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Full Length Research Paper

Antimicrobial resistance, serotypes and genotypes of *Streptococcus pneumoniae* isolates associated with ocular infection in China

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Streptococcus pneumoniae **is an important pathogen causing ocular infections, such as ophthalmia, dacrocystitis, conjunctivitis and corneitis. A total of 39** ***S. pneumoniae*** **isolates were isolated from the eyes of pediatric patients with ocular infection aged from 0 to 2 years old in a Chinese children's hospital. All tested isolates with penicillin minimum inhibitory concentration values ranging from ≤ 0.06 to 2.0 µg/ml were susceptible to penicillin and vancomycin. The resistance rates of the** ***S. pneumoniae*** **isolates to erythromycin, clindamycin, tetracycline and ttrimethoprim-sulfamethoxazole were 84.6, 84.6, 82.1 and 74.4%, respectively. While resistance rates of the tested isolates to rifampicin and chloramphenicol were 2.6 and 10.3%, respectively. 76.9% of the tested isolates were multi-resistant to antimicrobials tested. Twelve different serotypes were identified among 39** ***S. pneumoniae*** **isolates, in which serogroup 19, 14 and 8 accounted for 8, 4 and 3 isolates, respectively. However, serogroup 6, 23, 15 and 20 accounted for only 2 isolates, respectively. Eleven isolates were non-serotypeable (NT). The remaining 5 isolates belonged to serogroup 9, 18, 11, 12 and 17, respectively. The 7-valent pneumococcal conjugate vaccine (PCV7) showed lower coverage (17/39, 46.2%). 17 STs were identified among selected 20 isolates, among which 3 novel STs were first found. The international resistant clone Taiwan19F-14 and Spain23F-1 were found in the present study. Taken together,** ***S. pneumoniae*** **isolates from the eyes of pediatric patients with ocular infection younger than 2 years old shows a genetically diverse population.**

Key words: *Streptococcus pneumoniae*, antimicrobial resistance, serotypes, serogroups, pneumococcal vaccines, genotypes.

INTRODUCTION

Streptococcus pneumononiae is a common and important human pathogen associated with otitis media and invasive diseases, such as pneumonia, septicemia, meningitis (Gehanno et al., 2001). *S. pneumoniae* is also an important pathogen causing ocular infections, such as ophthalmia, dacrocystitis, conjunctivitis and corneitis (Parmar et al., 2003). However, there is no data available in the literatures on the antimicrobial resistance, serotype and genotype of the ophthalmic isolates of *S. pneumoniae* in China. It is important to determine the distribution of *pneumococcal* serotypes and genotypes in China as well as the prevalence of antimicrobial-resistant *pneumococci* associated with ocular infections. In this study, we evaluated the distribution of *pneumococcal* serotypes and determined the serotype coverage of pneumococcal vaccine formulas in China.

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Table 1. The antimicrobial resistance profiles of 39 *S. pneumoniae* isolates associated with ocular infection.

Antimicrobials	0 to 2 years (n=39)		
	R (%)	I (%)	S (%)
Penicillin	0	0	100
Vancomycine	0	0	100
Erythromycin	84.6	0	11.4
Clindamycin	84.6	0	11.4
Levofloxacin	0	7.7	92.3
Tetracycline	82.1	5.1	12.8
chloramphenicol	10.3	0	89.7
Rifampicin	2.6	0	97.4
Sulfamethoxazole-trimethoprim	74.4	12.8	12.8

R, resistance; I, intermediate; S, susceptibility.

MATERIALS AND METHODS

Bacterial isolates

From January 2009 to December 2010, a total of 39 *S. pneumoniae* isolates were collected randomly from the eyes of pediatric patients with ocular infections in department of ophthalmology of Shaoxing Municipal Women and Children Hospital with 400 beds in Shaoxing, Eastern China. 355 patients were enrolled in the present study. *S. pneumoniae* accounted for 11% of all isolated bacteria from eye. The age of the children in the study ranged from 0 to 2 years old. Each child came from one family and did not receive a pneumococcal conjugate vaccine. These children suffered from ocular infections such as dacrocystitis, conjunctivitis and corneitis.

