

SUMMARY

Approximately 448 million new cases of curable STIs (Chlamydia infection, gonorrhoea, syphilis and trichomoniasis) are reported annually with 75% to 80% occur in developing countries. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are two major STD causing pathogens. Signs and symptoms of the disease include conjunctivitis, urethritis, epididymitis, proctitis, and salpingitis. If untreated, infection leads to infertility, low fetal birth weight, premature delivery and pelvic inflammatory disorder. The infection can also disseminate via blood stream to other organs leading to pain in joints (arthritis), degeneration of heart valve, gonococcal meningitis etc. It is also a leading cause of neonatal blindness. Infection due *Neisseria gonorrhoeae* and *Chlamydia trachomatis* also facilitates acquisition of HIV and HPV. Approximately 70% to 80% women and 50% men remain asymptomatic. Co-infection of *Chlamydia* and *Neisseria* is readily reported and infection rates are higher in women in adolescents. Although commercial kits are available, their high cost and requirement of technical expertise make them far from use in developing countries like India. Culture method and gram stain remains the method of choice, but culture method takes 2-3 days for results to come before treatment could be recommended. This leads in persistence and spread of disease in the society. Thus WHO has recommended syndromic management of treatment of *Chlamydia* and *Neisseria*. There occurs overlap in the spectrum of symptoms due to infection by *Chlamydia* and *Neisseria* and with various other STI causing pathogens. Discharge and pain in lower abdomen are also common during pregnancy. The guidelines for syndromic management issued by WHO are neither specific nor sensitive for the two pathogens. Also infections by *Chlamydia* and *Neisseria* are highly asymptomatic thus treatment only on the basis of vaginal discharge syndrome not only misses out asymptomatic infections but may also leads to overtreatment. This further 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.

Penicillin and tetracycline were widely used antibiotics till 1980's, but due to emergence of resistant strains of *Neisseria*, these were discontinued in most of the countries. Thus, newer β -lactam antibiotics like ceftriaxone, cefixime, fluoroquinolones, ciprofloxacin,

ofloxacin, and levofloxacin are recommended for treatment. However, *Neisseria* with reduced susceptibility towards fluoroquinolones, ceftriaxone and cefixime are reported from different parts of the world. South East Asia is reported to be the centre of origin of several resistant strains probably due to syndromic management of gonorrhea. Continuous appearance of multi drug resistant strains as well as strains resistant to new drugs is alarming. Thus, there is an urgent need for economic, rapid, point of care test for co-detection of *Chlamydia* and *Neisseria* as well as an indepth understanding of gonococcal antibiotic resistance mechanisms is essential for the development of new and effective antimicrobial agents.

The major objective of this work was to develop an in-house PCR assay that is cost effective and has high sensitivity and specificity and rapid so that it can be used for co-detection of *C. trachomatis* and *N. gonorrhoeae* infection. Patients (n=812) having symptoms and expecting treatment were enrolled in the study. Using uPCR, dPCR and commercial assays our study suggests high prevalence of *Neisseria* (27.8%) and *Chlamydia* (26.3%) amongst symptomatic women seeking treatment. Most of the women were from age group of 18-30 years. In-house uniplex PCR (uPCR) was developed and evaluated against commercially available, Roche Amplicor MWP kit for detection of *Neisseria gonorrhoeae* using unique sequence of *orf1* gene as target. Our method was found to be 95.8% sensitive and 97.9% specific. Once uPCR was evaluated, dPCR was developed and standardized for co detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Uniplex PCR for *Chlamydia trachomatis* was already established in lab against *gyrA* gene. Samples (n=533) were evaluated for dPCR against uPCR. Discrepant samples (n=24) were resolved using Roche Amplicor MWP Kit. Based on the concordance between uPCR and Roche assay, these samples were scored positive for either *Chlamydia* or *Neisseria*. 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 %).

Lack of resources and technical expertise makes diagnosis of STDs difficult in rural areas of India. Thus, to make our method as test of choice, PCR premix-reagents (2X) have been stabilized such that assay reaction mixture can be kept at 4°C for upto 4 months, making it an easy assay to be carried out in peripheral laboratories where

deep freezers are not available. Further PCR amplification using gDNA isolated from dry swabs as mode of sample collection and transportation was also standardized. We found that dry swabs work equally good for collection of cervical swab samples as wet swabs (swabs in PBS with 1mM). Since 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, the assay so designed will be of great use for easy, rapid diagnosis and surveillance programs in developing countries.

