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Antibiotic susceptibility profile of *Haemophilus influenzae* and transfer of co-trimoxazole resistance determinants

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Abstract. Prior to the implementation of *Haemophilus influenzae* type b vaccination worldwide, *H. influenzae* has been one of the main causative agents of community acquired pneumonia and meningitis in children. Due to the lack of information on the characteristics of the *H. influenzae* isolates that have previously been collected in Malaysia, the *H. influenzae* were assessed of their microbial susceptibility to commonly used antibiotics. Emphasis was made on strains that were resistance to co-trimoxazole (SXT) and their mode of transfer of the antibiotic resistance determinants were examined. A collection of 34 *H. influenzae* isolates was serotyped and antimicrobial susceptibility tests were performed to 11 antibiotics. To the isolates that were found to be resistant to co-trimoxazole, minimum inhibition concentration (MIC) to SXT was performed using Etest while agar dilution method was used to measure the individual MICs of trimethoprim (TMP) and sulfamethoxazole (SUL). These isolates were also examined for presence of plasmid by PCR and isolation method. Conjugal transfers of SXT-resistant genes to SXT-susceptible hosts were performed to determine their rate of transfer. Result showed that 20.6% of the total number of isolates was serotype B while the remaining was non-typeable. Antimicrobial susceptibility profile of all the isolates revealed that 58.8% was resistant to at least one antibiotic. Majority of these isolates were equally resistant to ampicillin and tetracycline (29.4% each), followed by resistance to SXT (26.5%). From nine isolates that were found to be SXT-resistant, five contained plasmid/s. Conjugal transfer experiment showed that these five isolates with plasmid transferred SXT-resistance determinants at a higher frequency than those without. From these observations, it is postulated that plasmid is not involved in the transfer of SXT-resistance genes but presence of plasmid facilitates their transfer. The information obtained from this study provides some basic knowledge on the antimicrobial susceptibility pattern of the *H. influenzae* isolates and their mode of transfer of SXT-resistance genes.

INTRODUCTION

Haemophilus influenzae is one of the common causes of community-acquired respiratory infection affecting mostly young children and the elderly. In Malaysia, it was reported to be one of the causative pathogen for community acquired pneumonia (Hooi *et al.*, 2001; Liam *et al.*, 2001). There are six antigenically distinct capsular types (a-f) with the type b being the major cause of postneonatal childhood meningitis. In 2000, it was estimated that globally, there were 8.13 million of serious illness due to *H. influenzae* type b (Hib) (Watt *et al.*, 2009). Despite the high incidence, Hib was rarely detected in the recent years, possibly due to the widespread use of antibiotics which may have hidden the true prevalence of the disease (Lolekha *et al.*, 2000) and/or the implementation of *H. influenzae* type b (Hib) vaccine which was introduced in 2002 (Morris *et al.*, 2008). Prior to the vaccination programme, *H. influenzae* was the most common pathogen than *Streptococcus pneumoniae* that had caused lower

respiratory infections in very young children in Malaysia (Ngeow *et al.*, 1997).

Since the first identification of β-lactamase producing isolate in 1972, increasing numbers of *H. influenzae* isolates have been reported to be resistant to multidrugs (Hoban & Felmingham, 2002; Hu *et al.*, 2002; Harrison *et al.*, 2009). In many parts of the world, *H. influenzae* isolates resistant to β-lactam antibiotics are the most frequently reported followed by co-trimoxazole, a combination of trimethoprim (TMP) and sulfamethoxazole (SUL) (Lim *et al.*, 1994; Sahm *et al.*, 2000; Thornsberry & Sham, 2000). Several surveillance studies on antimicrobial resistance showed that co-trimoxazole (SXT)-resistant *H. influenzae* are frequently isolated worldwide (de Almeida *et al.*, 2006). In some countries in Asia, 60% of the SXT prescriptions were mainly for otitis media infection (Sahm *et al.*, 2000) while in Finland, 81% and 15% of all SXT were used for treatment for respiratory and urinary tract infections, respectively (Karpanoja *et al.*, 2008).

