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Biofilm formation in trimethoprim/sulfamethoxazole-susceptible and trimethoprim/sulfamethoxazole-resistant uropathogenic *Escherichia coli*

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ABSTRACT

Objective: To compare biofilm formation in trimethoprim/sulfamethoxazole (SXT)-susceptible *Escherichia coli* (*E. coli*) (SSEC) and SXT-resistant *E. coli* (SREC) isolated from patients with urinary tract infections, and study the motile ability and physical characteristics of the isolates.

Methods: A total of 74 *E. coli* isolates were tested for antimicrobial susceptibility with the disc diffusion assay. Based on the SXT-susceptibility test, the *E. coli* isolates were divided into SSEC ($N = 30$) and SREC ($N = 44$) groups. All *E. coli* isolates were examined for motile ability by using a motility test medium, and for checking biofilm formation a scanning electron microscope was used. Bacterial colony size was measured with a vernier caliper and bacterial cell length was measured under a light microscope. The bacterial growth rate was studied by plotting the cell growth (absorbance) versus the incubation time.

Results: The frequencies of non-motility and biofilm formation in the SREC group were significantly higher than that in the SSEC group ($P < 0.01$). The SREC bacterial cell length was shorter than that in the SSEC group [(1.35 ± 0.05) *vs.* (1.53 ± 0.05) μm, $P < 0.05$)], whereas the bacterial colony size and mid-log phase of the growth curve were not significantly different.

Conclusions: The present study indicated that biofilm formation and phenotypic change of uropathogenic *E. coli* can be attributed to the mechanism of *E. coli* SXT resistance.

1. Introduction

Urinary tract infections (UTIs) can develop into serious and potentially life-threatening infections of the kidney [1,2]. *Escherichia coli* (*E. coli*) is the most prevalent pathogen that causes community-acquired (about 80%) and hospital-acquired UTIs (more than 30%) [1–4]. Trimethoprim/sulfamethoxazole (SXT) is an antimicrobial combination drug that is widely used for the treatment of mild to moderate bacterial infections. It is recommended in areas where the prevalence of SXT–resistant pathogens does not exceed 20%. However, SXT resistance in *E. coli* has substantially increased from 17.9% in 2000 to 24.2% in 2010 in outpatients in the United States of America [5]. In addition, *E. coli* is often isolated from the urine of kidney stone patients with UTIs in the northeast of Thailand. These *E. coli* isolates are frequently found to be multidrug resistant [6].

The ability of *E. coli* to persist and grow as biofilms seems to be an important factor involved in both the severity of UTIs and antimicrobial resistance [7]. As the *E. coli* was protected within the bacterial extracellular matrix, antimicrobial agents were

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ineffective in eradicating the infection [8]. A previous study on uropathogenic *E. coli* isolates showed a significant correlation between biofilm formation and resistance to multiple antimicrobial drugs, such as ampicillin, amikacin, norfloxacin and SXT. Biofilm formation was increased in these *E. coli* isolates [9]. However, the previous studies did not exclude interfering factors, such as the ability of extended-spectrum β-lactamase (ESBL) producers, and did not select the *E. coli* isolates from the most compatible pattern of antimicrobial drug resistance. To obtain more information about the SXT resistance mechanism and its related factors, extensive analyses of changes in the physical characteristics as well as biofilm formation and motile ability are required. Therefore, the aim of this study was to compare biofilm formation in SXT-susceptible *E. coli* (SSEC) and SXT-resistant *E. coli* (SREC). The motile ability and physical characteristics, including bacterial colony size, cell length and growth rate, of the two groups were also evaluated.

2. Materials and methods

2.1. E. coli isolation and identification

Urine specimens from UTI patients were obtained from Srinagarind Hospital, Khon Kaen University between September 2012 and August 2013. Uropathogenic SXT-susceptible and SXT-resistant *E. coli* isolates were collected after identification at the Clinical Microbiology Laboratory, Srinagarind Hospital. The inclusion criteria were (i) pure isolation of *E. coli*; (ii) bacterial colony count $\geq 10^5$ CFU/mL; (iii) no multiple samples from the same patient; and (iv) non ESBL producer.

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility test on the selected *E. coli* isolates was performed by the disc diffusion method. Five antimicrobial agents were tested, which were amikacin (30 μg), gentamicin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg) and SXT (1.25/23.75 μg) (Oxoid Ltd., Basingstoke, England), according to the standard method of the Clinical and Laboratory Standards Institute [10]. Identification of ESBL-producing bacteria with resistance to third-generation cephalosporins was determined by the double-disc diffusion test. Based on the SXT susceptibility pattern, *E. coli* isolates were divided into two groups: SSEC (susceptible to all five antimicrobial agents) and SREC (susceptible to all antimicrobial agents except SXT) groups. Both of them were non ESBL producers.

2.3. Motility test

Each *E. coli* isolate was inoculated in the motility test medium (Bird Banding Laboratory, Maryland, USA) and incubated at 37°C for 24 h. A positive motility test was indicated by a turbid area extending away from the line of inoculation. A negative test was indicated by growth along the inoculation line.

2.4. Determination of biofilm formation by scanning electron microscope

Determination of biofilm formation with a scanning electron microscope was performed according to the modified method of Salo *et al* [7]. Each *E. coli* isolate was cultured in tryptic soy broth (TSB) (Oxoid Ltd., Basingstoke, England) overnight, and the concentration was adjusted to 0.5 McFarland standard (10^8 CFU/mL) with the same medium. One microliter of *E. coli* suspension was then subcultured in 40 μL of TSB in a sterilized 24-well plate; a sterilized glass slide (diameter 6 mm) was added into each well and incubated at 37 °C for 48 h. The culture medium was removed and the glass slide was fixed with 4% formaldehyde overnight. After removing the 4% formaldehyde, the cells on the glass slide were dehydrated with 25%, 50%, 75% and 96% ethanol for 20 min each at room temperature followed by air-drying. The cells were observed under a scanning electron microscope (HITACHI S-3000N, Hitachi Science Systems Ltd., Ibaraki, Japan). Positive biofilm formation was indicated by dense clusters of bacterial cells. A negative result was indicated by interspersed cells.