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, indigenous fluorescent based dark reader for detection. To look for relative load of infection, the intensity of fluorescence can be measured by ELISA reader. Looking for fluorescence 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 carry-over contamination. The present study offers an easy, rapid and economical NAAT-based assay to study the burden and pattern of infection by *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

Although multidrug resistance strains have been reported from Delhi and other parts of India, there is paucity of information on the mutational patterns of the above mentioned genes in these isolates. In the next objective of the work, we tried to envisage the plausible mechanism of antibiotic resistance in clinical isolates of *Neisseria gonorrhoeae*. Resistance could be plasmid mediated or chromosomal mediated. Plasmid mediated resistance is confined to penicillin and tetracycline. Mutations in various genes (*gyrA*, *parC*, *penA*, *mtrR*, *mtrE*, *porB* etc.) act synergistically for providing chromosomal

mediated resistance to *Neisseria*. Therefore, to understand how mutations in these genes contribute to resistance, we in present study explored the co-relation between mutations in various genes (*penA*, *mtrR*, *mtrE* and *porB*) responsible for antibiotic resistance with the change in MIC values for penicillin, tetracycline and ceftriaxone. Fluorescence assay and electrophoretic mobility shift assay were carried out to study the effect of mutations in MtrR on its biological activity. Attempts have been made to *in-silico* model the structure of MtrR to understand how mutations affect its interaction with DNA. Protein structure of wild type and mutant MtrR was compared to understand the structural effect of mutations. We also studied the binding of wild type and mutant MtrR with penicillin as well as DNA using both *in silico* approach and fluorimetric assay.

Plasmid mediated resistance to penicillin is one of the major mechanism and in India, we found African type of plasmid in all the resistant isolates. Multi drug chromosomal mediated resistance was found to be highly prevalent in clinical isolates. All the clinical isolates (n=28) studied carried mutation in one or more genes and played role in imparting resistance. PIB phenotype (88.88%; 24/27) was found to be far more prevalent than PIA phenotype for *porB* gene. None of the resistant isolate carried mutations in PIA phenotype (11.11%; 3/27). The mutations at G120 and A121 position of loop3 of the mature PorB protein has been documented for increased resistance to several antibiotics. Mutations either at G120/ A121 or in both amino acids was reported both in isolates resistant as well as reduced susceptible/susceptible for penicillin as well as tetracycline. A121S mutation was present in five resistant isolates only. We here report a novel mutation of N122K (present always along with A121S) residing in loop3 which may be responsible for altered pore size or permeability.

Mutations both within and outside the transpeptidase domain (AA340-570) of *penA* gene encoding for penicillin binding protein are responsible for providing resistance for penicillin and other beta lactams. An insertion of aspartic acid at 346th position in transpeptidase domain is shown to be associated with the altered structure and acylation of β lactam antibiotics. All the isolates showed Asp 346 insertion and mutations F504K, A510V and A516G. Mutant P551L was found to be one of the most frequent mutations while only two isolates had H541N mutation. In our study, we also found two novel mutations G550R, N562L and S567. We had also observed new substitution at I566N

along with the previously reported mutations, thus giving rise to new mutation patterns (Table 6). Mosaic pattern IX (11/26; 42.3%) and II (10/26; 38.46%) were most prevalent patterns observed in isolates from Delhi hospitals. Isolates which carried triad of mutations but were PPNG negative also showed either reduced susceptibility or were sensitive for penicillin supporting the previous contention that single mutations at F504K, A510V and A516G are not responsible for development of resistance against penicillin and ceftriaxone. It has been previously shown that substitutions at F504L and P551S lead to decreased penicillin acylation rates but mechanism of G542S has not been elucidated. Thus, in present study we had tried to elucidate mechanism using homology modeling and docking methods. Using crystal structure of PBP2 and modeling techniques, the mutant protein was generated and active sites were compared. It was observed that unlike wild type, serine 542 formed hydrogen bond with tyrosine 544 whose side chain points into the active site. This may alter the spatial arrangement of the tyrosine with respect to active site and thus is responsible for higher affinity of the mutant for penicillin.

Mutations in the structural genes of Mtr operon encoding for MtrCDE efflux pump or its regulatory protein (MtrR) as well as in promoter region could result in increased efflux of drug and thereby make the cell resistant to the said drug. MtrE encodes for outer membrane part of the efflux pump. K181E, K191R and I429S are mutations found in various clinical isolates. K181 resides in non conserved region of MtrE and is found in resistant as well as susceptible isolates suggesting that this mutation has no direct contribution in imparting antimicrobial resistance. K191R and I429S mutations reside in conserved region of MtrE. The above mutation resides in the lining of intraprotomer groove of the pump which binds with the MtrC and plays role in the channel formation and thus the function of the pump. Comparing the resistance pattern of isolates, it appears that K191R substitution may be crucial in imparting high resistance towards penicillin and tetracycline. We also compared the MIC values for tetracycline in PPNG positive isolates and found that isolates with K181E and K191R double mutants were less susceptible in comparison to K181E and K181E and I429S double mutant indicating some role of K191R in resistance.

The most common mutation observed in MtrR gene was H105Y present in 11 out of 25 clinical isolates and was mostly coupled with A/T deletion in promoter region

(10/11 isolates). This mutation resides in C Terminal of the protein possessing dimerization domain as well as ligand binding cavity. The G45D and A39T mutations in DNA binding domain of MtrR protein are known to provide high resistance against penicillin and tetracycline. Only G45D mutation was observed in clinical isolates in present study. In present work L33V, a novel mutation is also observed in two tetracycline resistant clinical isolates along with G45D mutation. To decipher the role of L33V mutation, we compared the MIC values of the isolates which possess similar mutation pattern for rest of the genes studied (*penA*, *mtrE*, *porB* and *mtrR* promoter region). Thus comparing the MIC value for tetracycline we found that single mutant had higher MIC value of 12 µg/ml as compared to 8 µg/ml possessed by the double mutants. This has led us to hypothesize that L33V is not advantageous to *Neisseria* with respect to antibiotic resistance. Although mechanism of action of G45D mutant MtrR is well recognized (i.e abrogation of binding of the protein), no direct evidence elucidating the mechanism of action H105Y mutation is known. We hypothesize two plausible mechanisms for the action of this mutation.