Most of the past reports of *H. influenzae* infections in Malaysia were mainly focused on retrospective and case studies. To date, no attempts have been made to study the characteristics of the *H. influenzae* strains that have been isolated in Malaysia since 1995. The mechanisms of transfer of ampicillin and tetracycline resistance determinants in *H. influenzae* have been well-documented (Dimopoulou *et al.*, 1992, 1997; Mohd-Zain *et al.*, 2004) as opposed to mechanisms involved in the transfer of SXT resistant genes as a package.

In this work, we examined the antimicrobial susceptibility profile of a collection of *H. influenzae* isolates and investigated whether plasmid is involved in transfer of the SXT resistant determinants within the same species. By understanding the molecular characteristics of the bacterium would provide some knowledge that could be used as an important platform in designing strategies to counter the development of antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Thirty-four isolates of *H. influenzae* collected by the Institute for Medical Research, Kuala Lumpur from 1995 to 2007 are listed in Table 1. The strains were grown on Chocolate II agar with 1% IsoLac™ (Becton-Dickinson, MD) and incubated in 5-10% CO_2 atmosphere for 48 hours. All the isolates were identified by standard methods including their requirement of X and V factors (Killian, 1976) and further confirmed using 16S rPCR (Quentin *et al.*, 1996). The products of the amplifications were sequenced and the compared to the DNA sequences stored in GenBank using the basic local alignment software (BLAST, National Center for Biotechnology Information, Bethesda, MD).

For antimicrobial susceptibility test, Muller Hinton (MH) agar supplemented with 15% haemin and 10% NAD was used. Brain heart infusion (BHI) broth and *Haemophilus influenzae* b (HIB) agar used in the study of conjugal transfer was supplemented with 15% haemin and 10% NAD. HIB agar was prepared according to Mohd-Zain *et al.* (2004). *Haemophilus influenzae* strain ATCC 49247 was included as quality control strain in the antibiotic susceptibility test.

Serotyping of *H. influenzae* isolates

The isolates were serotyped using classical slide agglutination test with *H. influenzae* polyvalent antiserum for presumptive identification followed by serotype-specific antiserum (Difco, USA).

Antibiotic susceptibility testing

Disk diffusion agar method was used to determine the susceptibility of the isolates to 11 antibiotics (BBL, USA) according to the guidelines recommended by the Clinical Laboratory Standard Institute (CLSI, 2002). The isolates that were found to be resistant to SXT were used to determine the minimum inhibition concentration (MIC) of the SXT using Etest strips (AB Biodisk, Sweden). Agar dilution method was used to determine the

Table 1. List of *H. influenzae* strains used in this study

	Strain	Source	Serotype	Phenotype (Resistance)
1	H150/95	CSF	B	–
2	H151/95	Eye swab	NT	–
3	H152/95	Sputum	NT	SXT
4	H153/95	Sputum	NT	SXT
5	H154/95	Sputum	NT	–
6	H155/95	Nasal	NT	C
7	H156/95	NA	NT	C, CXM
8	H157/95	Blood	B	–
9	H158/95	Blood	B	CXM
10	H159/95	Sputum	NT	–
11	H209/95	HVS	NT	–
12	H219/95	Sputum	NT	TE
13	H210/95	HVS	NT	–
14	H220/95	Sputum	NT	TE
15	H222/95	Sputum	NT	AMP, SXT
16	H223/95	Sputum	NT	AMP, SXT
17	H224/95	Sputum	NT	AMP, AMC
18	H225/95	Sputum	NT	–
19	H226/95	Sputum	NT	AMP, TE, SXT
20	H227/95	Sputum	B	AMP, TE
21	H228/95	Sputum	NT	–
22	H229/95	Blood	B	–
23	H252/95	Sputum	NT	SXT
24	H253/95	Sputum	NT	–
25	H254/95	NA	NT	–
26	H255/95	Sputum	NT	–
27	H256/95	Sputum	NT	–
28	H300/95	Sputum	NT	AMP, TE, SXT, S
29	H582/95	NA	B	AMP, C, TE
30	H603/95	Sputum	NT	TE
31	H597/96	Sputum	NT	TE, SXT
32	H607/98	Sputum	NT	AMP, C, SXT
33	H615/06	NA	B	AMP, TE
34	H620/07	NA	NT	AMP, TE

