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A simple nested polymerase chain reaction for differential identification of *usp*I and *usp*II genes encoding uropathogenic specific protein of uropathogenic *Escherichia coli*

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The gene encoding a protein which was designated as uropathogenic specific protein was identified on pathogenicity island of uropathogenic *Escherichia coli*. The *usp* gene is mainly classified into two types (*usp*I *and usp*II) depending on the difference in DNA sequence at the 3' region. A simple nested polymerase chain reaction assay was applied to differentiate *usp*I and *usp*II. The results indicate the presence of 22 *usp*I and 42 *usp*II genes positive isolates. This study will be useful for accuracy of uropathogenic specific protein pathogenicity island subtyping method for epidemiological study of uropathogenic *Escherichia coli*.

Key words: Uropathogenic *Escherichia coli*, uropathogenic specific protein, nested polymerase chain reaction, gene typing.

INTRODUCTION

A 4167-bp putative pathogenicity island (PAI) commonly associated with Uropathogenic *Escherichia coli* (UPEC) strains was identified while searching Zot (zonula occludens toxin) – like genes in these strains (Kurazono et al., 2000). Using molecular methods, a gene encoding a protein which was designated as uropathogenic specific protein (USP) was found on PAI. The *usp* gene is followed by three small open reading frame units (*orfUs*): *orfU1*, *orfU2* and *orfU3* of 98, 97 and 96 amino acids, respectively (Kurazono et al., 2000; Nakano et al., 2001).

The precise mechanism of USP on the urinary tract was not clear. In one report, it was pointed out that the USP protein and small OrfUs following this protein showed high homology to the S-type pyocins produced

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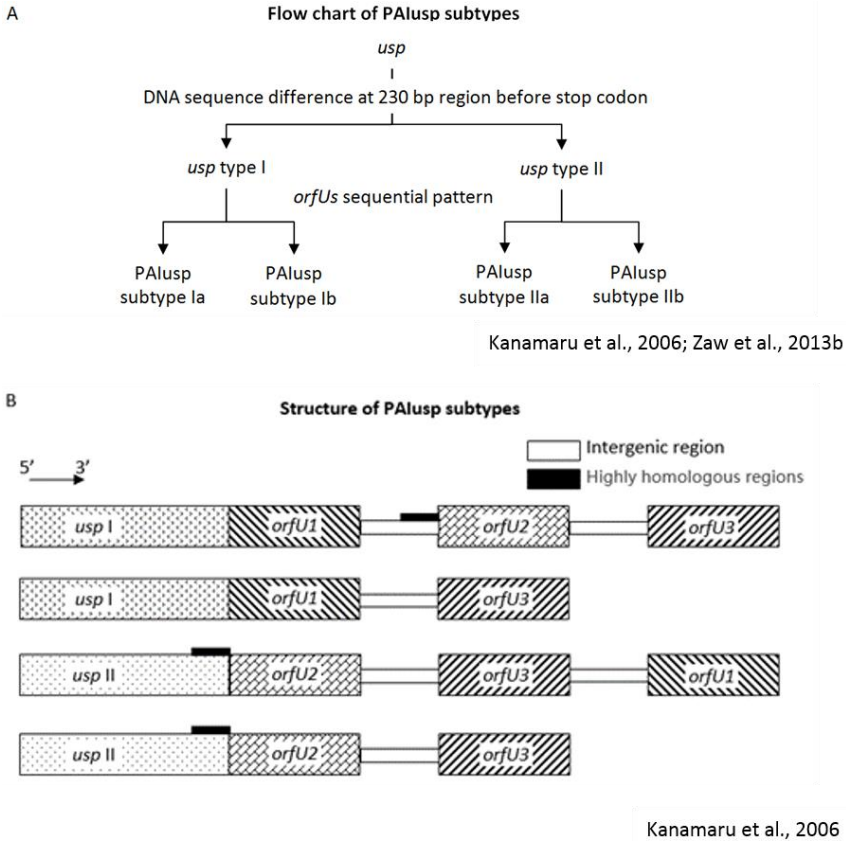

Figure 1. A. Flowchart of PAIusp subtypes classification**.** The flowchart was drawn for this study. The information for this flow chart was taken from references mentioned under the figure. **B.** Diagram showing structures of genes in each subtype of PAIusp. The black bars indicate the location of the highly homologous regions of 3' half of inter-genic region between *orfU1* and *orfU2* following *usp*I and 3' diverse region of *usp*IIs. This information was obtained from the alignment of DNA sequences from NCBI database gene bank accession no. AB056434 and AB056437 (Nakano et al, 2001).

