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Macrolide-Resistance Mechanisms in *Streptococcus pneumoniae* Isolates from Chinese Children in Association with Genes of *tetM* and Integrase of Conjugative Transposons 1545

Xuzhuang Shen, Hui Yang, Shangjie Yu, Kaihu Yao, Yonghong Wang, Lin Yuan, and Yonghong Yang

This study investigated macrolide-resistant *Streptococcus pneumoniae* carried by Beijing children presenting with respiratory tract infections. Nasopharyngeal *S. pneumoniae* strains were tested for sensitivity with 15 antibiotics and further analyzed for phenotypes of macrolide-resistant strains and by PCR for the macrolide-resistant genes *ermB*, *mefA*, *tetM*, and integrase of conjugative transposon (Tn1545) *intTn*. We found 185 strains of *S. pneumoniae* relatively highly resistant to erythromycin (78.9%), clindamycin (76.2%), tetracycline (86%), and SMZ-TMP (78.7%) but with relatively low resistance to amoxicillin (2.2%), cefaclor (15.5%), ceftriaxone (2.8%), and cefuroxime (14.1%). The 146 strains of erythromycin-resistant *S. pneumoniae* showed extensive cross-resistance to other macrolides like azithromycin (100%), clarithromycin (100%), acetylspiramycin (95.2%), and clindamycin (95.9%). Genes *ermB* and *mefA* were detected in all erythromycin-resistant strains, with *ermB*(+) 79.5%, *ermB* + *mefA*(+) 17.8%, and *mefA*(+) 2.7%. About 96.9% of tetracycline-resistant isolates were positive for *tetM*, compared to 26.9% of sensitive strains. Ninety percent of tetracycline-resistant strains were also erythromycin-resistant versus 11.5% of tetracycline-sensitive strains. The *intTn* gene was present in 87.6% of *S. pneumoniae* strains and correlated with erythromycin and tetracycline resistance. The close relationship between the conjugative transposon Tn1545 and the genes *ermB* and *tetM* is probably one of the important mechanisms explaining the multiple drug resistance of *S. pneumoniae*.

Introduction

Streptococcus pneumoniae is a major cause of childhood pneumonia, otitis media, meningitis, and septicemia. In recent years there has been an increasing trend worldwide of antibiotic resistance, with particular concern being expressed about drug resistance (DR) to commonly used antibiotic classes such as β-lactams and macrolides. Asia is a region of high prevalence for macrolide-resistant *S. pneumoniae*[13] and multiple resistance to several agents such as erythromycin and tetracycline is relatively common. The DR rates to clarithromycin, azithromycin, and clindamycin in Japan are 71.1%, 76.3%, and 36.8%, respectively[12] whereas the DR rate in Europe and North America is relatively low with DR rates for macrolides and clindamycin often below 35%.[4,23,15] Macrolides have been widely used in Chinese pediatric practice in recent years, due to their effective targeting of typical and atypical respiratory tract pathogens, yet as new macrolides become widely used in the clinic, resistance increases. In previous studies, we have shown that over 85% of *S. pneumoniae* clinical isolates from children in Beijing area were erythromycin resistant.[32]

S. pneumoniae macrolide-resistance mechanisms include: (a) 23S rRNA methylase encoded by DR gene *ermB*, which modifies the ribosome. The DR phenotype is MLSB, that is, cross-resistant to macrolides, lincosamides, and streptogramin B. The *ermB*-carrying strain displays a higher level of macrolide DR. (b) Active out-pumping system encoded by *mefA* is M phenotype of DR, that is resistant to 14- and 15-membered ring macrolides while sensitive to 16-membered ring macrolides. The *mefA*-carrying strains have a relatively lower macrolide resistance. (c) 23S rRNA, ribosomal protein L4 or L22 mutation. These strains bear no *ermB* or *mefA* gene. 1.5–1.8% of them belong to this mechanism.[6,21,2] Some reports have discussed the variations in regional distribution of macrolide resistance.[28,29,19,7,11,10,24,25,9] The *ermB* mutation is predominant in most European and Asian countries and *mefA* mutation in the United States and Hong Kong. Farrell reported that among macrolide-resistant *S. pneumoniae* in the

Laboratory of Microbiology and Immunology, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, Beijing, China.

