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Article

Distribution of *sul* genes and their variants in uropathogenic *Escherichia coli* isolated from two hospitals of Sabah

Zaw Lin[1]*, Yun Mei Lai[1], Myo Thura Zaw[1] and Ahmad Toha Samsudin[2]

[1]Department of Pathobiological and Medical Diagnostics, Faculty of Medicine and Health Science, Universiti Malaysia Sabah
[2]Department of Pathology, Hospital Queen Elizabeth, Kota Kinabalu, Sabah

*Corresponding author: Pathobiological and Medical Diagnostics Department, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, Jalan UMS, Kota Kinabalu, 88400, Sabah, Malaysia. Phone: 320000 Ext: 611428; E-mail: zawlin@ums.edu.my

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Abstract: Sulphonamides resistant strains are highly prevalent in uropathogenic *Escherichia coli* (UPEC) isolates. *Sul* genes encode sulphonamide resistance and are present on transferrable plasmids. Integrons (IGNs) are genetic elements containing integrase gene, *attl* site and gene cassettes which carry multiple antibiotic resistant genes. Class 1 integrons have been extensively studied because these were most prevalent among clinical isolates. In this study, UPEC isolates were determined for the antibiotic susceptibility patterns to four antibiotics commonly used for urinary tract infections, which include co-trimethoxazole (TMP-STX). Distribution of *sul* genes and integrase1 gene (*intI1*) was studied in TMP-STX resistant UPEC isolates by using multiplex polymerase chain reaction (mPCR). *Sul* genes variants were investigated by DNA sequencing of the whole open reading frame of *sul1* and *sul2* genes and PCR product of *sul3* gene. *Sul1*, *sul2* and *sul3* genes were prevalent in 37 (24.7%) of 150 UPEC isolates. *IntI1* is positive in 22 *sul* genes positive isolates. Of six isolates positive with *sul2* genes, *sul*2(a) and *sul2*(b) variants, which were described in the previous study, in the four isolates and the two isolates respectively were observed. This is the first mPCR which investigates the prevalence of three *sul* genes and *intI1* in the UPEC clinical isolates from two hospitals of Sabah.

Keywords: uropathogenic *Escherichia coli*; *sul* genes; integrase 1 gene; *sul2* variants; multiplex polymerase chain reaction; hospitals of Sabah

1. Introduction

The major problem in treatment of UTI is the resistance of UPEC to antimicrobial agents. The previous studies indicated that there is increasing trend of UPEC resistance to TMP-STX. In Europe, TMP-STX resistance rates among uropathogens increased from 0–5% before 1990 to 9–26% in 1999 and 2000 (Blahna *et al*., 2006).

After their clinical introduction in 1935, sulfonamides were used to treat bacterial and protozoal infections. Sulfonamides have generally been used in combination with diaminopyrimidines to overcome the rapid emergence of resistance since 1970s (Huovinen *et al*., 1995; Huovinen, 2001). The combination trimethoprim-sulfamethoxazole is still commonly used in clinical practice and one of the drugs of choice for the treatment of urinary tract infections.

Dihydropteroate synthase (DHPS) is an enzyme important in the folic acid biosynthesis pathway. Competition of sulfonamides with the structural analog p-aminobenzoic acid for binding to dihydropteroate synthase (DHPS), results in inhibition of the formation of dihydrofolic acid (Sko¨ld, 2000). Resistance to sulfonamides in *E. coli* can result from mutations in the chromosomal DHPS gene (*folP*) as well as the acquisition of an alternative DHPS gene (*sul*). The protein encoded by *sul* gene has a lower affinity for sulphonamides

(SwedbergandSko¨ld, 1980; Swedberg *et al*., 1993; Ra°dstro¨m and Swedberg, 1998; Vedantam *et al*., 1998; Sundstro¨m *et al*., 1998).

Three alternative sulfonamide resistance DHPS genes (*sul1*, *sul2* and *sul3*) in gram-negative bacteria have been described. The DNA sequences of *sul1* and *sul2* from *E. coli* are different from all the known chromosomal DHPS genes from *E. coli* and other bacteria and their origin remains unknown (Ra°dstro¨m and Swedberg, 1998). *Sul1* has been found on large conjugative plasmids carrying on class 1 integrons (Ra°dstro¨m *et al*., 1991; Antunes *et al*., 2005; Hammerum *et al*., 2006; Trobos *et al*., 2008). The *sul2* gene has also been recently found on a wide range of large conjugative plasmids as well as small non-conjugative plasmids (Hammerum *et al*., 2006; Trobos *et al*., 2008; Bean *et al*., 2009).

