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Comparison of the Activities among Three *SUL* Genes Present in Uropathogenic *Escherichia Coli*

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ABSTRACT

The three plasmid borne alternative dihydropteroate synthetase (DHPS) genes namely *sul1*, *sul2* and *sul3* genes were heterologous in amino acid sequence and have about 40 – 50% identity. However, they have same DHPS activity with disc diffusion zone size of 6 mm with suphamethoxazole disc in our previous study. *Sul1*, *sul2* and *sul3* genes were observed in sulphamethoxazole resistant uropathogenic *Escherichia coli* (UPEC). In this study, all the three genes were cloned into *E. coli* host and minimum inhibitory concentration (MIC) was investigated for each *sul* gene to compare the activities of *sul* genes. The MIC values of *E.coli* containing *sul2, sul1* and *sul3* genes inserted recombinant plasmid were observed to have 18.5 mg/ml, 18 mg/ml and 17.5 mg/ml respectively as mean value of five experimental results. Although comparable MICs were obtained as a result, the MIC value was highest in *E. coli* carrying *sul2* gene indicating that this DHPS enzyme activity of *sul2* was strongest among three *sul* genes.

INTRODUCTION

In the folic acid synthesis, DHPS is an important enzyme. Sulphonamides is similar in structure of para amino benzoic acid for binding of DHPS. This inhibited dihydropteroate synthetase activity[1]. Sulphonamides are the important antimicrobial agent for treatment of *E. coli* infections such as urinary tract infection (UTI). Mutations in DHPS gene in the chromosome results in resistance to sulphonamides in gram

positive bacterial, whereas the acquisition of plasmid-borne alternative DHPS gene caused resistance in gram negative bacterial including *E. coli*. Affinity of *sul* gene product to sulphonamides is low[2 - 4]. There are three plasmid-borne alternative DHPS genes namely *sul1*, *sul2* and *sul3* genes.

Sul1 is always observed on large conjugative plasmids carrying class 1 integrons. In the early report, *sul2* was commonly located on small non-conjugative plasmid but the updated study showed it to be present on large conjugative plasmid[5]. *Sul3* gene was first reported in pigs at Switzerland and it was consequently present in humans globally. *Sul3* gene has been observed in non-classic class 1 integron which was present on plasmid [5, 6]. This gene was first time reported in clinical UPEC isolate in 2003 at Sweden[7]. The previous report observed that *sul2* gene was the most common gene found in *E. coli* whereas *sul1* gene was also common followed by *sul3* gene which was rarely present in *E. coli*[8].

Sul1 and *sul2* from *E. coli* share 57% DNA level identity, and their origin remains unknown, as their sequences are totally different from all the known chromosomal DHPS genes from *E. coli* and other bacteria[9]. *Sul3* gene was 40.6% homologous in amino acid sequence to *sul2* from *E. coli* plasmid RSF1010[10], and 40.9% identical to *sul1* from *E. coli* plasmid R388[11, 12]. Although these three genes do not share amino acid identity, these were observed to have same alternative DHPS activity. However, potency of each *sul* genes was not well understood. The aim of this study is to test the potency of *sul1*, *sul2* and *sul3* gene on the sulfamethoxazole resistance activities. This is the first report to investigate the sulphonamides resistant activities of each *sul* gene.

MATERIALS AND METHODS

PCR Amplification of Complete DNA Sequence of *sul* Genes (*sul1*, *sul2* and *sul3*)

Gene bank data analysis was done for *sul* genes and the primers were designed to amplify the open reading frame of complete *sul* genes. The primer sequences were stated in Table 1 together with the size of amplicon (the open reading frame of genes). *Sul* genes were amplified under the PCR cycling condition listed in Tables 2. *Sul* genes were amplified from sulphonamides resistant UPEC and verified by DNA sequencing.

Table 1 Primer sets applied for the amplification of *sul* genes

Target gene	Sequence of Primers (5′ – 3′)	Size of amplicon (bp)
Sul1 WS	F: 5′ –ATG GTG ACG GTG TTC GGC –3′ R: 5′ –CTA GGC ATG ATC TAA CCC T –3′	840
Sul2 WS	F: 5′ –ATG AAT AAA TCG CTC ATC A –3′ R: 5′ –TTA ACG AAT TCT TGC GGT –3′	816
Sul3 WS	F: 5′ –ATG AGC AAG ATT TTT GGA ATC G –3′ R: 5′ –CTA ACC TAG GGC TTT GGA TAT T –3′	792

