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Sequencing and Expression of Fimbrial Gene of *Pasteurella multocida* B:2

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Abstract: The fimbrial gene of *Pasteurella multocida* B:2, isolated from cattle with haemorrhagic septicaemia was amplified, cloned, sequenced and expressed in a vector. Cloning of the fimbrial gene revealed a 435 bp band while sequencing revealed similar nucleotide sequencing to the *PtfA* gene of *P. multocida* A:3 isolated from chicken, except the five nucleotide changes at residues 171, 282, 363, 387 and 414. The fimbrial gene was found to encode a deduced protein of 144 amino acids. The expressed fimbrial protein was detected from 4 h post-induction with a molecular weight of 18-kDa.

Key words: Pasteurella multocida B:2, Fimbrial gene, Sequencing, Expression

Introduction

The fimbriae of *Pasteurella multocida* is a main surface structure that involves in adhesion to the host cells. Thus, it is correlated to the virulence of the organism (Glorioso *et al*., 1982). The fimbriae is a long proteinaceous appendage, approximately 6-7 nm in diameter and composed of a single repeated polypeptide subunit (Storm and Lory, 1993) with a deduced molecular mass from 15- to 20-kDa (Adler *et al*., 1999). It is similar, in morphology, to the type 4 fimbriae of other bacteria with respect to the diameter and length (Ruffolo *et al*., 1997).

The fimbriae of *P. multocida* serogroup A exhibits an extensive N-terminal amino acid sequences and have been designated as N-methylphenylalanyl fimbriae (MacDonald *et al*., 1993) and shares a high similarity with the amino acid sequence of type 4 fimbriae of other bacterial species. Thus, all type 4 fimbriae share a number of features, which include a characteristics short signal peptide, a hydrophobic domain at the amino terminus of the mature protein and a pair of cysteine residue at the carboxyl terminus of the protein, capable of forming a di-sulphide bridge (Storm and Lory, 1993).

To date, the fimbriae of *P. multocida* B:2 isolated from cattle with haemorrhagic septicaemia has never been studied. Therefore, this study reports on the sequence and expression of fimbrial gene of *P. multocida* B:2 isolated from cattle with haemorrhagic septicaemia.

Materials and Methods

Bacterial isolates: A total of 5 isolates of *P. multocida* serotype B:2, obtained from cattle with haemorrhagic septicaemia were used in this study. The identification of *P. multocida* was made using gram-staining and biochemical tests while the serotyping was confirmed by the Veterinary Research Institute, Ipoh, Perak.

Fimbrial Subunit Gene Amplification: The DNA of *P. multocida* B:2 was extracted using the commercially available genomic DNA purification system (Fermentas, Lithuania). The primers were designed to amplify the *ptfA* gene, based on the whole genome *P. multocida* serotype A3 obtained from GenBank database (AE004439). The fimbrial gene was amplified using two primers, EZ1 (5' GGA GTT TTA TAT GAA AAA AG 3') and EZ2 (5' ATG AAA CAT TAT GCG CAA AAT 3') (Biosyntech). The reaction mixture consisted of 1x PCR buffer, 0.2mM dNTP, 3mM $MgCl_2$, 0.75μM of primer EZ1 and EZ2 and 1 unit *Taq* polymerase with 10-100 ng of template DNA isolates. PCR amplification was performed in thin walled 0.2 ml PCR tubes in a thermal cycler (Mastercycler, Eppendorf). The amplification cycle used was 94°C for 4 min (94°C for 1 min, 50-55°C for 1 min, 72°C for 1 min) for 35 cycles followed by 72°C for 10 min. Amplification products were separated by electrophoresis in 1.5% agarose gels and visualised following staining with ethidium bromide (0.5 μg/ml).

Cloning of the PCR Products: The PCR products were purified using Wizard® PCR Preps DNA Purification System (Promega, USA). The purified product was ligated to a pCR®2.1 vector for sequencing while the pCRT7® expression vector was used to express the gene (TOPO TA Cloning, Invitrogen, USA) and the reaction was incubated at room temperature for 5 min. Two μl TOPO TA Cloning reactions were added to a vial of One Shot cells and mixture was incubated on ice for 30 min. The cells were heat shocked for 30 seconds at 42°C without shaking. The tube was then incubated on ice for 2 min. The transformed cells were recovered in 250 μl of room temperature SOC medium shaken horizontally at 37°C for 1 h. Fifty to one hundred μl of the transformed bacteria was spread onto LB plates containing 50 μg/ml ampicillin and 4 mg/ml X-gal. The plates were incubated at 37°C for 16 h.