Perreten and Boerlin described a new alternative sulfonamide resistance determinant called *sul3* which is probably acquired by *E. coli* from a distantly related organism. The presence of *sul3* on different plasmids in different *E. coli* clones, this gene was flanked by two insertional elements and a distinct DNA sequence of it suggested that it was a new *sul* gene (Perreten and Boerlin, 2003)

Rapid transmission of drug resistance in bacterial pathogens is the consequence of the widespread use of antibiotics as well as transfer of drug resistance determinants mediated by plasmids, transposons and gene cassettes in IGNs. The presence of variable no. of drug resistance genes in their gene cassettes makes the genetic organizations of IGNs diverse (Li *et al*., 2013). An IGN is defined as a genetic element that consists of three parts. The parts are the gene which encodes integrase that mediates site-specific recombination events, the site, *attI*, at which gene cassettes can be integrated by site-specific recombination and gene cassettes. Although IGNs themselves are not mobile, the integrase enzyme excises and integrates the gene cassettes from and into the IGN (Bennett, 1999; Fluit and Schmitz, 2004).

There are five classes of IGN and the first three classes are resistant integrons while class 4 and 5 are super-integrons. The most prevalent IGN is class I and it is present in gram-negative bacteria including *E. coli*. Class 1 IGNs have been extensively studied because these were most prevalent among clinical isolates as well as commensals out of three classes (Bass *et al*., 1999; Chang *et al*., 2000; Mazel *et al*., 2000; Naas *et al*., 2000; Sundeand Sorum, 2000)

Out of three *sul* genes, the *sul2* gene has been reported to be the most prevalent gene in *Escherichia coli*. This gene was highly conserved, and is usually carried on large conjugative resistance plasmids. *Sul2* genetic variation in *E.coli* isolates from animal, meat and human clinical samples and normal human was previously studied. Six point mutations were observed with four synonymous mutations and two non-synonymous mutations (Trobos *et al*., 2009). However, there were no reports of *sul1* and *sul3* genetic variants.

In this study, UPEC isolates were determined for the antibiotic susceptibility patterns to four antibiotics. Multiplex PCR of three *sul* genes and *intI1* gene was set up to screen TMP-STX resistant UPEC and application of this PCR method to detect three *sul* genes in UPEC isolates from two hospitals, Hospital Queen Elizabeth and Hospital Papar, of Sabah State, Malaysia. Most of the *sul* genes were present on IGN and detection of class 1 integrase was included in this study. In addition, DNA sequencing of three *sul* genes were undertaken to find out the variants of *sul* genes.

2. Materials and Methods

2.1. Samples and controls

One hundred and fifty UPEC isolates stocked in Microbiology Laboratory, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah were investigated for antibiotic susceptibility tests in this study. These UPEC isolates were derived from urine samples sent to microbiology laboratory (for culture and sensitivity) of two hospitals, Hospital Queen Elizabeth and Hospital Papar which are located around Kota Kinabalu, Sabah and we have checked for significant bacteriuria to include in the criteria for UPEC. The study was done on the urine samples collected between January and June, 2013.

Positive controls for *sul1, sul2, sul3, intI1* were pKTN117, pKTN118 and pKTN 119, pKTN 120 respectively. These plasmids were constructed with the PCR products of these genes, which were cloned into TopoTA plasmids. The genes inside the plasmids were sequenced, aligned with the sequences downloaded from the NCBI website and the DNA sequences were observed to be homologous. *Escherichia coli* ATCC 25922 was used as the negative control in this study.

2.2. Disc diffusion method

The stocked UPEC isolates were cultured on MacConkey agar and disc diffusion method was performed on Mueller–Hinton agar plates, according to Clinical and Laboratory Standards Institute (CLSI) guidelines to

perform antibiotic susceptibility test (AST) (CLSI, 2012).The antibiotic discs used in this study were co-trimethoxazole (TMP-STX), ciprofloxacin (CIP), gentamycin (GM) andcefotaxime (CTX).

