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BRIEF COMMUNICATION

Polymerase chain reaction-restriction fragment length polymorphism method for differentiation of *uropathogenic specific protein* gene types

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Abstract The putative pathogenicity island (PAI) containing the *uropathogenic specific protein* (*usp*) gene and three small open reading frames (*orfU1*, *orfU2*, and *orfU3*) encoding 98, 97, and 96 amino acid proteins is widely distributed among uropathogenic *Escherichia coli* (UPEC) strains. This PAI was designated as PAI*usp*. Sequencing analysis of PAI*usp* has revealed that the *usp* gene can be divided into two types — *usp*I and *usp*II — based on sequence variation at the 3′ terminal region and the number and position of *orfUs* differ from strain to strain. Based on *usp* gene types and *orfU* sequential patterns, PAI*usp* can be divided into four subtypes. Subtyping of PAI*usp* is a useful method to characterize UPEC strains. In this study, we developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to differentiate *usp* gene types. This method could correctly identify the *usp* gene type in *usp*-positive UPEC strains in our laboratory.
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Introduction

Kurazono et al[1] discovered a putative pathogenicity island (PAI) containing the gene encoding uropathogenic specific protein (*usp*) in uropathogenic *Escherichia coli* (UPEC) strain Z42. Located downstream of the *usp* gene are three small open reading frames (*orfU1*, *orfU2*, and *orfU3*) putatively encoding 98, 97, and 96 amino acid proteins, respectively. It has been demonstrated that the plasmid containing the *usp* gene enhances the infectivity of host *E. coli* strains in a mouse pyelonephritis model suggesting that the *usp* gene contributes to the pathogenesis of urinary tract infection (UTI) and the *usp* gene may encode the putative virulence factor of UPEC.[2] The *usp* gene is widely distributed in *E. coli* strains isolated from urinary tract infection as well as other extraintestinal infections.[2–4] This PAI is different from a PAI previously reported in UPEC strains and designated as PAI*usp*. Based on sequence homology, it is thought that uropathogenic specific protein (usp) encoded by the *usp* gene is a bacteriocin and the proteins encoded by small open reading frames downstream of the *usp* gene (designated as OrfU proteins) are immunity proteins for Usp.[5] A recent study has demonstrated that Usp is a non-specific DNase belonging to H-N-H nuclease family.[6]

Sequencing analysis of PAI*usp* in representative *E. coli* strains revealed that the *usp* gene can be classified into two types — *usp*I and *usp*II — based on sequence variation in 3′ end. In addition, the number of *orfUs* and position of *orfU* in relation to each other vary from strains to strains.[7] Detailed investigation of the PAI*usp* showed that *usp*I is linked with *orfU1* and *usp*II is linked with *orfU2*.[5]

PAI*usp* can be subtyped into four subtypes (Ia, Ib, IIa, and IIb) depending on *usp* gene type and sequential pattern of *orfUs* (Figure 1). Kanamaru et al[8] performed the subtyping of PAI*usp* in UPEC strains isolated in Japan using a polymerase chain reaction (PCR) method. Type IIa was found to be the most common subtype (42.4% of UPEC strains subtyped) followed by Type Ia (28.4%), Type Ib (9.8%), and Type IIb (2.7%). In their report, the method for differentiation of *usp* gene types was not described. However, identification of *usp* gene type is the first step in subtyping of PAI*usp*. So, in our study, we developed a PCR-restriction fragment length polymorphism (PCR-RFLP) method for differentiation of *usp* gene types.

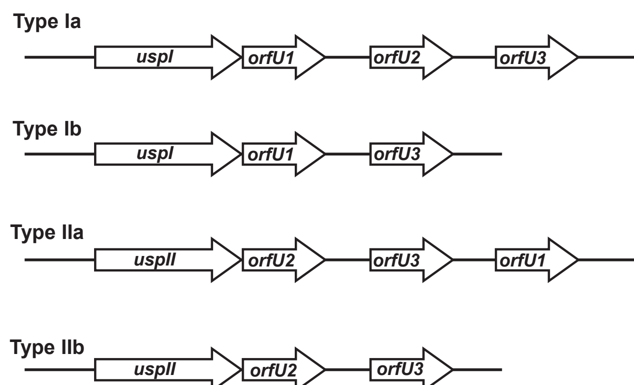

Figure 1. Structures of the *usp* gene and *orfUs* in each subtype of PAIusp. The arrows represent the length and transcriptional direction of the genes in PAIusp (Modification from Kanamaru et al., 2006).[8]

Materials and methods

Bacteria strains and media

Forty *usp*-positive UPEC isolates stocked in Microbiology Laboratory, School of Medicine, Universiti Malaysia Sabah, Malaysia were used in this study. Bacteria were grown on MacConkey agar and subsequently in tryptic soy broth at 37°C overnight.

