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Resistance of *Streptococcus pneumoniae* isolated from Lebanese patients between 2005 and 2009

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ABSTRACT

Introduction: *Streptococcus pneumoniae* is an important organism in view of its prevalence and ability to cause serious infections; its resistance to antimicrobial agents is increasing worldwide. The purpose of this study was to evaluate the patterns of resistance of *S. pneumoniae* to penicillin, macrolides and various other antibiotics in strains isolated from Lebanese patients.

Methods: 121 strains isolated between January 2005 and January 2009 from two university hospitals in Beirut were identified and tested for MIC determination using the E-test method. The presence of *erm*(B) and *mef*(A/E) genes was investigated using PCR.

Results: The majority of the strains (73.5%) were isolated from respiratory tract infections, 50.4% were isolated in winter, 15.7% were invasive strains, 61.9% came from male patients, and 68.5% from adults. Out of 121 isolates, 58 were susceptible to penicillin, 61 were intermediate, and 2 were fully resistant to this antibiotic. Amoxicillin-clavunanic acid and cefpodoxime showed 100% activity on all tested isolates. In general, the $MICs_{90}$ appear to fluctuate within the same range over the four years. The *erm*(B) *gene* was detected in 85.3% of the isolates, *mef*(A/E) in 19.5% whereas *erm*(A) was not detected in any of the macrolide resistant strains.

Discussion: The results of this study have important impact on the empirical antibiotic prescriptions; the increasing prevalence of resistance jeopardises the treatment choices posing a serious threat. Further surveillance and epidemiological serotyping are needed to monitor the local and regional resistance patterns and to track the spread route of resistance.

Keywords: Antibiotics, Resistance, *Streptococcus pneumoniae*

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Resistencia de cepas de *Streptococcus pneumoniae* aisladas en pacientes libaneses entre 2005 y 2009

RESUMEN

Introducción: *Streptococcus pneumoniae* es un organismo importante en tanto la prevalencia como la capacidad de causar infecciones graves; su resistencia a los antibióticos es cada vez mayor en todo el mundo. El objetivo de este trabajo fue evaluar los modelos de resistencia de *S. pneumoniae* a penicilina, macrólidos y varios otros antibióticos en cepas aisladas de pacientes libaneses.

Métodos: 121 cepas aisladas entre enero de 2005 y enero de 2009 en dos hospitales universitarios en Beirut se identificaron y comprobaron para la determinación de la concentración mínima inhibitoria por el método E-test. La presencia de los genes *erm*(B) y *mef*(A/E) investigó mediante PCR.

Resultados: La mayoría de las cepas fueron aisladas de infecciones del tracto respiratorio (73,5%). Cincuenta con cuatro por cien fueron aislados en el invierno, 15,7% fueron cepas invasivas, 61,9% procedían de pacientes de sexo masculino, y un 68,5% de adultos. De los 121 aislamientos, 58 fueron susceptibles a la penicilina, 61 fueron intermedios, y 2 fueron completamente resistentes a este antibiótico. Amoxicilina-ácido clavulánico y cefpodoxima mostraron 100% de actividad en todos los aislamientos estudiados. En general, las $CMIs_{90}$ parecen fluctuar dentro del mismo rango en los cuatro años. El gen *erm*(B) se detectó en el 85,3% de los aislamientos, *mef*(A/E) en 19,5%, mientras que *erm*(A) no se detectó en ninguna de las cepas resistentes a macrólidos.

Discusión: Los resultados de este estudio tienen un gran impacto en la prescripción antibiótica empírica en el Líbano. Una mejor vigilancia epidemiológica acompañada de un serotipado es imprescindible para el futuro.

Palabras clave: Antibióticos, Resistencia, *Streptococcus pneumoniae*

INTRODUCTION

Streptococcus pneumoniae is an important cause for bacterial infections[1] which can result in increased morbidity and mortality[2]. *S. pneumoniae* is the most common cause of bacterial acute respiratory tract infections which are the leading cause of mortality among children especially in developing countries[3]. Pneumococcal diseases have been reported in several countries of the Middle East and Arab world including Kingdom of Saudi Arabia, Egypt, United Arab Emirates, Yemen, Qatar, Kuwait, Jordan, Lebanon and Oman[4] where morbidity rates due to pneumococcal infections reached 38% in Yemen and Kuwait[4].