2.3. Determination of minimum inhibitory concentration

This procedure was performed using the agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The minimal inhibitory concentration (MIC) is defined as the lowest antimicrobial concentration able to totally inhibit bacterial growth. *E. coli* ATCC 25922 was used as a negative control for both disc diffusion method and MIC study (CLSI, 2012).

2.4. Multiplex PCR for detection of *sul1, sul2, sul3 and intI1* genes

Those isolates having resistance to TMP-STX were studied for detection of *sul1, sul2, sul3* genes and *intI1*by multiplex PCR. The bacterial isolates were inoculated in 3 ml of Luria-Bertani broth and incubated at 37˚C for 18 hrs. The bacterial DNA was extracted by boiling method (Abdallah *et al*., 2011; Ifeanyi *et al*., 2015).
The primer sets for *sul1*, *sul2*, *sul3* genes, *intI1* gene and 16srRNA gene were mentioned in Table 1 (Kerrn *et al*., 2002; Gundogdu *et al*., 2011). The 16s rRNA gene was amplified in each reaction as an internal positive control for confirmation of *E. coli*. The ingredients for multiplex PCR were 6 µL of DNA template, 1x PCR buffer, 3mM $MgCl_2$, 0.4mM dNTPs, 1.5U of Taq polymerase, 0.4 mM each of 16srRNA gene, *sul1*, *sul2* and *intI1* sets of primers, 0.8mM of *sul3* forward and reverse primers and sterile distilled water was added to make total 25 µl of PCR mixture. The PCR conditions were 94°Cx5min, 30 cycles of 94°C x 30s, 58°C x 30s, 72°C x 1min and a final extension of 72°C x 7min.
Amplification was performed using Applied Biosystems Thermocycler and First base *Taq* DNA polymerase. The sizes of PCR amplicons were checked by 1.5% TAE agarose gel electrophoresis and Alpha Imager® HP System gel documentation apparatus after the gel was stained with ethidium bromide.

2.5. PCR for *sul1* and *sul2* whole gene sequence for detection of *sul* gene variants

For *sul1* gene and *sul2* gene the whole sequence amplification, the primer sets*sul1*ws and *sul2*ws primer sets shown in Table 1 were used. The primers sets were taken from NCBI website taking care of the precautions in primer design. The PCR ingredients were 6 µL of DNA template, 1x PCR buffer, 3mM $MgCl_2$, 0.4mM dNTPs, 1.5U of Taq polymerase, 0.4 mM of each forward and reverse primers of *sul1*ws for *sul1*. The PCR conditions were 94°C x 5min, 30 cycles of 94°C x 30s, 56°C x 30s, 72°C x 1min and a final extension of 72°C x 5min. The PCR ingredients were the same for *sul2* gene with the exception that forward and reverse primers of *sul2*ws were used. The PCR conditions were 94°C x 5min, 35 cycles of 94°C x 45s, 54°C x 45s, 72°C x 1min and a final extension of 72°C x 5min. Amplification and gel documentation were the same as mPCR method. For the *sul3* gene DNA sequence, PCR products of mPCR method were used.

2.6. DNA sequencing of PCR products

DNA sequencing of PCR products was performed with Applied Biosystems highest capacity-based genetic analyzer using Big Dye® Terminator v3.1 cycle sequencing kit.

2.7. Alignment of DNA sequences to find single nucleotide polymorphisms (SNPs)

The resulting DNA sequences were analyzed with A plasmid Editor (ApE) software and nucleotide sequences were aligned with wild type *sul* gene sequences downloaded from NCBI website to detect *sul* genes variants and nucleotide sequence numbers were counted according to wild type sequences.

