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Research Article

MOLECULAR CHARACTERIZATION OF INDIAN ISOLATES OF PASTEURELLA MULTOCIDA BY RAPD-PCR

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ABSTRACT

The present study was conducted to determine the genetic relatedness among the Indian isolates of *Pasteurella multocida* (n=17) of buffalo origin by random amplified polymorphic DNA (RAPD)-PCR. All the three RAPD primers successfully typed all the 17 *P. multocida* isolates by assigning them to distinctive profiles. With single decamer, OPG-10 and OPG-13 primers used in the study, all the 17 isolates of *P. multocida* were divided into 3, 3 and 2 major clusters, respectively.

Key words: Haemorrhagic septicaemia, *P. multocida*, RAPD-PCR, genotyping

Haemorrhagic septicaemia (HS) is one of the most important bacterial diseases of Asian and African countries in terms of economic losses due to high morbidity and mortality in cattle and buffalo (Verma and Jaiswal, 1998; Singh *et al.*, 2008). This disease is caused by a Gram negative coccobacillary bacterial pathogen known as *Pasteurella multocida*. The diagnosis of HS is mainly based on clinical symptoms and identification of the organism from the suspected clinical specimens. Presently, Carter and Heddleston serotyping system is used for typing the isolates of *P. multocida* which divides it into five capsular serogroups (A, B, D, E and F) and 16 somatic serogroups (1-16) based on indirect haemagglutination and agar gel precipitation tests, respectively (Carter, 1955; Heddleston *et al.*, 1972). The conventional identification and serotyping is time consuming and requires skilled personnel and also the maintenance of battery of hyperimmune sera.

DNA-based identification and typing systems involving techniques like restriction endonuclease analysis (REA), ribotyping, pulse-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) methods such as enterobacterial repetitive intergenic consensus (ERIC-PCR) and random amplified polymorphic DNA (RAPD)–PCR are quick and can be used as confirmatory diagnosis within a short period (Townsend *et al.*, 1998; Miflin and Blackall, 2001; Shivachandra *et al.*, 2006). These techniques are applied in epidemiological studies aimed at recognizing outbreaks of infection, detecting the cross-contamination of strains between different avian and animal hosts and determining the origin of infection (Shivachandra *et al.*, 2006). Hence, the present study was undertaken to study the genetic relatedness of the isolates of *P. multocida* using RAPD-PCR.

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MATERIALS AND METHODS

Seventeen freeze dried cultures of *P. multocida* serotype B:2 of buffalo origin collected across the country and maintained in the Division of Bacteriology and Mycology, IVRI, Izatnagar were used in the study (Table 1). The purity and identity of the cultures were tested by morphological, cultural and biochemical tests. The biochemical tests were carried out as per the standard procedures described by Cowan and Steel (1970). The genomic DNA was isolated following the method of Wilson (1987) with slight modifications. The purity and concentration of each DNA sample was determined spectrophotometrically by measuring the optical density (O.D.) at 260 nm and 280 nm. All PCR

reactions were carried out with all the 17 isolates using the custom synthesized oligonucleotide primers (Table 2) (Chaslus-Dancla *et al.*, 1996; Brickell *et al.*, 1998; Dziva *et al.*, 2001). Dendrograms were derived from a matrix of similarity values by the unweighted pair group method using arithmetic averages by using NTSYSpc (Applied Biostatistics Inc. ABI) software.

Molecular Identification of *P. multocida*: The identification of *P. multocida* serotype B:2 was carried out using *P. multocida* species specific primers which amplify ~ 460 bp product (Townsend *et al.*, 1998), cap primers which produces ~760 bp product in serogroup B (Brickell *et al.*, 1998) and serotype B:2 specific primers which produces ~ 320 bp product (Table 2). Genomic DNA from *P. multocida* isolates was used as a template for multiplex PCR assay. PCR reaction in a volume of 25 µl consisted of each primer at a concentration of 3.2 µM, dNTPs (200 µM each), 1xPCR buffer, 2 mM MgCl2, 1U Taq DNA polymerase and template DNA. The PCR amplification was carried out with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 min. A 5 µl amplified PCR product was mixed with 1 µl of 6x ready to use gel loading dye (MBI, Fermentas)

Table 1
Details of the *Pasteurella multocida* isolates of buffalo origin used in the study

Isolate ID	Year of isolation	State	Carter type	Heddleston type
P52	1949	Uttar Pradesh	B	2
102	2001	Uttar Pradesh	B	2
115	2001	Uttar Pradesh	B	2
286	2002	Orrisa	B	2
288	2003	Orrisa	B	2
358	2004	Himachal Pradesh	B	2
390	2004	Himachal Pradesh	B	2
409	2005	Jammu & Kashmir	B	2
410	2005	Jammu & Kashmir	B	2
553	2006	Assam	B	2
606	2008	Punjab	B	2
610	2008	Punjab	B	2
614	2008	Punjab	B	2
652	2008	Assam	B	2
653	2008	Assam	B	2
816	2010	Gujarat	B	2
830	2011	Gujarat	B	2

