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Poor Correlation of Diversified MDR Genes in *Gonococci* Plasmids: Does Alteration in Chromosomal DEGs, PBP2 and Target Mutations Sufficient to Widespread Multi-Resistance in *Neisseria gonorrhoeae*?

Asit Kumar Chakraborty*

Department of Biotechnology and Biochemistry, Genetic Engineering Laboratory, Vidyasagar University, Midnapore, India

Abstract

Spread of ceftriaxone and ciprofloxacin resistant *gonococci* diseases demand new drug development as research have contradicted potent carbapenem and aminoglycoside antibiotics against *Neisseria gonorrhoeae* infections. Pubmed and GenBank analysis demonstrated only bla TEM and tetM genes present in *N. gonorrhoeae* plasmids where as in most MDR Enterobacteriaceae, hundred diversified beta-lactamase genes (blaOXA, blaCTX-M, blaCMY and blaNDM1) as well as many drug modified genes (aacA1/C1, catB3, aph, strA/B, sul1/2, aad, aph and aac(6')-1b) are frequent in plasmids and chromosome. Thus existing knowledge on *gonococci* mdr genes is limited and merely few chromosomal drug efflux genes (DEGs=ermAB, mtrCDE, macAB etc.) and penicillin binding proteins (PBPs=ponA and penA) have assigned as cause of multi-resistance. It appeared that *N. gonorrhoeae* had limited life cycle outside the host limiting conjugation with other MDR-bacteria to acquire mdr genes easily. BLAST-search confirmed that every MDR *N. gonorrhoeae* genome did carry mtrD RND transporter gene linked to mtrC outer membrane efflux gene (MFS) similar to *P. aeruginosa* mexAB-family transporters. Further, macA/B transporters are involved in macrolide drug efflux and many mutations in penA, gyrA, mtrR and porB genes are maximum in MDR strains although mtrF and norA efflux genes are infrequent. We argue that plasmid mediated multi-resistance in gonococcal diseases needs to be reinvestigated and mutation theory (penA, gyrA, mtrR) may not sufficient to prove the worldwide spread of multi-drug resistant STDs.

Keywords: MDR *gonorrhoea*; Chromosomal drug efflux genes; acrAB-mexAB; Penicillin binding proteins; blaTEM and tetM

Introduction

Sexually transmitted diseases (STD) play a critical role in society as most STDs are unnoticed and are delayed treatment due to drug unresponsiveness. *Gonorrhoea* transmission is globally increasing (62 million/year) as marriage age is increased from 20-25 year age group to 25-35 age groups due to higher education, job insecurity and high cost of urban houses [1]. Likely, most young people fall prey to uncontrolled sex partners with hygienic sub-standard and one contact with the mouth, penis, vagina, or anus of an infected sexual partner is sufficient for MDR *gonorrhoea* disease [2]. *Gonorrhoea* is caused by diplococcic gram (-) bacterium *Neisseria gonorrhoeae* which evade the epithelial cells of endocervix, urethra, rectum, oropharynx, nasopharynx and conjunctiva [3,4]. Major symptoms are exudation of pus from genitals as *gonococci* evade host defences by antigenic variation and capsule formation [5,6].

During our studies with MDR-bacteria of Kolkata water bodies (Ganga River), a huge blaTEM, blaCTX-M, acrAB, sul1, tetA/C, strA/B, mcr, cat3B, aac6'-1b, aacC2 etc MDR genes were detected in conjugated plasmids as found in most MDR Enterobacteriaceae studied worldwide [7]. However, our search of 20 bla (beta-lactamase) genes in *N. gonorrhoeae* plasmids and chromosomes failed except blaTEM [8,9] and tetM [10]. Single copy penA and ponA genes encoding penicillin binding proteins were located in chromosome but hardly satisfy with the all cephalosporin resistance in *N. gonorrhoeae*. It appeared that 85 years antibiotic chemicals insult in hosts had favoured many chromosomal changes over-expressing mutated porins, PBPs, rRNAs, gyrA/B and parC/E as well as DEGs (macA/B, mtrF, emrE, and mtrCDE) differing that found in many MDR Enterobacteriaceae which mostly mediated by large MDR conjugative plasmids [11].

Recently, we have published the MDR bacterial contamination in rain water, drain water, Ganga River water and Bay of Bengal sea water [7,12,13] and PubMed and GenBank search indicated diversified beta-lactamase genes with 500 mutations as well as many heterogeneities intent genes, aac genes and mex genes [13-15]. We noticed that nature of MDR genes implicated in gonococcus multi-resistance infections was different and interestingly, most potent carbapenem drugs (imipenem) were contradicted for gonococcal treatment. Few plasmids were recovered on BLAST search carrying only blaTEM beta-lactamase and tetracycline binding protein, tetM. There was no trace of OXA, CTX-M, and CMY, NDM1 type beta-lactamases, neither acetyl transferases (AAC), phosphotransferases (APH) nor drug transporter like acrAB and mexAB/CD/EF genes. Such MDR genes were very abundant (>95% all clinical isolates) causing multi-drug resistance in most Enterobacteriaceae were studied so far [7,12]. Recent outbreaks of pan drug resistant species suggested that mexAB/AcrAB types drug transporter like mtrCDE genes regulated by mtrR/tetR type repressor for over expression and multi-resistance although many mutations in porB, ponA, penA and gyrA genes have also been suggested [16,17,1].

