Chapter 6

DISCUSSION
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The efficacy of the different treatment regimens for gonococci is dependent upon the type, degree and prevalence of antibiotic resistant strains (Tapsall et al 1995). Antibiotic sensitivity tests have long been considered essentially retrospective since the majority of patients would already have been treated on the basis of Gram stained smear results. Nevertheless, they are important for epidemiological purposes and in planning appropriate therapy. Thus, it has become imperative to know the in vitro susceptibility of gonococci to available antibiotics in order to provide adequate and rational therapy. Pattern of gonococcal resistance to different antibiotics may vary in different areas. Knowledge of local resistance pattern may help the health care providers and health administrators to make decisions on appropriate cost-effective control of gonococcal infections. The control of gonococcal infection is important considering the high incidence of acute infections, complications and sequelae and its role in facilitating acquisition and transmission of HIV (Wasserheit 1992, Emmert et al 2000). Many antimicrobials were active against *N. gonorrhoeae* in the past. However, with the emergence and rapid increase of antimicrobial resistance only few antibiotics are now effective against *N. gonorrhoeae* in the past. Single dose therapy is the universal practice of choice in the treatment of uncomplicated gonorrhea. WHO and Center for Disease Control and Prevention (CDC) recommend a change in the treatment regimen when the prevalence of antimicrobial resistance exceeds five per cent for a specific antibiotic. (WHO 2006, Newman et al 2007). This cutoff point defines the requirement for an effective treatment in individuals and for the purposes of public health. By monitoring drug resistance in *N. gonorrhoeae*, standard treatment regimens can be optimized and wider
measures adopted for control of gonococcal disease. Thus, it is critical to continuously monitor antibiotic resistance in *Neisseria gonorrhoeae* and encourage research and development of new treatment regimens. The high prevalence of multidrug resistance caused by varied resistance mechanisms in *N. gonorrhoeae* limits the drug choice. Ongoing surveillance of antimicrobial resistance and discovery of new effective antibiotic therapy are warranted in endemic areas (Chen et al 2010). Gene on either plasmids or chromosomes controls the antimicrobial resistance. Chromosomal resistance results from the additive effect of mutations at several resistance loci and spreads more slowly than plasmid mediated resistance.

The Gonococcal Isolate Surveillance project in USA in 1993 reported 15.4% of the chromosomally mediated resistance to penicillin while the Australian Surveillance programme reported 8.3% of CMRNG strains in 1995. Highest percentage of CMRNG strains has been reported in the south-east Asian countries including Phillipines, Thailand and Indonesia. In a study carried in Bangkok in 1995, CMRNG was exhibited by 36% of the strains. In USA, the percentage of CMRNG increased annually from 0.5% in 1988 to 5.7% in 1999 (Eng et al 1997). The prevalence of penicillin was highest in South East Asia and Sub-Saharan Africa. In USA, in 1999, 21.1% of the isolates were PPNG strains. The prevalence of penicillin and PPNG recently from other parts of the world varied from 11.7% - 37.8% and 16%–86% (Rahman et al 2002, Zheng et al 2003, Ito M, et al 2004, Van Loo et al 2005, Yang et al 2006, De Jongh et al 2007, Lee H et al 2011, Jabeen et al 2011, Tanaka et al 2011, Fayemiwo SA et al 2011).

Various studies in India have also shown an alarming pace of resistance to penicillins. In 1966, 23.4% strains were reported to be penicillin resistant that subsequently increased to 74% in 1987 & 60.5% in 1989 (Pahwa et al 1987). The PPNG strain was first reported by Vijay Lakshmi et al from Madras in 1980 and then in 1984 three PPNG strains were reported from
Vishakhapatnam (Vijayalakshmi et al 1984, Rao et al 1974). The prevalence of *N. gonorrhoeae* was 2.3% in 1983 in Bombay & then increased to 16% in 1986 and to 17.88% in 1994 (Divekar et al 1999). In Delhi in 1994-1995, 5.3% of the isolates were found to be PPNG that increased to 8% in 1998 (Bhalla et al 1998). Study conducted by Ray et al (2000) showed high level of penicillin resistance from Hyderabad (79%) and Chennai (62.5%), while low level of resistance (20-33%) for penicillin was observed from isolates obtained from Kolkata, Nagpur and Pune (Ray et al 2005). Various studies in India have also reported penicillin resistance & PPNG varying from 11%-37.8% & 16%-86% (Khaki et al 2002, Bala M et al 2008). In our study we found 48.4% of the *N. gonorrhoeae* strains were resistant to penicillin and 15.6% of these were PPNG & these results were similar with the earlier findings reported.

