MOLECULAR CHARACTERIZATION OF VIRULENCE AND ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF UROPATHOGENIC *ESCHERICHIA COLI* FROM PATIENTS IN A TERTIARY HOSPITAL, SOUTHERN THAILAND

Monchanok Themphachana[1], Suttiporn Kongpheng[2], Pattamarat Rattanachuay[3], Saowapar Khianngam[4], Kamonnut Singkhamanan[5] and Pharanai Sukhumungoon[1]

[1]Department of Microbiology, Faculty of Science; [5]Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Songkhla; [2]Department of Clinical Pathology, Microbiology Laboratory, Maharaj Nakhon Si Thammarat Hospital, Nakhon Si Thammarat; [3]Department of Pre-clinic, Faculty of Science and Technology, Prince of Songkla University, Pattani campus, Pattani; [4]Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Phetchaburi IT Campus, Phetchaburi, Thailand

Abstract. Among uropathogens, uropathogenic *Escherichia coli* (UPEC) is the most common cause of urinary tract infection (UTI) worldwide, but clinical aspects due to this bacterial species is not fully understood in southern Thailand. Two hundred fifty-four UPEC isolates from patients admitted to Maharaj Nakhon Si Thammarat Hospital, southern Thailand were examined for crucial virulence genes, showing that 33.5% contained at least one of the virulence genes tested. Genes encoding P fimbria, cytotoxic necrotizing factor-1 and α-hemolysin constituted the majority (15.8%) carried by UPEC isolates. Phylogenetic group classification revealed that 57.5% of UPEC belonged to group D. Antimicrobial susceptibility tests showed that 70.5% and 65.1% of the isolates were resistant to ciprofloxacin and norfloxacin, respectively. Moreover, 50.0% of UPEC were capable of producing extended spectrum beta-lactamases. These findings should be of benefit for more appropriate treatment of UTI patients in this region of Thailand.

Keywords: uropathogenic *Escherichia coli*, antibiotics resistance, *cnf1*, *hlyA*, *pap*, Thailand

INTRODUCTION

While diarrheagenic *Escherichia coli* (DEC) plays a crucial role in the human intestine, extra-intestinal *E. coli* (ExPEC) possessing virulence factors, can co-occupy within the human gut and upon release causes severe forms of illnesses in other organs, such as neonatal meningitis, sepsis and pneumonia (Smith *et al*, 2007). Among ExPEC members, uropathogenic *E. coli* (UPEC) is very common (Smith *et al*, 2007; Manges and Johnson, 2012) and frequently is found to cause urinary tract infection (UTI) in patients over a wide range of age groups (Bashir *et al*, 2012). Infection

Correspondence: Pharanai Sukhumungoon, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
Tel: + 66 (0) 74 288322; Fax: + 66 (0) 74 446661
E-mail: pharanai82@gmail.com

caused by UPEC may lead to variable degrees of severity depending on organ infected, resulting in bacteriuria, cystitis and pyelonephritis (Foxman, 2002). Some cases of infections result in renal scars and are associated with long-term morbidity (Montini *et al*, 2011).

The crucial step of bacterial pathogenesis is the adherence of UPEC to the uroepithelial cells, mediated by adherence-associated virulence factors, of which P fimbriae (P-blood group antigen-associated fimbriae), encoded by *pap*, is one of the most important factors. P fimbriae are involved in the enhancement of tubular epithelium adherence at the early colonization step (Bien *et al*, 2012). In renal transplant patients P fimbriae-expressing UPEC leads to acute allograft injury (Rice *et al*, 2006).

In addition to P fimbriae, some UPEC strains contain other adherence-associated virulence factors, such as Afa adhesin (coded by *afa*) involved in chronic and recurrent UTIs (Le Bouguénec, 2005) and S fimbriae (based on its receptor, sialyl galactosides) (encoded by *sfaDE*) that is reported to be associated with meningitis and sepsis (Ott *et al*, 1986; Le Bouguénec, 2005). Other than adherence factors, toxins produced by UPEC, such as α-hemolysin (encoded by *hlyA*) and cytotoxic necrotizing factor-1 (CNF-1) (encoded by *cnf1*), are also essential virulence factors leading to the death of a wide variety of cells *in vivo* and *in vitro*, the latter including Chinese hamster ovary (CHO), Vero and HeLa cells (Caprioli *et al*, 1983; Ghadir *et al*, 2010).

