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Molecular insights of co-trimoxazole resistance genes in *Haemophilus influenzae* isolated in Malaysia

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Abstract. In the last few decades, co-trimoxazole (SXT), an antibacterial combination of trimethoprim and sulfamethoxazole, has been used for treatment of upper respiratory tract infection due to *Haemophilus influenzae*. The usage of this antibiotic has become less important due to emergence of SXT-resistant strains worldwide. Most reports associate SXT-resistance to the presence of variants of dihydrofolate reductase (DHFR) *dfrA* genes which are responsible for trimethoprim resistance; while the sulfamethoxazole (SMX) resistance are due to sulfonamide (SUL) genes *sul1* and *sul2* and/or mutation in the chromosomal (*folP*) gene encoding dihydropteroate synthetase (DHPS). This study aims to detect and analyse the genes that are involved in SXT resistance in *H. influenzae* strains that were isolated in Malaysia. Primers targeting for variants of *dfrA*, *fol* and *sul* genes were used to amplify the genes in nine SXT-resistant strains. The products of amplification were sequenced and multiple alignments of the assembled sequences of the local strains were compared to the sequences of other *H. influenzae* strains in the Genbank. Of the five variants of the *dhf*A genes, *dfrA1* was detected in three out of the nine strains. In contrast to intermediate strains, at least one variant of *folP* genes was detected in the resistant strains. Multiple nucleotide alignment of this gene revealed that strain H152 was genetically different from the others due to a 15-bp nucleotide insert in *folP* gene. The sequence of the insert was similar to the insert in *folP* of *H. influenzae* strain A12, a strain isolated in United Kingdom. None of the strains had *sul1* gene but *sul2* gene was detected in four strains. Preliminary study on the limited number of samples shows that the TMP resistance was attributed to mainly to *dfrA1* and the SMX was due to *folP* genes. Presence of *sul2* in addition to *folP* in seven strains apparently had increased their level of resistance. A strain that lacked *sul1* or *sul2* gene, its resistance to sulfonamide was attributed to a 15-bp DNA insert in the *folP* gene.

INTRODUCTION

Co-trimoxazole (SXT) an antimicrobial combination of trimethoprim (TMP) and sulfamethoxazole (SMX) have been used for treatment against *Haemophilus influenzae* infections for last few decades; however their use has decreased in recent years due to increasing numbers of *H. influenzae* strains resistant to SXT (Turnak *et al.*, 2001). In some countries however, SXT remains an alternative to treat patients sensitive to β-lactams and also because of its cost-effectiveness (Grant *et al.*, 2009). It is also being used as treatment for septic arthritis caused by non-capsulated *H. influenzae* (Quellec *et al.*, 2013). In Malaysia, recent reports on antibiotic resistance of *H. influenzae* strains are lacking, however, in other parts of the world, there are reports that co-trimoxazole resistance rank the highest in South Africa and India (Morobe *et al.*, 2013; Thomas *et al.*, 2013).

In enteric bacteria, resistance to TMP is mainly due to enzyme dihydrofolate reductase (DHFR) encoded by *dfr* genes,

which can be both chromosomal and plasmid-borne (Skold, 2001). The *dfr* genes are grouped into families A and B comprising *dfrA* and *dfrB*, respectively (Recchia *et al.*, 1997). The *dfrA* which consists of at least 20 genes has been identified in chromosomes of bacteria while some of its genes are found on mobile elements. Among the *dfrA* genes, five variants (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17*) are included in the common integron-carried genes which mediate widespread dissemination of TMP resistance (Grape *et al.*, 2005). In *H. influenzae*, mutation in the *dfrA* gene on the chromosome alters the enzyme structure which results in the loss of or a decrease in the level of TMP binding (de Groot *et al.*, 1996).

SMX is a sulfonamide (SUL) bacteriostatic antibiotic. SUL resistance in Enterobacteriaceae is usually attributed to the presence of dihydropteroate synthase (DHPS) variants encoded by *sul1* and *sul2* genes (Radstrom *et al.*, 1991). The *sul1* gene is mostly found linked to other resistance genes in integrons, while *sul2* is usually located on small plasmids (Skold, 2001). In *Neisseria meningitis* and *Escherichia coli*, resistance to SUL was mainly due to mutation of the chromosomal (*folP*) gene encoding DHPS (Skold, 2001). In *H. influenzae* strain Rd, *folP* gene was found duplicated on the genome with 135 kb nucleic acids apart. Both mechanisms i.e. acquisition of *sul2* genes and mutational changes in the chromosomal gene *folP*, occurred in strains of *H. influenzae* isolated in United Kingdom and Kenya (Enne *et al.*, 2002). In addition, the *sul2* genes of these strains were linked to streptomycin resistant genes, *strA* as well as *strB* (Radstrom & Swedberg, 1988).