by *Pseudomonas aeruginosa* (*P. aeruginosa*) strains and its immunity proteins (Parret and De Mot, 2002). Nuclease colicins need the specific immunity (inhibitor) proteins which make the colicins-producing bacteria to avoid committing suicide (Papadakos et al., 2012). These two literatures pointed out that USP is bacteriocin like protein and the OrfUs were immunity protein to protect *Escherichia coli (E. coli)* from the effect of USP. In one study, it was demonstrated that USP together with OrfU2 was applied to mammalian cells and genotoxic activity was observed (Nipič et al., 2013). OrfU3 (immunity protein – 3) had DNA and RNA binding activity and prevents suicidal effect of genotoxin USP on *E. coli* (Črnigoj et al., 2014). In the recent study, it was found that USP was purified after co-expression of *usp* gene and *orfU1* and characterization of USP was done. In the same study, like other bacteriocins, USP was observed to have non-specific DNase activity (Zaw et al., 2013).

Virulence genes are the molecular markers for epidemiological studies. For example *fimH* gene, *pap* gene and *usp* gene are commonly used for molecular epidemiology of UPEC (Dias et al., 2010; Bauer et al., 2002; Karimian et al., 2012). In the very recent study, the profiling of virulence genes like *tox*A, aprA, *rhl*AB, *plc*H, *las*B and *fli*C of *P. aeruginosa* isolated from patients with urinary tract infections (UTI) was done. The results showed variable distribution of virulence genes. The study highlights the virulence genes are useful diagnostic markers for clinical *P. aeruginosa* strains isolated from UTI (Sabharwal et al., 2014).

The reasons that *usp* gene encoding USP protein was predominant in UTI isolates, it was classified into two types due to the difference in DNA sequence and the sequential position of associated three *orfU*s immediately downstream of *usp* gene was not the same (that is orfU1*, orfU2 and orfU3* are in sequence, or *orfU2, orfU3 and*

Table 1. Nucleotide sequences of primers used in this study.

Name of primer	Primer sequences	Size of PCR products
Nest 1 primers	N1F:5'- GCT TTC ATC AGG AAC TCG CTG G –3' N1R:5'-TTA TCT CCT GTA GAA TTT CAT CAT G -3'	380 bp (this study)
*usp*I primers	MYF:5'-ATT CCC CCT ATG TCC CTG AG -3' MYR: 5'-TCC ACC ACC ATG TTC TAT A-3'	101 bp (this study)
*usp*II primers	MY2F: 5'-CAG GAT CCG GTG TTG ATA-3' MY2R: 5'-GCT GCC ACC ATA TTC AAC T-3'	161 bp (this study)

orfU1 are in sequence, or sometimes only two *orfU*s are present with different combination depending on isolates) make *usp* gene possible to be useful epidemiological marker. PAIusp subtyping method, which characterizes UPEC isolates from the molecular level, depends on (1) *usp* gene typing and (2) arrangement of 3 *orfU*s following *usp* gene and PAIusp subtypes are Ia, Ib, IIa and IIb (Figure 1A and B) (Kanamaru et al., 2006). However, researchers up to now relied on *orfU*s sequential order for PAIusp subtyping and this fundamental method of *usp* typing was not mentioned in their procedure. Therefore, simple nested PCR assay was introduced in this study with nest duplex PCR using newly designed primer sets for two *usp* genes to differentiate *usp*I and *usp*II.

MATERIALS AND METHODS

Ethical clearance was applied to University ethical committee and National Medical Research Registration (NMRR) and approval was already obtained. Sixty-four UPEC isolates positive for *usp* gene stocked in microbiology laboratory, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah were included in this study. These UPEC isolates were derived from urine samples sent to microbiology laboratories of Hospital Queen Elizabeth and Papar Hospital for culture and sensitivity during January to March, 2013. The two hospitals were located around Kota Kinabalu, Sabah, Malaysia. We have checked the urine samples for significant bacteriuria. Positive controls were the strains which were shown to have *usp* I and *usp* II genes by DNA sequencing and negative control was *E. coli* ATCC 25922.