Screening of Clones: Transformants were selected after 16 h and the extraction of plasmid was performed using Wizard® Plus Minipreps DNA Purification System (Promega, USA). Transfomants were subjected to digestion with EcoRI, and *Bam*HI and *Hin*dII enzymes to confirm the properties.

Sequencing of the Fimbrial Subunit Gene: Two positive clones of each isolates were selected for sequencing. All DNA sequencing was performed using the automated sequencer ABI Prism 377 (Perkin Elmer). The plasmids from the clones were mixed with BigDye mixture and primer before performing the extension by sequencing cycle. The M13 primer was chosen for the extension in opposite directions. The sequences were then analysed using Bioedit Software program.

Pilot Expression: A time course experiment was conducted to optimize the expression of the recombinant protein. Two ml of LB containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) were inoculated with a single recombinant colony. The culture was grown overnight at 37°C in orbital shaker incubator (225 rpm). On the following day, 20 ml of LB containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol were inoculated into the overnight culture. The culture was grown at 37°C with shaking (225 rpm) to an OD_{600} = 0.5 to 0.8. One ml of cell culture was removed prior to the IPTG induction and centrifuged before the supernatant was discarded. The cell pellet was re-suspended with distilled water and frozen at −20°C. This was regarded as zero time sampling. IPTG was added to a final concentration of 1 mM (0.02 ml of 1 mM IPTG stock to 20 ml culture) and the cells were left growing in the incubator. After 1 h of incubation, 1 ml of the culture was removed and centrifuged before the supernatant was discarded. The cell pellet was re-suspended in 60 μl distilled water and frozen at -20°C. Samples of the culture were taken at 1-hour interval for 4 to 8 hours. When samples for all time points were collected, each pellet was re-suspended in 60 μl distilled water and frozen at −20°C.

Detection of Recombinant Protein by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): The technique of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is routinely employed to estimate the molecular weight of proteins due to its convenience, speed and cost effectiveness. All samples were analysed under SDS-PAGE by using 12% (w/v) resolving gel and 4% stacking gel for detection of the expressed protein of fimbrial gene. The SDS-PAGE was conducted in a Mini-Protein® II Electrophoresis Cell (BIO-RAD, USA).

Analysis of the Expressed protein by Western Blot: After SDS-PAGE, the polypeptides were transferred onto 0.45 μm nitrocellulose membrane using either a mini Trans-Blot Cell (Bio-Rad, USA) or Hoefer TE22 Mighty Small Transphor Electrophoresis (Hoefer, USA). For immunoblotting, the membrane was blotted with antiserum against the fimbriae of *P. multocida* as primary antibody and with horseradish-peroxidase-labelled anti-rabbit immunoglobulin (IgG) (Promega, USA) as secondary antibody. The membrane was incubated in a substrate solution containing methanol, 4-chloro-1-napthol (Sigma, USA), TBS pH7.5 and 31% hydrogen peroxide.

Results

Cloning of Fimbriae Gene Into pCR® 2.1 vector and pCRT7® vector (TOPO TA Cloning): All isolates produced a single product of approximately 453 bp, which contained the fimbrial subunit gene (Fig. 1). The recombinant plasmid, probably positive clones were confirmed by producing two separate bands, the vector and the insert. The positive colonies of recombinant pCR®2.1 vector (~3.9 kb) and fimbriae gene (~435 bp) were confirmed by digestion with *EcoR*I and sent for sequencing (Fig. 2). However, the recombinant plasmid for expression were digested with *Bam*HI and *Hin*dIII and produced 2.8 kb derived from the pCRT7® vector and ~435 bp of fimbriae gene as shown in Fig. 3.

Sequence Analysis of the Fimbrial Gene: The nucleotide sequences of all isolates used in this study were comparable to each other even though the isolates originated from different outbreaks and locations. There were, however, five nucleotide changes in residues 171, 282, 363, 387 and 414 of the sequence when they were compared to the *PtfA* gene of *P. multocida* A:3 from Genbank (AE004439). The nucleotide sequence and the predicted amino acid sequence of the fimbrial preprotein are presented in Fig. 4 and 5. The fimbrial gene was found to encode a deduced protein of 144 amino acids. All isolates were found to have a large central disulphide loop by having two pairs of cycteine residues. The first pair was at residues 62 and 72 and the second pair was at residues 131 and 143 that were conserved in all aligned sequences (Fig. 5).