CSF: cerebrospinal fluid; HVS: High vaginal swab, NT: Non-typeable, NA: Data not available
SXT: Co-trimetroxazole (25 µg/ml); AMP: Ampicillin (30 µg/ml); AMC: Amoxicillin (30 µg/ml); C: Chloramphenicol (30 µg/ml); TE: Tetracycline (30 µg/ml); S: Streptomycin (25 µg/ml); CXM: Cefuroxime (30 µg/ml)

MIC of trimethoprim (TMP) and sulfonamide (SUL) by carrying out two-fold dilutions of TMP and sulfamethoxazole in the ratio of 0.5/9.5 µg/ml and 32/608 µg/ml, respectively.

Detection of plasmid

Screening for plasmids, both integrative as well as excised, was performed according to the methods of Leaves *et al.* with some modifications (Leaves *et al.*, 2000). Briefly, in each PCR reactions (20 µl) contained 10x PCR buffer, 2 mM dNTP mix, 25 mM $MgCl_2$, 100 pmol/µl each of forward and reverse primers, 2 units of Taq polymerase (Qiagen, Germany), template DNA and sterile water. Plasmid isolation by alkaline 'mini-prep' method was performed as described by Kado and Liu (1981) (Kado *et al.*, 1981).

Conjugative mobilization

Transfer assay were carried out from SXT-resistant strains (donors) to SXT-sensitive strains (recipients). Donors were grown on the supplemented MH agar supplemented with 10% NAD and 1.25 µg/ml of TMP and 23.75 µg/ml SUL (in a ratio of 1:20) and incubated for 8 h. Recipients with other antibiotic markers were grown on MH agar with the corresponding antibiotics.

A dense suspension of the cells was made in 1 ml of BHI broth and the cells were harvested by centrifugation at 8,000 rpm (Eppendorf, Japan). The pellet was resuspended in the broth to a final OD of approximately 0.5 MacFarland's Standard. To 1 ml of BHI broth, 4 ml of donor cells and 40 µl of recipient cells were added. The cells were gently mixed and 100 µl of the mixture was spread onto duplicate plates of HIB agar without antibiotics. The inoculum was allowed to dry in a short-incubation (5-10 minutes) at 37°C prior to incubation in CO_2 for 8 hours. The mated cells were harvested with BHI broth to a volume of approximately 1 ml. Serial dilutions were carried out to determine the viable counts by plating on selective BHI agar containing antibiotics for enumeration of colony-forming unit (cfu) of transconjugants, donors and recipients. The rate of transfer is measured by the number of transconjugants per donor cell.

RESULTS

Serotypes and antimicrobial susceptibility of *H. influenzae* strains

Of the 34 *H. influenzae* strains, seven (20.6%) were serotype B whereas the remainders were non-typeable (NTHI) (Table 1). Most (58.8%) of the NTHI were isolated from sputum. The result of the antibiotic susceptibility test showed that 20 (58.8%) of the total isolates were resistant to at least one antibiotic with 12 (35.3%) isolates were resistant to more than one antibiotic. Most of the isolates were resistant to ampicillin (29.4%), tetracycline (29.4%), SXT (26.5%), chloramphenicol (11.8%), rifampin (8.8%) and cefuroxime (5.9%) (Table 2). All strains were susceptible to cefotaxime and ciprofloxacin.