Table 2
Details of different primers used in the study

Primer code	Name of primer	Sequences (5'-3')
KMT1T7	PM-PCR (F)	ATCCGCTATTTACCCAGTGG
KMT1SP6	PM-PCR ®	GCTGTAAACGAACTCGCCAC
Cap-PCR	CAPA-F	TGCCAAAATCGCAGTCAG
Cap-PCR	CAPA-R	TTGCCATCATTGTCAGTG
Cap-PCR	CAPB-F	CATTTATCCAAGCTCCACC
Cap-PCR	CAPB-R	GCCCGAGAGTTTCAATCC
Cap-PCR	CAPD-F	TTACAAAAGAAAGACTAGGAGCCC
Cap-PCR	CAPD-R	CATCTACCCACTCAACCATATCAG
KTSP61	B:2 Specific (F)	ATCCGCTAACACACTCTC
KTT72	B:2 Specific (R)	AGGCTCGTTTGGATTATGAAG
OPG 10	RAPD primer	AGGGCCGTCT
OPG 13	RAPD primer	CTCTCCGCCA
S.D. Primer	RAPD primer	GATCGCCCGC

and analyzed on 2% agarose gel containing 0.5 µg/ml ethidium bromide along with 100 bp DNA ladder (MBI-Fermentas) using 1x TAE electrophoresis buffer. The gels were visualized and photographed under UV-gel documentation system (Alpha Innotech Corp., USA).

RAPD-PCR: RAPD-PCR was carried out separately with OPG10, OPG13 and single decamer primers (Table 2) as per the following method. The reaction mixture was prepared with 50 ng of purified genomic DNA, 0.8 µl of 10 mM dNTP mix, 40 pmol of each primer and 1.5 U of *Taq* polymerase in 1x PCR buffer. The PCR condition for amplification were followed as initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 6 min. Amplified fragments from all the three primers were electrophoresed in 1.5% agarose gel.

Analysis and Comparison of Fingerprint Profiles: Photographs were scanned with a Hewlett-Packard IIcx flatbed scanner and Deskscan1 software (Hewlett-Packard, Boise, ID, USA) to create a tagged image format file for computer analysis. Images of fingerprint profiles were analyzed and similarity between all the possible pairs of fingerprint profiles within a serotype was calculated by the cluster analysis module of the NTSYSpc software using the Dice coefficient and a band position tolerance of 0.5%. Dendrograms were derived from a matrix of similarity values by the unweighted pair group method using arithmetic averages. Isolates that had ~97.3% similarity based upon the data combined from analysis of fingerprint

profiles were considered identical.

A data matrix composed by the numerals 1 and 0 was built on the presence (1) or absence (0) of any fragment appearing in each isolate. Only distinct and prominent bands were scored and used in assigning isolate specific pattern. The size of the bands which differed by 5% on the different gels were considered as same band. The genetic similarity or diversity of isolates was analyzed by computerized software NTSYSpc (Applied Biostatistics Inc. ABI), which works on the principles of UPGMA clustering methods. The profiles thus generated by each typing technique were compared and correlated to find out the extent of genetic similarity or diversity amongst the isolates.

RESULTS AND DISCUSSION

The isolates exhibited cultural and biochemical properties similar to the previous reports on *P. multocida* (Carter and Chengapa, 1981). The genomic DNA of all the 17 isolates of *P. multocida* was isolated and its concentration was found to be 90-100 µg/ml. The purity of isolated DNA was found between 1.6-1.8. All the isolates were confirmed by species specific, capsular and serotype B:2 specific PCR which gave amplified products of ~ 460 bp, 760 bp and 320 sizes bp (Figs.1 and 2). All the three RAPD primers successfully typed all the 17 isolates by assigning them to distinctive profiles.

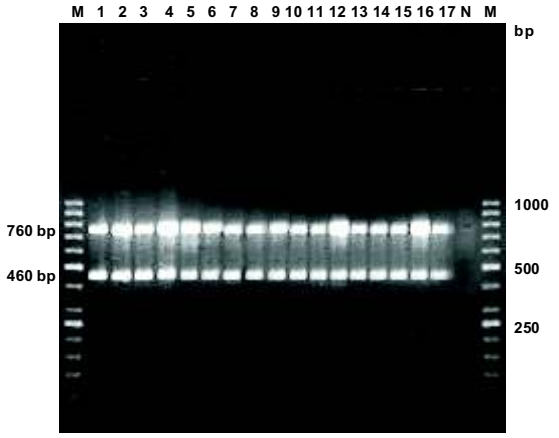

Fig 1. Multiplex PCR for different isolates of *P. multocida* Lane M=50 bp DNA ladder; Lanes 1-17=Isolates of *P. multocida* serotype B of buffalo origin; Lanes N=Negative control.

