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Research Article

Macrolide Resistance Phenotype among the Tetracycline-Resistant Isolates of *Streptococcus Pneumoniae*

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Abstract

Streptococcus pneumoniae caused serious invasive infections such as pneumonia, meningitis, septicemia of children less than five years old and caused high rate of morbidity and mortality. Multi-drugs resistance phenomena worsen the treatment option of the disease. One hundred and thirty two isolates detected from pediatric pneumonia and meningitis patients admitted three hospitals in Dhaka city were included this study. Ninety five of 207 (46%) *S. pneumoniae* isolates were tetracycline resistant (MIC, ≥8µg/ml). Among the isolates, ten isolates showed erythromycin resistance (MIC, ≥1µg/ml). These erythromycin resistant strains were investigated for macrolide resistance phenotype. Eight of six (80%) isolates were partially inducible (iMcLS) and the rest two was efflux (M) mediated resistance phenotype. Real time PCR revealed *erm* and *mef* genes from the inducible and efflux-mediated phenotype respectively. Tetracycline resistant isolates showed higher rate of resistance to azithromycin, erythromycin, clindamycin, chloramphenicol, co-trimoxazole and penicillin compared to tetracycline susceptible isolates. Importantly, almost ninety five percent of tetracycline resistant pneumococcal isolates showed multidrug resistant. Twenty two different serotypes were found among our 207 pneumococcal isolates. Serotype 19F showed highest frequency (n=25), followed by serotype 14 (n=13), 13 (n=4), 23F (n=6), 6A (n=5), 6B (n=4) and 7B (n=5). Among the macrolides resistance phenotype, more than 80% are *erm* mediated inducible macrolide resistance.

Keywords: Streptococcus pneumonia; Pneumococcus; Macrolide; Tetracycline; Antibiotic resistance

Introduction

Penicillin has been regarded as the drug of choice for treatment of pneumococcal infections for a long time. Macrolide antimicrobial are often the major alternative for treatment of patients allergic to penicillin or when clinical failure is observed [1]. There are three recognized mechanisms of resistance to macrolides; target modification, inactivation of the antibiotic and active efflux of the drugs [2,3]. The best-known resistance mechanism is the production of an enzyme that methylates the ribosomal binding sites. The former, prevalent mechanism usually depends on a posttranscriptional methylase mediated modification of 23S rRNA encoded by the *erm*B gene [4-6]. Ribosomal methylation leads to co-resistance to Macrolides, Lincosamides and StreptograminB (MLS_B) compounds, and is known as the MLS_B phenotype [1,2]. ErmB gene can be expressed either constitutively, with high-level resistance to all MLS antibiotics ($cMLS_B$ phenotype) or inducibly (iMLSB phenotype). An efflux-mediated mechanism reducing the intracellular macrolides concentration to sub-toxic levels is associated with a resistance pattern (M phenotype) characterized by low-level resistance to only 14- and 15-membered macrolides among MLS antibiotics due to membrane proteins encoded by *mefE* gene [1,3,7]. In *S. pneumoniae*, tetracyclin resistance is predominantly due to ribosomal protection, i. e., the production of cytoplasmic proteins encoded by *tet* (M) or less often, other *tet* genes capable interacting with the ribosome and masking it insensitive to tetracyclin inhibition [1,8,9]. In *S. pneumoniae* the association of erythromycin resistance and tetracycline resistance may be due to Tn*1545* and related conjugative transposons, which encode erythromycin resistance via *erm*B and tetracycline resistance via *tet*M and also kanamycin resistance via *aphA3* [1,10]. In the current study, we report for the first time about macrolide resistance pneumococcal isolates with their resistance phenotypes.

Materials and Methods

Bacterial strains

Sixty-one tetracyclin resistant (MIC; ≥8µg/ml) *S. Pneumoniae* included this study were obtained from 132 [invasive (n=55) and colonized (n=76)] isolates collected from pediatric pneumonia and meningitis cases between 1999-2002 from three hospital in Dhaka city. Strains were identified by optochin susceptibility, bile solubility test and Polymerase Chain Reaction (PCR) to detect lytA gene from optochin resistant strains.

Antimicrobial susceptibility

Susceptibility to antimicrobial drugs was determined by disc diffusion method and Minimum Inhibitory Concentration (MIC) by microdilution technique according to National Committee for Clinical Laboratory Standard (NCCLS) guidelines [11]. Mueller-Hinton II broth (BBL Microbiology systems, Coceysville, MD.) supplemented with 3% sheep lysed blood was used as the test medium and S. pneumoniae ATCC 49619 was used for quality control. Antimicrobial drugs used were azithromycin, erythromycin,

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Table 1: Antibiotic resistance patterns of tetracycline resistant versus tetracycline susceptible pneumococcal isolates (N=207).