Pilot Expression of Recombinant Protein: The recombinant plasmid was transformed into BL21 and the expected band of the expressed fimbrial protein was found to be between 15- and 20-kDa, starting at 4 h post-induction. The intensity of the band was found to increase and the highest expression of the protein was detected after 6 h post-

induction with the protein weighed approximately 18-kDa, which was analyzed using Gel Image Analysis (Syngene Tool Ver.2.10.03, UK) (Fig. 6).

Analysis of the Fimbrial Protein by Western Blot: Following Western blotting, no band appeared from 0-6 h but a faint band was observed at 8 h post-induction (Fig. 7). The calculated size of the protein was approximately 18-kDa (Syngene Tool Ver.2.10.03, UK).

Discussion

The amplification of the fimbrial gene was conducted by using a set of primer, which was designed based on the reference sequence of the whole genome *P. multocida* serotype A3 from avian (Barbara *et al.*, 2001). Similarly, Doughty *et al.* (2000) reported that the complete *ptfA* gene from *P. multocida* serotype A1 has been amplified successfully by inverse PCR and a 432 bp band was identified. Subsequently, the PCR product in this study was purified and later successfully cloned into pCR® 2.1 vector and pCRT7® vector (TOPO TA cloning). Both of vectors are highly efficient whereas amplified PCR products into a plasmid with one step cloning strategy but pCRT7® vector are designed for high level protein expression and simplified protein purification in *E. coli*. White colonies, likely to be probable positive clones from recombinant plasmid (pCR® 2.1 + insert), were then selected, screened and used for sequencing. It was found to be similar to the *PtfA* gene of serotype A:3 with 100% amino acid identities (Doughty *et al.*, 2000). The results from the present study demonstrated that the fimbrial subunit gene is conserved among the different isolates of *P. multocida*.

Following expression, a 18kDa band was identified. This indicates the presence of the desired protein of *P. multocida* in the *E. coli* host, which has capability to express the fimbriae gene of *P. multocida* B:2. However, the location of

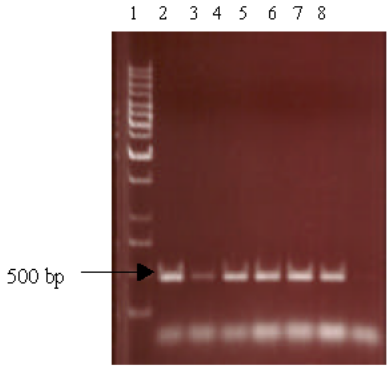

Fig. 1: Agarose gel electrophoresis of the amplified DNA of *P. multocida* B:2. Lane 1- 100 bp marker; Lane 2- reference strain; Lane 3, 4, 5, 6- *P. multocida* B:2 fimbrial subunit gene; Lane 7- negative control.

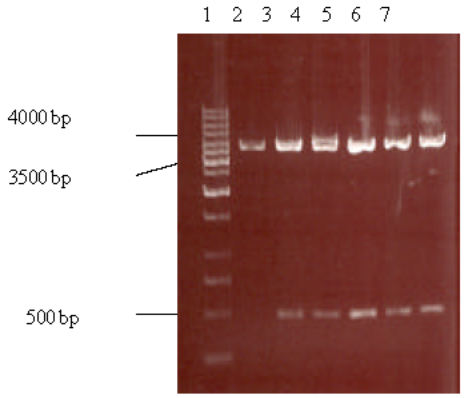

Fig. 2: Restriction endonuclease analysis (*EcoRI*) of the positive transformant colonies (pCR® 2.1 + fimbrial subunit gene). Lane 1- 1 kb DNA marker (Fermentas, Lithuania); Lane 2-vector only (3.9 kb); Lane 3-7-positive clones, vector (3.9 kb) with insert (435 bp).