- MtrR is known to bind as homodimer to its promoter. Mutation H105Y may affects the dimer formation and thus its binding with DNA.
- H105Y may alter the binding of antibiotics in ligand binding cavity. The binding of antibiotics like penicillin may induces conformational change and thus its binding with the DNA.

With a view to understand the functional implications of mutant MtrR, we decided to check whether mutations in MtrR are responsible for any conformational change in the protein and if so, does it affect its interaction with DNA and small ligands like penicillin. Therefore, to identify a plethora of processes in which MtrR has some role to play, we selected the following objectives:

- Bioinformatics approach to study the change in the conformation of MtrR and its interaction with penicillin and promoter DNA
- Comparative analysis of the DNA binding activity of wild type and mutant MtrR protein using purified protein.

To achieve these objectives, *in-silico* modelling of wild type and mutant MtrR proteins was carried out using swiss PDB server and Discovery studio. Conformational change was studied by superimposing mutant protein over wild type. Change in conformation majorly in Helix 1 (N terminal) and helix 4 (C terminal) was observed. Size of the ligand binding cavity decreased from 356 Å³ of wild type to 256 Å³ in H105Y mutant. This change is in contrast to G45D mutant wherein a minor increase is observed in the volume of the cavity (364 Å³). We also observed that presence of penicillin has an effect on MtrR conformation and/or DNA binding as suggested by fluorescence spectrophotometric results. The assays were performed using purified wild type and H105Y mutant MtrR titrated with increasing concentration of penicillin which showed shift in lambda max is more pronounced in case of mutant. Appearance of second peak is observed around 420 nm in case of H105Y mutant MtrR. Both of these results suggested altered conformation of the mutant. We then docked penicillin in wild type as well as H105Y mutant MtrR to check if penicillin affects MtrR conformation by directly binding to it. Change in pose and thus interacting partners was observed in wild type and Mutant MtrR. On comparing the various parameters, H105Y mutant appears to possess higher affinity for penicillin probably due to hydrogen bond formed by substituted Tyrosine 105 thus stabilizing the protein penicillin complex.

Next we checked the effect of the mutation on DNA binding of MtrR using EMSA, fluorescence spectrophotometric assays and *in silico* analysis. Electrophoretic mobility gel shift assay performed using crude lysate of over expressed wild type, mutant G45D and mutant H105Y showed that higher concentration of mutant H105Y protein is required to bind with its promoter DNA to cause visible gel shift. Competition assays suggested that far less DNA is required to compete with mutant protein as compared to wild type suggesting H105Y has either low DNA binding affinity or increased rate of dissociation. Fluorescence spectrophotometry experiments clearly indicated that unlike wild type MtrR, H105Y mutant MtrR failed to bind effectively to its binding site on the promoter. The binding affinity of the mutated MtrR with promoter was about 1.13 folds lower than that of wild type. Although change is small, it could be possible that two mechanisms act synergistically and

binding is effectively altered in the presence of penicillin. Further to decipher the change in hydrogen bond profile of protein DNA complex assembly, MtrR was docked with the promoter DNA. Since homodimer binds to the promoter, we first docked monomers of wild type and the mutants to form homodimers. Homodimer of H105Y mutant was most stable energetically, but increased centre to centre distance between helix 1 and helix 1' of two monomers suggested altered alignment of the two monomers on the pseudo-repeat binding site on the DNA which may reduce its binding with DNA as compared to that of wild type MtrR. To check this hypothesis, protein DNA docking was performed using refined monomer form from the dimer complex. Best pose was screened using structural similarity with QacR (complexed with its promoter), another member of TetR family. No optimal binding pose was obtained for G45D mutant as expected as this amino acid is critical for DNA binding. For H105Y mutant, two best poses which imperfectly resembled that of wild type and QacR DNA complex were selected. This may be explained in the light that due to increased centre to centre distance, residues of helix3 are not able to reside close to DNA residues in second half site of pseudodirect repeat of the promoter. Hydrogen bond analysis shows formation of different hydrogen bond between wild type DNA complex and H105Y mutant DNA complex. Based on these results, we envisage that although different hydrogen bonds are formed between H105Ymutant MtrR and DNA complex, they may be sufficient to provide only partial stability to the complex which may account for the observed protein-DNA binding using EMSA and fluorescence spectrophotometry. It remains to see whether mutation in H105Y affects the rate of dissociation from DNA which will indirectly result in increased expression of the operon. Thus even a single amino acid change outside the DNA binding domain causes a conformational change in the binding domain of MtrR both in the presence and absence of penicillin which may be responsible for altered DNA-protein interaction.