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Short Communication

Molecular characterization of *Pasteurella multocida* isolates using RFLP-PCR of *ompH* gene and RAPD analysis

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Development of molecular techniques as an alternative to serotyping is being undertaken for an important veterinary and opportunistic human pathogen *Pasteurella multocida* (Deressa *et al*. 2010, Subaaharan *et al*. 2010). Among the genotyping studies, restriction fragment length polymorphism (RFLP) and random amplification of DNA (RAPD) have proven valuable for genomic fingerprinting of the pathogen. *ompH* gene encoding major outer membrane protein H (OmpH) in the envelope of *P. multocida* was used for molecular typing and epidemiological study (Jabbari and Esmaelizadeh 2005). The present study was aimed at evolving a newer and rapid technique in the typing of *P. multocida* isolates using RFLP-PCR of *omp*H gene and RAPD analysis.

Bacterial isolates: The *P. multocida* isolates were obtained from Department of Veterinary Microbiology, Madras Veterinary College, Chennai, India. The field isolates of buffalo (PB) belonged to capsular type B, while field isolates of sheep (PS 01–03), goat (PG), turkey (PT) and chicken (PP) belonged to capsular type A. The vaccine strain P52 of *P. multocida* (serotype B: 2) was obtained from Division of Standardization, Indian Veterinary Research Institute, Izatnagar, India. The vaccine strain and the isolates were maintained on blood agar medium and cultured in brain-heart infusion (BHI) broth.

DNA extraction: The DNA was extracted from the isolates as per the standard protocol of phenol: chloroform extraction.

RFLP analysis: The *ompH*-specific primers *(OmpH*-1 and *OmpH*–2) for the gene amplification were custom synthesized based on the sequence of *ompH* gene of *P. multocida* serotype B: 2, Accession no. EU 016232 (Singh 2008). The sequences of the two primers encoding the truncated open reading frame (ORF) were as follows:

OmpH-1: 5' TCAGGATCCCAGCAACAGTTTA CAATCAAGA- 3'- 31 mer

OmpH-2: 5'- CTACCCGGGTTAGAAGTGTACG CGTAAACCA- 3' - 31 mer

PCR mixtures consisted of 20 ng of *P. multocida* genomic DNA, 25 pmol of each primer, 0.1 mM dNTPs, 1×PCR buffer, and 2.5 units of *Taq* DNA polymerase in 50 µl volume. Thirty-five cycles of amplification consisting of denaturation for 15s at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C was performed with an initial denaturation of target DNA at 94°C for 5 min and final extension of 10 min at 72°C. The RFLP reaction was carried out by digesting the PCR-amplified products with EcoR I, Hind III and Pst I and separting on 1.5% agarose gel.

RAPD analysis: The RAPD reaction was performed in 50 µl consisting of 20 ng template DNA, 10 × PCR buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 1.25 U of Taq DNA polymerase, and 1 µM of OPA-11 primer (5'-CA AT CG CC GT- 3') (Ozbey *et al.* 2004). The optimized PCR cycling conditions were, 50 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min and products were analysed in 1.5% agarose gel.

Analysis of the RFLP-PCR and RAPD products: RFLP-PCR and RAPD analysis were carried out with NTSYSPC programme. The data were converted to a two-dimensional rectangular data matrix of binary codes for all *P. multocida* isolates (1 for presence of product band; 0 for absence of product band). The distances obtained were subjected to similarity coefficient analysis and an unweighted pair-group mean arithmetic method (UPGMA) was used to construct the dendrograms.

RFLP-PCR Profiles: All isolates possessed one restriction site for ECoR I, HinD III and Pst I excluding PS–01 which did not have a restriction site for ECoR I and Pst I. PV, PB, PG, PP and PT showed similar pattern on digestion with ECoR I and Pst I giving bands of 520 bp and 440 bp with