All the SXT-resistant strains (n = 9) that comprised two intermediates, were non-typeable. The MIC of SXT measured by Etest was 32 µg/ml for the resistant strains and 1 µg/ml for the intermediate strains. The MIC of TMP ranged from 2 to 16 µg/ml while SUL ranged from 38 to 304 µg/ml. The MIC of TMP/SXT for the susceptible strains was <0.5 /9.5 µg/ml.

Conjugal transfer of antibiotic resistance determinants and detection of plasmid

Highest rate of transfer of SXT determinant was observed in strain H222/95 (Table 3).

Table 2. Antimicrobial susceptibility profile of *H. influenzae* determined by disc diffusion

Antibiotic		Concentration (µg/ml)	Percentage (%)		
			R*	I*	S*
Co-trimetroxazole	SXT	25	26.5	5.9	67.6
Ampicillin	AMP	30	29.4	14.7	55.9
Amoxicillin	AMC	30	2.9	0	97.1
Chloramphenicol	C	30	11.8	11.8	76.4
Tetracycline	TE	30	29.4	14.7	55.9
Rifampin	RD	5	8.8	5.9	85.3
Streptomycin	S	25	2.9	8.8	88.3
Erythromycin	E	15	0	5.9	94.1
Cefuroxime	CXM	30	5.9	0	94.1
Cefotaxime	CTX	30	0	0	100
Ciprofloxacin	CIP	5	0	5.9	94.1

* Interpretation according to CLSI; R = resistant; I = intermediate; S = sensitive

Table 3. Detection of plasmid and transfer of SXT-resistant genes from by conjugation

Strain (Donor)	Presence of Plasmid	Conjugation Transfer of SXT-resistant Genes				
		Phenotype of Donor	Recipient	Selection Marker	Phenotype of Transconjugant	Rate of Transfer
H152/95	–	SXT	H224	AMP	SXT, AMP	2.8×10^{-2}
H153/95	–	SXT	H224	AMP	SXT, AMP	1.2×10^{-2}
H222/95	+ (i)	SXT, AMP	H155	C	SXT, C	2.0
H223/95	+ (e)	SXT, AMP	H155	C	SXT, C	1.0
H226/95	+ (e,i)*	SXT, AMP, TE	H155	C	SXT, C	0.7
H252/95	–	SXT	H224	AMP	SXT, AMP	1.4×10^{-3}
H300/95	+ (e)	SXT, AMP, TE	H155	C	SXT, C	1.1
H597/96	–	SXT, TE	H224	AMP	SXT, AMP	7.1×10^{-3}
H607/98	+ (e,i)	SXT, AMP, C	H219	TE	SXT, TE	0.6

SXT: co-trimoxazole; AMP: ampicillin; TE: tetracycline; C: chloramphenicol
+: plasmid detected; – : no plasmid detected; (i): Integrated plasmid; (e): Excised plasmid
*: plasmid isolated

From the nine SXT-resistant isolates, PCR detected five isolates that carry plasmid but by isolation, only isolate was positive for plasmid (Table 3). Of the five isolates, two had both excised as well as integrative plasmids. It was observed that the strains that possessed plasmid/s produced higher number of transconjugants compared to those without.

DISCUSSION

Haemophilus influenzae resistant to antibiotics remains a growing problem worldwide involving high impact on the cost of medical treatment. The pattern of antimicrobial resistance varies from one region to another. Thus, it is important to gather information on the local resistance pattern which could contribute to some knowledge for selection of appropriate empirical antibiotic therapy. In this present study, although the numbers of *H. influenzae* isolates examined were small and does not really reflect the true incidence of *H. influenzae* infections in Malaysia, nevertheless, the information obtained in this work provides some insights on the antibiotic susceptibility pattern and the mobility of the SXT resistance genes of *H. influenzae* isolates that were collected since 1995.