Microbiologic methods

Presumptive *S. pneumoniae* isolates were picked based on typical colony morphology, α-hemolysis, and Gram stain. Identification of *S. pneumoniae* was performed by optochin sensitivity and bile solubility tests. Thirty-nine isolates of *S. pneumoniae* were isolated from ophthalmic specimens (predominantly from dacryocystisis and conjunctivitis) over a period of 24 months. The tested isolates were stored at -70°C in 1 ml aliquots of trypticase soy broth with 10% glycerol and were revived before antimicrobial susceptibility testing, serotyping and genotyping were carried out.

Antimicrobial susceptibility

Minimum inhibitory concentrations (MICs) of penicillin for all tested isolates were determined using E-test (AB Biodisk) strips according to manufactory's instruction. The antimicrobial susceptibility of the tested isolates to erythromycin, clindamycin, vancomycin, rifampicin, levofloxacin, tetracycline, sulfamethoxazole-trimethoprim, and chloramphenicol was determined by disk diffusion method in accordance with guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010).

According to the criteria of CLSI (CLSI, 2010), the *S. pneumoniae* isolates causing non-meningitis with a penicillin MIC of > 8 µg/ml were defined as penicillin-resistant. *S. pneumoniae* ATCC 49619 was used as a quality-control strain for antimicrobial susceptibility testing. *S. pneumoniae* isolates with resistance to more than 3 different classes of antimicrobial agents were defined as multidrug-resistant isolates. All data were analyzed using WHONET software (version 5.3).

S. pneumoniae serogrouping/serotyping and vaccine coverage

All tested isolates were serogrouped by latex agglutination using Pneumotest-latex kits (Statens Serum Institute, Copenhagen, Denmark) according to the manufacture's instruction. Potential coverage by 7-valent conjugate pneomococcal vaccine (PCV7), which covered serotypes 4, 6B, 9V, 14, 18C, 19F,and 23F, was determined by calculating the percentage of isolates belonging to the sero-groups/types included in the vaccines. Serogroups 19, 23, 6, 7, 9, and 18 were considered to belong to19F, 23F, 6B, 7F, 9V, and 18C, respectively.

Extraction of total DNA

S. pneumoniae isolates were cultured on blood agar overnight. 3 to 4 fresh bacterial colonies were suspended in 150 µl of sterile distilled water and incubated in water at 37°C for 30 min. All extracted DNA was purified following the instruction of the Genomic DNA Extraction kit (Genecore, China). The DNA was stored at -20°C and prepared for PCR detection.

MLST typing

Multilocus sequence typing of the *S. pneumoniae* isolates has been conducted by amplifications of internal fragments of the seven housekeeping genes of *S. pneumoniae*. The PCR products of seven housekeeping genes tested for MLST typing were purified and sequenced. The numbers of alleles and sequence types were assigned using online database (http:// *S pneumoniae*.mlst.net/).

RESULTS

Antimicrobial susceptibility testing

The antimicrobial resistance profiles of the *S. pneumoniae* isolates were shown in Table 1. A total of 39 *S. pneumoniae* isolates were isolated from the eyes of pediatric patients with ocular infection aged from 0 to 2 years old in a Chinese children's hospital. All tested isolates with penicillin minimum inhibitory concentration values ranging from ≤ 0.06 to 2.0 µg/ml were susceptible to penicillin and vancomycin. The resistance rates of the *S. pneumoniae* isolates to erythromycin, clindamycin, tetracycline and trimethoprim-sulfamethoxazole were 84.6, 84.6, 82.1 and 74.4%, respectively. While resistance rates of the tested isolates to rifampicin and chloramphenicol were 2.6 and 10.3%. 76.9% of the tested isolates were multi-resistant to antimicrobials tested. Only 10.3% were susceptible to all tested antimicrobials. One isolate was multi-resistant to five antibiotics including rifampin but susceptible to chloramphenicol and levofloxacin. The most frequent resistance pattern among the tested isolates with multi-resistance was multi-resistant to erythromycin, clindamycin, tetracycline, and trimethoprim-sulfamethoxazole.

Table 2. Serotypes and penicillin MICs for 39 *S. pneumoniae* isolates.