History of drug development and drug failure against gonococcal diseases

Every medicine had been prescribed since the discovery of penicillin antibiotics in 1928 by Alexander Flaming and thereafter by Dr. Selman Waksman discovered over twenty antibiotics including streptomycin. So

***Corresponding author:** Asit Kumar Chakraborty, Department of Biotechnology and Biochemistry, Genetic Engineering Laboratory, Oriental Institute of Science & Technology (OIST), Vidyasagar University, Midnapore 721102, West Bengal, India, Tel: 91+9339609268; E-mail: chakraakc@gmail.com

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sulphonamides was introduced in 1930, sulfa-drugs in 1940, penicillin's in 1943, tetracycline in 1945, streptomycin and chloramphenicol in 1949, erythromycin in 1952, ciprofloxacin in 1965 and so on had been prescribed for gonococcus infections. Sadly, gonococcus drug resistance appeared as early as in 1958 but confirmed in 1976 when blaTEM beta-lactamase gene was recovered from gonococcal plasmid in Asia and Africa [18,19]. Streptomycin and tetracycline resistance appeared between 1958-1962 followed by aminoglycosides resistance in 1980 [20], ciprofloxacin resistance in 1985 [21] and azithromycin resistance between 1995-1999 [22]. So modified derivatives of penicillin like cefixime, ceftriaxone and cefotaxime (cephalosporins) were in centre stage of gonococcus infections for decades [23]. Tetracycline was useless as tetM gene was discovered in plasmids and ciprofloxacin became useless when gyrA, parC and parE mutations were confirmed in 1990. But cefixime was removed as blaCTX-M gene was confirmed in gonococcal plasmids. Many mutations in the membrane porin genes (porB) were implicated in cephalosporins, tetracycline and aminoglycosides drug resistance.

The recent emergence of the first *N. gonorrhoeae* "superbug" strain in Japan (H041/ MLST ST7363) was shown to exhibit extremely resistance to all antibiotics including cefixime (MIC=8 μg/ml), and ceftriaxone (MIC=2-4 μg/ml) as well against other antibiotics [24,25]. *N. gonorrhoeae* F89 strain was isolated in France with high resistance properties and also in Spain. *Gonococci* were also acquired various different types of antimicrobial resistance (AMR) due to drug inactivation, modification of drug targets, changing permeability barriers modifying porin genes and drug efflux genes like ermAB, macAB, mtrCDF and norA [26]. macAB gene was implicated in macrolide drug resistance as well as farB and mtrF. Chromosomal-mediated resistance to penicillin involves modification of the penicillin binding proteins (PBP2/1 or penA and ponA genes coupled with mutations in porins (porB gene).

Treatment options of gonococcal infections

In 2000 ciprofloxacin resistance first reported in Hawaii of USA, followed by drug resistance among homosexuals in 2004. Ciprofloxacin resistance was increased 0.6% in 2001 to 6.7% in 2007 among homosexuals. In 2006 13.8% all clinical *N. gonorrhoeae* were ciprofloxacin resistant leading to withdraw of ciprofloxacin for *gonorrhoea* treatment in 2007 [26]. In 2010 ceftriaxone plus azithromycin or doxycycline were recommended for gonococcal treatment. But new cases of gonococcus infections in the USA were increased to 820, 000 demonstrating the need for new drug development. The activity of broad spectrum fluroquinolones in gonococcal treatment were reported as ciproflox acin<ofloxacin<norfloxacin< levofloxacin<lomofloxacin<gemiflox acin. Patients who can tolerate neither ceftrioxane nor ciprofloxacin, spectinomycin in 2 g i.m. single dose has been recommended. Gentamycin+Azithromycin or Gemifloxacin (320 mg)+Azithromycin (2 g) are used in many cases but 20-30% patients might suffer nausea and vomiting [27]. As the situation in the USA is very grim for STD, newly experimental drugs like ETX0914 (Entasis Therapeutics) may be cleared by FDA very soon. Matsumoto et al. have revealed that synthetic efflux pump inhibitor, D13-9001 acts synergistically with aztreonam, ciprofloxacin, and erythromycin against the MexAB-OprM mediated MDR *Pseudomonas aeruginosa* and PAβN acts synergistically, especially with erythromycin and polymyxin B [28]. This result was indicated that drug efflux inhibitors might be used against MDR gonococcal diseases.

Carbapenem drugs as choice against *Neisseria gonorrhoeae*

Carbapenem drugs must be recommended against *N. gonorrhoeae* infections as merropenem was found very successful against gram (-) infections. However, recent reports showed that a single dose of imipenem-cilastatin cured 116 of 122 men with uncomplicated *N. gonorrhoea* as blaKPC/blaVIM type of class B beta-lactamases were absent in such clinical isolates. Further study indicated that penA gene coding for PBP2 with mutations in A501, G545 and P551 might contribute to extended spectrum cephalosporins resistance in *N. gonorrhoeae*. Similarly, rpsJ gene of ribosomal protein S10 and mtrR mutations may also involve in aminoglycoside resistance [29,30]. In 2007, about 350000 cases of gonococcus infections were reported in the United States which was increased few fold in recent years indicating the importance of new drug development. Unemo M et al. studied XRD gonococcal strain H041 and F89 with high level ceftriaxone resistant and ertapenem appeared promising drug. However, result indicated carbapenem drugs had failed to give superior pharmaco kinetic parameters to clear gonococcal infections as compared in ceftriaxone, the best drug recommended yet.

Results

Complete genome sequencing of *Neisseria gonorrhoeae*

The first complete genome of *N. gonorrhoeae* was done in 2000 and the strain NCCP11945 was done in 2001 [31]. Many laboratory strains were sequenced and many mdr genes were detected in complete genome [32]. Recently, WHO supported Sanger Institute of UK has completed many full length genome sequencing of MDR Neisseria reference clones and many mutations are identified in penA, ponB, porB, mtrR and gyrA mdr genes (Table 1).