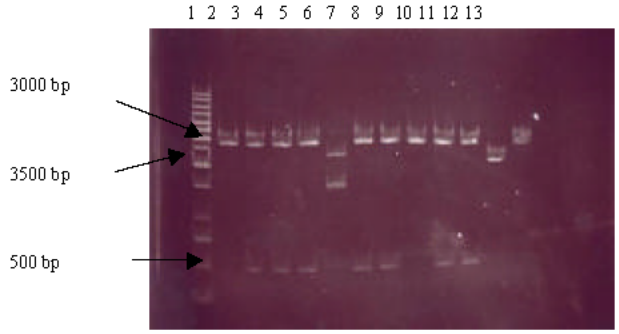

Fig. 3: Restriction endonuclease analysis (*Bam*HI and *Hin*dIII) of the positive transformant colonies (pCRT7® + fimbrial subunit gene). Lane 1- 1 kb DNA marker (Fermentas, Lithuania); Lane 2- vector only (2.8 kb); Lane 3, 4, 5, 7, 8, 10 and 11- positive clones, vector (2.8 kb) with insert (435 bp); Lane 6, 9, 12 and 13- negative clones.

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                    10         20         30         40         50         60         70
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3          atgaaaaaag ccattttctt ttcgattaaa aaagggttta cgctaattga attaatgatt gtcattgcca
PMTB (6:B)          .......... .......... .......... .......... .......... .......... ..........
2744 (6:B)          .......... .......... .......... .......... .......... .......... ..........
3027 (6:B)          .......... .......... .......... .......... .......... .......... ..........
PMX (6:B)           .......... .......... .......... .......... .......... .......... ..........
3030 (6:B)          .......... .......... .......... .......... .......... .......... ..........
                    80         90         100        110        120        130        140
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3          ttattgcgat tttagccacg attgccgtgc catcttatca aaattataca aaaaaagcgg caatctctga
PMTB (6:B)          .......... .......... .......... .......... .......... .......... ..........
2744 (6:B)          .......... .......... .......... .......... .......... .......... ..........
3027 (6:B)          .......... .......... .......... .......... .......... .......... ..........
PMX (6:B)           .......... .......... .......... .......... .......... .......... ..........
3030 (6:B)          .......... .......... .......... .......... .......... .......... ..........
                    150        160        170        180        190        200        210
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3          attattgcaa gccgctgctc cttatcgtgc tgaggtagaa ctttgtatct ataatacaaa tagtacaacg
PMTB (6:B)          .......... .......... .......... C......... .......... .......... ..........
2744 (6:B)          .......... .......... .......... C......... .......... .......... ..........
3027 (6:B)          .......... .......... .......... C......... .......... .......... ..........
PMX (6:B)           .......... .......... .......... C......... .......... .......... ..........
3030 (6:B)          .......... .......... .......... C......... .......... .......... ..........
                    220        230        240        250        260        270        280
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3          aattgtaatg caggaagtca tggtattcgt gctgatgctg cagcaagtaa aaaatattta aaagccatca
PMTB (6:B)          .......... .......... .......... .......... .......... .......... ..........
2744 (6:B)          .......... .......... .......... .......... .......... .......... ..........
3027 (6:B)          .......... .......... .......... .......... .......... .......... ..........
PMX (6:B)           .......... .......... .......... .......... .......... .......... ..........
3030 (6:B)          .......... .......... .......... .......... .......... .......... ..........
                    290        300        310        320        330        340        350
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3          cagttaaagc tggggtaatt actgtcactg gacaagggag cttggaagga attagctata cgctgacagc
PMTB (6:B)          .G........ .......... .......... .......... .......... .......... ..........
2744 (6:B)          .G........ .......... .......... .......... .......... .......... ..........
3027 (6:B)          .G........ .......... .......... .......... .......... .......... ..........
PMX (6:B)           .G........ .......... .......... .......... .......... .......... ..........
3030 (6:B)          .G........ .......... .......... .......... .......... .......... ..........

                    360        370        380        390        400        410        420
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3          gagtggtaac gcagctcaag gtgtttcttg gacagttaat tgtggtgctc aatctgacat tttcccagca
PMTB (6:B)          .......... ..T....... .......... ......G... .......... .......... ...T......
2744 (6:B)          .......... ..T....... .......... ......G... .......... .......... ...T......
3027 (6:B)          .......... ..T....... .......... ......G... .......... .......... ...T......
PMX (6:B)           .......... ..T....... .......... ......G... .......... .......... ...T......
3030 (6:B)          .......... ..T....... .......... ......G... .......... .......... ...T......
                    430        440
                    ....|....| ....|....|
Ref 1 (A:3          ggattttgcg cataa
PMTB (6:B)          .......... .....
2744 (6:B)          .......... .....
3027 (6:B)          .......... .....
PMX (6:B)           .......... .....
3030 (6:B)          .......... .....
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Fig. 4: Alignment of nucleotide sequences from fimbrial gene from different isolates of *Pasteurella multocida* B:2.