PROTEKT US (2001–2002), 68.7% possessed *mefA*, 16.8% of isolates harbored *ermB*, and 12.2% possessed *ermB* + *mefA*. In Japan, *mefA* gene was detected in 44.8% and expressed as M type; 55.2% *ermB* genotype and phenotype MLS. Among these, there was only one strain of cMLS and the rest were all of iMLS type; in Taiwan, 65% cMLS type, 0.4% iMLS type, and 34.6% M type; In European counties, *ermB* gene 73%; in Canada, *ermB* and *mefA* genes were 46.5% and 48.8%, respectively. The tetracycline-resistant *S. pneumoniae* is mainly due to the *tet*-encoded protective effects of protein ribosome. That is, this type of protein is capable of interacting with the ribosome to render the bacterial strain insensitive to the bacteriostatic effects of tetracycline. *tetM* is the most common DR gene in *S. pneumoniae* and the next most common is *tetO*.[17,1]

The erythromycin- and tetracycline-resistant genes of *S. pneumoniae* are often carried by the conjugative transposon. For example, the *ermB* gene may be carried by such transposons as Tn1545, Tn917, and Tn3872.[16,31] *tetM* gene is located on the transposon Tn1545, Tn5251 or Tn916.[3] With a close relationship with the combined erythromycin- and tetracycline-resistant *S. pneumoniae*, the conjugative transposon Tn1545[27,20,8] may carry erythromycin DR gene (*ermB*), tetracycline DR gene (*tetM*), and kanamycin DR gene (*aph3′-β*). Through the mechanism of incisional integration, it may perform direct conjugative transposition among the same and different species of bacteria without the plasmid participation and integrate into the recipient chromosomes or different plasmid loci.[26] As the integrase gene of transposon, the *intTn* of Tn1545, is able to encode the essential integrase to integrate the transposon into a new locus. Measuring *intTn* is the common testing method of Tn1545. At present, there are few research reports on the relationship between the macrolide DR, tetracycline DR, and Tn1545 transposon in clinical isolates of *S. pneumoniae* among the Chinese children.

In the present study, the authors confirmed the prevalence of macrolide- and tetracycline-resistant *S. pneumoniae* isolated from the nasopharyngeal secretions of Chinese children with respiratory infections, analyzed the presenting characteristics of DR strains for *ermB*, *mefA*, and *tetM* genes, and illustrated their relationship with Tn1545 transposon integrase gene.

Materials and Methods

Materials

1. Source of bacterial strains: 185 strains of *S. pneumoniae* were collected during 2001–2003 from the nasopharyngeal secretions of 0–5 years old Beijing children suffering from acute URT infections.

2. Sensitivity testing: the *E*-test paper strip (AB Biodisk, Sweden) included penicillin, erythromycin, amoxicillin, cefaclor, ceftriaxone, and cefuroxime; drug sensitivity paper disk includes tetracycline, chloramphenicol, SMZ-TMP, ciprofloxacin, and oxacillin. The standard antibiotic products include azithromycin (15-membered ring macrolides), clarithromycin (14-membered ring macrolides), acetylspiramycin (16-membered ring macrolides), and clindamycin (lincosamide) (Chinese Testing Institute for Drugs and Biological Products). Paper disks of drug sensitivity include erythromycin 15 μg/disk and clindamycin 2 μg/disk paper disks purchased from OXOID Inc. (UK).

3. Taq enzyme, dNTP, PCR primer, and cetyltrimethylammonium bromide (CTAB) were purchased from Shanghai Sangon Biological Engineering & Technology and Service Co., Ltd.; Tris saturated phenol from Beijing DingGuo Biotechnology Development Center; and proteinase K from German SERVA Inc.

4. QC bacterial strains *S. pneumoniae* ATCC49619 was used as the strain for QC purposes to verify the *E*-test, agar dilution and Kirby Bauer (KB) sensitivity tests.