Resistance to tetracycline was first reported from Philadelphia in 1985 & then spread to France, Spain, Netherlands, Canada, England, Central Africa and India. In 1992, 10% TRNG isolates were reported in Central Africa (Vn Dyck et al 1992). Its prevalence in USA in 1999 was 5.8% (Eng TR et al 1997). In the WHO Western Pacific study, TRNG were widely but unevenly distributed. In 1998, particularly high proportions of TRNG were seen in Singapore (84%), the Solomon Islands (74%) and Vietnam (35.9%), continuing a pattern observed in earlier years (Tapsall 2001). In all other regions TRNG distribution was below 10 per cent. Reports of high level tetracycline resistance were also documented in Africa, Europe and Netherlands (Tapsall J 2001). TRNG strains were also identified in the WHO South East Asia Region, and Thailand alone accounted for about 16 per cent of isolates in 1994-1997 (Tapsall 2001). Other studies have reported varying range from 9%-77.6% (De Jongh M et al 2007, Lee et al 2011, Jabeen et al 2011, Tanaka et al 2011).
A study from Delhi in 1998 reported 28% of isolates resistant to tetracycline (Bhalla et al 1998). In 2000/2001, Ray et al reported resistance varying from 0-45.6% in three centers of India (Hyderabad, Nagpur and Pune) (Ray et al 2005). A recent study at Delhi reported 8.9% tetracycline resistance (Bala M 2008). In our study we found 58% of isolates were resistant to tetracycline, which was higher compared to earlier reports from India.

Due to no or less resistance seen, cephalosporins were preferred for the last 10 years for management of gonorrhoea. However, several recent reports from Japan indicated much higher MIC values for cephalosporins (Ito et al 2004, Tanaka et al 2006, Yokoi et al 2007, Osaka et al 2008). Similar results were also documented from other countries like China, Hong Kong, Taiwan, Europe, US and Africa (GRASP 2008, Tapsall et al 2008, Tzelepi et al 2008, Wang et al 2007, Moodley et al 2006). A surveillance report from India, wherein isolates collected from different laboratories of India, Bangladesh, Nepal and Sri Lanka during 1999-2001, documented significant increase in the isolates with decreased susceptibility to ceftriaxone (Ray et al 2000).

In India, Bala et al reported nine isolates with ceftriaxone MIC of 0.064 mg/l among the 382 isolates studied during 2002-2006 (Bala M et al 2007). Only few studies have reported resistance to ceftriaxone (Camara et al 2012, Unemo et al 2012). In our study we found all the strains were susceptible to ceftriaxone, cefixime & spectinomycin.

Typing of gonococcal strains, based on their antibiotic susceptibility, nutritional requirements, serogroups and serovars play an important role in studying the epidemiology of outbreaks of infections with resistant strains & for monitoring changes in the phenotypes of gonorrhea with regards to geographic areas, race, treatment failure & other factors (Daneilsson 1983).
The number of different auxotypes that have been reported by other studies is between 3-16 (Knapp et al 1985, Whittington et al 1985, Coghill DV et al 1987, Danelsson D et al 1983, Agarwal SK et al 1992, Khaki P et al 2007). Although, NR and proline requiring auxotypes often predominate, other auxotypes also have been reported from other parts of the world (Whittington 1985, Yvert et al 1985, Coghill et al 1987, Brett et al 1992) Non-requiring and proline requiring auxotypes were also common auxotypes in Mumbai & Delhi (Divekar et al 1999, Khaki et al 2007). In our study too, the most prevalent auxotype was NR (45.3%), followed by proline requiring (32.8%) auxotypes. Restriction of certain auxotypes to different geographical areas can help in global epidemiological studies. In our study we found few auxotypes which have been reported earlier from Delhi and Mumbai which indicates that there is a pool of circulating strains in our community.

In our study, 26.5% N.gonorrhoeae isolates belonged to WI (IA) serogroup, 48.4% to WII/III (IB) serogroup and 25% isolates were of combination of WI and WII/WIII. Data showed that serogroups WII/III & WI/WII/WIII were most prevalent in Delhi while WI was found to be the most prevalent in Pune.