Many MDR genes were poorly assimilated in gonococcal plasmids and chromosome

Many MDR genes were found in single conjugative plasmids (50-500 kb) that had been fully sequenced from *Escherichia coli, Salmonella enterica* and *Pseudomonas aeruginosa*. At least 20 types β-lactamase genes (TEM, OXA, CMY, NDM1, KPC, VIM, IMP, FOX, ACC etc.) with ten thousands mutations were detected in many Enterobacteriaceae plasmids [33]. As we compared with the available very few *N. gonorrhoeae* plasmids, only blaTEM-1 and blaTEM-135 genes were detected and two mutations were predominant as demonstrated in Table 1 [34-38]. Further only tetM gene [22] was found but no tetA, tetC, catB3, strA/B, sul1/2, acrAB, mdtA, mcr-1, vanA, arr3, aacA1/C1, aphA4, aadA2 etc genes highlighted in BLAST search. Our search however, was confirmed acrB/mexB RND drug transporter (mtrD) as important candidate of MDR gene in *N. gonorrhoeae* which actively transport drugs as tripartite protein complex involving acrA or mexA (mtrC) as well as TolC or OprM like membrane proteins (mtrX) as demonstrated in Table 2. Figure 1 demonstrated the seq2 BLAST sequence similarity between acrB of Salmonella and mexB of Neisseria which was known as mtrD. The mechanisms of drug resistance in *N. gonorrhoeae* are different and depicted in Figure 2. The correct positions of different mdr genes and DEGs was depicted in Figure 3 pinpointing the localization of mtrCD-mtrX (mexAB-oprM type), mtrF, macAB and ermAB (farAB) mdr drug-efflux genes in *Neisseria gonorrhoeae* FA19 (accession number CP012026; nt. 1-2232367).

Molecular mechanisms of penA genes in *N. gonorrhoeae*

Penicillin-resistant gonococcus strains had many point mutations in the penA gene which encodes PBP2 that binds strongly β-lactam antibiotics decreasing effective drug concentration and thus increasing AMR [39,40]. Penicillin binding protein (PBP2) with P551S and F504L mutations and aspartic acid insertion after amino acid 345 greatly

Major MDR plasmids sequenced in *Neisseria gonorrhoeae*					
Accession	Plasmid	Size, bp	Strain	MDR Gene	Protein id
L12242	pOZ101	2175	2903	tetM/644aa	nt. 213-2144
LT591899	P2/ Tn916	42004	WHO_G	tetM/639aa	SBO57235
LT591912	P3/ Tn916	42004	WHO_N	tetM/644aa	SBO57858
GU479466	pEP5289	42004	5289	tetM/644aa	ADF36634
LT592147	P2	5598	WHO_O	blaTEM/286aa	SBO58240
GU479464	pEP5050	7825	5050	tetM/644aa	ADF36620
NC_019211	pEM1	4868	GP08MUS	blaTEM/286aa	YP_006960556

Table 1: Localization of only two *mdr* genes in plasmids of *Neisseria gonorrhoeae*.

Survey of MDR Genes in the *Neisseria gonorrhoeae* Complete Genome (Protein Ids are given)								
Accession	Strain no	mtrD	penA	ponA	macB	ermB	porB	mtrF
AE004969	FA 1090	mexB	AAW90178	AAW88860	AAW90081	AAW90307	AAW90430	AAW90016
CP012028	35/02	AKP14429/8	AKP14613	AKP15416	AKP14514/3	AKP14822/1	AKP15153	AKP14432
CP012027	FA6140	AKP12794	AKP12982	AKP13752	AKP12880	AKP13148	AKP13281	AKP12798
CP012026	FA19	AKP10807	AKP11052	AKP11771	AKP10893	AKP1159	nd	nd
CP003909	MS11	EEZ48388	EEZ48220	EEZ47070	EEZ48314	AGU85171	EEZ48806.2	EEZ48387
CP001050	NCCP11945	AF30251	ACF30471	ACF28840	ACF30348	ACF30680	ACF31060	ACF30250
CP016016	34530	ANJ50411	ANJ50573	ANJ49290	ANJ50489	ANJ50724	ANJ51042*	ANJ50416
LT592161	WHO_Y	SBO69656	SBO73065	SBO60086	SBO70245	SBO74513	SBO76109	SBO69682
LT592159	WHO_U	SBO68533	SBO70018	SBO58843	SBO69230	SBO71116	SBO72388	SBO68571
LT592153	WHO_Z	SBO69419	SBO71709	SBO60042	SBO70015	SBO74544	SBO76145	SBO69401
LT591897	WHO_F	SBO21786	SBO22365	SBO18214	SBO22019	SBO22721	SBO23078	SBO21795
LT591901	WHO_ L	SBO49030	SBO56999	SBO47370	SBOo52892	SBO56120	SB)55148 -	SBO49018