The aim of this work was to detect the presence of the genes that are involved in SXT resistance in strains of *H. influenzae* isolated in Malaysia. Information on the molecular mechanism of SXT-resistance would provide some platform to formulate strategies to counter the development of bacterial resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A collection of 34 *H. influenzae* strains available at the Institute for Medical Research, Kuala Lumpur were used in this study. These strains were collected from 1995 to 2007. Seven of these strains were serotype B and the other 27 strains were non-typeable strains (Mohd-Zain *et al.*, 2012). These strains were grown on Chocolate II agar with 1% IsoLac™ (Becton-Dickinson, MD) and incubated for 48 hours in 5-10% CO_2 atmosphere.

Antibiotic susceptibility test

Disk diffusion agar method was used to determine the susceptibility of the 34 strains to SXT (TMP-SMX, 25 µg/ml; BBL, USA) according to the guidelines recommended by the Clinical Laboratory Standard Institute (CLSI, 2002). *Haemophilus influenzae* strain ATCC 49247 was included as quality control strain in the antibiotic susceptibility test. The isolates that were found to be resistant to SXT were used to determine the minimum inhibition concentration (MIC) of the combined TMP-SMX using Etest strips (AB Biodisk, Sweden). Additionally, a strain susceptible to SXT was included as a negative control.

Detection of antibiotic resistance genes by PCR

Preparation of DNA templates for PCR was according to Enne *et al.* (2002). Detection of five TMP resistance *dfrA* variant genes (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *drfA17*) by PCR was performed as described (Grape *et al.*, 2007). For the detection of *folP*, five sets of primers (DHPS1 to DHPS5) were used (Enne *et al.*, 2002). Primers DHPS1 and DHPS2 were derived from *folP* genes on *H. influenzae* Rd while DHPS3 and DHPS4 were obtained from *H. influenzae* strain A12. Primer DHPS5 was designed to detect an insertion in *folP* genes similar to *H. influenzae* strain A12. A single fragment

generated from amplification of *folP* using this primer would indicate that the strain carries two copies of *folP* of the same size. The sequences of primers to detect SUL resistance genes *sul1*, *sul2* and streptomycin resistance genes, *strA* and *strB*, were also obtained from the work of Enne *et al.* (2002). The primer sequences are shown in Table 1.

All PCR reactions were performed as described (Enne *et al.*, 2002; Grape *et al.*, 2007). The PCR products were viewed using Agilent 2100 DNA Bioanalyser system (USA) following the manufacturer's protocol.

DNA sequence determination

All of the PCR amplification products were purified using MinElute PCR kit (Qiagen, Germany) and the DNA fragments were sequenced by First Base Sdn. Bhd., Kuala Lumpur. These sequences were assembled using BioLign version 2.0.9 (*http://en.bio-soft.net/dna/BioLign.html*) and confirmed of the respective genes using BLAST tool (*http://www.ncbi.nlm.nih.gov*). Multiple alignments of the sequences of the test strains with *H. influenzae* strains Rd and A12 were carried out using CLUSTALW (*http://www.ebi.ac.uk*).

Table 1. Oligonucleotide primers used in PCR for detection of resistant genes

Primer	Sequence (5' – 3')	Annealing Temp (°C)	Reference
dfrA1F	TGGTAGCTATATCGAAGAATGGAGT	52	Grape *et al.*, 2007
dfrA1R	TATGTTAGAGGCGAAGTCTTGGGTA	52	
dfrA5F	AGCTACTCTTTAAAGCCTTGACGTA	55	
dfrA5R	GTGTTGCTCAAAAACAACTTCG	55	
dfrA7F	ACATTTGACTCTATGGGTGTTCTTC	55	
dfrA7R	AAAACTGTTCAAAAACCAAATTGAA	55	
dfrA12F	GAGCTGAGATATACACTCTGGCACT	60	
dfrA12R	GTACGGAATTACAGCTTGAATGGT	60	
drfA17F	ACCTCAAGTGAACAGTAGACAAAT	55	
drfA17R	TCTCTGGCGGGGGTCAAATCTAT	55	
DHPS1F	CCACCAAAATCACTCTAA	52	Enne *et al.*, 2001
DHPS1R	ATGCATAATACAAACAGG	52	
DHPS2F	TAGAAGAGGGGGCGACAA	50	
DHPS2R	ATAAAACCATCAGGCATT	50	
DHPS3F	TGATAGCGGACAGTTTTT	50	
DHPS3R	TCATTGATTTGCGAGATA	50	
DHPS4F	CGTCCGTCATTCCTTTAT	52	
DHPS4R	ACTGCCTATCACTCTCTG	52	
DHPS5F	TGGAAGAAGGGGCGACAA	55	
DHPS5R	ACTACTGGCACAACACGA	55	
sul1F	CCGATATTGCTGAGGCGGACT	58	
sul1R	CCAACGCCGCTTCAGCTT	58	
sul2F	TCGTCAACATAACCTCGGACAG	55	
sul2R	GTTGCGTTTGATACCGGCAC	55	
strAF	CAACTGGCAGGAGGAACA	55	
strAR	CGCAGATAGAAGGCAAGG	55	
strBF	TTCTCATTGCGGACACCT	57	
strBR	GGCATTGCTCATCATTTG	57	

