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Multiplex PCR for the detection of putative virulence factor genes *mviN* and *vacB* in *Pasteurella multocida* B: 2 strains

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ABSTRACT

Vacuolating cytotoxic activity in the mouse macrophage cell line RAW 264 was reported in *Pasteurella multocida* B: 2 strains associated with haemorrhagic septicaemia in buffaloes and cattle. A putative virulence-related gene *mviN* was reported in the sequenced genome of *P. multocida* serotype A (avian strain Pm70). MviN is an integral membrane protein that belongs to MATE (multi-antimicrobial extrusion) family. The present study was undertaken to detect the presence of putative virulence- related genes, viz. *vacB* and *mviN* among *P. multocida* B: 2 strains (8). Sequence information for the *vacB* gene was obtained from the sequenced genome of *P. multocida* serotype A (avian strain Pm70). Oligonucleotide primers were designed for these genes and synthesized. All the 8 *P. multocida* B: 2 strains, 1 *P. multocida* type A 3 (bovine) and 1 *P. multocida* type D (pig) strain had both of these genes of the expected size (1.5 Kb for *mviN* and 2.4 Kb for *vacB*) as confirmed by PCR. *E. coli* K12 used as negative control did not show any band. Both these putative virulence-related genes were amplified in a single reaction mixture using multiplex PCR. The expected size (1.5 Kb for *mviN* and 2.4 Kb for *vacB*) of both the genes was obtained. This is the first report of the detection of these virulence-related genes among *P. multocida* B: 2 strains. Besides providing additional targets for diagnostic applications, the identification and subsequent analysis of virulence-related genes in *P. multocida* B: 2 strains may help in improving our understanding of molecular mechanism of adaptation, survival and virulence of *P. multocida* B: 2.

Key words: Detection, *Pasteurella multocida*, PCR, Virulence factor genes

The pathogenicity of *Pasteurella multocida* serotype B: 2, causative agent of haemorrhagic septicaemia that accounts for huge economic losses to the cattle industry, is not clearly defined. Bacterial virulence determinants are the components and products of the bacterial cells that have the potential to harm the host. These are conveniently divided into those that are cell associated i.e cell surface components (fimbriae, lipotechoic acid, exo-polysaccharides-capsule and slime and OMPs) and those that are extracellular i.e the classic exotoxin and the extracellular enzymes. Various possible virulence factors are the capsule, endotoxin, OMP, iron binding systems, heat shock proteins, neuraminidase production and antibody cleaving enzymes. Pratt *et al.* (2000) demonstrated production of lipase, as a potential virulence factor by clinical isolates of *P. multocida*. No single virulence factor has been associated with the variation in virulence among strains of *P. multocida*. HS associated strains of *P. multocida* produced a vacuolating cytotoxin encoded by the *vacB* gene. This cell associated and extracellular vacuolating cytotoxic activity of *P. multocida* B and E strains caused large cytoplasmic vacuoles in mouse peritoneal macrophages and in mouse macrophage cell line, and eventually cell death, in contrast to A and D strains (Shah *et al.* 1996). Detection of new putative virulence factors would aid in better understanding of the pathogenesis of this deadly disease. Nucleic acid amplification (NAA) technology especially polymerase chain reaction (PCR) has completely revolutionized the detection and characterization of DNA from different sources. Multiplex PCR is a variant of PCR in which 2 or more loci are simultaneously amplified in the same reaction. This study was envisaged with the aim to detect new putative virulence factor genes i.e *mviN* and *vacB* in a single reaction using multiplex PCR.

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MATERIALS AND METHODS

P. multocida B: 2 strains, viz. 90016, 85020, 90025, 8402, 86006 and 85009 originally from cases of haemorrhagic septicaemia in cattle in Sri Lanka; A porcine isolate, *P. multocida* CW5519/93 (serotype D), a bovine strain of *P. multocida* (serotype A3), a bovine strain of *Mannheimia haemolytica* and *Escherichia coli* K12 were included as controls. All strains were stored as lyophilized cultures in the Institute of Infection, Immunity and Inflammation, University of Glasgow.

Preparation of chromosomal DNA: Cells from 1 ml of overnight cultures were harvested by centrifugation for 1 min at 13,000 rpm and washed once in sterile dH_2O. DNA was prepared by boiling the washed cells for 15 min and then snap cooling on ice. DNA was stored at –20°C until use.

PCR primers: During primer design, care was taken to avoid any overlap, potential internal secondary structure, compatible Tm values and where possible a GC clamp was engineered at the 3' end of the primer. Primers (desalted and deprotected) were obtained from Invitrogen, UK and resuspended in filter sterile distilled water to give final concentration of 25 pmol/µl for PCR. Table 1 shows the PCR primers used in this study.