Pneumococcal isolates (n)	Number bacterial strains resistant to (%)							
	MDR*	Em	Az	Clin	Pen*	Cm*	Sxt	Cip
Tetracycline-resistant (n=96)	24 (25%)	9 (8%)	6 (6%)	7 (7%)	15 (16%)	15 (16%)	86 (90%)	2 (2%)
Tetracycline-susceptible (n=111)	1(1%)	1 (1%)	1 (1%)	1 (1%)	1(1%)	0 (0%)	85 (77%)	4 (4%)

*P-value statistically significant.

Table 2: Macrolide resistance phenotypes, serotype and antimicrobial resistance patterns of *S. pneumonia.*

Strain ID.	Macrolide phenotype	Macrolide Genotype	Minimum inhibitory concentration (µg/ml)							Serotype
			Ery	Az	Clin	Tet	Pen	Cm	Sxt	
1[i]	$iMcLS_B$	*erm*	32	≥256	32	16	0.25	2	16	7B
2[c]	$iMcLS_B$	*erm*	32	≥256	32	32	0.5	2	16	7B
3[i]	$iMcLS_B$	*erm*	32	≥256	32	32	0.5	2	32	9V
4[c]	$iMcLS_B$	*erm*	32	≥256	32	32	0.25	2	32	9V
5[i]	$iMcLS_B$	*erm*	16	0.5	32	32	0.5	2	16	14
6[c]	$iMcLS_B$	*erm*	16	≥256	16	1	0.5	2	8	19F
7[c]	$iMcLS_B$	*erm*	16	0.75	32	32	0.5	1	16	19F
8[i]	$iMcLS_B$	*erm*	32	≥256	32	32	0.25	2	16	6A
9[c]	M	*mef*	2	1.0	0.25	8	0.06	2	0.5	13
10[i]	M	*mef*	1	0.75	0.06	8	0.03	2	0.5	NT

I: Invasive Isolates; C: Colonized Isolates; NT: Non Type able.

clarithromycin, clindamycin, chloramphenicol, cotrimoxazole, ciprofloxacin, penicillin and tetracycline.

Detection of resistance phenotype

Macrolide resistance phenotypes were determined by Erythromycin-Clindamycin-Rokitamycin Triple Disc (ECRTD) test (Oxoid, UK). In order to easily differentiate, within erythromycin-resistant pneumococci, a triple-disc test was set up by adding a rokitamycin disc (30 mg; BBL) to the erythromycin and clindamycin discs of the conventional ECDD (erythromycin-clindamycin double disc) test [12]. The erythromycin disc was placed at the center of the agar plate with the clindamycin and rokitamycin discs placed 15 to 20 mm apart on either side. The iMcLS strains were characterized by no significant zone of inhibition around either the erythromycin or the clindamycin disc, in line with their resistance to both drugs, but presented a zone of inhibition around rokitamycin that was blunted on the side proximal to the erythromycin disc, in line with the inducibility of their rokitamycin resistance. By the ECDD test these strains would be identified as cMLS, no clindamycin zone of inhibition being appreciable. The true cMLS phenotype characterized by the absence of a significant zone of inhibition around the three discs, and the M phenotype characterized by susceptibility to clindamycin and rokitamycin with no blunting of the relevant zones of inhibition [12].

Gene detection by Real time PCR

Erythromycin resistance gene *erm* and *mef* was detected by reverse time Polymerase Chain Reaction (PCR). For detection of erm and mef primers described by Trieu-Cuot et al., and by Tait–Kamradt *et al.*, with the sequences (*erm*) 5′-

CGAGTGAAAAAGTACTCAACC-3′ (positions 362 - 382) and 5′

GGCGTGTTTCATTGCTTGATG-3′ (positions 978 - 958) and (*mef*) 5′-

AGTATCATTAATCACTAGTGC-3′ (positions 57-77) and 5′-

GTAATAGATGCAATCACAGC-3′ (positions 551-532) was selected [13].

Serotyping

All isolates were serotyped by capsular swelling test using specific antisera (Statens Serum institute, Copenhegan, Denmark).

