDS) to treat N. gonorrhoeae and C. trachomatis was 96.5% and 91.8% respectively. However, the specificity was only 76.3% and 72.5%, respectively (Choudhry et al. 2010). Our test was more specific as well as detected both the pathogens in single test thus could be considered better that proposed by Shilpee and co workers (2008). Most of the data available is from hospitals and STI clinics but a large population goes undetected due to social stigma attached to infection especially among vulnerable population of minors, pregnant women and adolescents. Thus, there is an urgent need of community based screening for STIs in India.

To further enhance the sensitivity and specificity of our test, molecular beacon were designed against the amplified region of orf1 and gyrA gene. Since molecular beacons can detect single nucleotide change, it thus also enhances the specificity of the test. Many commercial kits like Roche MWP kit uses labeled probe as the detection method. Their detection method is based on indirect ELISA and thus is time consuming (3hrs), cumbersome and requires technical expertise. Various other detection assays also use molecular beacons but most of these involve automated real time technologies. These kits generally remain out of reach in developing countries. In the present study, detection of amplified PCR product is done directly in PCR tube using a simple to use, indeginous fluorescent based dark reader for detection. To look for relative load of infection, the intensity of flourescence can be measured by ELISA reader. Looking for flourescence by directly putting PCR tubes on dark reader is far
less time consuming than running agarose gel and/or PCR-ELISA and requires no technical expertise. The prime advantage of using closed tube is it minimizes chances of cross contamination and carryover contamination. In the present study we have also standardized 2X master mix and stored it at 4°C. Stability of mastermix was checked at intervals 0-6 months. This helps in storage of reagents and performance of PCR assays in peripheral laboratories with minimal resources.

The present study offers an easy, rapid and economical NAAT-based assay to study the burden and pattern of infection. This not only reduces the cost, turnaround time but also the pain and harassment that patient has to undergo by revisiting the clinic, as both the pathogens will be tested in the same sample. We found that our test was 95% sensitive and 97% specific for Neisseria gonorrhoeae using orf1 gene whereas sensitivity and specificity has already been established for Chlamydia trachomatis in our previous studies (Patel et al. 2010). We further modified uPCR to dPCR which was found to be 85.7% sensitive and 97% specific. We have observed that our assay performed with similar specificity and sensitivity even when the dry swab samples were collected and transported at ambient temperature. Premix was also stabilised at 4°C for 4 months. Thus, we recommend collection of dry swabs for routine diagnosis as it not only reduces the cost of transport medium but also makes the collection and transportation of samples from field to nearest laboratory less cumbersome (Feng et al., 2010)
1.7 BIBLIOGRAPHY


van Bergen JE, Spaargaren J, Gotz HM, Veldhuijzen IK, Bindels PJ, Coenen TJ, Broer J, de Groot F, Hoebe CJ & other authors (2006b). Population prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in the Netherlands. Should asymptomatic persons be tested during population-based Chlamydia screening also for gonorrhoea or only if chlamydial infection is found? *BMC Infect Dis* **6**, 42.