3. Results

3.1. Results of AST and MIC value

One hundred and fifty UPEC isolates were tested for antibiotic susceptibility patterns with four antibiotics commonly used for UTI, which were mentioned in the method. Out of 150 isolates, forty, five, four and three isolates were resistant to TMP-STX, gentamycin, ciprofloxacin and cefotaxime, respectively. In case of MIC determination for the TMP-STX resistant isolates, it was observed to be 128/2432 µg/mL

3.2. Results of mPCR setting up and screening of *sul* genes and *intI1 g*ene in TMP-STX resistant UPEC isolates

After setting up of multiplex PCR (Figure 1), all the TMP-STX resistant isolates were investigated for *sul* genes and *intI1 g*ene. The distribution of *sul* genes were mentioned in Table 2. Presence of at least one of the *sul* genes was observed in 37 (92.5%) of 40TMP-STX resistant isolates. *Sul2* gene was most prevalent (68%), *sul1* gene

was 52% and *sul3* gene was the least (16%). Of total 22 isolates positive for *intI1*, 11 were *sul1* positive, 13 were *sul2* positive and 5 were *sul3* positive (Table 2).
There was absence of isolate having *sul1*, *sul2* and *sul3* genes together. An interesting finding was no coexistence of *sul1* and *sul3* in the studied UPEC isolates.

Table 1. Primer sequences for *sul* genes, *intI1* gene, 16srRNA gene for *E. coli*, *sul1*ws and *sul2*ws.

Target gene	Sequence of Primers	Expected size of PCR products
Sul1	F: 5'-CTTCGAT GAGAGCCGGCGGC-3'	433 bp
	R: 5'-GCAA GGCGGAAACCCGCGCC-3'	(Kerrn *et al*., 2002)
Sul2	F: 5'- GCGCTCAAGGCAGATGGCATT-3'	293 bp
	R: 5'-GCGT TTGATACCGGCACCCGT-3'	(Kerrn *et al*., 2002)
Sul3	F: 5'-GAGCAAGATTTTTGGAATCG-3'	790 bp
	R:5'- CATCTGCAGCTAACCTAGGGCTTTGGA-3'	(Gundogdu *et al*., 2011)
IntI1	F: 5'-CAGTGGACATAAGCCTGTTC-3'	160 bp
	R: 5'-CCCGAGGCATAGACTGTA-3'	(Gundogdu *et al*., 2011)
16S rRNA (*E.coli*)	F: 5'-GCGGACGGGTGAGTAATGT-3'	200 bp
	R: 5'-TCATCCTCTCAGACCAGCTA-3'	(Kerrn *et al*., 2002)
*Sul1*ws	F: 5'-ATGGTGACGGTGTTCGGCA-3'	840 bp
	R: 5'-CTAGGCATGATCTAACCCTC-3'	(this study)
*Sul2*ws	F: 5'-ATGAATAAATCGCTCATCA-3'	816 bp
	R: 5'-TTAACGAATTCTTGCGGT-3'	(this study)

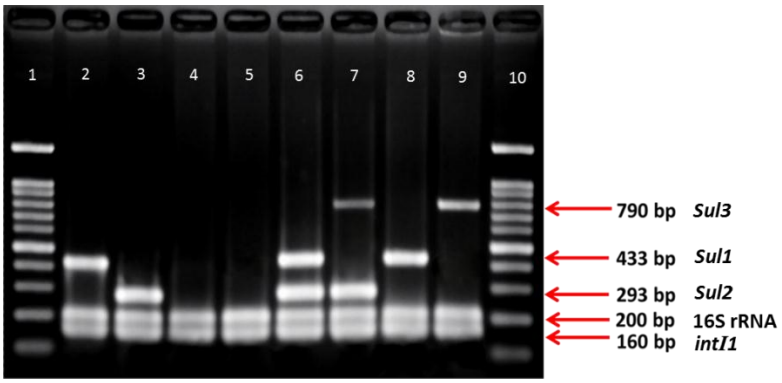

Figure.1. Gel electrophoresis picture of multiplex PCR for three *sul* genes, *intl 1*gene and 16s rRNA of *E. coli.* All the sample lanes are 16s rRNA and *intI1* gene positive. Lane 2,8 are*sul1* positive isolate. Lane 3 is *sul2* positive isolate. Lane 4 and 5 have no *sul* genes. Lane 6 is *sul1* and *sul2* positive isolate. Lane 7 is *sul2* and*sul3* positive isolate. Lane 9 is *sul3* positive isolate. Lane 1 & 10 are100bp ladder molecular marker. The sizes of PCR products are 433bp for *sul1* gene, 293bp for *sul2* gene and 790bp for *sul3* gene while 200bp for 16s rRNA gene and 160bp for *intI1* gene.