In southern Thailand, previous study has shown that there are high numbers of patients suffer from UTI daily and the elderly patients constitute a large proportion (Themphachana *et al*, 2014). Information regarding bacterial virulence and their antimicrobial resistant profiles is essential for public health measures. Thus, this study characterized UPEC virulence factors and their antimicrobial resistance profiles isolated from patients admitted to Maharaj Nakhon Si Thammarat Hospital, southern Thailand.

MATERIALS AND METHODS

Bacterial collection

Bacterial isolates were obtained from patients admitted to Maharaj Nakhon Si Thammarat Hospital, during July - December, 2014. All isolates were identified as *E. coli* by standard biochemical assays (FDA, 2012) and were confirmed by PCR amplifying *E. coli uidA* (Heninger *et al*, 1999). All UPEC isolates were kept as stock in 10% glycerol at -80ºC. The protocol was approved by Ethics Committee of the Faculty of Medicine, Prince of Songkla University, Thailand (EC 58-234-19-2).

PCR-based detection of virulence genes

PCR template was prepared by a boiling method (Pannuch *et al*, 2014). In brief, a single colony was inoculated into 3 ml of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) and incubated at 37ºC for 6 hours with aeration. One ml aliquot of bacterial culture was boiled for 10 minutes, then immediately immersed on ice for 5 minutes, sedimented and supernatant diluted ten-fold in sterile deionized water. Solution was kept at -20ºC until used.

Oligonucleotide primers employed in PCR amplification of UPEC virulence genes, *pap* (P fimbriae), *afa* (Afa adhesion), *sfaDE* (S fimbriae), *cnf1* (cytotoxic necrotizing factor-1), and *hlyA* (α-hemolysin), were listed in Table 1. PCR was conducted in a 25-μl mixture consisting of 0.4 μM

each primer pair, 0.1 mM dNTPs, 1X Go-*Taq* DNA polymerase buffer (Promega, Madison, WI), 0.5 U Go*Taq* Flexi DNA polymerase and 2 μl of DNA template. Thermocycling (conducted in T100™ Thermal Cycler, Bio-Rad, Hercules, CA) conditions were as follows: 95°C for 3 minutes; followed by 35 cycles of 94°C for 1 minute, 58°C for 50 seconds (for *sfaDE*, *cnf1* and *hlyA*), 60°C for 40 seconds (for *pap* and *afa*) or 60°C for 50 seconds (for *uidA*), and 72°C for 1 minute or 1.20 minute for *hlyA*; and a final step at 72°C for 5 minutes. Amplicons were analyzed by 1.0% agarose gel-electrophoresis, stained with ethidium bromide and visualized using WSE-5200 Printgraph 2M gel imaging system (ATTO, Tokyo, Japan).

PCR-based phylogenetic group determination

Investigation of phylogenetic group employed PCR amplified fragments of UPEC *chuA*, *yjaA*, and TspE4.C2 as previously described (Clermont *et al*, 2000). Uni-plex PCR was performed using primers listed in Table 1. A 25-μl mixture consisted of the same concentration of components as described above except for specific oligonucleotide primers to each gene. Thermal cycling conditions were as follows: 95°C for 3 minutes; followed by 35 cycles of 94°C for 50 seconds, 54°C for 50 seconds, and 72°C for 30 seconds; with a final step at 72°C for 5 minutes. Amplicons were also analyzed as mentioned above.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed by the disk diffusion method (CLSI, 2014) using 11 antimicrobial agents: amikacin (30 μg), cefoperazone/sulbactam (75 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), norfloxacin (10 μg), piperacillin/tazobactam (100/10 μg), meropenem (10 μg), and ertapenem (10 μg) (Oxoid, Hampshire, UK).

Extended spectrum β-lactamase (ESBL) production

Production of ESBL was determined by the double disc synergy test (DDST), using 3 types of third-generation cephalosporins (3GC), namely, ceftazidime (30 μg), ceftriaxone (30 μg), and cefotaxime (30 μg), along with amoxicillin-clavulanic acid (AMC) (20/10 μg) (CLSI, 2014). In short, bacterial culture was inoculated on Mueller-Hinton agar (MHA) plate. Then, three 3GC were placed on MHA 15 mm apart from AMC and the plate was incubated at 37°C for 18 hours. Increase in zone diameter (synergism) of 3GC towards AMC was considered as positive for ESBL production.

Statistical analysis

Data were computerized using SPSS for Windows version 11.0 (SPSS, Chicago, IL). ANOVA was used to analyze for significant differences among the virulence genes carried by a UPEC strain, for comparison of virulence gene types detected in the UPEC strains, and for comparison of phylogenetic groups. Significance was set at p-value < 0.05.