Our data indicates that there are a wide variety of serovars of Neisseria gonorrhoeae in India. Among the serogroup WI, the serovars Aost (47%) was the most prevalent, followed by Ast (29.4%) and Arost(17.5%). The seovar Boprt (54.8%) was the most common in the serogroup WII/III followed by Bopty(12.9%), Boptv(9.6%). Previous studies indicate similar results (Yvert et al 1985, Tzanakaki et al 1989, Brett et al 1992), the serovars Arost and Arst belonging to serogroup WI and Boprt and Broyrt belonging to serogroup WII/III were the most common serovars (Coghill et al 1987, Palomares et al 1990). However, Dillon et al. (1987)
reported that serovars Arost and Arot in the serogroup WI and serovars Boprt and Brpyust in the 
serogroup VII/III were most prevalent in Jamaica. In the present study, sixteen strains that cross 
reacted with both Av and Bx reagents (Av/Bx) were detected. This result is consistent with 
previous studies (Yvert et al 1985, Brett et al 1992) in which the same Av/Bx strain has been 
reported.

Our results showed an association between auxotype or serotype and penicillin 
sensitivity. The strains belonging to AUH group were sensitive to penicillin whereas Boprt 
strains were resistant to penicillin and tetracycline. Most of our PPNG strains were non requiring 
and apparently belonged to WI group.

The discriminatory index for antibiotic susceptibility, auxotyping and serotyping was 
found to be 0.78, 0.68 and 0.88. The results of our study showed that serotyping in combination 
with auxotyping (A/S typing) provided greater discrimination between isolates than the use of 
only one of these techniques. A/S typing provided a highest discriminatory index of 0.97% which 
is an acceptable level of discrimination in a typing method.

Quinolones have been widely used to treat infections caused by *N. gonorrhoeae* since the 
1980s because of their strong antibacterial activities, comparatively low costs and convenience 
of oral administration (Wang B et al 2006). Resistance to ciprofloxacin and ofloxacin emerged in the late 1980s and early 1990s when it was detected sporadically among gonococcal strain 
isolated from patients in south-east Asia and other countries including USA, UK and Australia 
(WHO 1997, Ye S et al 2002). In the recent years, there have been many reports on increasing 
number of quinolone resistant strains in many countries (Yang et al 2006, Martin et al 2005). In 
India, the use of ciprofloxacin, as the first-line therapy for gonorrhoea started in 1990 (Tapsall
Resistance to norfloxacin soon appeared in 1996 from New Delhi, India (Bhalla 1998). By the end of 2000, a burst in ciprofloxacin-resistant isolates was observed in India (Bala M et al 2003, Divekar et al 1999, Ray et al 2005). A high level of resistance to ciprofloxacin was observed in the recent studies (Bala M et al 2007, Bala M et al 2008, Kulkarni et al 2011). Most of the studies have looked for ciprofloxacin resistance only and limited studies have tested resistance against other members of the quinolone group such as ofloxacin, lomefloxacin, norfloxacin (Zhang et al 2009, Hovhannisyan et al 2007) and gatifloxacin (Koroku et al 2007). Newer quinolones with different resistance pattern & activities against gyrA and parC have been reported (Shultz et al 2001). Therefore, in the present study we compared the antibiotic susceptibility pattern of N. gonorrhoeae to six different quinolone antibiotics and observed the mutations in gyrA and parC. A high level of resistance (95 to 98%) to various quinolones was observed except 17.1% strains were resistant to gatifloxacin and 68.7% strains showed intermediate resistance. This low resistance to gatifloxacin has not been reported in the limited studies that employed gatifloxacin. In our study, we observed that the strains showing resistance to all the antibiotics had high MIC value (≥8 ug/ml).

Quinolones have a bactericidal effect when these bind to two target enzymes, DNA gyrase & topoisomerase IV, which are essential for DNA replication within cell. DNA gyrase (gyrA) mutations have shown to play an important role in the development of quinolone resistance in gonococci, and simultaneous parC mutations play a complementary role in increasing the level of resistance (Su X et al 2001, Vernel-Pauillac et al 2009, Belland et al 2011, ). We also found that strains resistant to all antibiotics & with high MICs (≥8 ug /ml) showed mutations in both gyrA and parC, whereas, mutations in gyrA region was seen in the strains resistant to ≤4 antibiotics & with low MIC values. Shultz et al. (2001) also reported that
the quinolone resistance mutations were found to have positive correlation with MICs of ciprofloxacin.