Because of the time required to identify the organism, assess its significance, and test the susceptibility of the strain isolated from patients with community-acquired respiratory tract infections, mainly in critical financial conditions, antimicrobial therapeutic choices are usually empirical[5]. Therapeutic options to treat pneumococcal infections are challenged by the increasing prevalence of antibiotic-resistant strains which may lead to treatment failure[6]. Resistance of *S. pneumoniae* to antibiotics has increased rapidly worldwide, with considerable geographical variations in both genotypes and phenotypes[1, 7]. Moreover, penicillin non- susceptible isolates, which are isolates that exhibit total or intermediate levels of resistance, can be sometimes multidrug resistant[8]. Unfortunately, data regarding endemic antimicrobial resistance are unavailable in many parts of the world, especially from areas where over-the-counter antibiotic use is common as in Lebanon[9].

Initially, all *S. pneumoniae* isolates were susceptible to penicillin and β-lactams were considered the treatment of choice for *S. pneumoniae* infections. Since the 1960s, resistance to these agents started to be reported[10]; and by the late 1970s, resistance to penicillin and other antimicrobial agents has increased rapidly worldwide where geographical variations in both genotypes and phenotypes have been observed[1, 11]. Resistance to macrolides also spread alarmingly and most probably due to the increased use of macrolides to treat community-acquired pneumonia especially after recommending it for penicillin allergic patients[12]. In Lebanon, resistance to erythromycin increased from 11.5% in 1997 to 43% in 2004[13].

Unfortunately, in Lebanon, data is still limited and no studies have addressed the mechanism of resistance of clinical isolates in the country. This study has investigated for the first time the genotypes of resistant strains as opposed to previous studies that conducted in the country which have concentrated on studying the phenotypes only.

MATERIALS AND METHODS

Isolates of *S. pneumoniae* were collected at Saint Georges Hospital-UMC in Beirut and at Dahr El Bashek Governmental University Hospital between January 1st 2005 and January 1st, 2009 and were subject to the phenotypic and/or genotypic studies. These isolates were obtained from fresh clinical specimens from different body sites including invasive and noninvasive strains. Strains coming from the same patient, same body site and having the same susceptibility profile within a period of three months were considered as duplicates and those coming from throat or nose swabs of healthy carriers were not included in the study.

Identification of *S. pneumoniae*. Definitive identification of *S. pneumoniae* was based on colonial morphology, hemolytic activity on blood agar medium, catalase test, optochin susceptibility, agglutination test (Quellung reaction- Slidex Pneumo kit Sigma 50), bile solubility and biochemical profile using the API 20 Strep Kit (Sigma 25- IVD- Biomerieux).

Susceptibility testing. The antimicrobial disk susceptibility tests were performed as recommended by the Clinical and Laboratory Standards Institute[14] and susceptibility was assessed accordingly[15]. *S. pneumoniae* isolates were subcultured from frozen glycerol stocks onto blood agar plates and incubated in 5% CO_2 overnight. They were incubated in Brain Heart Infusion for 15 minutes before culturing them on blood agar. They were sub-cultured once more by streaking onto appropriate agar plate and incubated overnight. The following day, colonies from the second agar plate were directly re-suspended in brain heart infusion and incubated for 15 minutes. The cell suspension was adjusted to match the 0.5 McFarland turbidity standards. Within 15 minutes, a sterile cotton swab was dipped into the adjusted suspension, and then taken out of the fluid, and any excess inoculum was removed by rotating the swab against the inside of the tube. Each blood agar plate was inoculated by streaking such a swab over its entire surface three times, rotating the plate approximately 60 degrees each time. Finally the rim of the agar was inoculated. *S. pneumoniae* isolates and the QC organism were tested against the following antibiotics using E-test (AB-biodisc, Sweeden): penicillin, amoxicillin-clavulanic acid, cefuroxime, cefaclor, cefpodoxime, azithromycin, clarithromycin, cefprozil, ciprofloxacin, and ofloxacin

To test for multi- drug resistance (MDR), the strains isolated over the period of 3 years (2006 to 2008) were tested against the following antibiotics: penicillin G, macrolides, trimethoprim-sulfamethoxazole (SXT), tetracycline, clindamycin, and chloramphenicol. In this study, MDR was defined as resistance to two or more antibiotics[16].