Table 2: Confirmation of MexA/B proteins in all *Neisseria gonorrhoeae* genome: MexAB efflux pump is also detected in strain numbers.

```
SE:acrB    MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPAVTISATYPGADAKTVQDT  60
           M  FFIDRPIFAWVI+I I+ AG   I  LPV+QYP++A P +T+ A YPGA A+ ++ +
NG:mexB    MAKFFIDRPIFAWVISIFIIAAGIFGIKSLPVSQYPSVAAPTITLHAIYPGASAQVMEGS  60

SE:acrB    VTQVIEQNMNGIDNLMYMSSNSDSTGTVQITLTFESGTDADIAQVQVQNKLQLAMPLLPQ  120
           V  VIE+NMNG++ L YMS+++DS+G+  ++LTF   TD ++AQV+VQNKL   +  LP
NG:mexB    VLSVIERNMNGVEGLDYMSTSADSSGSGSVSLTFTPDTDENLAQVEVQNKLSEVLSTLPA  120

SE:acrB    EVQQQGVSVEKSSSSFLMVVGVINTDGTMTQEDISDYVAANMKDPISRTSGVGDVQLFGS  180
            VQQ GV+V K+ S+FLM+V ++++D   + E+++DY   N+   + R  GVG V+LFG+
NG:mexB    TVQQYGVTVSKARSNFLMIV-MLSSD-VQSTEEMNDYAQRNVVPELQRIEGVGQVRLFGA  178

SE:acrB    QYAMRIWMNPTELTKYQLTPVDVINAIKAQNAQVAAGQLGGTPPVKGQQLNASIIAQTRL  240
           Q AMRIW++P +L  Y L+  DV +A+ AQN Q++AG +G  P V+GQ + A++ AQ +L
NG:mexB    QRAMRIWVDPKKLQNYNLSFADVGSALSAQNIQISAGSIGSLPAVRGQTVTATVTAQGQL  238

SE:acrB    TSTDEFGKILLKVNQDGSQVRLRDVAKIELGGENYDVIAKFNGQPASGLGIKLATGANAL  300
            + +EFG ++L+ N DGS + L+DVAK+ LG E+Y    + NG   +G+ + L+   NA+
NG:mexB    GTAEEFGNVILRANTDGSNIYLKDVAKVGLGMEDYSSSTRLNGVNTTGMAVMLSNSGNAM  298

SE:acrB    DTATAIRAELKKMEPFFPPGMKIVYPYDTTPFVKISIHEVVKTLVEAIILVFLVMYLFLQ  360
            TA A++  L  +E +FP GM    PYDT+ FV+ISI +V+ TL+EA++LVF+VMYLFLQ
NG:mexB    ATAKAVKERLAVLEKYFPQGMSWKIPYDTSKFVEISIEKVIHTLIEAMVLVFVVMYLFLQ  358
```

Figure 1: Seq-2 sequence similarity between Salmonella enterica and Neisseria gonorrhoeae mexB proteins. Major GenBank data have demonstrated mtrCD as mexAB related proteins which have similarity to acrAB drug transporters as well.

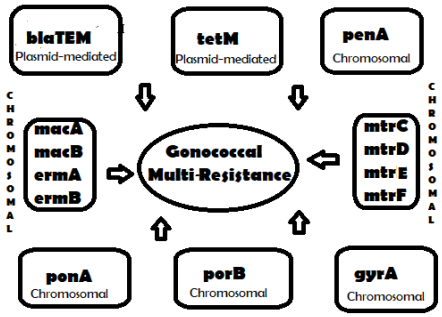

Figure 2: MDR Genes implicated in multi-resistance of *Neisseria gonorrhoeae* mediated STD. We question where are hundreds *cat, tet, strAB, sul1/2, aacA1, aacC2, blaOXA, blaCTX-M, blaNDM1, mdr* genes that frequently have observed in other species MDR plasmids and chromosome?

affected the use of cephalosporins as treatment options [41]. Further analysis of penA genes found more mutations than expected (I312M, V316T, N328T, S342A, S353T, R412Q, A502V, F504L, N513Y, G543S, A550T, P552S/L and K556Q) (Figure 4). Multidrug-resistant H041 and WHO_Z strain penA genes are highly mutated with >50 mutations (protein ids. BAK19153, SBO71709) as compared to WHO_L strains (protein id. SBO56999) that has acquired few mutations (A402V, F405V, A411V, A417T, and G543S) as compared to wild type strain (protein id. AAA25463). Cephalosporin resistant *N. gonorrhoeae* strain A3210 penA protein (protein id. ANI26527) has moderate mutations at the C-terminal as compared to MS11 strain. Similarly, many mutations in penC gene have been implicated as AMR inducer (Q172E, N648S, N432S, N648S, S341N, D494N and S341S) implying

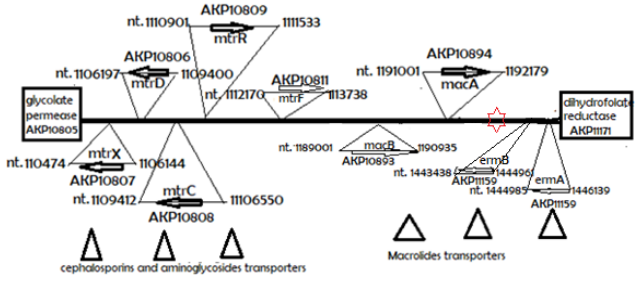

Figure 3: Localization of mtrCD-mtrX (mexAB-oprM type), mtrF, macAB and ermAB (farAB) mdr drug-efflux genes in *Neisseria gonorrhoeae* FA19 (accession number CP012026; nt. 1-2232367).

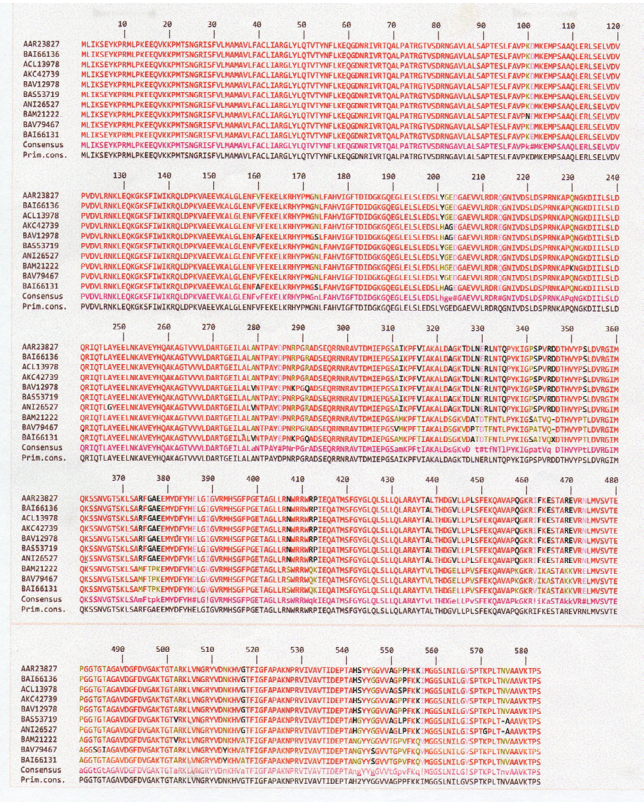

Figure 4: Multiple alignment of few penA proteins of *Neisseria gonorrhoeae* to demonstrate many mutations.