WS - whole sequence

Table 2 PCR conditions performed for the amplification of *sul* genes

	Sul1 WS	*Sul2 WS*	*Sul3 WS*
Initial	95°C for 5 minutes	95°C for 5 minutes	94°C for 5 minutes
Cycles	35	35	30
Denaturation	95°C for 30 seconds	95°C for 30 seconds	94°C for 30 seconds
Annealing	55°C for 30 seconds	55°C for 30 seconds	58°C for 30 seconds
Extension	72°C for 30 seconds	72°C for 30 seconds	72°C for 30 seconds
Final extension	72°C for 7 minutes	72°C for 7 minutes	72°C for 7 minutes
Stop	Hold at 4°C	Hold at 4°C	Hold at 4°C

Cloning of *sul* Genes into TA Cloning Vector

The PCR products were then cloned into TA cloning vector using a TArget Clone™ kit (TOYOBO, Tokyo, Japan). The reaction mixture for ligation was prepared and ligation was incubated at 24°C for 10 minutes according to the instruction of the manufacturer.

Transformation of Recombinant Plasmids

The recombinant DNA was transformed into the Competent Quick DH5α *E. coli* (TOYOBO, Osaka, Japan). Transferred 3 µL of recombinant DNA was transferred into 100 µL of competent cell and then the mixture was incubated in ice for 30 minutes. The mixture was heat shocked for 30 seconds in 42°C, transferred the tube into ice again and incubated for at least 2 minutes. The mixture was added into 900 µL of SOC (Super Optimal broth with Catabolite repression) medium and incubated at 37°C for 1 hour. One hundred µL of the mixture was then plated on MHA (Müller-Hinton agar, Becton Dickinson, USA) plates containing ampicillin and sulphamethoxazole, and incubated overnight at 37°C. Among the colonies on MHA agar, 6 colonies were selected for PCR to confirm *sul* genes were inserted in the TA vector.

Minimum Inhibitory Concentration (MIC) Determination of *E. coli* Containing Recombinant Plasmid Inserted with *sul1*, *sul2* and *sul3* Genes

MIC determination was performed according to the guidelines of Clinical laboratory Standards Institute[13]. Inoculum was prepared by inoculated a single colony of the *sul* gene positive transformed *E. coli* into 3 mL MHB (Müller-Hinton broth, Becton Dickinson, USA) and incubated at 37°C for 2 hours. The inoculum was adjusted with MHB to 5×10^5 CFU/mL. Adjusted inoculum was added to the vials containing MHB and sulphamethoxazole solution in the volume stated in Table 3.

Table 3 Content of serial Mueller Hinton Broth for M.I.C. determination

Final concentration of the mixture in 1 mL	Volume added (µL)		
	Sulphamethoxazole	**MHB**	**Inoculum**
0.0 mg/mL	–	500.0	500
5.0 mg/mL	25.0	475.0	500
6.0 mg/mL	30.0	470.0	500
7.0 mg/mL	35.0	465.0	500
8.0 mg/mL	40.0	460.0	500
9.0 mg/mL	45.0	455.0	500
10.0 mg/mL	50.0	450.0	500
12.0 mg/mL	60.0	440.0	500
12.5 mg/mL	62.5	437.5	500
13.0 mg/mL	65.0	435.0	500
13.5 mg/mL	67.5	432.5	500
14.0 mg/mL	70.0	430.0	500
14.5 mg/mL	72.5	427.5	500
15.0 mg/mL	75.0	425.0	500
15.5 mg/mL	77.5	422.5	500
16.0 mg/mL	80.0	420.0	500
16.5 mg/mL	82.5	417.5	500
17.0 mg/mL	85.0	415.0	500
17.5 mg/mL	87.5	412.5	500

18.0 mg/mL	90.0	410.0	500
18.5 mg/mL	92.5	407.5	500
19.0 mg/mL	95.0	405.0	500
20.0 mg/mL	100.0	400.0	500
25. 0 mg/mL	125.0	375.0	500

Absorbance at 600 nm was measured before and after overnight incubation by using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Waltham, USA). The experiment was repeated for five times and each measurement was repeated five times. The growth of the competent *E. coli* with recombinant inserted were determined by the change of absorbance measured. Competent *E. coli* without recombinant insertion was used as the growth control.

RESULTS

Amplified *sul* genes were verified by DNA sequencing and the sequences were deposited in NCBI GenBank. Accession number of *sul1*WS is MH765657, while *sul2*WS and *sul3*WS are MH765655 and MH765653, respectively.

Growth curve was plotted from the change of absorbance and average value of five times measurement was used. Among all three *sul* genes, sul2 needed 18.5 mg/mL of sulfamethoxazole to inhibit the growth while *sul1* and *sul3* required 18.0 mg/mL and 17.5 mg/mL respectively (Table 4). The growth was dropped drastically in the concentration of sulfamethoxazole at 12.0 mg/mL for all three genes (Figure 1). Competent *E. coli* without recombinant inserts did not grow in MHB containing sulfamethoxazole. All transformed and untransformed competent *E. coli* were grown in the sulfamethoxazole-free MHB.