Serotypes	MICs (μg/ml)								Total
	≤0.064	0.125	0.25	0.50	0.75	1.0	1.5	2.0	
6B	2								2
9V	1								1
14	2			1		1			4
18C	1								1
19F	1			2	3	1		1	8
23F	1					1			2
Nontypeable (NT)	10			1					11
15	1						1		2
20	2								2
8	3								3
11	1								1
12	1								1
17	1								1

Distribution of serogroups/types and vaccine coverage

The predominant serotype among the tested isolates was serotype 19 which accounted for 20.5% (8/39), followed by serotype 4 accounting for 10.3% (4/39) and serotype 8 accounting for 7.7% (3/39). However, serogroup 6, 23, 15 and 20 accounted for only 2 isolates, respectively. Eleven isolates were non-serotypeable (NT). The remaining 5 isolates belonged to serogroup 9, 18, 11, 12 and 17, respectively.

In our analysis of vaccine coverage, we assumed that serogroups 19, 23, 6, 9 and 18 were considered to belong to 19F, 23F, 6B, 9V, and 18C. Based on this assumption, the 7-valent conjugate vaccine coverage was reported to be 46.2% (18/39) among the tested isolates from eyes in China younger than 2 years old.

MICs to penicillin for different serotypes

The penicillin MICs for 27, 1 and 11 isolates was 0.06, 2.0 μg/ml and 0.5 to 1.5 μg/ml. No penicillin-resistant *S. pneumoniae* (PRSP, MIC ≥ 8 μg/ml) were found according to the criteria recommended by CLSI (CLSI, 2010). Penicillin MICs varied among the leading serotypes, among which 91% of NT serotype isolates with penicillin MIC ≤ 0.06 μg/ml, 88% of serotype 19F isolates with penicillin MIC 0.5 to 2.0 μg/ml and a serogroup 19 isolate with penicillin MIC= 2.0 μg/ml. The isolates with different serotypes and their penicillin MICs were listed in Table 2.

MLST typing

Among 20 isolates selected for MLST typing, 17 different sequence types (STs) were identified, of which 3 novel STs including ST6990, ST6991 and ST6992 were found and deposited in the MLST database. The STs were showed in Table 3. The most frequent ST was ST320 accounting for 3 isolates, followed by ST505 accounting for 2 isolates. The remaining STs accounted for only one isolate.

DISCUSSION

Streptococcus pneumoniae is a commensal pathogen of the human nasopharynx. Colonization precedes any pneumococcal disease, and colonized individuals furthermore serve as a reservoir for horizontal spread of the organism in the community (Bogaert et al., 2004; De Lencastre and Tomasz, 2002). *S. pneumoniae* has been reported as one of the most common causes of bacterial corneal ulceration in previous studies undertaken in developing countries (Srinivasan et al., 1997). Despite prompt treatment with appropriate antibiotics, endophthalmitis caused by *S. pneumoniae* is associated with a poor visual prognosis (Miller et al., 2004)

In China, use of the 7-valent PCV has been licensed in 2008, but little is known about the distribution of pneumococcal serotypes. Of the 39 isolates tested, 28 (71.8%) could be typed effectively by using the chessboard system of serotyping. In contrast to the serotype distribution among the ophthalmic isolates in India (Mathews et al., 2000), serogroups 19 and NT occurred most frequently in the present study, .The serogroups/types identified among ophthalmic isolates in the present study are more sporadic and the unserotypeable isolates were of high proportion. In recent years, several conjunctivitis outbreaks caused by nontypeable (NT) strains of *S. pneumoniae* have been

Table 3. STs among 20 selected *S. pneumoniae* isolates.