Components of PCR: The reaction mixture (25 µl) contained 1 µl of each primer (25 pmol), 0.5 µl of template DNA and 22.5 µl of 1.1 × ReddyMix PCR Master Mix (1.5 mM $MgCl_2$) (AB gene). The PCR assays were performed in 0.5 ml microfuge tubes.

Conditions of PCR: Amplification was done in a thermal cycler using different cycles of denaturation, annealing, extension for different genes in this study (Table 2).

PCR for species and type specific identification of P. multocida strains: All the 6 *P. multocida* B: 2 strains, 1 each of *P. multocida* Pm A: 3, *P. multocida* Pm D strain and *E. coli* K 12 were tested against *P. multocida* species and *P. multocida* B: 2 type specific primers (Table 1). *E. coli* K 12 was used as the negative control.

PCR for identification of vacB and mviN genes: The nucleotide sequences of *P. multocida* genes encoding putative virulence factors were retrieved from the published *P. multocida* genome sequence (May *et al*. 2001) and BLAST searches were conducted to select those virulence factor genes that display no homology with other DNA sequences at GenBank. Oligonucleotide primers were designed from these genes and synthesized by Invitrogen, UK.

PCR conditions were standardized for these 2 genes. The size of *mviN* gene is 1.5 Kb and that of *vacB* is 2.4 Kb. All the *P. multocida* B: 2 strains (n=6), *P. multocida* type A (bovine) and *P. multocida* type D (pig strain) were tested for these 2 virulence factor genes.

Multiplex PCR for mviN and vacB genes: Multiplex PCR is a variant of PCR in which 2 or more loci are simultaneously amplified in the same reaction. Putative virulence factor genes *mviN* and *vacB* were amplified in a single reaction mixture.

Analysis of amplicons: The amplified products (8 µl) were mixed with 2 µl of 6-fold loading buffer and electrophoresed in 1% (w/v) agarose gel. The 1 Kb DNA ladder was used as DNA molecular weight markers.

Table 1. PCR primers used in this study

Primer name	Primer sequence	Reference
KMT1T7	ATC CGC TAT TTA CCC AGT GG	Townsend *et al*. (1998)
KMT1SP6	GCT GTA AAC GAA CTC GCC AC	Townsend *et al*. (1998)
KTSP61	ATC CGC TAA CAC ACT CTC	Townsend *et al*. (1998)
KTT72	AGG CTC GTT TGG ATT ATG AAG	Townsend *et al*. (1998)
MVINF	AAT CCA TGG CGT TGT TAT CAC GCA TTC TAG GC (*NcoI)*	May *et al*. (2001)
MVINR	ACG CTC GAG TCC TTT AGT GGT TAA ATG ATG TTT ACG (*XhoI)*	May *et al*. (2001)
VACBF	AAT CCA TGG CAA AAA TCA AAA ACA ATC CAC ATT TG (*NcoI)*	May *et al*.(2001)
VACBR	ACG CTC GAG CTT TCT CTT TTT AGC TGA TTT CTT AC (*XhoI)*	May *et al*. (2001)

Table 2. PCR conditions for different gene amplifications

Gene	Initial denaturation temp. (°C)	PCR Cycle				Final extension (°C)
		Denaturation (°C)	Annealing (°C)	Extension (°C)	No. of cycles	
P. multocida	95 ×4 min	95 × 1 min	55 × 1 min	72 × 1 min	30	72 × 9 min
P. m B: 2	95 × 4 min	95 × 1 min	55 × 1 min	72 × 1 min	30	72 × 9 min
mviN gene	94 × 4 min	94 × 30s	46 × 30s	72 × 2 min	40	72 × 9 min
vacB gene	94 × 4 min	94 × 30s	43 × 30s	72 × 2 min	40	72 × 9 min
Multiplex PCR	94 × 4 min	94 × 30s	43 × 30s	72 × 2 min	40	72 × 9 min

(mviN and vacB)

Gel preparation and electrophoresis: Agarose (type II-A medium EEO) was suspended in 0.5 × tris-borate EDTA (TBE) buffer at concentration of 0.7% and heated until the agarose was completely dissolved. The solution was allowed to cool (hand-hot) and ethidium bromide was added to a final concentration of 0.5 µg/ml. A gel tray was prepared by taping the edges with adhesive tape and the gel cast to the desired thickness. Upon setting, the gel was immersed in 0.5 × TBE buffer in a horizontal submarine electrophoresis tank. A powerpack was used to provide a constant voltage corresponding to 1–5 volts/cm ^{2}. Electrophoresis was carried out until the marker dyes migrated an appropriate distance.