Methods

Antibiotics drug sensitivity testing. The *E*-test was carried out in accordance with the manufacturer's instruction for determining the minimal inhibitory concentration (MIC) of penicillin, erythromycin, amoxicillin, cefaclor, ceftriaxone, and cefuroxime against 185 strains of *S. pneumoniae*. Sensitivity to azithromycin, clarithromycin, clindamycin, and acetylspiramycin was assessed using the agar dilution method in accordance with the protocol NCCLS M7-A5. The drug sensitivity of tetracycline, chloramphenicol, SMZ-TMP, ciprofloxacin, and oxacillin was evaluated using the Kirby Bauer (KB) method in accordance with the protocol NCCLS M2-A7. Antibiotic sensitivity was classified according to the 2002 version of the NCCLS standard, except for acetylspiramycin for which no judgment value exists, sowe used the 2000 version of French SFM (sensitive strain MIC $\leq$ 1 μg/ml and DR strain MIC $\geq$ 8 μg/ml) as the judgment standard for this antibiotic. *S. pneumoniae* ATCC49619 was used as the control strain for the *E*-test, agar dilution, and Kirby Bauer (KB) sensitivity tests.

DR induction test for macrolides (KB method)[18]. The paper disks of erythromycin and clindamycin were placed 20 mm apart onto MH agar plates containing 5% defibrinated goat blood. The plates were then incubated with the resistant bacterial strains with McFarland turbidity of 0.5 at 35°C and 5% CO_2 for 18–24 hr. The test results showed that the bacterial inhibition circles on both paper disks had diameters within the ranges for DR and for the structural type of resistant phenotype cMLS, which showed sensitivity to clindamycin by the same method. However, the bacterial inhibition circle became blunt closer to the erythromycin side for the induction type of resistant phenotype iMLS. This phenotype showed sensitivity to clindamycin with no blunted bacterial inhibition circle closer to the erythromycin side.

Polymerase chain reaction testing. PCR was used to detect the 185 strains of macrolide-resistant *S. pneumoniae* genes *ermB*, *mefA*, and *tetM*.[4,22] The primers employed to detect *ermB*, *mefA*, and *tetM* were 5′ GAAAAGGTACTCAAC CAAATA 3′, 5′ AGTAACGGTACTTAAATTGTTTAC 3′; 5′ AGTATCATTAATCACTAGTGC 3′, 5′ TTCTTCTGGTAC TAAAAGTGG 3′; and 5′ GTGGACAAAGGTACAACGAG 3′, 5′ CGGTAAAGTTCGTCACACAC 3′, respectively.

Detection of *S. pneumoniae* conjugative transposon Tn1545 integrase gene intTn[5]. PCR was also used to detect this transposon. The primers were 5′ GCGTGATTGTATCT CACT 3′and 5′ GACGCTCCTGTTGCTTCT 3′. The length of PCR product was 1,046 bp. The PCR amplification product was treated as the same as above. The electrophoretic testing results of each gene are summarized in Fig. 1.

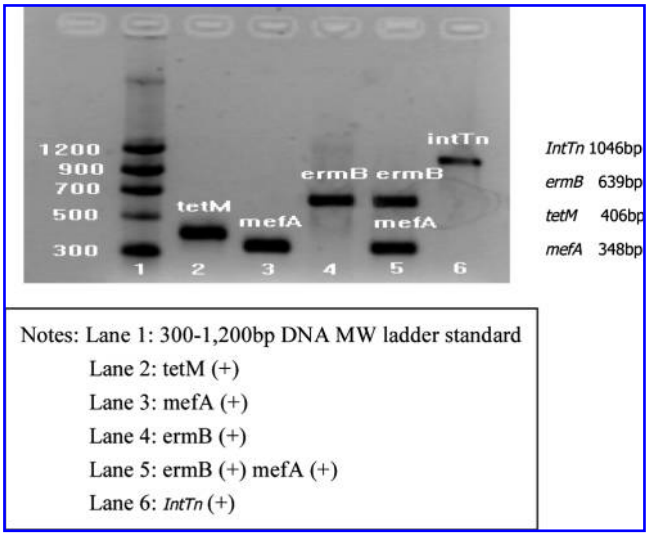

FIG. 1. Electrophoretic results of PCR products for five genotypic strains.

Statistics. Statistical processing software, type: SPSS 11.0, was used and the chi-square test was employed to analyze significance, with a significant difference set at probability $p < 0.05$.

Results

The drug sensitivity of the 185 isolated strains of *S. pneumoniae* to 15 antibiotics is shown in Table 1. The DR rates for macrolides, clindamycin, tetracycline, and SMZ-TM were relatively high at 78.9%, 76.2%, 86%, and 78.7%, respectively. The rates for amoxicillin, cefaclor, ceftriaxone, and cefuroxime were relatively lower at 2.2%, 15.5%, 2.8%, and 14.1%, respectively. Among these, 143 strains were resistant to both erythromycin and tetracycline, while 23 strains were sensitive to erythromycin and tetracycline. The MICs of isolated strains for azithromycin, clarithromycin, acetylspiramycin, and clindamycin were tested by the agar dilution method, yielding DR rates between 77.3% and 78.9%. About 95.2% of erythromycin-resistant strains were resistant to all tested macrolides, with both MIC50 and MIC90 being at or above 256 mg/L.