Detection of the resistance genes. Genomic DNA was prepared using Qiagen DNA extraction kit as described by manufacturer's instructions, and stored at -20°C. Macrolide resistance genes (*mef*A, *mef*E, *erm*A, and *erm*B) were detected by PCR, followed by separation on an agarose gel and staining with ethidum bromide and UV exposure. Amplification reactions were performed in 25µl reactions containing 0.5U Taq DNA polymerase (Fermentas), Taq buffer, 50ng DNA template, 100ng of each primer. After an initial denaturation cycle at 95°C (4 min), 30 amplification cycles were performed as such: denaturation at 95°C (30 sec), primer annealing (1 min, temperature specific to each primer pair) and extension at 72°C (1 min). A final elongation step was performed at 72°C for 10 min. Primer sets specific for the amplification of *erm*A, *erm*B[17], *mef*A[17], *mef*E[18], and *pbp1a*, *pbp2b*, *pbp2x*[19] were used. Gel electrophoresis was performed to

Table 1 **Antimicrobial susceptibility of 121 *S. pneumoniae* isolated at SGH-UMC and DBH-GUH between 2005 and 2008**

		All isolates				Penicillin G susceptible				Penicillin G intermediate			
		n	MIC_{50}	MIC_{90}	S	n	MIC_{50}	MIC_{90}	S	n	MIC_{50}	MIC_{90}	S
Antibiotic	Year		(mg/L)	(mg/L)	(%)		(mg/L)	(mg/L)	(%)		(mg/L)	(mg/L)	(%)
Amoxicillin-clavulanic acid													
	2005	37	0.06	0.75	100.0	21	0.02	0.06	100.0	16	0.50	0.88	100.0
	2006	30	0.09	0.75	100.0	13	0.02	0.06	100.0	17	0.25	0.85	100.0
	2007	26	0.09	0.50	100.0	7	0.02	0.08	100.0	18	0.18	0.58	100.0
	2008	28	0.02	0.75	100.0	17	0.02	0.11	100.0	10	0.16	0.75	100.0
Claritromycin													
	2005	37	0.06	4.20	74.30	21	0.06	14.00	75.00	16	0.09	>256	73.30
	2006	30	0.06	0.78	58.60	13	0.09	1.73	76.90	17	>256	>256	43.80
	2007	26	0.05	0.09	61.50	7	0.06	1.64	85.70	18	>256	>256	50.00
	2008	28	0.03	0.06	67.90	17	0.03	0.08	94.10	10	>256	>256	40.00
Azithromycin													
	2005	37	0.25	3.80	71.40	21	0.25	68.80	75.00	16	0.50	>256	66.60
	2006	30	0.25	1.50	73.90	13	0.25	5.00	76.90	17	>256	>256	41.20
	2007	26	0.25	0.75	53.80	7	0.25	9.80	85.70	18	>256	>256	38.90
	2008	28	0.19	0.50	66.60	17	0.19	0.50	94.10	10	>256	>256	30.00
Cefuroxime													
	2005	37	0.08	2.00	70.30	21	0.02	0.05	100.0	16	0.22	2.00	37.50
	2006	30	0.19	2.00	66.70	13	0.05	0.24	100.0	17	0.75	2.00	41.20
	2007	26	0.09	1.50	76.60	7	0.02	0.08	100.0	18	0.19	1.65	72.20
	2008	28	0.03	1.40	81.50	17	0.02	0.06	100.0	10	0.22	2.00	60.00
Penicillin													
	2005	37	0.08	1.20	54.10	21	0.02	0.08	100.0	16	0.50	1.50	0.00
	2006	30	0.16	1.05	43.30	13	0.06	0.09	100.0	17	0.38	1.50	0.00
	2007	26	0.35	1.00	26.30	7	0.06	0.09	100.0	18	0.63	1.00	0.00
	2008	28	0.02	0.53	57.10	17	0.01	0.02	100.0	10	0.19	0.75	0.00
Cefaclor													
	2005	37	0.25	9.60	59.50	21	0.13	1.50	85.70	16	3.50	14.00	25.00
	2006	30	0.50	8.00	63.30	13	0.19	1.28	84.60	17	1.50	8.00	47.00
	2007	26	0.75	8.00	61.50	7	0.25	0.83	85.70	18	1.00	8.00	55.50
	2008	28	0.38	6.00	75.00	17	0.13	0.38	100.0	10	1.50	6.20	50.00
Cefprozil													
	2005	37	0.19	2.00	90.90	21	0.05	0.09	100.0	16	1.25	3.00	81.30
	2006	30	0.32	2.00	93.30	13	0.06	0.23	100.0	17	0.75	2.40	88.20
	2007	26	0.44	2.00	100.00	7	0.06	0.18	100.0	18	0.50	2.00	100.0
	2008	28	-	-	-	17	-	-	-	10	-	-	-
Cefpodoxime													
	2005	37	-	-	-	21	-	-	-	16	-	-	-
	2006	30	-	-	-	13	-	-	-	17	-	-	-
	2007	26	0.09	0.38	100.00	7	0.02	0.08	100.0	18	0.25	0.38	100.0
	2008	28	0.02	1.00	100.00	17	0.02	0.18	100.0	10	0.25	1.00	100.0
Ofloxacin													
	2005	37	1.75	4.00	72.90	21	2.00	4.00	66.70	16	1.50	3.20	87.50
	2006	30	1.50	2.20	86.70	13	1.50	2.80	84.60	17	1.50	2.00	94.10
	2007	26	1.50	3.00	84.60	7	1.50	3.40	71.40	18	1.50	2.00	94.40
	2008	28	1.50	3.00	78.60	17	2.00	3.00	70.60	10	1.50	2.00	100.00