Preparation of DNA template

Bacteria grown in tryptic soy broth overnight were harvested by centrifugation at 6149 *g* for 10 minutes. Bacterial cells were suspended in sterile distilled water. DNA extracted by the boiling method was used as the template for PCR.

PCR

Each PCR reaction was prepared as 50 μL reaction mixture containing 5 μL of template DNA, 1 μL of dNTPs (10 mM), 5 μL of 10× buffer, 2 μL of each primer (10μM), 34.5 μL of distilled water and 0.5 μL of *Taq* polymerase (5 U/μL) (Takara Bio Inc, Shiga, Japan). The sequence of primers is shown in Figure 2. PCR was performed with a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, USA) and began with initial denaturation at 95°C for 5 minutes followed by 30 cycles of amplification (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) and final extension at 72°C for 10 minutes. PCR products were checked by electrophoresis in 1% agarose gel and ethidium bromide staining. ExactMark 100 bp DNA ladder (1st BASE Singapore Ltd, Singapore) was used as the molecular size marker.

RFLP analysis

PCR products (6 μL) were mixed with 1 μL *Hpa*II (Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 μL 10× buffer and 11 μL distal led water and incubated at 37°C for 3 hours. The digested DNA was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining.

DNA sequencing

DNA sequence analysis was performed at First BASE Laboratories Sdn. Bhd. using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA).

Results

Nakano et al[7] reported that the *usp* gene can be divided into two subtypes, *usp*I and *usp*II, depending on sequence variations at the 3′ terminal 230 bp region. To develop the PCR-RFLP method for differentiation of *usp* gene types, *usp*I and *usp*II sequences were retrieved from the database.

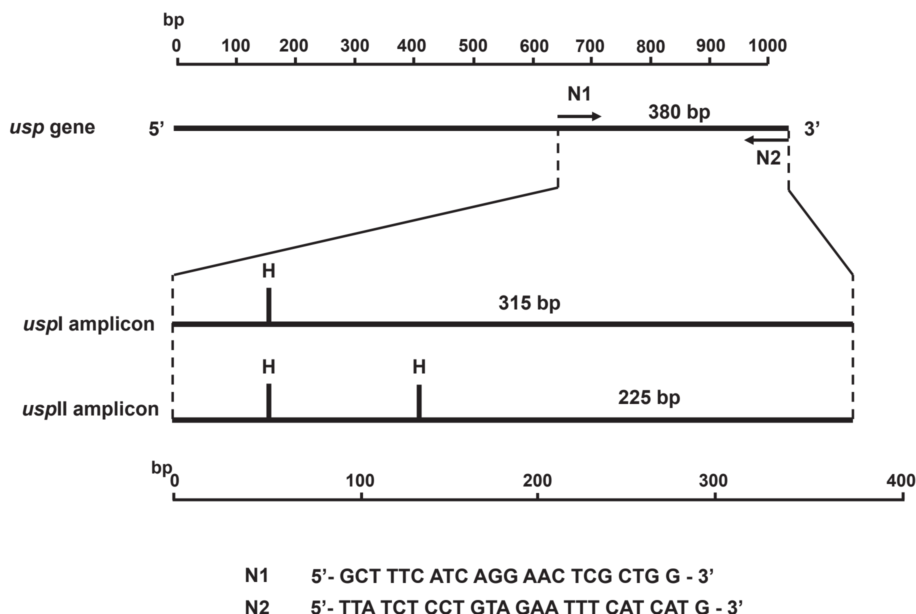

Figure 2. Schematic diagram of the *usp* gene showing primer binding sites and a restriction map of 380 bp *usp*I and *usp*II amplicon showing *Hpa*II sites.

The restriction map of *usp*I and *usp*II sequences was analyzed using A plasmid Editor and the 3′ terminal 380 bp region of the *usp* gene was selected for PCR-RFLP analysis. We designed a set of primers to amplify this region (Figure 2). As *usp*I and *usp*II contain one *Hpa*II site and two *Hpa*II sites in this region, respectively, *Hpa*II digestion of this region would allow the identification of *usp* gene subtypes based on the size of fragments generated. The *usp*I would produce two fragments including the 315 bp fragment and *usp*II would produce three fragments including the 225 bp fragment (Figure 2). DNA extracted from 40 *usp*-positive UPEC strains was subjected to PCR amplification and all strains give the PCR products with an expected size of 380 bp (Figure 3A). When the PCR products were digested with *Hpa*II and analyzed by agarose gel electrophoresis, 15 strains showed a 315 bp band indicative of *usp*I and 25 strains showed a 225 bp band indicative of *usp*II (Figure 3B). For the validation of this PCR-RFLP method, the nucleotide sequence of the 3′ terminal 380 bp of the *usp* gene in these strains was checked against the representative *usp*I and *usp*II sequence in databases. The strains giving a 225 bp band and a 315 bp band on RFLP analysis have a nucleotide sequence almost identical to *usp*II and *usp*I, respectively. These findings indicate that this method can correctly identify the *usp* gene types and, out of 40 strains, 25 strains possessed the *usp*II gene and 15 strains carried the *usp*I gene.