The macrolide-resistance induction results showed the cMLS phenotype was the most common among 146 drug-resistant strains, accounting for 95.9%. The iMLS phenotype appeared in only two strains and M type in only 4. The results for drug-resistant phenotypes and MICs were consistent with each other.

The relationship between the macrolide-resistant genotype and macrolide resistance is shown in Table 2. The *ermB* and *mefA* genes were detected in 146 strains of erythromycin resistance. Among these, the *ermB*(+) was 79.5%, *mefA*(+) only 2.7% and *ermB*(+) plus *mefA*(+) 17.8%; Neither *ermB*(-) or *mefA*(-) drug-resistant strains were detected. In the drug-resistant strains mediated by *ermB* or combined *ermB* + *mefA*, the MIC values for erythromycin, azithromycin, and clarithromycin were all ≥ 64 μg/ml. Four strains were found to show DR, which was due to the presence of *mefA* gene. The MIC values of erythromycin, azithromycin, and clarithromycin were 1–32 μg/ml. No *ermB* or *mefA* was detected in 39 macrolide-sensitive strains.

The relationship between the *tetM* genotype and tetracycline resistance is shown in Table 3. Of the 159 tetracycline-resistant strains, 154 (96.9%) were found to carry the *tetM* gene, compared to only seven (26.9%) of the 26 strains sensitive to tetracycline ($\chi^2 = 90.702$, $p < 0.01$). The distribution of erythromycin-resistant strain in tetracycline resistant and sensitive groups is listed in Table 3. The rates of erythromycin resistance in tetracycline-resistant group was 90%, which was higher than that of in tetracycline-sensitive group (11.5%) ($\chi^2 = 82.555$, $p < 0.01$).

The resistant rates in the group of strains that have the *intTn* gene were 88.3% for erythromycin, 94.4% for tetracycline, and 86.2% for sulphamethoxazole/tremethoprim, while the resistant rates in the group of strains that do not have the *intTn* gene were 13% for erythromycin, 26.1% for tetracycline, and

Table 1. Sensitivity Test of 185 *Streptococcus pneumoniae* Isolates

Method	*Antibiotics*	R%	*MIC_{50} (μg/ml)*	*MIC_{90} (μg/ml)*
E-test	Penicillin[a]	24.9	0.023	0.75
	Erythromycin	78.9	512	512
	Amox	2.2	0.016	0.75
	Cefaclor	15.5	0.38	24
	Ceftriaxone	2.8	0.032	0.75
	Cefuroxime	14.1	0.032	3
Agar dilution method	Azithromycin	78.9	256	256
	Clarithromycin	78.9	256	256
	Clindamycin	76.2	256	256
	Acetylspiramycin	77.3	128	256
Disk diffusion method	Tetracycline	86	–	–
	Chloramphenicol	26.4	–	–
	Sulph/trim	78.7	–	–
	Ciprofloxacin	55.7	–	–

[a]Penicillin include intermediary susceptible.
R, resistant; Amox, amoxicillin; Sulph/trem, sulphamethoxazole/tremethoprim.

Table 2. Correlation Between Macrolide-Resistance Genes and Macrolides and Clindamycin Resistance

		No. of isolates sensitivity to MIC (μg/ml)																	
		Erythromycin			*Azithromycin*			*Clarithromycin*			*Acetylspiramycin*				*Clindamycin*				
Gene type (no.)	R%[a]	≤0.25	0.5–32	64–128	R%	≤0.5	1–32	64–128	R%	≤0.25	0.5–32	64–128	R%	≤1	2–32	64–128	R%	≤0.25	0.5–32	64–128
*Erm*B (116)	79.5	0	0	116	79.5	0	0	116	79.5	0	0	116	79.5	0	31	85	78.1	2	1	113
MefA (4)	2.7	0	4	0	2.7	0	4	0	2.7	0	4	0	0	4	0	0	0	4	0	0
*Erm*B + *mef*A (26)	17.8	0	0	26	17.8	0	0	26	17.8	0	0	26	17.8	0	1	25	17.8	0	0	26
N[b] (39)	0	39	0	0	0	39	0	0	0	39	0	0	0	39	0	0	0	39	0	0

[a]Erythromycin resistant%.
[b]Neither *ermB* nor *mefA* was detected.