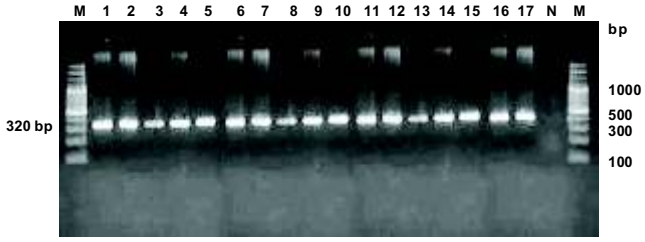

Fig 2. *P. multocida* serotype B:2 specific PCR. Lane M=100 bp DNA ladder; Lanes 1-17=*P. multocida* serotype B:2 isolate of buffalo origin; Lane N=Negative control

Single Decamer (SD) Primer: A random amplification of *P. multocida* isolates using a single decamer primer produced recognizable bands in the range of ~800 bp to 2.8 kbp (Fig. 3). Eight bands were selected for comparison of each isolate with other isolates. RAPD-PCR fingerprinting with SD primer divided the isolates into three major clusters i.e. SD1, SD2 and SD3 while SD1 was further divided into two subclusters (Fig. 3a).

OPG-10 Primer: A random amplification of *P. multocida* isolates using OPG-10 primer produced

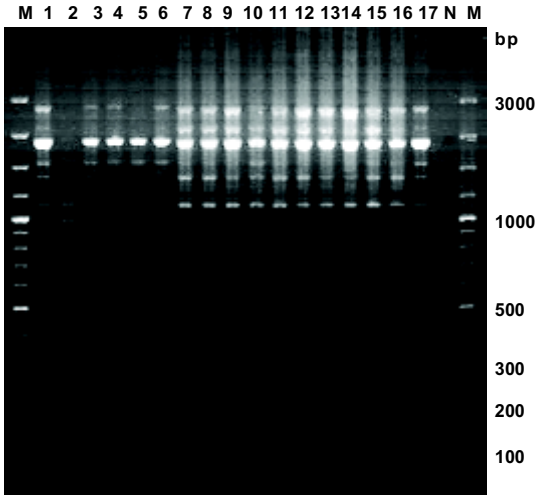

Fig 3. RAPD-PCR using SD Primer. Lane M=100bp DNA ladder; Lanes 1-17=Amplified products from different *P. multocida* isolates=Lane N=Negative control

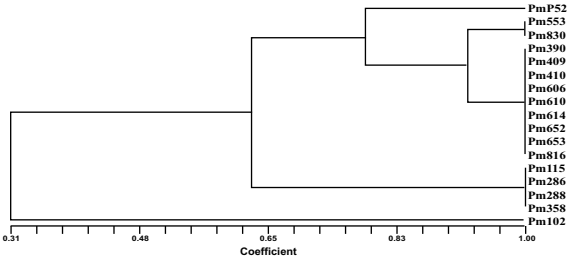

Fig 3. RAPD dendrogram analysis with SD primer

recognizable bands in the range of ~400 bp to 3.1 kbp (Fig. 4) and eight bands were selected for comparison. RAPD-PCR fingerprinting with OPG-10 primer divided all 17 isolates into 3 distinct clusters: 10a, 10b and 10c with both 10a and 10b having two subclusters (Fig. 4a).

OPG-13 Primer: A random amplification of *P. multocida* isolates using OPG-13 primer produced recognizable bands in the range of ~250 bp to 3.6 kbp (Fig. 5) and ten bands were selected for comparison. RAPD-PCR fingerprinting with OPG-13 primer divided the isolates into two clusters (Fig. 5a).

Polymerase chain reaction (PCR) assays are emerging as alternative tools for identifying the pathogen and are regarded as rapid, specific, sensitive and capable of early detection with relatively simple methodology (Blackall and Miflin, 2000; Townsend *et al.*, 2001). Various molecular techniques like RAPD-PCR, REA, PFGE, repetitive extragenic palindromic (REP)-PCR, ERIC-PCR, ribotyping and multi-locus enzyme electrophoresis are used routinely for characterization of *P. multocida* (Maiden *et al.*, 1998).