The limitation of RFLP analysis is that a single nucleotide change would result in a loss of the restriction site, leading to incorrect typing. However, the *Hpa*II sites used in this

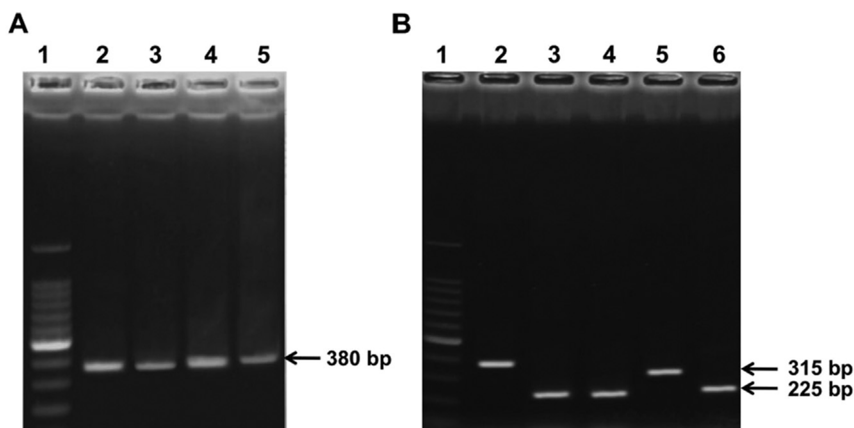

Figure 3. (A) Agarose gel electrophoresis of polymerase chain reaction (PCR) amplicon after amplification with primers mentioned in Figure 2. Lane 1, 100 bp ladder; Lane 2—5, 380 bp amplicons. (B) Agarose gel electrophoresis of DNA fragments after digestion of PCR amplicon with *Hpa*II. Lane 1, 100 bp ladder; Lane 2, undigested amplicon; Lane 3, 4, 6, 225 bp fragment indicating *usp*II; Lane 5, 315 bp indicating *usp*I.

method are present in all of the strains examined in this study.

Discussion

The *usp* gene is widely distributed in UPEC strains and considered to be the epidemiological marker of UPEC. PAI*usp* can be classified into four subtypes depending on the *usp* gene type and *orfU* sequential pattern. Previously Kanamaru et al[8] applied PAI*usp* subtyping to characterize UPEC strains. PAI*usp* Subtype Ia and Subtype IIa containing all three of the *orfUs* are frequently found in UPEC strains belonging to the phylogenetic group B2. There was a close association between PAI*usp* Subtype Ia and UPEC isolates from prostatitis patients. In addition, each PAI subtype was associated with specific O serogroups; Subtype Ia with O4, O22, and O25; Subtype Ib with O75, and Subtype IIa with O1, O6, O16, and O18.

In their study, PCR assay based on the *orfU* sequential pattern was used for PAI*usp* subtyping without taking into consideration the *usp* gene type.[8] However, identification of *usp* gene type is the important step in subtyping of PAI*usp* and should be used together with *orfU* sequential pattern.

At first, we have tried to develop a duplex PCR method to differentiate *usp*I and *usp*II. This duplex PCR method is based on the heterogeneity of the 3′ 230 bp-region upstream of the stop codon in *usp*I and *usp*II. This duplex PCR assay yielded a single distinct band with *usp*II isolates. However, two bands were produced with *usp*I isolates because the heterologous 230 bp-region in *usp*II is nearly identical to the intergenic region between *orfU1* and *orfU2* of *usp*I isolates. In other words, the duplex PCR method is not applicable in differentiation of *usp* gene type. Instead, a PCR-RFLP method was developed for the subtyping of the *usp* gene in this study. This method should be used in conjunction with the method developed by Kanamaru et al[8] in subtyping PAIusp and will be valuable in further characterizing UPEC strains for epidemiological studies.

Conflicts of interest

The authors in this study do not have any conflicts of interest to declare.

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