RESULTS

Antimicrobial susceptibility of *H. influenzae* strains

By using disk diffusion method, nine strains were found resistant to SXT. Etest carried out on these nine strains showed that the MIC of SXT against the resistant strains ranged from 1 to > 32 µg/ml whereas the MIC against the susceptible strain (H209) and the reference strain (ATTC 49247) was 0.5 µg/ml and 0.064 µg/ml, respectively (Table 2).

Detection of antibiotic resistant genes

Table 2 summarizes the results of the detection of genes for TMP and SUL (SMX) resistance in the strains. No resistance genes were detected in either the SXT-susceptible strain (H209) or the ATTC strain 49247. Except for *dfrA1* gene, none of the strains showed detected *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17*. The *dfrA1* genes of the three positive strains were sequenced and found to be identical to each other. BLAST analysis of the *dfrA1* sequences were 100% identical to the trimethoprim resistant DHFR of *Staphylococcus aureus* (Genbank accession number NP_877983.1).

Using DHPS primers, *folP* was detected in all of the resistant strains, except the two intermediate resistant strains. Interestingly, four strains (H152, H222, H223, H226 and H607) were found to have a combination of *folP* genes from both strains Rd and A12. This is because the primers used in this study, DHPS1 and DHPS2 originally derived from *folP* of *H. influenzae* Rd while DHPS3 originated from *H. influenzae* A12. Conversely, the *folP* gene of H252 and H253 were similar to the *folP* of strain Rd only.

Multiple alignment of the nucleotide sequence of *folP* gene amplified by DHPS1 revealed a 15-bp insert in the *folP* of H152 (Fig. 1). This insert was identical to the insert of the *folP* gene of *H. influenzae* A12 as detected by primer DHPS5. This was confirmed by nucleotide analysis of the *folP* gene of H152 which showed 99% similarity to the insert in the *folP* of A12.The presence of a single band fragment of 118-bp in H152 detected by DHPS5 suggests that H152 carried two copies of *folP* genes of the same size. The insertion of the amino acid had caused the sequence of the *folP* in H152 to be genetically different from the other strains of *H. influenzae*.

Table 2. MIC of SXT in *H. influenzae* strains and detection of the appropriate antibiotic resistance genes by PCR

Strain	Origin	MIC[a] (µg/ml)	TMP^R *dfrA*[b]	Detection of genes by PCR				
				SUL^R			STR^R	
				folP[c]	*sul*1	*sul*2	*str*A	*str*B
H152	Kuala Lumpur	>32	+ (1)	+ (1,2,3,4,5)	–	–	–	–
H153	Kuala Lumpur	1	–	–	–	–	–	–
H222	Penang	>32	–	+ (1,3)	–	+	+	–
H223	Penang	>32	–	+ (1,3)	–	+	+	–
H226	Penang	>32	–	+ (1,2,3)	–	+	+	–
H252	Penang	32	–	+ (1,2)	–	–	–	–
H300	Penang	>32	+ (1)	+ (1)	–	+	+	–
H597	Kuala Lipis	3	–	–	–	–	–	–
H607	Alor Setar	>32	+ (1)	+ (1,3)	–	+	+	–
H209	Kuala Lumpur	0.5	–	–	–	–	–	–
49247	ATCC	0.064	–	–	–	–	–	–

[a]MIC of SXT by Etest (TMP-SMX, 25 µg/ml); S = ≤ 0.5 I = 1-2 R = ≥ 4;
[b]The parenthesis refers to the *dfr*A variant numbers (1, 5, 7, 12 and 17)
[c]The parenthesis refers to the DHPS primer numbers (1, 2, 3, 4, 5)