RESULTS

Bacterial collection

A total of 254 UPEC isolates were collected from patients' urine midstreams or urine catheters from the wards at Maharaj Nakhon Si Thammarat Hospital. One hundred forty-nine (59%) isolates were from female and 105 (41%) from male patients (Table 2). Patients ≥ 61 years of age had the highest proportion (65%) of UPEC infection, while the lowest (4%) was in patients aged 16-30. A high proportion (97 isolates, 38%) of UPEC was obtained from

Table 1
Oligonucleotide primers used in this study.

Gene	Virulence factor	Primer	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
pap	P fimbriae	pap3	GCAACAGCAACGCTGGTTGCATCAT	336	Yamamoto *et al*, 1995
		pap4	AGAGAGAGCCACTCTTATACGGACA		
afa	Afa adhesin	afa1	GCTGGGCAGCAAACTGATAACTCTC	750	Le Bouguénec *et al*, 1992
		afa2	CATCAAGCTGTTTGTTCGTCCGCCG		
sfaDE	S fimbriae	sfaDE-F	CTCCGGAGAACTGGGTGCATCTTAC	408	Le Bouguénec *et al*, 1992
		sfaDE-R	CGGAGGAGTAATTACAAACCTGGCA		
cnf1	Cytotoxic necrotizing factor-1	cnf1-F	GGCGACAAATGCAGTATTGCTTGG	552	Yamamoto *et al*, 1995
		cnf1-R	GACGTTGGTTGCGGTAATTTTGGG		
hlyA	α-hemolysin	hly1	AACAAGGATAAGCACTGTTCTGGCT	1,177	Yamamoto *et al*, 1995
		hly2	ACCATATAAGCGGTCATTCCCGTCA		
uidA	β-glucuronides	uidA-F	ATCACCGTGGTGACGCATGTCGC	486	Heninger *et al*, 1999
		uidA-R	CACCACGATGCCATGTTCATCTGC		
chuA	Heme transport	chuA1	GACGAACCAACGGTCAGGAT	279	Clermont *et al*, 2000
		chuA2	TGCCGCCAGTACCAAAGACA		
yjaA	Unknown	yjaA1	TGAAGTGTCAGGAGACGCTG	211	Clermont *et al*, 2000
		yjaA2	ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	Unknown	TspE4.C2-1	GAGTAATGTCGGGGCATTCA	152	Clermont *et al*, 2000
		TspE4.C2-2	CGCGCCAACAAAGTATTACG		

Table 2
Demographic data of UPEC-infected patients from Maharaj Nakhon Si Thammarat Hospital, southern Thailand, July-December, 2014.

Category	Number of *E. coli* isolates (%) (n = 254)
Sex	
Male	105 (41)
Female	149 (59)
Age (year)	
≤15	18 (7)
16-30	11 (4)
31-45	20 (8)
46-60	41 (16)
≥61	164 (65)
Hospital unit	
Out-patient	97 (38)
In-patient	
Pediatrics	7 (3)
Surgery	53 (21)
ICU	4 (1.5)
Internal Medicine	89 (35)
Special clinic	4 (1.5)
Specimen	
Midstream urine	133 (52)
Catheter urine	121 (48)
ESBL producer	
Positive	127 (50)
Negative	127 (50)

out-patients, 89 isolates (35%) from internal medicine ward, while only 4 (1.5%) isolates from intensive care unit (ICU) and special clinic. The numbers of UPEC isolated from midstream urine and catheter urine samples were comparable, 52% and 48%, respectively. Interestingly, 50% of UPEC isolates were ESBL producers.

Presence of virulence genes

Of the 254 UPEC isolates, 169 (66.5%) did not harbor any of the 5 virulence genes tested, while 38 (15%), 18 (7%), 25 (10%), and 4 (1.5%) isolates possessed 1, 2, 3, and 4 genes, respectively (Fig 1A). Forty (16%) isolates carried *pap*, *cnf1* and *hlyA*, 24 (9.5%) *sfaDE* and 21 (8.3%) *afa* (Fig 1B).

Phylogenetic group examination

Of the 254 UPEC, 146 isolates (57.5%) belonged to phylogenetic group D ($p<$ 0.05). Fifty-one isolates (20%) belonged to phylogenetic group B2, 28 (11%) to phylogenetic group B1, and 29 (11.5%) to phylogenetic group A (Fig 2).