2.5. Evaluation of bacterial colony size

A single colony of each *E. coli* isolate was cultured in TSB and incubated for 24 h. After incubation, the *E. coli* suspension was adjusted to 0.5 McFarland standard. Fifty microliters of *E. coli* suspension was then spread onto MacConkey agar (Oxoid Ltd., Basingstoke, England) and incubated for 24 h. The diameters of all colonies on the spread plate were measured with a vernier caliper.

2.6. Evaluation of bacterial cell length

A sterile needle was used to pick up bacteria from a single colony on the same spread plate that was used for the bacterial colony size evaluation. The needle was suspended in 1 μL of distilled water, which was smeared on a glass slide (1.2 cm × 1.2 cm) and stained with 0.25% safranin O. The length of each cell in one field of view was measured under a light microscope (Nikon ECLIPSE 80i Microscope, Nikon Corporation, Japan).

2.7. Mid-log phase of bacterial growth curve

All *E. coli* isolates were cultured on MacConkey agar for 24 h. A single isolated colony from each strain was used to prepare a bacterial suspension of 0.5 McFarland standard in TSB. These suspensions were inoculated in a 96-well plate (200 μL/well) (Nunclon™ Delta Surface, Thermo Fisher Scientific, Jiangsu, China), and the optical density at 570 nm was recorded every 15 min for 12 h by a Tecan Sunrise plate reader (Tecan, Austria).

2.8. Ethics

This study was approved by the Ethics Committee of Khon Kaen University (HE551307).

2.9. Statistical analysis

All data were reported as mean ± SE of the mean. Statistical analysis was performed using SPSS software (version 17). To test differences between two groups, *Chi*-square and student's *t*-tests were used. *Chi*-square test was used to determine the relationship between biofilm formation and SXT resistance. A *P*-value less than 0.05 was considered to be statistically significant.

3. Results

According to the selection criteria, 74 *E. coli* isolates were included in this study. Based on the SXT susceptibility pattern, the *E. coli* isolates were divided into two groups: SSEC (30 isolates) and SREC (44 isolates). To minimize biofilm formation from other interfering factors, none of the studied *E. coli* isolates were ESBL producers and the SREC isolates were susceptible to all antimicrobial agents except SXT. The motile ability and biofilm formation of the two groups were shown in Table 1. Compared to the SSEC group, the SREC group showed a statistically significant lower frequency for the motile ability ($P < 0.01$) but higher frequency for biofilm formation ($P < 0.01$). Additionally, the data obtained from the both groups demonstrated positive correlations between the biofilm formation and SXT resistance ($P < 0.05$). The SREC cell length [(1.35 ± 0.05) μm] was shorter than that of the SSEC group [(1.53 ± 0.05) μm, $P < 0.05$]; whereas, the diameters of bacterial colony and mid-log phase of the growth curve were not significantly different in both groups (Table 2).

Table 1
Motile ability and biofilm formation of SSEC ($N = 30$) and SREC ($N = 44$) groups.

Parameters	SSEC group [*N* (%)]	SREC group [*N* (%)]	*P*-value
Motile ability	15 (50.00)	7 (15.91)	$P < 0.01$
Biofilm formation	17 (56.67)	39 (88.64)	$P < 0.01$

Table 2
Bacterial colony size, bacterial cell length and time of mid-log phase of SSEC ($N = 30$) and SREC ($N = 44$) groups.

Groups	Diameter of bacterial colony (mm)	Bacterial cell length (μm)	Time of mid-log phase of growth curve (h)
SSEC group	2.97 ± 0.09	1.53 ± 0.05*	3.91 ± 0.10
SREC group	2.89 ± 0.10	1.35 ± 0.05	3.68 ± 0.09

*: Statistically significant compared to SREC group ($P < 0.05$).

4. Discussion

The results from this study indicated that the incidence of biofilm formation among SREC in UTI patients from the northeast of Thailand is high (approximately 88.64%; 39 out of 44 isolates). This data were consistent with a previous report that demonstrated biofilm formation in 83% of uropathogenic *E. coli* in South India [9]. In addition, our data supported this report which indicated that biofilm formation was correlated to multidrug resistance, especially SXT resistance. The ability to form a biofilm in *E. coli* is an important factor in persistent infection and resistance to antimicrobial agents [7,11].

The present study revealed that the SREC group had a shorter bacterial cell length, higher frequencies of non-motility and biofilm formation than those of the SSEC group. The induction of biofilm formation in the SREC group would inevitably result in these phenotypic changes. Bacteria within a biofilm are phenotypically different from their planktonic forms, and they activate many genes that can alter their susceptibility to antimicrobial agents [11,12].

In conclusion, the present study has shown that the incidence of biofilm formation among SREC in UTI patients from the northeast of Thailand is high, and that biofilm formation in these *E. coli* isolates was associated with SXT resistance. The shorter cell length of the SREC isolates indicated phenotypic change that alters the susceptibility to antimicrobial agents. Further study on the uropathogenic *E. coli* biofilm formation may provide information about the mechanism of SXT resistance.

Conflict of interest statement

We declare that we have no conflict of interest.

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