DNA sequence of nest 1 forward and reverse primers and newly designed forward and reverse primers for *usp*I and *usp*II genes are shown in Table 1 with their sizes of PCR products expected. The primer designation was performed following the guidelines by Burpo (2001).

The bacterial isolates were inoculated in 3 ml of Luria-Bertani broth and incubated at 37°C for 18 h. The bacterial DNA was extracted by boiling method (Abdallah et al., 2011; Ifeanyi et al., 2015). In case of primary PCR, 5 µl of DNA as template was added to PCR reaction mixture (25 µl) containing 10 µm each primer, dNTPs 10 mmol, 1x buffer and 1 unit of Taq polymerase (Takara). PCR was done in Applied Biosystems Thermocycler with PCR conditions as initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, extension at 72°C for 30 s. The size of PCR products was checked by florosafe in a 1.5% agarose gel, and recorded by gel documentation apparatus Alpha Imager® HP System. For nest PCR, which was duplex PCR, the PCR ingredients were same as above mentioned procedure with the exception of 0.1 µl nest 1 product was used as the template and two primer sets were added. PCR conditions were initial denaturation at 94°C for 5 min, 15 cycles of denaturation at 94°C for 10 s annealing at 55°C for 10 s, extension at 72°C for 20 s and 2.5% agarose gel was used to check PCR products. It took less than 2 h after conditions were standardized for the whole nested PCR.

RESULTS AND DISCUSSION

In the previous studies on USP, the epidemiologically important PAIusp subtyping method was used to characterize the UPEC isolates. There is absence of gene typing method which is more fundamental step when compared with the sequential order of *orfUs* in their study (Kanamaru et al., 2006). In this study, the set of primers which could amplify the region where *usp*I *and usp*II have heterologous sequences were designed for the primary PCR. For the nest PCR, the two primer sets, each having the most heterologous sequences in *usp*I and *usp*II genes in the 3' regions were used for duplex PCR which could differentiate two genes. PCR products sizes were convenient for the gel electrophoresis by 1.5% and 2.5% agarose gel, which were 380 bp for the primary PCR and 161 bp and 101 bp for the nest PCR respectively. All the 64 samples were positive in the primary PCR. This primary PCR amplify the regions which include where the two genes are heterologous. This PCR has advantage in leaving the intergenic region which will disturb the following nest PCR and this information will be explained in the discussion. In the nest PCR, 22 samples were positive for *usp*I gene and 42 were positive for *usp*II gene. The primer pairs have succeeded in differentiation of two genes. The 7 UPEC positive isolates for *usp* Nested PCR were shown in Figure 2A and B.

Up to now, there were two reports about the nature and action of USP. One study proved that it has the non-specific DNase activity (Zaw et al., 2013). It was observed in the other study that it has genotoxic effect when applied to mammalian cells (Nipič et al., 2013).