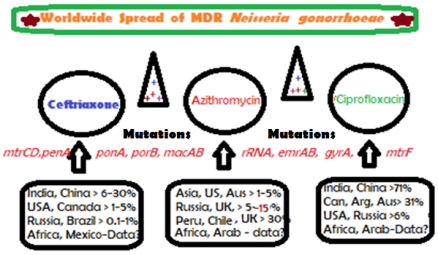

Figure 5: High rate worldwide emergence of multidrug-resistance STD diseases. The 2014 GASP (WHO) data indicated a massive spread of gonococcal diseases with all three best drugs (ceftriaxone, azithromycin and ciprofloxacin) resistant species. India and China got worse hit including American and European countries and also African countries whose data was limited.

multi-resistance was associated with other *mdr* genes like potent drug transporter, mtrCD [42]. Ceftrioxane resistance was implicated for mutations in multiple loci of *Neisseria gonorrhoeae* isolates at the PIB, PBP2 and mtrR genes [43,44]. Recently, many mutations in blaTEM gene like M182T, P14S/L, G228S and Q269K were found in gonococcal plasmids with 8%, 4.5%, 1.3% and 0.6% frequency respectively but 80% isolates were blaTEM-1 variant like blaTEM-135 (18). The wide spread of ceftriaxone, azithromycin and ciprofloxacin was depicted in Figure 5 and India had worse hit. Analysis suggested that *N. gonorrhoeae* has no beta-lactamase genes like blaCTX-M, blaOXA, blaNDM1, and blaKPC those are highly involved in *Escherichia coli, Pseudomonas aeruginosa*. *Acinetobacter baumannii*, *Staphylococcus aureus* and *Salmonella enterica* multi-resistance inactivating penicillin, cephalosporin and carbapem drugs. Further, many drug acetyl transferases are assembled in MDR conjugative plasmids but no has genes has been implicated in *N. gonorrhoeae* [45]. Thus penA gene mutations and rearrangement theory still hold promise but contradictory to believe all beta-lactams resistance and a role of drug transporters (mtrCDE and emrAB) is presumptive as compared to mexAB/CD genes of *Pseudomonas aeruginosa*. Importantly, Neisseria meningitis penA gene mutations are also implicated in multi-resistance with 8-13 mutations like V5I, T69V, V81A, E107K, N129S, L131I, N150D, K178Q, K1179I, D183K and L195R (protein ids. WP_002234448, WP_002246789, WP_061726051). Figure 6 demonstrated the mutations of 301-360 amino acids of penA protein in different drug resistant strains and such changes keeping the drug binding normal or higher indeed unique. PenA protein of Neisseria flavecens and Neisseria dentiae have 37 and 55 mutations suggesting gene alteration is common phenomenon in bacterial species in response to adaptation. The mutation theory for PenA mutations in cephalosporins resistance may correlate with changes in α and β helical structures as shown in Figure 7. Such demonstration is important but not conclusive.

Molecular mechanism of ponA genes in *N. gonorrhoeae*

PenA gene and PonA genes encode penicillin binding protein

Figure 6: BLAST Seq2 alignment of 301-360 amino acids of penA protein of different strains of *Neisseria gonorrhoeae* demonstrating the chimera gene formation producing pan cephalosporin drug resistance isolates.

Figure 7: Secondary structure alteration of PenA protein of MDR Neisseria gonorrhoeae. H, E and T mean Helix, Sheet and Turn conformations.

type-2 and type-1 (PBPs) that also has been implicated in ceftrioxane and other cephalosporins resistance in *N. gonorrhoeae* [46]. For the inactivation of penicillin and cephalosporin drugs, ponA and penA genes must be over-expressed. Genome analysis suggested that a single gene was present with promoter activation. Biochemical characterization indicated that mutant enzyme could neutralize ceftrioxane more efficiently and thus >20 mutations accumulated in ponA genes giving multi-resistance and mutations are frequent similar to many mutations of blaOXA gene in other MDR Enterobacteriaceae [10].

Molecular mechanisms of porB genes in *N. gonorrhoeae*

PorB type genes were implicated in normal entry of drug and chemicals into *N. gonorrhoeae* cells. However, it was proved that mutations in the porB gene were sufficient to show the cefixime and imipenem resistance in *N. gonorrhoeae* due to reduced drug entry (Figure 8). Most isolates (282/289, 98%) contained the porB1b (NEIS2020) allele associated with decreased susceptibility to β-lactams and tetracycline with AMR conferred through non-synonymous substitutions in loop III of PorB [47]. A total of 31 distinct loop III regions were identified with those containing G120K and A121D/N mutations associated with resistant MIC values to penicillin and tetracycline. Interestingly, only 3/289 (1%) isolates contained amino acid substitution D526N found in pilQ (NEIS0408) associated with decreased susceptibility to cefixime and ceftriaxone, However, these isolates lacked mosaic penA (NEIS1753), mtrR (NEIS1635) and porB1b (NEIS2020) mutations (Figure 7). PilQ gene association in multi-resistance to be confirmed. Thus various penA, mtrR, porB and ponA mutations in *Neisseria gonorrhoeae* isolates were demonstrated with reduced susceptibility to cefixime or ceftriaxone (Tables 1 and 2).