Antimicrobial susceptibility test

One hundred seventy-nine UPEC isolates (70.5%) were resistant to ciprofloxacin, and 164 strains (65%) to norfloxacin both second-generation fluoroquinolones

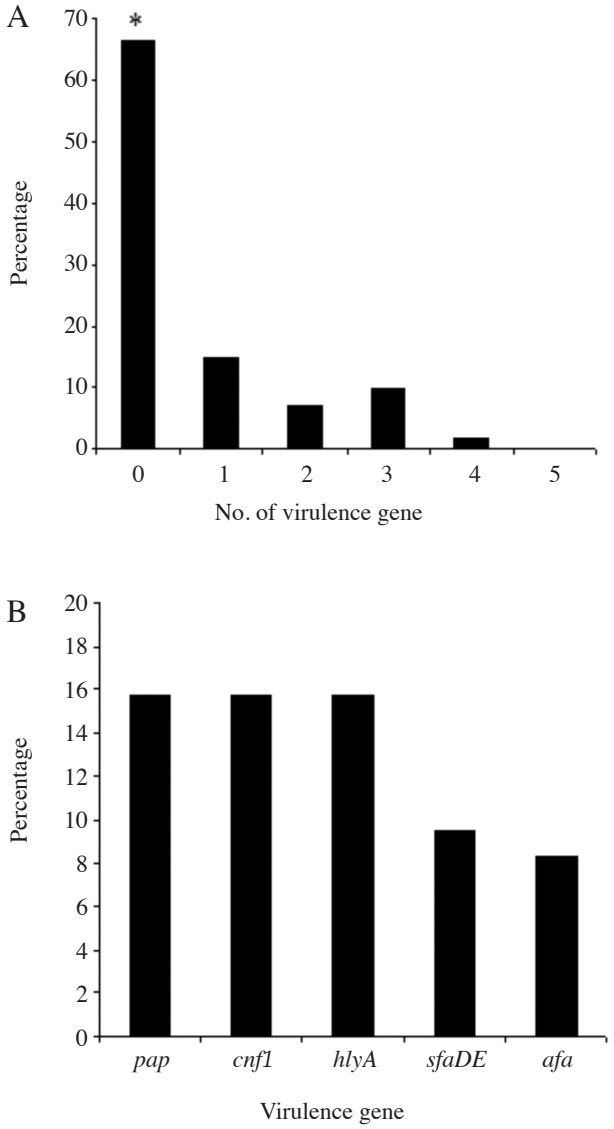

Fig 1–Numbers (A) and types (B) of virulence genes found in UPEC strains isolated from patients, Maharaj Nakhon Si Thammarat Hospital, southern Thailand, July-December 2014. Type of virulence gene was identified by PCR using gene specific primers. *p-value <0.05; n = 254.

(Table 3). Moreover, 105 (41%) strains were resistant to ceftriaxone, a third-generation cephalosporin. All strains were susceptible to meropenem and ertapenem, members of carbapenem antibiotics.

DISCUSSION

As the female urethral structure is about 5-fold shorter (4 cm) than the male (Zacharin, 1963; Kohler *et al*, 2008), UTI has been considered as one of the illnesses affecting females more than males, with 10%-20 % of women experiencing UTI once in their lifetime (Johnson and Stamm, 1989). About 90% of UTI result from UPEC infections (Johnson and Russo, 2002).

On Phuket Island, southern Thailand, between February and September, 2013, UPEC is the cause of 68% of all UTI cases, with 72% of UPEC infections occurring in females (Themphachana *et al*, 2014). In our study, however, there were comparable numbers of UPEC infections between the two sexes. This may have been due to the large number of catheter urine samples. Although disinfection using povidone-iodine (PVP-I) was used for catheterization processes and this PVP-I could effectively decrease bacterial contamination, a low immunity level in an elderly might not be able to withstand bacterial infections, resulting in high numbers of infections in catheterized patients.

Apart from the bacterial adherence processes, destruction of uroepithelial cells caused by toxins is an essential step in pathogenesis. Cnf-1 has been reported to be produced by 40% of UPEC in France (Hofman *et al*, 2000), but lower prevalences in other areas of the world have been shown (Bashir *et al*, 2012). In this study, *cnf1* was found at a prevalence of 16%.