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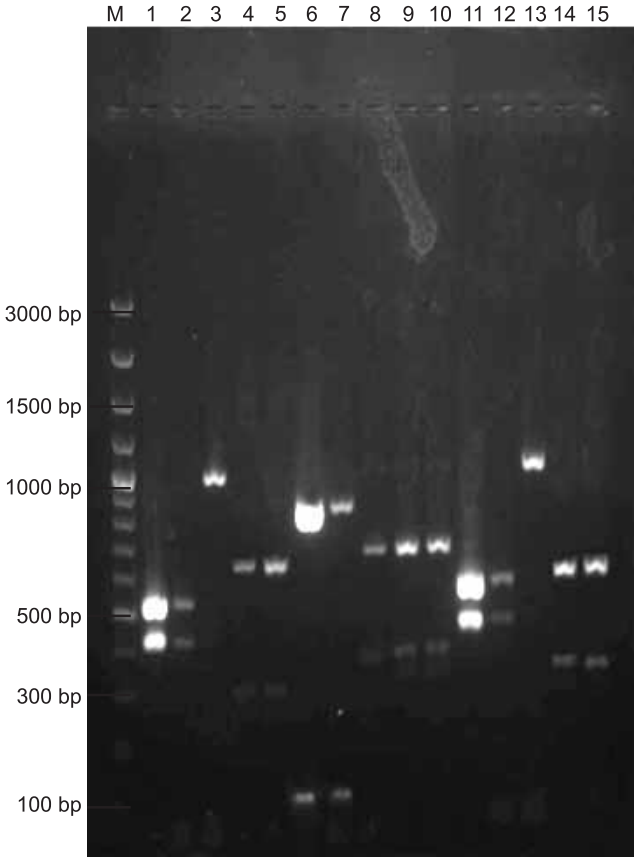

Fig. 1. Electrophoretic profiles of *P. multocida* isolates following PCR-restriction fragment length polymorphism of *ompH* gene, Lane M–100 bp marker, Lanes 1, 6, 11– RE digested product of PV, Lanes 2, 7, 12– RE digested product of PB, Lanes 3, 8, 13– RE digested product of PS-01, Lanes 4, 9, 14– RE digested product of PS-02, Lanes 5, 10, 15– RE digested product of PS-03, Lanes 1–5– PCR products digested with ECoR I, Lanes 6–10– PCR products digested with HinD III, Lanes 11–15–PCR products digested with Pst I.

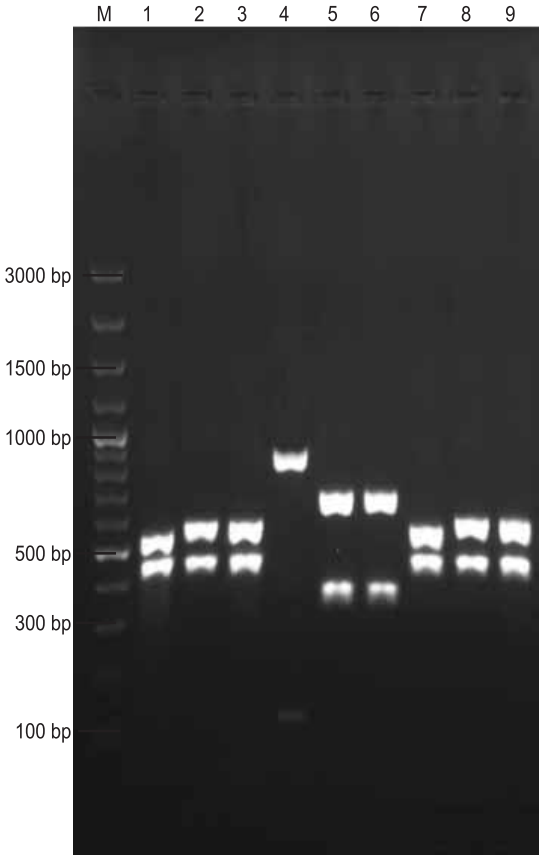

Fig. 2. Electrophoretic profiles of *P. multocida* isolates following PCR-restriction fragment length polymorphism of *ompH* gene. Lanes 1, 4, 7– RE digested product of PG; Lanes 2, 5, 8– RE digested product of PP, Lanes 3, 6, 9– RE digested product of PT, Lanes 1–3– PCR products digested with ECoR I, Lanes 4–6– PCR products digested with HinD III and Lanes 7–9– PCR products digested with Pst I, Lane M–100 bp marker.

ECoR I and 530 bp and 430 bp with Pst I (Fig. 1). On digestion with HinD III, PV, PB and PG gave similar restriction pattern with bands corresponding to 840 bp and 120 bp. While all sheep isolates produced bands corresponding to 680 bp and 280 bp, PP and PT produced bands corresponding to 620 bp and 340 bp (Fig. 2). Among sheep isolates, PS-02 and PS-03 gave similar pattern with all 3 enzymes but PS-01 differed on digestion with ECoRI and PstI by having no restriction sites (Fig. 1).

The 3 sheep isolates can be easily differentiated from rest isolates by any one enzyme ECoR I, Hind III and Pst I and can be differentiated among themselves using either ECoR I or Pst I. PV, PB, PG, PP and PT cannot be differentiated by ECoR I and Pst I. But, digestion with HinD III resulted in 2 groups. One group comprised PV, PB and PG, while other consisted of PP and PT. Further demarcation between PV, PB and PG cannot be made with the enzymes.