S (%) = percentage of susceptibility
The data of the two penicillin-resistant *S. pneumoniae* isolates are not shown in this table.

Table 2 MICs (mg/L) of the two *S. pneumoniae* isolates resistant to penicillin

	Penicillin	Amox/clav	Cefuroxime	Cefaclor	Cefpodoxime	Azithromycin	Clarithromycin	Ofloxacin
Isolate 1	2	0.5	1.5	12	1	0.38	0.064	3
Isolate 2	2	3	3	6	1	256	256	1

Amox/clav= amoxicillin/clavulanic acid

find out whether PCR has amplified the correct gene. DNA and standard 1 ug of 100 bp DNA Ladder (Fermentas) were loaded to the wells of the agarose gel then electrophoresed at 100V for 45 minutes. The mixture loaded in the wells of the agarose consisted of 10 ul of the PCR product with 5ul loading dye (Fermentas). Agarose gels were removed from the TBE buffer and placed on an Ultra-Violet transilluminator system (DIGI DOC-IT System TM) for imaging and interpretation.

RESULTS

A total of 121 isolates of *S. pneumoniae* which were associated with various pneumococcal diseases were collected at Saint Georges Hospital University Medical Center in Beirut and Dahr El Bashek Governmental University Hospital- Beirut, between January 1, 2005 and January1, 2009.

Strain distribution. The number of isolates varied among the seasons where 21 (17.5%) strains were isolated in fall, 61 strains (50.4%) in winter, 25 (20.6%) in spring and 14 (11.5%) in summer. The population included 19 (15.7%) invasive strains isolated from blood and CSF. Of the 121 strains, 89 (73.5%) were collected from respiratory tract specimens, 17 (14.0%) were isolated from blood cultures, 7 (5.7%) were isolated from middle ear aspirates, 2 (1.6%) were isolated from cerebrospinal fluid, and 6 (4.9%) were isolated from other sites like pus, abdominal and eye secretions. Of the 121 pneumococcal isolates, 75 (61.9%) were isolated from male patients and 46 (38.0%) from female patients, 37 (30.5%) were isolated from children and 83 (68.5%) from adults.

Antimicrobial susceptibility testing. MICs of 121 isolates of *S. pneumoniae* isolated at SGH-UMC and DBH-GUH between 2005 and 2009 are shown in table 1. The MIC_{90} of clarithromycin and azithromycin were significantly high in the penicillin intermediate group as compared to the "penicillin susceptible" and "all isolates" groups. In general, MIC_{50} and MIC_{90} were found to be higher in the "penicillin intermediate" group except for ofloxacin where lower MICs and higher percentages of susceptibility were found. The percentages of susceptibility of "all isolates" to penicillin as determined by the MICs show an important decrease in 2007 when compared to the other years (26% of susceptibility). The $MICs_{90}$ appear to fluctuate within the same range over the four years with exceptions with clarithromycin and azithromycin.