Molecular mechanisms of drug efflux genes in *N. gonorrhoeae*

Chromosomal mtrCDE, FarA/B (ermA/B), macA/B, mtrF, and norM type's drug efflux pumps have been implicated in tetracycline and cephalosporin resistance in *N. gonorrhoeae* [48]. Most strains have all such genes but norM (Table 2). Over expression of the MtrC-MtrD-MtrE efflux pump due to an mtrR mutation is required for chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. However, mtrR de-repression due to C120T mutation in upstream of mtrC, have activated mtrCDE efflux pump in maintaining high AMR for many aminoglycosides and macrolides [49]. We presented the mutations of promoters of mtrR and mtrCD locus but mutations were rare (Figure 9) and also the hyper resistance WHO_Z strain showed only three mutations in mtrR (Figure 10). Interestingly, no mutations in the TATATAAT box of mtrCD and mtrR between WHO_L and WHO_Z strains indicating mutation theory of promoters failed (Figure 11). Our recent data suggested that acrAB and tetA highly activated in Kolkata MDR-bacteria [50,51] and have similarities to *Pseudomonas aeruginosa* mexAB, mexCD and mexEF drug transporter genes. In *Escherichia coli* acrA-acrB-TolC contributed the drug efflux pump giving resistance to beta-lactams, quinolones and aminoglycosides [51]. Analysis suggested that in *N. gonorrhoeae* mtrCDE multiple drug efflux genes were activated to show the XDR pattern drug resistance although it lacked many bla genes and aac, aad, aph types' aminoglycosides modifying genes as well as highly abundant strA/B, tetA/C, sul1/2 and catB3 genes. The drug efflux genes like macA/B, ermA/B, norA, and mtrF, were also implicated in gonococcal drug resistant and were sequenced in most genomes of MDR *N. gonorrhoeae* (Table 2). However, penA, ponA like penicillin binding proteins likely inactivated the penicillin drugs and porB protein mutation further restricted the entry of drugs into *N. gonorrhoeae* cytoplasm. VanA gene cluster implicated in vancomycin resistance and tetA/C type drug efflux genes involved in tetracycline resistance. Surprisingly, no blaOXA, blaDHA, blaIMP, blaKPC, blaSPM and blaNDM1 or blaCTX-M genes were not detected in genome analysis of *N. gonorrhoeae*. Thus we concluded mtrCDE of *N. gonorrhoeae* are highly required for XDR type drug resistance having similarity 66% similarity to both *Escherichia coli* acrAB and *Pseudomonas aeruginosa* mexAB drug efflux proteins (Figure 1).

Search of *N. gonorrhoeae* FA1090 genome (accession no. AE004696) indicated that acrA type gene was located (acriflavin transporter, Protein Id. AAW90013) but there was no mention for acrB gene. Blast search of AE004969 sequence did indicate few homologies at nt.1325311-1324757 and nt. 1323646-1323188 corresponding to the nt. 3305-3862 and nt. 4973-5401 of *Escherichia coli* acrB gene (accession no. U00734 at nt. 2312-5463) with 68% and 62% similarities respectively. This indicated a real acrB type RND transporter gene in *N. gonorrhoeae*. Search further indicated that a 268aa protein of mtrD (mexB) indeed was located in *N. gonorrhoeae* genome downstream of mtrC between nucleotides 1323095-1326298. This protein is similar to mtrD protein in other *N. gonorrhoeae* genes like strain 35/02 (accession no. CP012028) with few mutations (T397I,

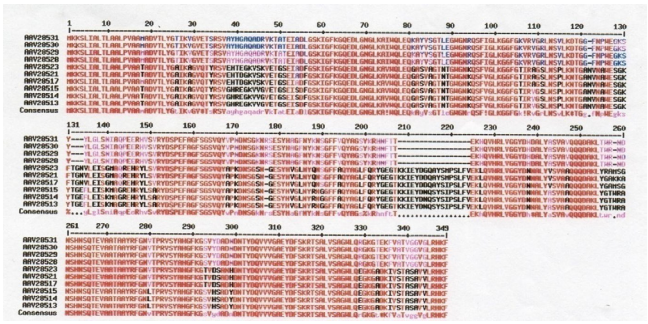

Figure 8: Multi-alignment of few porB membrane channel proteins of *Neisseria gonorrhoeae* to demonstrate isomers (porB1a and porB1b) and many mutations.

Figure 9: A 201 bp upstream sequence of penA gene of strain FA19 was compared by BLAST search.

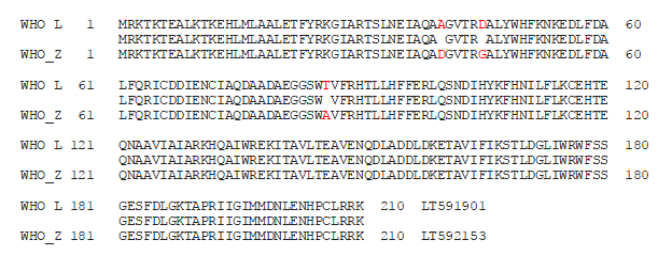

Figure 10: Comparison of amino acid sequence of mtrR repressor of *N. gonorrhoeae* MDR strain WHO_L and PDR strain WHO_Z.