From the collection of *H. influenzae*, NTHI were observed to be more common than the serotype B, whereby, majority (61.7%) of the NTHI isolates were isolated from sputum. This observation is in agreement to the report that NTHI causing invasive diseases was rare in Malaysia (Lim *et al.*, 1994). To our knowledge, there is no current published report available on the invasive *H. influenzae* diseases in Malaysia since 1999, during which, it was reported that serotype b was accounted to 75 clinical cases with meningitis, pneumonia and septicaemia (Nik Khairulddin *et al.*, 1999). It is unclear whether incidence of invasive Hib infections in Malaysia has truly declined since the introduction of Hib vaccination programme in 2000, because there is no published data available on the current status of Hib infections. Elsewhere, implementation of Hib conjugated vaccination to children of <2 years of age has been shown to have eliminated Hib disease in Gambia (Adegbola *et al.*, 2005) and reduced incidences of meningitis in Bangladesh and Senegal (Adegbola *et al.*, 2005; Baqui *et al.*, 2007; Fleming *et al.*, 2011).

Although infections caused by NTHI are considered to be less significant than Hib, the importance of NTHI should not be disregarded. NTHI not only able to adhere and invade respiratory epithelial cells, it is also

able to form biofilms which reduces the effectiveness of an antibiotic therapy (Gallagher *et al.*, 2006; Erwin *et al.*, 2007).

Similar to the earlier reports, the susceptibility pattern of the *H. influenzae* isolated in Malaysia prior to 1995 did not differ from the present work. Ampicillin remains to be most frequent antibiotic to be resistant to the *H. influenzae* isolates, followed by chloramphenicol and SXT (Choo *et al.*, 1990; Nik Khairulddin *et al.*, 1999). The majority of the multiresistant strains (10 of 12) in this present study were co-resistant with ampicillin. Similar to many other reports, ampicillin-resistance genes commonly co-exist with tetracycline-resistance genes (Shen *et al.*, 2007; Saha *et al.*, 2008; Kunthalert *et al.*, 2009). Besides tetracycline-resistant genes, SXT-resistant gene co-existing with ampicillin-resistant genes was also equally common. This observation is comparable to the other countries whereby ampillicin and SXT resistance among *H. influenzae* were highly detected (Jones *et al.*, 2002).

Although SXT is not the first line of antibiotics for treatment of respiratory tract infections in Malaysia (Cheong *et al.*, 2004), it is recommended by WHO as an alternative in β-lactam-allergic patients for the treatment of non-severe childhood pneumoniae (Grant *et al.*, 2009). From this study, the persistent resistance to SXT accounts to the reduced in its popularity for *H. influenzae* infections.

Based on the present study, only five isolates of SXT-resistant strains possess plasmid/s. This observation suggests that SXT-resistance determinants are not necessarily plasmid-mediated but presence of the resistance genes on plasmid/s facilitate/s the transfer of the SXT-determinants in comparison to those without plasmid. In *H. influenzae*, trimethoprim resistance has been demonstrated to be due to mutation of the chromosomal dihydrofolate reductase (*dfr*) genes (de Groot *et al.*, 1996) whilst two mechanisms have been recognised to be responsible for sulfonamide resistance; i.e. by alteration of short insertion in chromosomal *folP* gene and acquisition of dihydropteroate synthase (DHPS) encoded by *sul2* (Enne *et al.*, 2002). In sulfonamide-resistant enteric bacteria (Skold, 2001) and the *H. influenzae* isolates examined by Enne *et al.* (2002), *sul2* was plasmid-borne. In this present study, it was found that although all the isolates were sulfonamide resistant, not all possess plasmid. Hence, we can postulate that the sulfonamide resistant in the SXT-resistance was attributed to alternation of short insertion in the *folP* gene rather than by plasmid which carries the *sul2*. In addition, conjugation may not be the common mechanism of transfer of SXT-resistance genes between strains of *H. influenzae*.

In conclusion, majority of *H. influenzae* that have been isolated in Malaysia were non-typeable and more than one-third were equally resistant to ampicillin and tetracycline, followed by SXT. In the SXT-resistant strains, it was confirmed that plasmid was not involved in the transfer SXT resistance genes; however, its presence has shown to facilitate the transfer of the resistance genes.

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