Visualization of DNA: An UV transilluminator was used to visualize the ethidium bromide stained DNA. Images were stored electronically. Images were printed using a videographic printer; where appropriate, photographs were captured with a camera using a flatbed scanner.

RESULTS AND DISCUSSION

Since the advent of PCR technique in 1985, it has been used effectively with the basic principle of *in vitro* nucleic acid amplification through repetitive cycling. PCR plays a critical role in clinical laboratory, as rapid and specific detection of micro-organisms has provided remarkable advances in the diagnosis of infectious agents, particularly in cases where the presence of an organism has significance (Relman and Persing 1996). All the 8 *P. multocida* B: 2 strains- Pm 90016, 85020, JRMT 1, 90025, 86006, 85009, 8402, and CW 5519/93 at Division of Infection and Immunity were confirmed as *P. multocida* and *P. multocida* B: 2 using species and type specific primers. Pm A3 and Pm 762 (type D pig strain) were positive for genus specific primers but negative for B: 2 type specific primers. *E. coli* K 12 was the negative control with no band with either of the primers. Size of the gene of *P. multocida* was 460 bp and that of *P. multocida* B: 2 was 590 bp (Fig.1). These results agree with those of previous reports by Kasten *et al*. (1997) and Townsend *et al*. (1998).

The main aim of this study was to establish a multiplex PCR for the detection of 2 putative virulence factor genes, viz. *mviN* and *vacB*. Transcriptional regulators play an important role in the adaptation and survival of bacteria and may display genus, species or subspecies specificity. Earlier investigations on a food-borne pathogen *Listeria monocytogenes* (Liu *et al*. 2003 and 2004) and nonpathogenic *L. innocua* (Liu *et al.* 2003) indicated that the presence of species and virulence specific transcriptional regulator genes in bacterial genomes is not uncommon.

The *mviN* gene was first identified in *Salmonella* Enterica serovar Typhimurium as a gene in a chromosomal region required for virulence in a mouse model of typhoidlike disease (Benzamin *et al*. 1991, Carsiotis *et al*. 1989). MviN is involved in murein synthesis in *E. coli* (Azusa *et al.* 2008).

All the 8 *P. multocida* B: 2 strains, Pm type A (bovine) and D (pig strain) had both these genes of the right size as confirmed by PCR. The size of *mviN* gene is 1.5 Kb and that of *vacB* is 2.4 Kb (Fig. 2). Both *mviN* and *vacB* genes could be amplified by conditions set for *vacB* gene (Fig. 2). Putative virulence factor genes *mviN* and *vacB* were amplified in a single reaction mixture. When equal volume of forward and reverse primers for both the genes were added in PCR mix, 1.5 Kb gene corresponding to *mviN* gene could be amplified

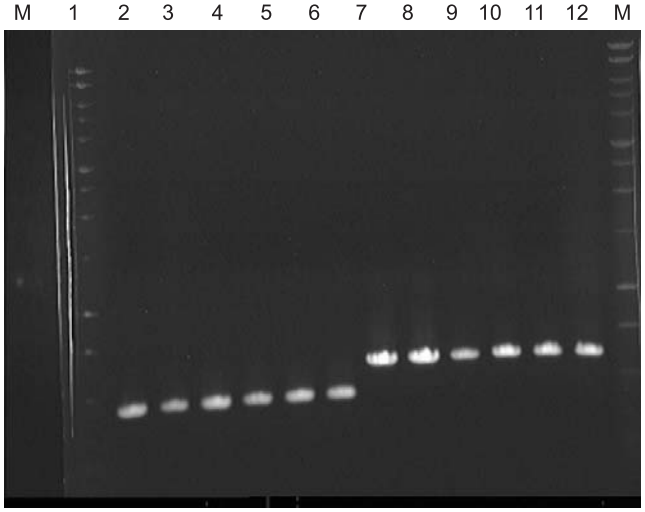

Fig. 1. Agarose gel showing genus and type specific bands in *P. multocida* B: 2 strains. M: 1 Kb DNA marker (Promega) lanes 1, 7: Pm85020; lane 2, 8: Pm90016; lane 3, 9: CW55519/93; lane 4, 10: Pm 86006; lane 5, 11: Pm85009; lane 6, 12: Pm8402; lanes 1–6: Species specific primers; and lanes 7–12: *P. multocida* B: 2 type specific primers.