Table 2. Distribution of *sul* genes in TMP-STX resistant isolates and *intI1* positive isolates which were TMP-STX resistant.

Types of Isolates	*sul1*	*sul2*	*sul3*	*sul1*& *sul2*	*sul1*& *sul3*	*sul2*& *sul3*	*sul1*, *sul2* & *sul3*	Total no. of *sul* genes	Total no. of isolates tested
UPEC	8	12	4	11	0	2	0	37 (*22.8%)	162
intI1 Positive isolates	5	5	3	7	0	2	0	22	-

Footnote - *Percentage of *sul* genes in total UPEC isolates

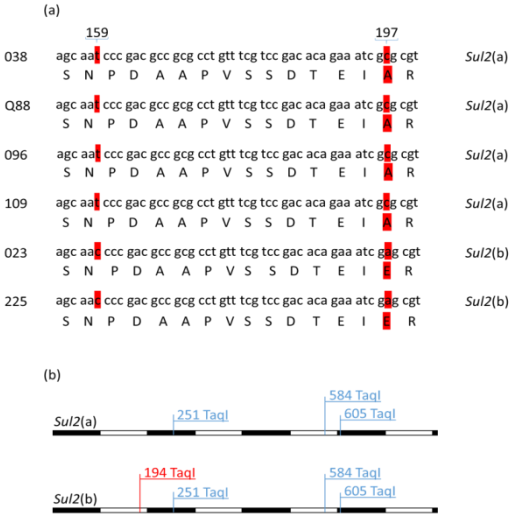

Figure 2. Six UPEC isolates with two *sul2* variants and differentiation of these variants by PCR-RFLP analysis (a) Nucleotide sequence alignment among six isolates with two *sul2* variants, *sul2*(a) and *sul2*(b)is shown. Isolates no. 038,Q88,096,109 have *sul2*(a) variants and isolates no. 023 and 225 have*sul2*(b) variants with change of amino acid from A to E (non-synonymous nucleotide change at 197 NP) (b) Restriction map of *sul2*(a) variant and *sul2*(b) variant are shown for PCR-RFLP analysis by *Taq*I restriction enzyme to differentiate *sul2* variants. *Sul2*(b) variant has extra-*Taq*I restriction site.

3.3. Investigation of *sul* genes variants

Altogether, six of each *sul* genes were undertaken for DNA sequence analysis and all the *sul1* and *sul3* genes were totally identical to the sequence of NCBI downloaded respective sequences. However, two isolates positive with *sul2* genes have one synonymous mutation at nucleotide position (NP) no.159 (T to C variant) and another non-synonymous mutation at NP no.197 (C to A variant), which changed from amino acid alanine to glutamic acid. Trobos *et al*. (2009) have named *sul2*(a) for T at NP 159 and C at NP 197 (TC variant) and *sul2*(b) for C at NP 159 and A at NP 197 (CA variant), which were observed in their study. As we followed the system described in that study, *sul*2(a) and *sul2*(b) variants in the four isolates and the two isolates respectively were observed among six *sul2* genes positive isolates sequenced (Figure 2 (a)). Mutation at nucleotide sequence no.197 could be investigated by PCR-RFLP analysis with *Taq* I restriction enzyme giving rise to 194 bp DNA fragment in case of *sul2*(b) variant gene while 251 bp DNA fragment was observed in *sul2*(a) variant (data not shown) (Figure 2 (b)).

4. Discussion

This is the first study to use multiplex PCR for detection of three *sul* genes in human UPEC isolates obtained from hospitals of Sabah. There has been a study on three *sul* genes in *E. coli* isolates from healthy humans, pork and pigs in Denmark and clinical samples were not included and the study did not mention that multiplex PCR was used. However, the literature has shown one PCR method for the four genes *sul1*, *sul2*, *sul3* and *intI1* indicating that their PCR was multiplex one (Hammerum *et al*., 2006).

In addition, Gundogdu *et al*. (2011) studied all three *sul* genes in UPEC. However, in their PCR *sul1* and *sul2* were detected in one PCR reaction while *sul3* was detected in another PCR reaction.