Isolates	Serotype	aroE	GDH	GKI	recP	SPI	XPT	DDL	ST
S6	6	7	13	1	6	6	6	14	4542
S8	19	15	16	19	15	6	20	26	236
S9	9	15	17	4	16	6	1	17	280
S10	NT	93	5	7	113	17	28	70	6990
S11	NT	46	8	2	10	6	1	22	505
S12	19	4	16	19	15	6	20	1	320
S18	NT	46	8	2	10	6	1	22	505
S20	23	4	4	2	4	4	1	1	81
S21	14	8	13	14	4	6	4	14	876
S35	19	4	16	19	15	6	20	1	320
S40	NT	8	5	6	1	9	10	14	1931
S61	NT	10	13	34	16	15	28	31	2752
S64	19	4	16	19	15	6	20	1	320
S76	NT	10	12	2	1	6	1	14	4450
S79	NT	5	5	6	16	9	1	14	4560
S80	NT	10	13	82	16	6	1	17	6991
S96	15	4	16	19	15	17	20	1	4465
S102	9	15	60	34	16	9	14	262	4216
S104	NT	7	203	40	1	6	1	22	3805
S105	19	8	5	275	1	2	11	14	6992

occurrence of conjunctivitis. Although NT isolates rarely cause invasive infection, they are frequently implicated in cases of pneumococcal conjunctivitis. Haas et al. (2011) have reported high proportion of nontypeable *Streptococcus pneumoniae* isolates among sporadic, nonoutbreak cases of bacterial conjunctivitis. Kojima et al. (2006) reported that penicillin resistance is spread among NT *pneumococci* typically associated with ophthalmic infections. And the serotypes included in PCV-7 now represent 46.2% (18/39) of strains isolated from eyes of pediatric patients with ocular infections in China. These findings have implications for the potential use of the 7-valent PCV in China.

Resistance to at least one antimicrobial is prevalent among ocular bacterial pathogens (Haas et al., 2011). The resistance of *S. pneumoniae* to antimicrobials varies over time, different regions, serotypes, and source of the specimen. Penicillin is one of the most commonly used drugs to treat *S.* p*neumoniae* infections. According to current breakpoint, all of our isolates with penicillin MIC ≤ 8 μg/ml were determined as PSSP. However, penicillin MICs for 5 isolates ranged 1.0 to 2.0 μg/ml. Among the 15 ophthalmic NT PSSPs, 12 (80.0%) had unaltered *pbp* genes; while alteration in either *pbp1a* or *pbp2b* was apparent in the remaining 3 (20.0%) isolates (Kojima et al., 2006). Interestingly, a predominance of high resistance to tetracycline, erythromycin, clindamycin, and trimethoprim-sulfamethoxazole was found in the present study, which suggested that these antimicrobials are not suitable for clinical treatment of pneumococcal infections in China. MDR isolates were common in our study, which is consistent with other previous reports (Song et al., 2004; Xue et al., 2010). However, markedly in contrast to these agents, nearly all isolates were shown to be susceptible to fluoroquinolones, and only 7.7% (3/39) had intermediate resistance to levofloxacin. This observation had also been reported from Hong Kong (Ip M et al., 2007). The reason may be that fluoroquinolones are not currently approved for children, except for some specific indications (Bowlware and Stull, 2004).

There is a paucity of data in recent literature on the prevalence of pediatric bacterial conjunctivitis (Patel et al., 2007). To our knowledge, this is the first study of pneumococci isolated from eyes in China in which MLST has been performed. The genotyping of 20 selected isolates showed that the pneumoccal population was highly heterogeneous. Of the 17 STs identified in this study, 14 have been described in other countries except 3 novel STs first found in the present study, including 2 of the 26 international resistant pneumococcal clones currently accepted by PMEN, Spain23F-1(ST81) and Taiwan23F-14 (ST236). ST280 and ST4542, ST876 and ST505 belong to subgroups of CC-ST156 (Spain9V-3) clone, SLV of CC-ST199 (M254-15B) clone and DLV of CC-ST180 (M264-3) clone, respectively. Although our 2 ST505 strains were NTs because of their mucoid appearance, ST180 and ST505 always show serogroup 3 positively associated with acute otitis media and acute conjuncitivitis, but with a low potential for cause invasive disease (Shouval et al., 2006).

All *S. pneumoniae* isolates in our study were recovered from children aged 0 to 2 years, The purpose of this study was to determine the susceptibility pattern, serotypes and genotypes of *S. pneumoniae* isolated from eyes of pediatric patients with ocular infections in China. The knowledge of population structure in *S. pneumoniae* isolates may have epidemiological and clinical implications. Further surveillance is needed in light of these findings, which hold important implications for treatment and management of ophthalmic infections caused by *S. pneumoniae*.

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