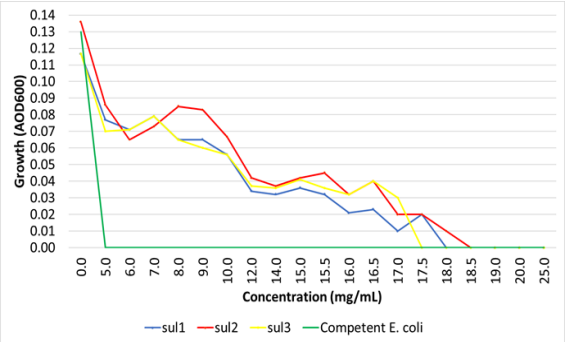

Figure 1 Graph for the growth of the competent *E. coli* with carried of different *sul* genes in different concentration of sulfamethoxazole

Table 4 Growth of the *E. coli* containing recombinant plasmids with different *sul* genes in different concentration of sulfamethoxazole

Concentration	Growth (A_{OD600})			
	Sul1	**Sul2**	**Sul3**	**Competent *E. coli***
0.0 mg/mL	0.12	0.14	0.12	0.13
5.0 mg/mL	0.08	0.09	0.07	0.00
6.0 mg/mL	0.07	0.07	0.07	0.00
7.0 mg/mL	0.08	0.07	0.08	0.00
8.0 mg/mL	0.07	0.09	0.07	0.00
9.0 mg/mL	0.07	0.08	0.06	0.00
10.0 mg/mL	0.06	0.07	0.06	0.00
12.0 mg/mL	0.03	0.04	0.04	0.00
14.0 mg/mL	0.03	0.04	0.04	0.00
15.0 mg/mL	0.04	0.04	0.04	0.00
15.5 mg/mL	0.03	0.05	0.04	0.00

16.0 mg/mL	0.02	0.03	0.03	0.00
16.5 mg/mL	0.02	0.04	0.04	0.00
17.0 mg/mL	0.01	0.02	0.03	0.00
17.5 mg/mL	0.02	0.02	0.00	0.00
18.0 mg/mL	0.00	0.01	0.00	0.00
18.5 mg/mL	0.00	0.00	0.00	0.00
19.0 mg/mL	0.00	0.00	0.00	0.00
20.0 mg/mL	0.00	0.00	0.00	0.00
25. 0 mg/mL	0.00	0.00	0.00	0.00

DISCUSSION

Antibiotics resistance acts by different mechanism at the genetic level. Fuoroquinolone resistance occurs mainly by mutations at the quinolone resistant determining region (QRDR) in *gyrA* and *parC* genes of chromosomal level. However, plasmid mediated quinolone resistance (PMQR) was also present e.g. *qnr* genes. Mutations at chromosomal level gave rise to high level resistance when compared to PMQR. Mutations at chromosomal level have consistent mutations in two genes which are mutations at amino acid 83 and 87 of gyrA gene and mutations at amino acid number 80 and 84 in *parC* gene[14].

However, in case of sulphonamides resistance, mutation at *folP* gene at chromosomal level is relatively rare in Gram-negative bacteria including *E. coli* so that research in that field is uncommon whereas plasmid borne alternative DHPS gene, *sul* genes, were distributed widely and level of drug resistance is high[8]. Although there are three *sul* genes in *E. coli*[8] and other Gram-negative bacteria up to now, these are heterologous at the amino acid level with consequent difference at nucleotide level[12] whereas their potency of drug resistance was nearly the same as shown by disc diffusion method[8]. Since the difference in amino acid levels is about 40 – 50%[12], it is worthwhile to study the comparison of the potency levels of the *sul* genes, although we know that there is not much difference in the potency between these genes based on the results of disc diffusion.

In the wild type *E. coli* strains, the size of the plasmids, the copy number of plasmids and other associated proteins in the host and the plasmid affect the level of expression and influence the drug resistant activity of each *sul* gene. To compare easily, we tried to clone each *sul* gene into same vector in the study and the recombinant plasmids were transformed into the same *E. coli* host for further expression of each gene. The activity of each DHPS enzyme was then compared for MIC by means of OD at Absorbance 600 and subculture method on culture plate. To reduce the experimental error, the same procedure was performed for five times and the mean value was taken as result and the plot was drawn with the concentration of sulfamethoxazole and OD at Absorbance 600.

In the previous study, there was no variant in *sul1* and *sul3* genes while *sul2* gene has 2 variants and also the most commonly distributed *sul* genes in *E. coli* isolates[8]. Therefore it may be necessary to test the other variant of *sul2* gene to draw the firm conclusion. However, we can conclude *sul2* gene has the strongest drug resistant activity in comparison with other two *sul* genes.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests in publishing this article.

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