A661V and I1020V). It has 49% similarity to Salmonella enteric acrB protein (protein id. AKO22252) and 49% similarity to *Escherichia coli* acrB protein (protein id. AAA67135) but has 49% and 40% similarities to mexB and mexF protein of *Pseudomonas aeruginosa* respectively. We concluded that most *N. gonorrhoeae* genomes (strains; WHO_Z, WHO_U, NCCP11945, FDAARG_260, MS11, FA6140, 35/02, and FA 1090 etc.) have efflux genes that likely kick out drugs (penicillins and carbapenems, fluroquinlones and DNA intercalators like doxorubicin). The nature of TolC and oprM types periplasmic membrane fusion proteins in *N. gonorrhoeae* to be studied but mexX/AmrX were located downstream of mtrD (protein id. AAW90013) signalling the authentic mexA-mexB-oprM type drug efflux system common in all drug resistant *N. gonorrhoeae* and designated as mtrC-mtrD-mtrX (Figure 3).

MacA/B genes were predominant in *N. gonorrhoeae* genome and study indicated that such proteins actively transported kanamycin, tobramycin, erythromycin and streptomycin type drugs giving multi-resistance. mtrD and mtrF crystal structure were developed recently to address the drug pump mechanisms [52]. AbgT drug transporter was frequently found in gonococcal chromosome [53]. It appears a genetic island was involved in multi-resistance involving many drug and metabolite transporters. A 515 a long farB or ermB transporter (protein id. EEZ44389, KMY25942, ACF30680) was also implicated in drug resistance in *gonorrhoeae* (accession no. CP001050) (Table 2).

Mechanism of tetracycline resistance in *N. gonorrhoeae*

Tetracycline resistance associated with the tetM plasmid [54]. TetM protein of *E. coli* is 639aa long and could bind strongly 30S ribosomal proteins releasing to tetracycline from ribosome. So the mechanism of other Tet proteins like tetA and tetC tetracycline drug effluxs are different as those pump to remove tetracycline from bacterial cytoplasm preventing to cause sufficient interaction with ribosome to stop protein synthesis [55]. TetM gene was located in many plasmids and provides rationally to tetracycline resistance but presence of wide spread plasmid-mediated tetA/B/C like genes should be re-evaluated in *gonorrhoeae* species.

Mechanism of DNA gyrase and DNA topoisomerase IV genes mutations in *N. gonorrhoeae*

DNA gyrase has two subunits (gyrA and gyrB) and is involved in DNA supercoiling regulating DNA replication, recombination and transcription. Ciprofloxacin inactivates gyrase stopping cell division but mutations and modification in 3-D structure in the gyrA and gyrB

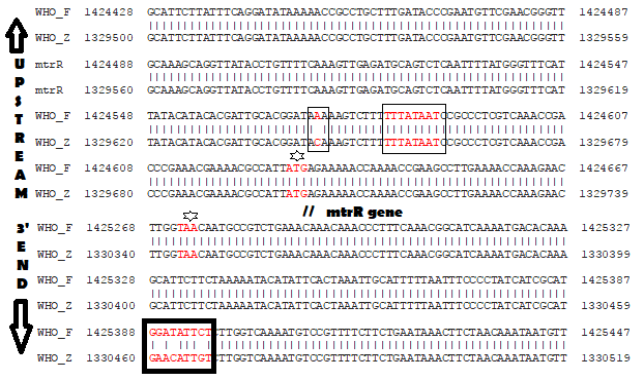

Figure 11: Comparison of mtrR gene upstream and downstream between MDR strain WHO_F and PDR strain WHO_Z.

subunits reduces the binding efficiency with fluroquinolones antibiotics. Mutations in gyrA (S91F, D95G/A/N/Y), parC (D86N, S87R/I/N and S88P) and parE (G410V) were implicated in ciprofloxacin and norfloxacin resistance [56]. parC and parE are DNA topoisomerase IV gene subunits and also are involved in chromosome remodelling and DNA topology [57]. However, higher lipophilic fluroquinolones like moxifloxacin and gatifloxacin have low response to such mutations and still are used in gonococcal infections.

Mechanism of rRNA mutations in multi-resistance of *N. gonorrhoeae*

Mutation C2599T in 23S rRNA was found in 25/289 (9%) isolates and these *N. gonorrhoeae* had azithromycin MIC values >8 µg/ml. One isolate, MUNG19, had mutation A2143G, 23S rRNA allele 456, and had resistant MIC to azithromycin, 256 µg/ml. Spectinomycin resistances was conferred through deletion of codon 27 and, subsequent L28E substitution in rpsE (NEIS0149) allele 83 or mutation C1186T in 16S rRNA allele 1538. Two isolates, ATL0121 and MUNG18, were found with either of these mutations with confirmed case of AMR.

Discussion

The real time sequencing of *Neisseria gonorrhoeae* genome (accession no. CP012028) indicated the presence of MacA (protein id. AKP14513 nt. 425862-427796 complement) and PBP2 (protein id.AKP14613; nt. 539173-540921 complement) mdr genes. But further analysis suggested that mtrD (protein id. AKP14428), mteC (protein id.AKP14429) drug transporter under the regulation of mtrR (protein id.AKP14430), two ABC transporters (protein ids.AKP16180, AKP14470) and 23S/16S rRNA methyl transferases (protein ids. AKP16100, AKP14615), phospho-N-acetyl muramoylpentapeptidetransferases (protein id. AKP14608) were present in the genome and likely contributed to the AMR.

Similarly, penA gene (protein id. SBO59320; nt. 1331021-1332259 complement) and abgT antibiotic resistant protein (protein id. SBO68571; nt. 1333781-1335349) were located in *N. gonorrhoeae* strain WHO_U (accession no. LT592159). However, mtrC (protein id.SBO68545; nt. 1331021-1332259 complement), mtrD (protein id.SBO68533) and mtrE (protein id. SBO68524; nt. 1326350-1327753) antibiotic transporters were accumulated at the same locus and likely controlled by acrR (protein id. SBO68553) in case of mtrC and pdhR (protein id.SBO68504) in case of mtrE transporters (Lee SG et al.). A 522aa long abgT transporter (protein ids. SBO68571, SBO69438, and SBM96603) for aminobenzoate-glutamate involved in foliate biosynthesis was implicated as drug transporter in *N. gonorrhoeae* and had been sequenced, over-expressed and crystal structure was elucidated. Similarly, mtrF transporter was implicated in AMR in *gonorrhoeae* as found in many genomic fragments (accession nos. AF176821. EQ973013, DS999940, FMSZ01000045 and GG749376).