Results

Antimicrobial susceptibility

Resistance rates of azithromycin, erythromycin, clindamycin, chloramphenicol, cotrimoxazole, ciprofloxacin and penicillin were 6%, 8%, 7%, 16%, 90%, 2%, and 16%, among tetracycline resistant and 1%, 1%, 1%, 0%, 70%, 4% & 1% among tetracycline susceptible pneumococcal isolates (Table 1). Tetracycline resistant pneumococcal isolates exhibit higher rates of resistance to commonly used antimicrobial agents compared to tetracycline susceptible isolates. Consistently, 25% of tetracycline resistant isolates were multi-drug resistant compared to 1% among tetracycline susceptible isolates (Table 1). Importantly, 25 (96%) of 26 multi-drugs resistant isolates belongs to tetracycline resistant group indicating a higher degree of correlation between tetracycline resistance and multi-drugs resistant phenotype.

Macrolide resistance phenotype

On the basis of Erythromycin-Clindamycin-Rokitamycin Triple-Disc Test (ECRTDT) 8 of the 10 erythromycin resistant strains were denoted as MLSB and another two was assigned to the M phenotype. All of the eight MLSB phenotypes were assigned to partially inducible (iMcLSB). Real time PCR revealed that ten isolates possess macrolide

resistance gene (either *erm* or mef). Eight (80%) of ten isolates have erm and rest of the two contain mef gene.

Serotyping

Twenty-two different serotypes were detected among the tetracycline resistant isolates, of which 11 represented by at least three isolates. The most numerous serotype was 19F (n=25), followed by 14 (n=13), 13 (n=6), 23F (n=6), 6A (n=5), 6B (n=4) and 7B (n=5). Among the 10 erythromycin resistant isolates, 7B (n=2), 9V (n=2) and 19F (n=2) covered 60% (6/10). Other four isolates were 6A, 13, 19F and non-type (Table 2).

Discussion

In *S. pneumoniae*, tetracycline resistance is predominant due to ribosomal protection, i. e., the production of cytoplasmic proteins encoded by *tet* (M) or other tet genes capable interacting with the ribosome and masking it insensitive to tetracycline inhibition [7,8]. In addition to ribosomal protection another mechanism known as efflux is involve in reducing the intracellular tetracycline concentration to sub-toxic levels through the membrane protein encoded by the gene *tet* (K) or *tet* (L) [8]. Similarly an efflux-mediated mechanism involve in pneumococcal erythromycin resistance by reducing the intracellular macrolide concentration to sub-toxic levels through the membrane protein encoded by the gene *mef* [7]. In clinical isolates of *S. pneumoniae*, tetracycline resistance is frequently associated with erythromycin resistance. Several studies from America (USA, Canada) and Europe (Spain, Italy) have shown >60% and >80% respectively. In our study, only 8% tetracycline resistant isolates were erythromycin resistant but 33% strains were erythromycin resistant among the Multidrug Resistant (MDR) (including tetracycline) pneumococcal isolates. Erythromycin resistance rate is higher (8%) among tetracycline resistant pneumococcal than tetracycline susceptible isolates (1%) in our study indicates a low association between tetracycline and erythromycin resistance. This low association between tetracycline and erythromycin resistance in our study suggest that the less frequency of presence of pneumococcal population transposons, typified by Tn*1545*, thought to result from the insertion of resistance determinant, such as *erm* (B) for erythromycin and *aphA3* for kanamycin, into primitive gram-positive conjugative transposons carrying *tet* (M) and the integrase gene *int-Tn*, typified by Tn*916* [8,14]. Erythromycin resistance phenotype in our study (80% MLS and 20% M phenotype) was comparable to European study in Italy, Spain, Turkey [12,15,16] and dissimilar to USA [17,18] and Canadian [19] study where M phenotype was prevalent. Resistance rates of penicillin, chloramphenicol, clindamycin, erythromycin, azithromycin, and cotrimoxazole are higher in tetracycline resistant isolates than that of tetracycline susceptible isolates but there was an exception for ciprofloxacin. Twenty five (96%) of 26 MDR isolates were tetracycline resistant reflecting that the MDR phenomenon is associated with tetracycline resistance. The most numerous serotypes in our study; 19F (n=25), followed by 14 (n=13), 13 (n=6), 23F (n=6), 6A (n=5), 6B (n=4) and 7B (n=5)]. Our serotypes distribution of tetracycline resistant pneumococcal isolates were similarity to study conducted in Italy and Germany, prevalent serotypes were 23F, 19A, 19F, 6B, 14 [13,15].

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