The present study was undertaken with the objective to identify and characterize the buffalo isolates of *P. multocida* serotype B:2 recovered from different parts of the country over a long period by employing RAPD-PCR. Using single decamer, OPG-10 and OPG-13 primers, all 17 isolates of *P. multocida* were divided into 3, 3 and 2 major clusters, respectively with different band sizes. Results of the present study are in accordance with the previous findings by various workers who have also reported considerable variations among the isolates of *P. multocida* by RAPD analysis (Dziva *et al.*, 2001; Dutta *et al.*, 2001; Davies *et al.*, 2004; Ozbey *et al.*, 2004; Hotchkiss *et al.*, 2011). Ozbey *et al.* (2004) reported that RAPD recognized two and three distinct profiles among 46 *P. multocida* isolates from cattle and sheep, respectively. Results of the RAPD assay in this study demonstrated little genetic heterogeneity among *P. multocida*. Similarly, Dziva *et al.* (2001) typed 81 *P. multocida* isolates of animal origin by both capsular typing and RAPD

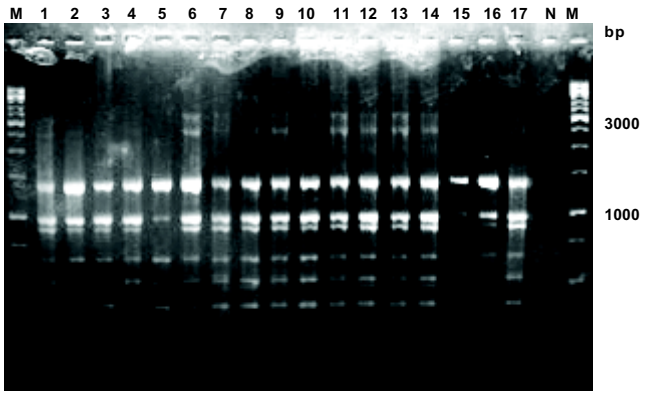

Fig 4. RAPD PCR using OPG 10 primer. Lane M=1kb ladder; Lanes 1-17=PCR amplification of different *P. multocida* isolates; Lane N=Negative control

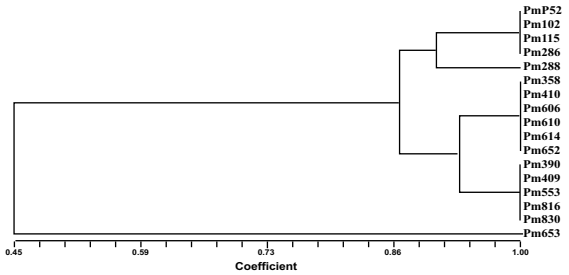

Fig 4 a. RAPD dendrogram analysis with OPG 10 primer

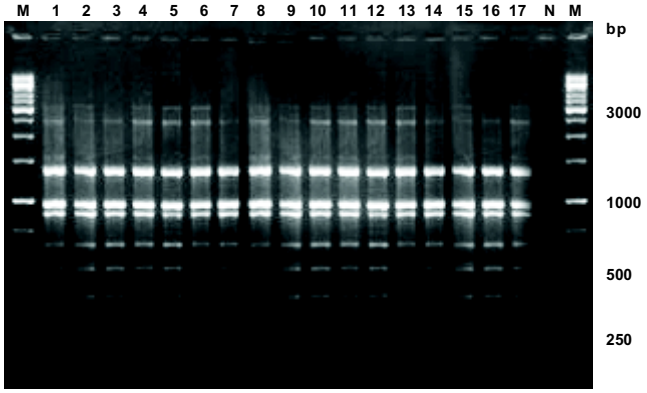

Fig 5. RAPD PCR using OPG 13 primer. Lane M=1kb ladder; Lanes 1-17=PCR amplification of different *P. multocida* isolates; Lane N=Negative control

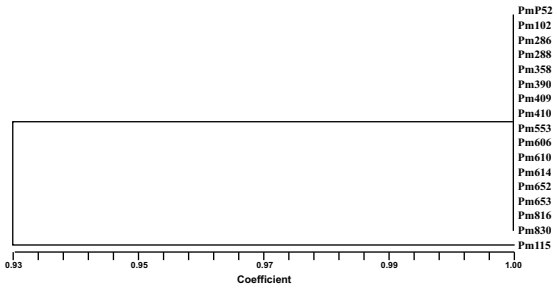

Fig 5 a. RAPD dendrogram analysis with OPG 13 primer

analysis and reported nine different groups with identical RAPD profiles (100% similarity) and significant relationship between phenotypes and RAPD profiles. Further studies of *P. multocida* isolates from other species and/ or disease conditions may provide a clear picture of the genetic diversity among the isolates of *P. multocida* and would also be helpful to find out the chances of host specific lineages of this organism.

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