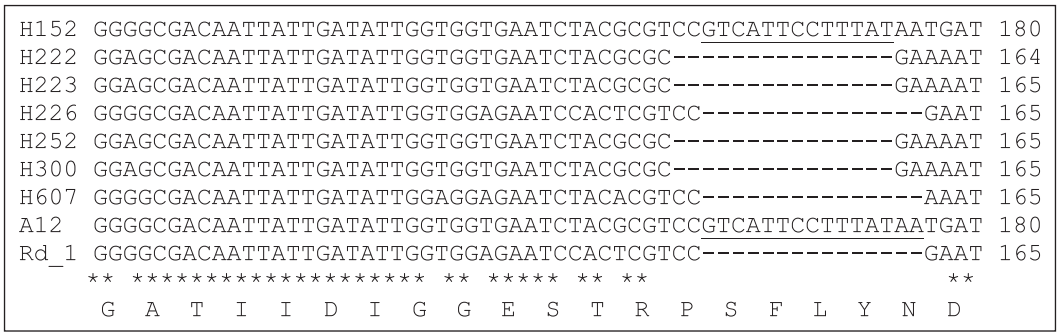

Figure 1. Multiple alignment of nucleotide sequence of a part of *folP* gene of *H. influenzae* strains amplified by DHPS1 which revealed a 15-bp insert in strain H152 similar to *H. influenzae* strain A12. The bottom line denotes the deduced amino acid sequence

It was also observed that all the nine strains lacked *sul1* gene, but five of the strains had *sul2* which were also positive for *strA* genes (Table 2). Gene *strB* was however, not detected in any of the strains. The nucleotide sequences of *sul2* and *strA* genes in the five strains were 100% identical to their respective genes.

The results obtained also showed that five strains possessed both *folP* and *sul2* genes while two strains (H152 and H252) had only *folP* gene. All of these five strains showed a high level of resistance to SUL, but it was also observed that although H152 lacked *sul* gene, the level of resistance to SUL was comparable to those having both *folP* and *sul2*.

DISCUSSION

In this study, the *dfrA1* gene was observed in only three of the strains. The DNA sequence of the *dfrA1* gene was identical to the DHFR of *S. aureus*. This suggests that only *dfrA1* gene of Enterobacteriaceae are common in TMP-resistant srains of *S. aureus* and *H. influenzae*. The assumption by Grape *et al.* (2007) that all five variants of *dfrA* genes in *E. coli* present in most Gram-negative bacteria do not include *H. influenzae* strains. The limited number of strains examined and the failure to explore the other remaining 15 *dfrA* genes could not provide definitive information on the *dfrA* genes of *H. influenzae*.

In a previous study, the resistance to SUL in *H. influenzae* was reported to be attributed to a mutation of the DHPS gene as well as to the acquisition of *sul* genes (Enne *et al.*, 2002). The resistance to SUL in the *H. influenzae* strains examined in this work was mainly due to *folP* rather than to *sul* genes because all the SXT-resistant *H. influenzae* possessed at least one *folP* gene. Intermediate resistant strains, however, lacked *folP* gene. The presence of *folP* in all of the resistant strains examined in this study provided evidence that *folP* was the main resistance determinant for SUL resistance.

Nucleotide sequencing of *folP* genes showed that all the strains except H152, had *folP* gene similar to Rd. Comparative analysis of the amino acid of the *folP* gene showed that strain H152 was genetically closer to strain A12 due to the presence of the 15-bp nucleotide insertion. Similar to the *H. influenzae* isolates of United Kingdom and Kenya (Enne *et al.*, 2002), the *folP* gene on the chromosome of H152 were of the same size. It is interesting to find a geographically distant strain to have an insert with DNA sequence identical to that of A12. It is likely that the nucleotides may have been the result of a recombination or a transfer from other bacterial species.

Similar to the findings of Enne *et al.* (2002), *sul1* was not detected in any of the strains. The *sul2* gene of strains examined by Enne *et al.* (2002) was found to be linked to *strA* and *strB*. The present data however,

showed that the *sul2* was linked only to *strA*; implying that the *sul2* gene were independent of *strB* genes.

Enne *et al*. (2002) also demonstrated the role of 15-bp insertion in mediating a high level of resistance to SUL. This justifies the reason for the strain H152 despite lacking in *sul* gene, yet showing comparable level of resistance, was compensated by the 15-bp insert. Similar to the strains isolated in United Kingdom and Kenya, two of the strains did not have any *sul* genes. As postulated by Enne *et al*. (2002), mutation in the chromosomal DHPS gene could have contributed to the resistance in strains lacking both *sul1* and *sul2*. The absence of both *sul* genes well as *folP* in intermediate resistant strains justifies the reason for their low resistance to SUL. This findings thus support the 15-bp insert plays a role in the increasing the level of SUL resistance.

In conclusion, the mechanisms involved in SXT resistance amongst Malaysian *H. influenzae* strains were mainly attributed to *folP*. The high level of resistance to SUL in a strain that lacked either *sul1* or *sul2* gene was attributed to a 15-bp DNA insert in *folP* gene.

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