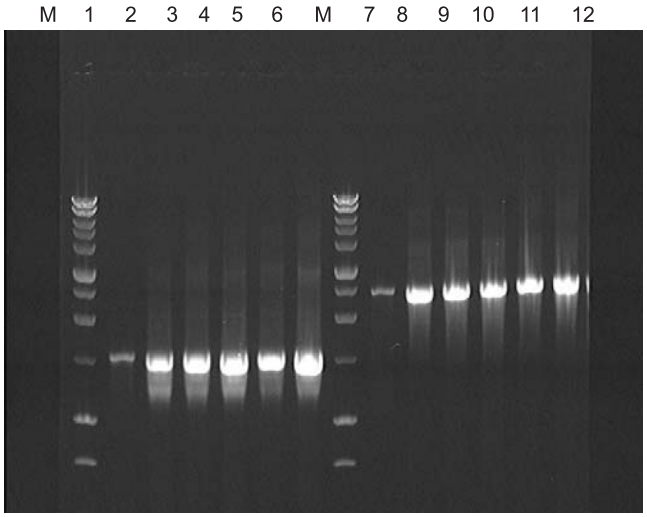

Fig. 2. Agarose gel showing presence of *mviN* and *vacB* genes in *P. multocida* strains M: 1 Kb DNA Ladder (Promega); lane 1,7: Pm A: 3; lane 2, 8: Pm 86006; lane 3, 9: Pm 85009; lane 4, 10: Pm 8402; lane 5, 11: CW5519/93; lane 6, 12: Pm 762; lanes 1–6: *mviN* gene; and lanes 7–12: *vacB* gene.

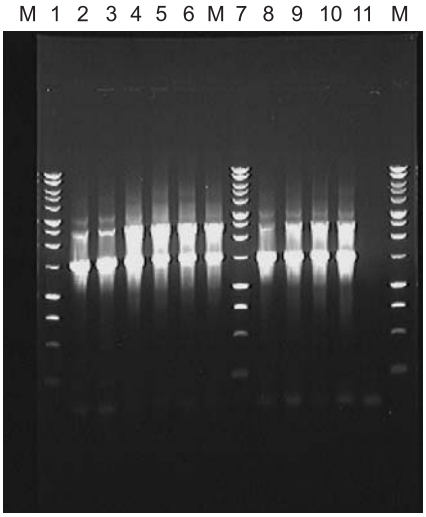

Fig. 3. Multiplex PCR showing *mviN* and *vacB* genes. M: 1 Kb DNA Ladder (Promega) lane 1: PmA: 3; lane 2: Pm 85020 ;lane 3: Pm 90016; lane 4: Pm 90025; lane 5: Pm 86006; lane 6: Pm JRMT12; lane 7: Pm 85009; lane 8: Pm 8402; lane 9: CW5519/93; lane 10: Pm762 (Pig strain); lane 11: *E. coli* K12.

but *vacB* gene could not be amplified. On decreasing the concentration of *mviN* gene primers to 0.25 µl (forward and reverse primers each) both genes could be amplified in all *P. multocida* B: 2 strains, *P. multocida* type A and D strains (Fig.3).

MviN is involved in murein synthesis in *E. coli* (Azusa *et al.* 2008). MviN is an integral membrane protein. It belongs to member of the MATE (multi antimicrobial extrusion)-like superfamily comprising integral membrane proteins. Members of the MATE family also function as drug/sodium antiporters. These proteins also showed to mediate resistance to a wide range of cationic dyes, fluoroquinolones, aminoglycosides and other structurally diverse antibodies and drugs (Omote *et al*. 2006). MATE proteins were also implicated in the production of polysaccharides, such as RfbX (Wzx) (Feldman *et al.* 1999) and WzxE (Rick *et al.* 2003), which were implicated in *E. coli* O antigen biosynthesis, *Bacillus subtilis* SpoVB, which is involved in spore cortex biosynthesis (Popham and Stragier 1991, Vasudevan *et al*. 2007), and eukaryotic RFT1, which is required for the translocation of the dolichyl diphosphate-Man5GlcNAc2 intermediate, an oligosaccharide complex used in protein glycosylation, from the cytosolic side of the ER membrane to the lumen during the biosynthesis of dolichyl diphosphate-Glc3Man9GlcNAc2 (Helenius *et al*. 2002). These results suggested that MviN may be involved in the transmembrane transport of peptidoglycan precursors across the inner membrane. This transport process was recently reconstituted *in vitro* (van Dam *et al*. 2007), which will enable further characterization of the function of MviN. Alternatively, MviN may be indirectly involved in peptidoglycan synthesis. For example, it may play a role in folding or localization of other proteins involved in peptidoglycan synthesis because the precursors accumulated by a wide variety of treatments that inhibit peptidoglycan synthesis or that block cell division (Lara *et al.* 2005). Further analyses are necessary to clarify its specific role.

Besides providing additional targets for diagnostic applications, the identification and subsequent analysis of these putative virulence factor related genes in *Pasteurella multocida* may help improve our understanding on the molecular mechanisms of *Pasteurella multocida* adaptation, survival and virulence.

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