Based on the dendrogram (Fig. 3) the isolates were grouped into clusters I and II with 97% of similarity. Cluster

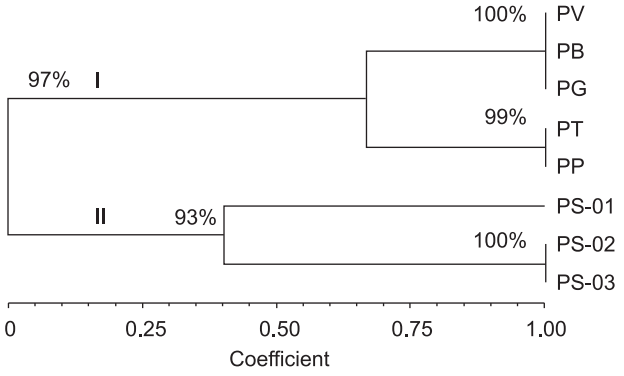

Fig. 3. Dendrogram showing genetic relatedness of *P. multocida* isolates constructed considering all bands of RFLP – PCR OF *ompH* gene; Percentage of similarity is shown at the nodes.

I consisted of 2 groups, group 1 (PB, PV and PG) showing 100% similarity. PP and PT showed 99% similarity and constituted group 2. Cluster II included all sheep isolates

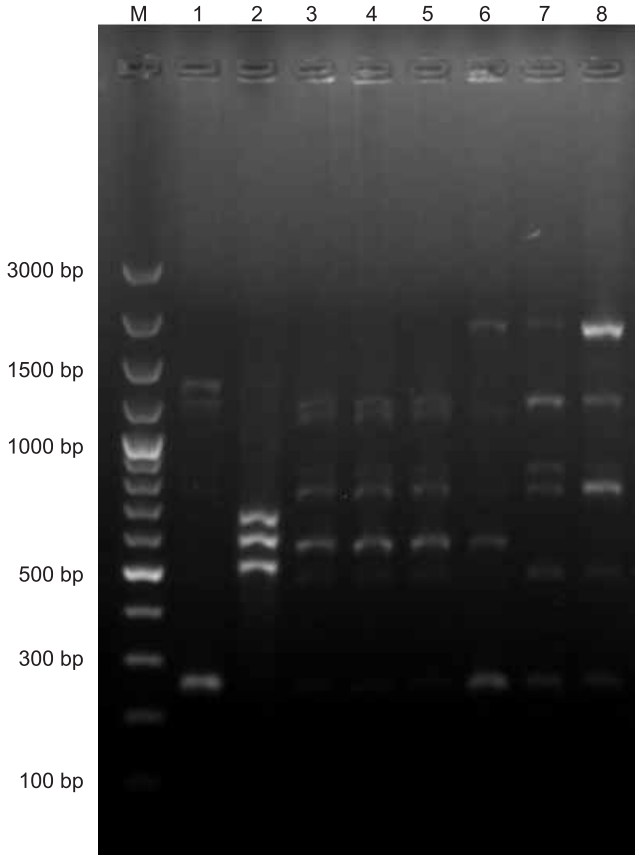

Fig. 4. Electrophoretic profiles of *P. multocida* isolates following random amplification of polymorphic DNA analysis, Lane M– 100 bp marker; Lane 1–8: Isolates PV, PB, PS-01, PS-02, PS-03, PG, PP and PT, respectively.

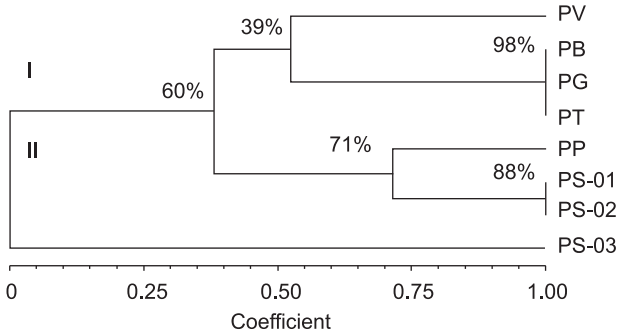

Fig. 5. Dendrogram showing genetic relatedness of *P. multocida* isolates constructed considering all bands of RAPD analysis percentage of similarity is shown at the nodes.

and divided into 2 groups with 93% of similarity. Group 1 consisted PS-01 and group 2 consisted PS-02 and PS-03 with 100% similarity. The similarity percentage based on the dendrogram correlated well with the findings of the RFLP-PCR pattern based on visual examination.