In *E. coli* strain J96 (Blum *et al*, 1995), *cnf1* was found to be inserted into the pathogenicity Island V (PAI-V) downstream of *hlyA* (Blanco *et al*, 1992; Bashir *et al*, 2012), and expression of these two toxin genes in the same time offers advantages to the pathogen. In our study, 30/40 UPEC strains examined carried both *cnf1* and *hlyA*, confirming the notion that *cnf1* and *hlyA* are linked together (data not shown).

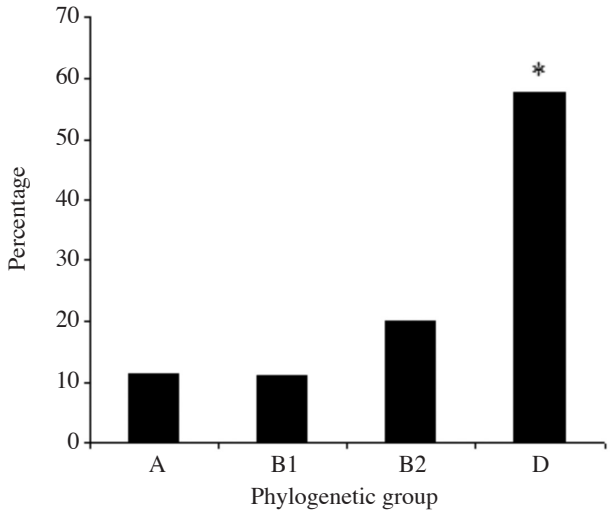

Fig 2–Phylogenetic groups of UPEC strains isolated from patients, Maharaj Nakhon Si Thammarat Hospital, southern Thailand, July-December 2014. Types of genes in phylogenetic groups were identified by PCR using gene specific primers. *p-value < 0.05; n = 254.

Fluoroquinolone-resistant bacteria have attracted recent attention because of their high prevalence including in UPEC (Dalhoff, 2012). Ciprofloxacin-resistant *E.coli* was almost non-existent until the mid 1990s and slowly have increased to 1.2% in 1998, to 2.5% in 2001 (Karlowsky *et al*, 2002) and to 5.5% in 2004 (Zhanel *et al*, 2006). Although low levels of ciprofloxacin resistance are currently low as 21% and 0-4.1% in Iran and South Korea, respectively (Asadi *et al*, 2014; Yun *et al*, 2014), in southern Thailand 58% of norfloxacin-resistant UPEC have been reported on Phuket Island (Themphachana *et al*, 2014). In the present study, fluoroquinolone resistance in patients admitted to a 1,000-bed tertiary hospital in Nakhon Si Thammarat Province, southern Thailand was as high as 70% and 65% for ciprofloxacin and norfloxacin, respectively. A study

Table 3
Antimicrobial susceptibility of UPEC from patients, Maharaj Nakhon Si Thammarat Hospital, southern Thailand, July-December 2014.

	No. of positive bacterial isolates (%) (n = 254)										
	AK	SCF	CAZ	CRO	CIP	CN	IPM	NOR	TZP	MEM	ETP
UPEC	41 (16)	44 (17)	105 (41)	128 (50)	179 (70)	99 (39)	3 (1)	164 (65)	33 (13)	0 (0)	0 (0)

AK, amikacin; SCF, cefoperazone/sulbactam; CAZ, ceftazidime; CRO, ceftriaxone; CIP, ciprofloxacin; CN, gentamicin; IPM, imipenem; NOR, norfloxacin; TZP, piperacillin/tazobactam; MEM, meropenem; ETP, ertapenem.

conducted in 28 hospitals across Thailand in the last decade also showed an upward trend of ciprofloxacin resistance from 45% to 51% (Polwichai *et al*, 2009). Among the reasons explaining the high level of ciprofloxacin resistance are the high rates of prescription of this drug, or alternatively, its inappropriate use (Lina *et al*, 2007). Thus, fluoroquinolone antibiotics should not be prescribed further for UTI treatment for patients in southern Thailand. On the other hand, carbapenem antibiotics, such as meropenem or imipenem/cilastatin, should be used instead because of the still very low level of bacterial resistance. Furthermore, these two carbapenem antibiotics are also considered as safe.

In summary, it is clear that there are a large number of patients with UTI in southern Thailand and infections in most of them are caused by UPEC carrying a number of virulence factors belonging to phylogenetic group D and having high levels of antimicrobial resistance, in particular to the fluoroquinolones. In addition, 50% of the UPEC isolates produce ESBL, which can compromise the therapeutic processes, with the possibility of higher risk of mortality. This study provides information that hopefully is beneficial for better treatment of patients with UTI leading to their more rapid recuperation and would be useful for the public health system.

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