PonA gene encoding penicillin binding protein-1 (protein id. AAB52536) was cloned (accession no. U72876). Genome sequencing of many *N. gonorrhoeae* genome was confirmed the mutational activation PBP1/2 causing acute penicillin resistance (see accession nos. CP000150, LT592146/ 50/ 53/ 57/59, LT591898 and LT591901/ 4/8). A Canadian study indicated 252 *N. gonorrhoeae* strains real-time PCR chromosomal DNA was positive 100% for ponA, and penA, 99.6% for mtrR, and 95.2% for porB [58]. But we doubt that plasmid-mediated blaCTX-M, tetC, strAB, sul1/2, aacA4, aacC1 must be overlooked due to lack of proper plasmid isolation from *N. gonorrhoeae*. BLAST analysis of genomic sequence (Ch-1) of WHO_Z PDR strain of *N. gonorrhoeae*

did not find such genes (accession number used: X75761 for tetA, KC590080 for tetC, EF516991 for cat, KM877269 for sul1, AP012056 for sul2, D90119 for norA, NG_050417 for mcr-1, KR047792 for vanA, J01749 for amp, X92506 for CTX-M, AF297554 for KPC, KC539430 for NDM1, AF227505 for OXA1 and JN207493 for OXA23),

Kubanov et al. studied recently 124 *N. gonorrhoeae* strains obtained from 9 regions in Russia using *N. gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST), an antimicrobial susceptibility test according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria and an oligonucleotide microarray for the identification of mutations in the penA, ponA, rpsJ, gyrA and parC genes responsible for penicillin-G, tetracycline, and fluoroquinolone resistance [34]. NG-MAST analysis showed a diversified population of *N. gonorrhoeae* in Russia with 58 sequence types, 35 of which were described for the first time. The STs 807, 1544, 1993, 5714, 9476 and 12531, which were typical for some Russian Federation regions and several countries of the former Soviet Union, were represented by five or more isolates. Preliminarily susceptible (G-807, G-12531) and resistant (G-5714, G-9476) geno-groups were revealed. Chen et al. in China also correlated the mutations in Non-mosaic penA alleles with A501T and G542S alterations, an H105Y alteration in mtrR gene and an A102D/N alteration in porB1b gene with decreased susceptibility or resistance to ceftrioxane (Figure 10) [58].

Chen et al. have been reported cefixime and ceftriaxone resistant isolates of a strain cluster of ST4378, a genotype that differs in the porB sequence by only one nucleotide from ST1407, in Taiwan during April 2006 to June 2012 [59]. Genes involved in β-lactam binding proteins (ponA), quinolone resistant genes (gyrA and parC) and multidrug transporter regulatory genes (mtrR, porB1b and pilQ) were sequenced with many polymorphisms. The adenine deletion in the 13bp promoter region associated with increased expression of the MtrCDE efflux pump was found in 178/289 (62%) isolates (proNEIS1635 allele 3) and was associated with mutations in many of the other AMR loci including penA (NEIS1753), ponA (NEIS0414) and porB (NEIS2020). However, *Acinetobacter baumannii* or Proteus mirabilis type MDR genomic islands with aad, sul1, blaTEM, aph, and strAB mdr genes have not detected in *N. gonorrhoeae* genome (see, accession nos. KU743384, NJ439039). Recently, WHO reported the active increase in antibiotic-resistance *N. gonorrhoeae* with very difficult or impossible to cure using cephalosporins? Of the 77 countries surveyed, 97% resistant to ciprofloxacin, 81% resistant to azithromycin and 66% resistant to cephalosporins [9,24,51].

Conclusion

MDR mechanisms are thus quite different in *Neisseria gonorrhoeae* as reflected by accumulation of mutations in chromosomal penicillin binding proteins and porin genes [50]. Also different *mdr* genes like that cat and bla genes are rarely seen in plasmids or chromosome of *N. gonorrhoeae* except blaTEM and tetM. However, many drug efflux genes (macB, farB, mdtA, ABC) were implicated in AMR of *N. gonorrhoeae* and inhibitors of efflux pumps may best choice for drug development against MDR *N. gonorrhoeae* infections [28]. Then why most early proton drug efflux genes like tetA or tetC did not assimilated in gonococcal plasmids? MtrtCDE drug efflux likely acrAB/mexAB types and thus is an important target for new drug development. Plasmid mediated over expression is important for MDR but we see only tetM and blaTEM in few plasmids are activated although mtrR activation has been demonstrated in mtrCDE efflux genes. In most MDR Enterobacteriaceae (*E. coli, S. enterica*, *P. aeruginosa*, *A. baumannii)* thousands large plasmids with 5-15 *mdr* genes and 10-20 TRA conjugative proteins have been reported including many IS-elements. G20 Nations have agreed at Meeting at Berlin (May 2017) and recent Meeting at Humberg (July 2017) to act together to control anti-microbial resistance and to develop new drug against superbugs. Higher derivatives of fluoroquinolones (JNJ-Q2; WQ-3810) were found effective to kill ciprofloxacin resistant *N. gonorrhoeae* [60,61]. We hope crystal structure is pivotal to understand anti-metabolite transporters structure and functions for new drug development against *N. gonorrhoeae* [62]. We demand a new direction in gonococcal research to identify new plasmids and related mdr genes directing new control measures for STDs [63,64].

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