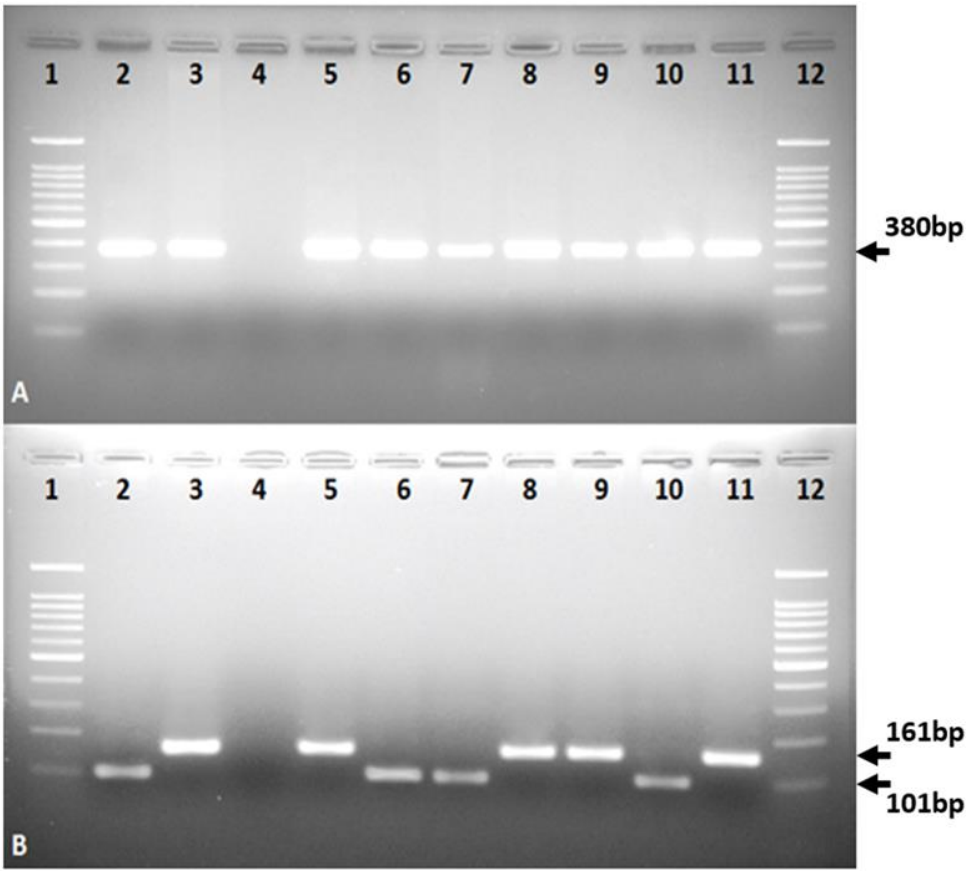

Figure 2. Nested PCR assay for differentiation of *usp*I and *usp*II . A. Gel electrophoresis picture of primary PCR product of UPEC isolates containing *usp* gene. Lane 2 and 3 are isolates for positive control *usp*I, *usp*II respectively. Lane 4 is an isolate used as negative control lacking of *usp* gene. Lane 5 to 11 were isolates investigated in this study all giving rise to 380 bp PCR products. Lane 1 and 12 were 100bp molecular markers. B. Gel electrophoresis picture of Nest PCR product having *usp*I or *usp*II. Lane 2 and 3 are positive control *usp*I, *usp*II showing 101 bp and 161 bp respectively. Lane 4 is negative control. Lane 6, 7 and 10 are PCR products *usp*I with the 101 bp DNA fragment size and lane 5, 8, 9 and 11 are PCR products *usp*II gene having 161 bp in size. Lane 1 and 12 were 100 bp molecular markers.

Although the action of USP was concluded by different researchers according to their findings, the value of *usp* gene and its *orfUs* following this gene is classification of UTI isolates into four PAIusp subtypes which are of epidemiologic significance.

Structural and diversity of *usp* genes indicated that *usp*I and *usp*II were heterologous at 3' end in the previous study (Nakano et al., 2001). This diversity occurred at 3' 230 bp region upstream of stop codon. Because of this diversity and sequential orientation of *orfU*s, epidemiologically important PAIusp subtypes were well known. PAIusp subtype IIa was highly prevalent in the studies in Japan (Kanamaru et al., 2006). However, PAIusp subtyping relied on sequential orientation of *orfU*s 1, 2 and 3 in that study. It is necessary to lay down the method for *usp* gene typing. If PAIusp subtyping pattern becomes different from currently classified four types in new isolates, *usp* gene typing will be helpful in characterization of UPEC isolates.

PCR-RFLP and direct duplex PCR were tried for *usp* gene typing. Although these are part of this study, these were not mentioned in this report. However, the first method depends on *Hpa* II restriction site and if there are single nucleotide polymorphisms (SNPs), typing can be mistaken. In case of duplex PCR, the *usp*II gene is highly homologous to 3'intergenic region between *orfU1* and *orfU2* of *usp*I gene (black bars in Figure 1B) giving rise to two reactive bands giving wrong impression of having two gene types in the detection of some *usp*I isolates. In this nested PCR assay, we can leave the intergenic region in primary PCR so that nest PCR can provide the correct gene type. We succeeded in the gene typing method for

*usp*I *and usp*II using simple and rapid nested PCR assay by taking advantage of principle of nested PCR.

This is the first study for gene typing of *usp*I and *usp*II, which is the basic step in epidemiologically important PAIusp subtyping. In the previous work, PAIusp subtyping was done depending on *orfUs* sequential patterns without gene typing. Gene typing of *usp*I *and usp*II is difficult because of the abovementioned reasons. However, nested PCR is valuable for overcoming these obstacles so that this study has contributed to the accurate method of PAIusp subtyping if there is extra subtype beyond the currently existing four subtypes.

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Conflict of interest

The authors have no conflict of interest to disclose.

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