RFLP of *ompH* gene exhibited extensive restriction site heterogeneity, which may be suitable for fingerprinting of *P. multocida* isolates. The patterns produced had much simpler profile than those produced by genomic DNA fingerprinting allowing easy visual analysis. Based on the above said results, with the exception of isolate PG the *P. multocida* serotype A and serotype B can be distinguished. Even among the isolates of *P. multocida* serotype A, clear distinction between species can be made using RFLP-PCR of *ompH* gene. Jabbari and Esmaelizadeh (2005) reported that combination of different restriction patterns of EcoR I, HinD III and Cfo I divided 25 isolates of avian *P. multocida* into 7 RFLP types. They also found extensive restriction site heterogeneity in the *ompH* gene. Similar findings were reported by Antony *et al.* (2007) when they performed REA of amplified products of OmpH-PCR with Dra I and HinF I enzymes. Dra I generated distinct profiles for the three serotypes A:1, A:3 and B:2 while enzyme HinF I generated patterns similar in A:3 and B:2 but distinct from A:1 suggesting REA of amplified products of OmpH-PCR with Dra I offers a novel technique for differentiation of various serotypes of *P. multocida*.

RAPD Profiles: 5 different profiles were found in this study (Fig. 4). The band pattern of PV differed from that of buffalo isolate and also from isolates of capsular type A. The total number of bands generated was 6 corresponding to 1477, 1376, 893, 782 and 235 bp. PB showed a distinct profile from the vaccine strain by having 6 RAPD bands corresponding to 1477, 1376, 1107, 680, 587and 505 bp. All sheep isolates showed similar profile having identical RAPD bands. The total number of bands generated was 7 corresponding to 1376, 1262, 893, 782, 587, 505 and 235 bp. PG showed a distinct profile from vaccine strain and sheep isolates by having five RAPD bands corresponding to 1915, 893, 782, 587and 235 bp (Fig. 4). The chicken and turkey isolates showed a similar profile by having 5 bands corresponding to 1915, 1660, 893, 782 and 235 bp (Fig. 4).

Based on the dendrogram (Fig. 5) the isolates were grouped into clusters I and II with 30% of similarity. Cluster I comprised of 2 groups with each group having 2 sub-groups. The group 1 had 60% similarity with group 2. Group 1 comprised 2 sub-groups, i.e. sub-group 1 and sub-group 2. Sub-group 1 was composed of PV and sub-group 2 was composed of all sheep isolates with 35% similarity. The percentage of similarity between the sheep isolates was 98%. The group 2 also comprised 2 sub-groups viz. sub-group 1 and sub-group 2. Sub-group 1 was composed of PG and sub–group 2 was composed of PP and PT with 71% similarity. The percentage of similarity between PP and PT was 88%. The cluster II comprised only of PB. The RAPD analysis further discriminated the isolates PV, PB and PG which were not distinguished by RFLP-PCR of *ompH* gene.

Based on the findings, with the exception of isolates PG and PV the *P. multocida* serotype A and serotype B can be distinguished. These results are in agreement with those of a previous study which reported the presence of 7 distinct profiles for *P. multocida* strains by the use of 2 random

primers (Chaslus-Dancla *et al*. 1996). Dziva *et al.* (2001) typed 81 *P. multocida* isolates of animal origin by both. Ozbey *et al.* (2004) observed 5 different profiles among *P. multocida* isolates of cattle and sheep using random primer OPA-11. Venkatesan *et al*. (2006) reported differences among *P. multocida* isolates were discernible by RAPD analysis, whereas conventional bacteriological methods failed to differentiate these isolates.

The development of a DNA-based technique for differentiation of serotypes could provide an alternative to conventional serotyping system.

SUMMARY

Seven field isolates and one vaccine strain of *Pasteurella multocida* were used in molecular characterization using RFLP-PCR and RAPD. The field isolate of buffalo (PB) and vaccine strain (PV) belonged to capsular type B while field isolates of sheep (PS 01-03), goat (PG), turkey (PT) and chicken (PP) belonged to capsular type A. RFLP-PCR of amplified *ompH* gene with ECoR I, HinD III and Pst I yielded 4 different profiles that distinguished *P. multocida* serotype A and serotype B with the exception of goat isolate. Among the isolates of *P. multocida* serotype A, a clear distinction was made using RFLP-PCR of *ompH* gene. To determine the genetic differences between the *P. multocida* isolates, a random primer OPA-11 was used in RAPD. Based on the amplified products in agarose gel and dendrogram construction, 5 different profiles were found. The *P. multocida* serotype A and serotype B were distinguished, with the exception of goat isolate and vaccine strain.

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