26.1% for sulphamethoxazole/tremethoprim. There was a statistically significant difference ($p < 0.01$) between the two groups for DRs with the *intTn* gene positive group showing higher resistance overall.

A total of 10 possible genotype/resistance relationships were investigated (see Table 4). The most common combination was *intTn* + *tetM* + *ermB*, accounting for 58.4%. Over 99% of the strains showing this genotype were resistant to erythromycin and tetracycline. The second most common combination, found in 26 strains was *intTn* + *tetM* + *ermB* + *mefA*. This genotype and the *intTn* + *tetM* + *ermB* genotype accounted for 72.4% of total number, with further four strains carrying *intTn*, *tetM*, and *mefA* simultaneously. Among 143 strains resistant to erythromycin and tetracycline, 140 strains (97.9%) were *intTn* positive and 133 (93%) were co-carriers of *intTn*, *tetM*, and *ermB*. Conversely, of the 23 erythromycin- and tetracycline-sensitive strains, only six (26.1%) were *intTn* positive genotypes.

Discussion

Of the 185 *S. pneumoniae* strains collected during the 2002–2003 study period, 79.5% were erythromycin-resistant, demonstrating a serious erythromycin resistance problem in the Beijing area of China. More importantly, *S. pneumoniae* is also showing significant resistance to newer macrolides such as azithromycin and clarithromycin. Furthermore, in the present study, resistance rates of erythromycin-resistant strains to azithromycin, clarithromycin, acetylspiramycin, and clindamycin were also all over 95%. This obviously presents a serious therapeutic challenge for antibiotic treatment in the Beijing area. The high rates of macrolide resistance amongst clinical isolates from this region are almost certainly due to the selective pressures created by overuse of these and other antibiotics in this region. Despite doubts in the literature about the clinical significance of *in vitro* drug sensitivity, some reports[14,30] have demonstrated the causal relationship between macrolide resistance and failed clinical therapy.

In terms of genetic factors, of the 185 *S. pneumoniae* strains isolated from nasopharyngeal swabs of pediatric URT cases in the Beijing area, *ermB* and/or *mefA* were detected in all the erythromycin-resistant strains. The *ermB*(+) strain was the most predominant accounting for 79.5% of erythromycin-resistant strains with the next most common being *ermB*(+) plus *mefA*(+) strain (17.8%), and *mefA*(+) strain (2.7%). In agreement with previous reports, these results suggest that the carrier state of macrolide-resistant *S. pneumoniae* among the children in Beijing area is caused by target modification and active out-pumping. Of these two mechanisms, the former is predominant. There are additional reports[18,4] claiming that there is correspondence between the genotype and phenotype of erythromycin resistance, that is, the MLSB phenotypic strain carries only *ermB* while M phenotype only *mefA*. As demonstrated by our research, the MLSB phenotypic strain may contain *ermB* and co-carry *ermB* + *mefA* genotypes while only *mefA* is detected in M phenotypic strain.

In China, tetracycline has been rarely used in clinical pediatrics over the past two decades, yet the tetracycline resistance levels for *S. pneumoniae* are still quite serious. This may be related to the overuse of tetracycline in agriculture and edible animals and relative stability of DR against this class of antibiotic.[28] The present study demonstrated that the

Table 3. Correlation of Tetracycline Resistance of 185 Isolates with Resistance Gene (*tetM*) and Erythromycin Resistance

		Genotype (%)		*Isolates*	
Isolates (%)	n	*tetM* positive	*tetM* negative	*Resistant to erythromycin*	*Sensitivity to erythromycin*
Resistant to tetracycline	159	154 (96.9%)	5 (3.1%)	143 (90.0%)	16 (10.0%)
Sensitivity to tetracycline	26	7 (26.9%)	19 (73.1%)	3 (11.5%)	23 (88.5%)
χ^2		90.702		82.555	
p-value		<0.01		<0.01	

main cause of tetracycline resistance is due to the close relationship of *tetM*-carrying isolated strains and tetracycline resistance. No *tetM* was detected in five strains of tetracycline resistance. The possibility of other existing drug-resistant genes needs to be considered. For example, non-expression of *tetO* and *tetM* genes. The exact reasons are the subject of ongoing further study.