The known quinolone resistance associated mutations in *N. gonorrhoeae* include Ser-91→Phe, Asp-95→Gly, Asp-95→Asn in *gyrA* and Glu-91→Gly in *parC* though in a few studies mutations at other codons 67, 75, 84, 120 in *gyrA* and at 85, 86, 87, 88, 92, 100, 116 in *parC* have also been reported (Dewi et al 2004, Ilina et al 2008, Zhang et al 2009, Unemo et al 2009). A majority of our strains (95.3%) also had the same mutations as reported by others.

Only few studies have reported ≤10 mutation patterns, while two studies (Chaudhry et al 2002, Dewi et al 2004) have reported as many as 19 and 22 mutation patterns. Mutation analysis on our strains revealed 7 different patterns.

Of the 64 strains that we studied, two strains were obtained from MSM patients and these strains showed Ser-91-Thr mutation which was not seen in the other strains. This mutation has been reported in earlier studies but the information on the source of strains was not available in these studies (Shultz TR et al 2001, Ilina EN et al 2008, Wang B et al 2006). Thus it would be worthwhile to study if this mutation is group specific. This has been the first report of this mutation from India.

Since the discovery of the MtrR repressor and the realization that it controls *mtrCDE* expression in *N. gonorrhoeae*, several studies have examined the impact of *mtr* mutations on gene expression and antibiotic resistance endowed by the MtrC-MrD-MtrE efflux pump (Pan 1994). These mutations include missense mutations that cause radical amino acid changes (e.g., Ala-39→T or Gly-45→Asp) in the MtrR DNA-binding domain or mutations that impact the C-terminal region of MtrR-His-105→Tyr and the Glu-202→Gly mutation that was isolated from experimentally infected mice (Warner *et al.*, 2007) & from clinical specimens.
In general, these coding sequence mutations elevate gonococcal resistance to structurally diverse hydrophobic antimicrobials by 2- to 4-fold. In contrast, promoter mutations are also frequently isolated in the absence of coding sequence mutations, and some enhance antimicrobial resistance by ≥ 10 fold. Mutations in the mtrR with the resultant overexpression of the MtrRCDE efflux system are reportedly implicated in chromosomally mediated low-level resistance to penicillin and tetracycline and reduced susceptibility to fluoroquinolones (Zarantonelli et al 2001, Veal et al 2002). Here, we have reported quinolone resistant *N. gonorrhoeae* carrying single or multiple mutations in the *mtrR* gene of the MtrRCDE efflux system.

In this study, we observed 48.4% *N. gonorrhoeae* isolates showed mutations in the mtrR region. Our results showed that nearly 50% of the isolates showed mutations in the *mtrR* gene.

Sequence analysis of the *mtrR* coding region showed the presence of mutations which were also described by others (Vereshchagin et al 2004, Dewi el 2004). In the current study, we observed Leu-47→Pro, Thr-86→Ala, Arg-98→Gln mutation which has not been reported earlier. The mutation Gly-45→Asp was found in 26.5% of *N. gonorrhoeae* isolates. Others have reported Gly-45→Asp mutations varying from 17.8% to 42.1% (Bet et al 2004, Vereshchagin et al 2004). Mutations in *mtrR* with the resultant over expression of the MrCDE efflux pump are reportedly implicated in reduced susceptibility to fluoroquinolones (Veal et al 2002), we also found a good correlation between the *mtrR* mutations (i.e., Gly-45→Asp, Ala-39→Thr, Tyr-105→His) and the quinolone resistance. *N. gonorrhoeae* isolates resistant to various quinolones showed multiple mutations in the *mtrR* region. The strains resistant to all antibiotics were significantly (p<0.01) associated with presence of multiple mutations in the *mtrR* genes.
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Phylogenetic analysis of the strains for gyrA and parC gene showed that the strains isolated from Pune were different than the strains isolated from other regions which may be partially explained by the fact that the mutation Asp-95→Asn was restricted to Pune strains only. Phylogenetic analysis of the mtrR gene showed diversity among the strains isolated from different regions.