Multiple- resistance in *S. pneumoniae*. This phenomenon was observed in 57% of the isolates. The patterns of Multi-Drug Resistance are shown in figure 1 where values are expressed in percent among the resistant strains. Twenty nine percent of the isolates were simultaneously resistant to penicillin, trimetho-

Table 3 MIC ranges of isolates having either *erm*(B) alone or *erm*(B) with *mef*(A/E)

	MIC range (mg/l)	
Antimicrobial	Strains with *erm*(B) alone	Strains with both *erm*(B) and *mef*(A/E)
Penicillin	0.032-1.0	0.5-2.0
Cefuroxime	0.016-1.5	1.0-6.0
Cefaclor	0.25-6.0	4.0-32
Cefpodoxime	0.016-2.0	0.75-2.0
Amox-clav	0.016-3.0	0.75-3.0
Azythromycin	0.75->256	>256
Clarithromycin	0.75->256	>256
Ofloxacin	0.75-3.0	1.5-3.0

Amox/clav= amoxicillin/clavulanic acid

prim-sulphametoxasole and macrolides. All the macrolide-resistant isolates were also resistant to penicillin and trimethoprim-sulphametoxasole except four isolates. In addition, all the penicillin- susceptible isolates were also susceptible to amoxicillin-clavulanic acid, cefuroxime, and cefaclor except one isolate that was intermediate-resistant to cefaclor. Moreover, the 2 penicillin resistant isolates were non-susceptible to cefuroxime and cefaclor. Amoxicillin-clavulanic acid and cefpodoxime were the only antibiotics with 100% of susceptibility among all isolates including penicillin- susceptible and non-susceptible isolates.

Detection of *erm*(B) and *mef*(A/E) genes. Out of the 41 macrolide intermediate and resistant strains, *erm*(B) was detected in 35 (85.3%) isolates, *mef*(A/E) was detected in 8 (19.5%) isolates (all of these isolates contained *erm*(B) simultaneously). The *erm*(A) gene was not detected in any of the macrolide resistant strains. All the *erm*(B)- positive isolates showed high level resistance to clarithromycin except 3 isolates that showed full susceptibility to this antibiotic. Table 3 shows clearly that MICs of the isolates harbouring both *erm*(B) and *mef*(A/E) were more elevated than those harbouring *erm*(B) alone.

DISCUSSION

A total of 121 isolates of *S. pneumoniae* causing various pneumococcal diseases were collected from Saint Georges Hospital University Medical Center and Dahr El bashek Governmental University Hospital in Beirut between 2005 and 2009. The majority of the strains were collected from respiratory tract specimens (73.5%) and this is in line with other studies that report the highest proportion of isolates from the respiratory tract specimens[20, 21, 22]. The distribution of the strains per season shows that most of the isolates were recovered in winter (50.4%) which extends from December till February, the year's coldest months. This could be attributed to the increased crowding of people in closed environments during winter, which favours the spread of microorganisms from person to person[23].

For the past 60 years, antimicrobial chemotherapy has been the mainstay of medical intervention against infectious diseases caused by bacterial pathogens[24]. Resistance to antibiotics has become a worldwide phenomenon in *S. pneumoniae* since the 1980s[21,24]. The prevalence of resistance is known to vary widely, with patterns dependent on geographical area. Studies from the Arab countries reported high rates of penicillin non-susceptibility which reached 64% in Kuwait[20], 55.4% in Saudi Arabia[4], and 37% in Egypt[9]. In Lebanon, between 1989 and mid 1996, *S. pneumoniae* showed a stable but high rate of susceptibility to penicillin (76-88%)[25]. A survey by Karam Sarkis et al., reported a variation of penicillin non- susceptibility ranging between 49.6% and 69% between the years 1997 and 2004[13]. In our study, 54% of the isolates were penicillin non-susceptible with MIC ranging between 0.004 and 2 mg/l. With respect to β-lactam, this resistance was accompanied with resistance to cefaclor and cefuroxime. This is not surprising, as penicillin resistance in *S. pneumoniae* is the result of alterations in penicillin binding proteins, and all β-lactams and β-lactams like agents bind at least to some extent to the same penicillin binding proteins[10].