The phenomenon of combined erythromycin and tetracycline resistance is quite common in China. As revealed by our findings, majority of isolated strains (77.3%) were resistant to both erythromycin and tetracycline, suggesting that the transposon Tn1545 may be widespread. To clarify this point we tested for the *intTn* gene in our 185 strains of *S. pneumoniae*. The results showed that the *intTn*(+) rate in erythromycin plus tetracycline-resistant strains was 97.9%; and co-carriage of *intTn*, *tetM*, and *ermB* accounted for 72.4% of isolated strains, resulting in a combined erythromycin/tetracycline resistance prevalence of 93.0%. These figures suggest that Tn1545 or Tn916–Tn1545 family have close relationship with *tetM* and *ermB*, as confirmed by the finding that over 99% of *intTn* + *tetM* + *ermB* co-carrying strains were resistant to both erythromycin and tetracycline, clearly suggesting that transposon Tn1545 is capable of carrying the erythromycin-resistant gene *ermB* and tetracycline-resistant gene *tetM*.

This finding is similar to those of Montanar *et al*.[17] and Seral *et al*.[26] Furthermore, the present study also found that the DR rates for the *intTn* gene positive group to erythromycin and tetracycline were markedly higher than those for *intTn* gene negative group. Within this group, four strains of *S. pneumoniae* carried *intTn* + *tetM* + *mefA* and had no *ermB* gene. There are also similar reports in Spain, Italy, and UK.[17,1,26] It is currently understood that there is no obvious link between *mefA*, *Tn1545*, *intTn*, and tetracycline resistance gene *tetM*. The tetracycline resistance of these strains may be mediated by transposon Tn916.[17]

In summary, children from the Beijing area with URT infections showed high nasopharyngeal carriage rates for erythromycin-resistant *S. pneumoniae*, some isolates of which were also highly resistant to newer macrolides and clindamycin. The phenotype of macrolide-resistant *S. pneumoniae* is mainly in forms of cMLS. The main mechanisms of action for erythromycin and tetracycline resistance are, respectively, target modification encoded by *ermB* and ribosomal protection encoded by *tetM*. Close relationships existing between conjugative transposon Tn1545 and *tetM* and *ermB* are probably one of the important mechanisms explaining the multiple DR of *S. pneumoniae* to macrolides and tetracycline. During the empiric therapy for pediatric respiratory tract infections, such antibiotics as macrolides and clindamycin must be used with great care to preserve their efficacy. In addition, resistance pattern monitoring of the common pediatric pathogens should be strengthened.

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Table 4. Correlation Between Genotypes and Antibiotic Resistance

InTn	TetM	ErmB	mefA	*No. (%)*	*Ery*	*Tetra*	*Chl*	*Pen*	*Sul/trim*	*Ceft*
Gene resistance% (except for oxacillin, nonsensitive) (n = 185)										
+	+	+	−	108 (58.4)	100	99.1	37	13	90.7	0.9
+	+	+	+	26 (14.0)	100	100	3.8	69.2	96.2	15.4
+	+	−	−	18 (9.7)	0	66.7	18.8	11.1	41.2	0
−	−	−	−	17 (9.2)	0	0	0	17.6	11.8	0
+	−	+	−	5 (2.7)	100	60	20	60	80	0
+	+	−	+	4 (2.2)	100	100	0	25	100	0
−	+	−	−	3 (1.6)	0	100	66.7	33.3	66.6	0
−	+	−	−	2 (1.1)	100	100	0	100	100	0
+	−	−	−	1 (0.05)	0	100	100	100	100	0
−	−	+	−	1 (0.05)	100	100	0	100	0	0
Total				185 (100)	78.9	86	26.4	24.9	78.7	2.8

Ery, erythromycin; Tetra, tetracycline; Chl, chloramphenicol; Pen, penicillin; Ceft, ceftriaxone; Sul/trim, sulphamethoxazole/trimethoprim.

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Address reprint requests to:
Prof. Yonghong Yang
Beijing Children's Hospital
Capital Medical University
56 South Lishi Road
Beijing 100045
P.R. China

E-mail: yyh66@vip.sina.com