Wu *et al*. (2010) studied on 109 TMP-STX resistant *E. coli* strains isolated from pig feces, pig carcasses and human stools. In their study, 26 isolates from human stool were resistant to TMP-STX. Of these isolates, *sul2* gene was the most prevalent gene followed by *sul1* gene. There was no coexistence of *sul1* and *sul3* in these human isolates. *Sul1* and *sul2* coexistence was higher while *sul2* and *sul3* genes were together in lesser no. of isolates. These above mentioned findings were consistent with the findings in our study. Teichmann *et al.* (2014) indicated that *sul2* gene was highly prevalent in TMP-STX resistant UPEC isolates up to 67% in their study. In this study, *sul2* genes were present in 68% of TMP-STX resistant isolates showing that *sul2* genes was more prevalent than two other *sul* genes.

IGNs are capable of collecting of promoter-less gene cassettes through the actions of specialized site-specific recombination enzymes, *intl*. By mean of these mobile elements such as plasmids and transposons, IGNs can be horizontally transferred drug resistant genes between bacterial species commonly within the *Enterobacteriaceae*

family (Hall *et al*., 2003). Therefore, in this study screening of *intI1* gene was undertaken together with *sul* genes in mPCR assay.

In the Danish study on *E. coli* isolates from UTI cases and bacteremia cases, *sul1*, *sul2* and *intI1* gene were included in the multiplex PCR and *sul2* gene was the most common gene among these. The study did not include the *sul3* gene. In that study, 97% of TMP-STX resistant isolates had carried *sul* genes and 96% of *sul1* gene positive strains had *intI1*gene (Kernn *et al*., 2002). In case of Class 1 integron, *sul1* gene was commonly located at the 3' conserved region which is downstream of gene cassettes (Carattoli, 2001).In our study, 92.5% of TMP-STX resistant isolates had positive for one of *sul* genes and 53% of *sul1* gene positive strains were positive for *intI1* gene.

Trobos *et al.* (2009) had studied on *sul2* genetic variation in *E. coli* isolates from animal, meat and human clinical samples and normal human. Six point mutations were observed in 68 samples. The most common variant is *sul2*(a) and the second common variant was *sul2*(b) in their study. TC variants rarely contained an extra non-synonymous mutation at position 427 (A instead of G) and this mutation was named *sul2*(c) in that study and this variant was observed in two isolates. Other three synonymous mutations occurred at 288, 489 and 672 nucleotide positions and each mutation was present in only one isolate.

Presence of the *sul2* gene without much genetic variation in both human and animal isolates suggests horizontal transfer of the gene. Because only two to three point mutations, it did not indicate genetic drift and has been concluded by researchers that this had occurred within a short time (Sorum H and L'Abee-Lund, 2002). As an alternative hypothesis, it can be assumed that DHPS enzyme cannot tolerate many changes to its amino acid sequence (Trobos *et al*., 2009).

In this study, TMP-STX resistant UPEC were studied for distribution of *sul1*, *sul2* and *sul3* genes and their variants because we have observed that *sul* genes positive isolates had consistent AST pattern and MIC values. These data support the information there is no much genetic variation of *sul* genes at the DNA level. However, the previous work in Denmark indicated that there were *sul2* variants. Therefore it will be an interesting information if we find *sul* gene variants in our study.

Regarding two *sul2* gene variants, the two variants observed in this study were included and the most common variants found in the other study (Trobos *et al*., 2009). The observed variants we observed in this study were common in human clinical isolates in their study and *sul2*(a) variant is more prevalent compared with variant *sul2*(b) in both studies. Although geographical regions were far away between Denmark and Sabah, Malaysia, it is surprising that the common two variants existing in *sul2* gene in the two countries were consistent. We need further information on *sul2* gene variants to draw a firm conclusion.

5. Conclusions

Up to date, there were no mPCR technique which can detect three *sul* genes and *intl1* in isolates from UTI cases of hospitals in Malaysia. This study will be beneficial for the microbiologists to detect sulphonamide or TMP-STX resistant strains because PCR technique provides rapid and reliable results. UPEC isolates in this study were not highly resistant to gentamycin, ciprofloxacin and third generation cephalosporin indicating that antibiotics restriction policy was well controlled in the hospitals of Sabah. Furthermore, *sul2*(a) and *sul2*(b) variants could be investigated by PCR-RFLP analysis using *Taq* I restriction enzyme.

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Conflict of interest

None to declare.

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