Due to increasing reports on the failures of antimicrobial treatment caused by macrolide resistant pneumococcal isolates, macrolides resistance in *S. pneumoniae* has become a matter of

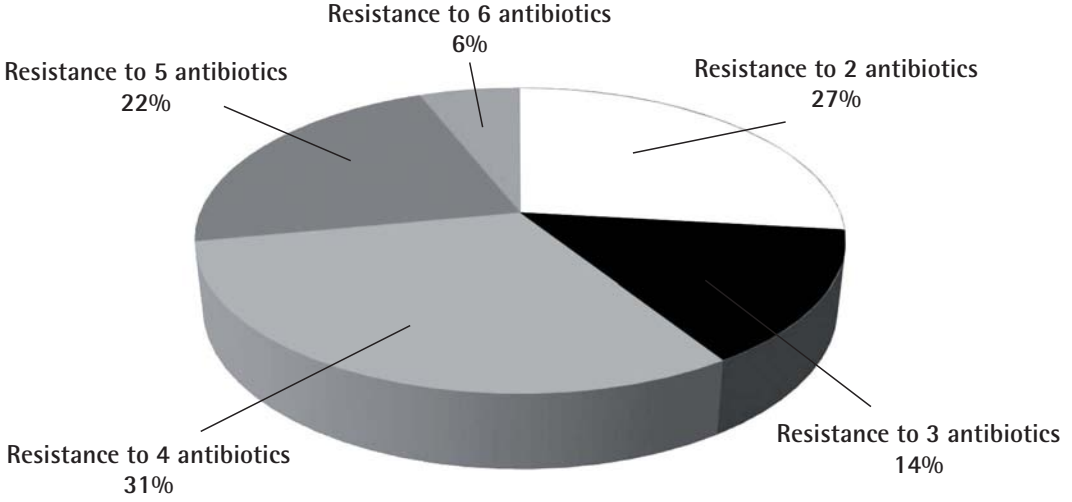

Figure 1 | **Percentage of Multi-Drug resistence in *S. pneumoniae* isolates**

growing concern especially in the last two decades[1]. Previous surveys in Lebanon reported variation in susceptibility to macrolides ranging between 11.5% and 43% between 1997 and 2004[13]. Our isolates showed percentages of non-susceptibility to clarithromycin varying between 25.7 and 41.4. Clarithromycin MICs were of great concern as strains were either fully susceptible or fully resistant giving the impression that resistance to macrolides is growing sharply rather than gradually.

On the other hand, multidrug resistance in *S. pneumoniae* is well documented all over the world. As a result, most antibiotics are becoming less efficient in combating bacteria responsible for serious infections. Most of the isolates recovered in this study (57%) were concomitantly resistant to two or more different families of antibiotics including penicillin, macrolides, sulphametoxasole, tetracycline, clindamycin and chloramphenicol. The emergence of such strains is problematic because it restricts the selection of drugs that can be used to treat severe pneumococcal infections. One potential explanation for the increase in high- level penicillin resistance and multi- drug resistance is the continuous proliferation of a few "fit" clones that are highly penicillin resistant and/or multidrug resistant[10].

Eight isolates contained both *erm*(B) and *mef*(A/E) genes. The MIC to these isolates of various antibiotics was compared with isolates having only *erm*(B) gene. Those with both determinants had higher MICs for most of the antibiotics tested especially β-lactams, trimethoprim-sulfamethoxazole, and macrolides. This has also been similarly reported by Pedrosa et al. in 2008[26].

Six (14.6%) isolates of our series with a macrolide-resistance phenotype were negative for the *erm(B)* and *mef*(A/E) genes. It has been described that other mechanisms found to confer macrolide resistance in pneumococci are mutations in the 23S rRNA or alterations in the ribosomal proteins L4 and L22 and a methylase encoded by the *erm*(A) (subclass *ermTR*) gene[27,28]. On the other hand, while the presence of *erm*(B) confers high level resistance to macrolides, 3 isolates were found to carry this gene, however, susceptible to macrolides. This result is not easy to interpret, and although it was previously reported that *erm*(B) can be associated to low level resistance[29], it requires more investigation involving more strains with this profile.

The results of this study have important impact on the empirical antibiotic prescriptions since the increasing prevalence of resistant organisms jeopardizes the treatment choices and poses a serious threat. Further surveillance and epidemiological serotyping need to be done to monitor the local and regional resistance patterns and to track the spread route of resistance organisms.

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