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                    10        20        30        40        50        60        70
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3      MKKAIFFSIK KGFTLIELMI VIAIIAILAT IAVPSYQNYT KKAAISELLQ AAAPYRAEVE LCIYNTNSTT
PMTB (6:B)      .......... .......... .......... .......... .......... .......... ..........
2744 (6:B)      .......... .......... .......... .......... .......... .......... ..........
3027 (6:B)      .......... .......... .......... .......... .......... .......... ..........
PMX (6:B)       .......... .......... .......... .......... .......... .......... ..........
3030 (6:B)      .......... .......... .......... .......... .......... .......... ..........
                    80        90        100       110       120       130       140
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Ref 1 (A:3      NCNAGSHGIR ADAAASKKYL KAITVKAGVI TVTGQGSLEG ISYTLTASGN AAQGVSWTVN CGAQSDIFPA
PMTB (6:B)      .......... .......... .......... .......... .......... .......... ..........
2744 (6:B)      .......... .......... .......... .......... .......... .......... ..........
3027 (6:B)      .......... .......... .......... .......... .......... .......... ..........
PMX (6:B)       .......... .......... .......... .......... .......... .......... ..........
3030 (6:B)      .......... .......... .......... .......... .......... .......... ..........
                ....|
Ref 1 (A:3      GFCA.
PMTB (6:B)      .....
2744 (6:B)      .....
3027 (6:B)      .....
PMX (6:B)       .....
3030 (6:B)      .....
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Fig. 5: Alignment of predicted amino acid sequences of fimbrial subunit gene from different isolates of *Pasteurella multocida* B:2.

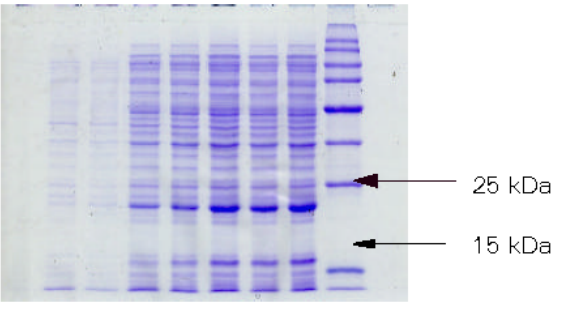

Fig. 6: Detection of the expressed fimbriae in *E. coli* using SDS-PAGE from 4-8 hours. Lane 1- BL21 *E. coli*; Lane 2- uninduced recombinant protein; Lanes 3, 4, 5, 6 and 7 represent 4, 5, 6, 7 and 8 hours post induction (induced with IPTG); Lane 8- Protein marker (Promega, USA). Long arrow pointed at the expressed fimbriae.

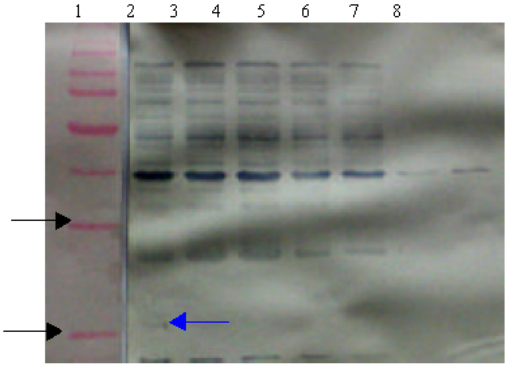

Fig. 7: Western blot analysis of fimbriae of *P. multocida* B:2. Lane 1– protein marker (Promega, USA); lane 2 – BL21 *E. coli* transformed with recombinant plasmid at 8 h post-induction; Lanes 3, 4, 5, 6 represent 7, 6, 5 and 4 hours post-induction respectively. Arrow indicates the band that represent fimbrial gene.

protein expression in *E. coli* was uncertain. It may be expressed intracellularly within the cytoplasm or extracellularly within the outer membrane fraction. Therefore, if it is expressed intracellularly, the translocation and assembly process of the fimbrial protein may be incomplete. Since then, when the anti-*E.coli* serum was used, no bands were observed through out the 8-hour experiment. This clearly shows that the expression of the endogenous fimbrial gene of *E. coli* was fully shut off.

Acknowledgement

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