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Identification of Animal Pasteurellosis by PCR Assay

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ABSTRACT:

Diagnosis of pasteurellosis has become difficult, as there are five different capsular types and 16 somatic types. Molecular techniques like PCR are adapted nowadays for rapid and accurate diagnosis in early stage of the disease and also it provides useful information for epidemiological studies. The present study was conducted to study the efficiency of polymerase chain reaction (PCR) in the identification of *P. multocida* isolates and evaluation of different PCR methods viz., (i) PCR using genomic DNA (ii) PCR using culture lysate and (iii) PCR by colony touch method. In the present study *P. multocida* specific PCR was performed by using KMT1SP6 and KMT1T7 oligos. These oligos amplified the genomic DNA from *P. multocida* isolates only. All the three methods produced PCR amplified product at 460 bp and colony touch method was found to be the best method.

Keywords:
Culture lysate, genomic DNA, *Pasteurella multocida,* PCR .

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INTRODUCTION

The various forms of pasteurellosis caused by *Pasteurella multocida* are the major health problem for livestock population worldwide. Diagnosis of pasteurellosis has become difficult, as there are five different capsular types and 16 somatic types. Molecular techniques like PCR are adapted nowadays for rapid and accurate diagnosis in early stage of the disease and also it provides useful information for epidemiological studies. Pasteurellosis has high impact on economic status of Indian farmers. The overall incidence rate of haemorrhagic septicaemia (HS) was reported as 6.4 per lakh population during 1974-86, resulting in losses exceeding ten million rupees annually (Dutta *et al.*, 1990; singh *et al.*, 1996).

Isolation and identification of *P. multocida* from specimens like fresh tissues or heart blood followed by the performance of various biochemical and serological methods have been used to study *P. multocida.* These include catalase, indole, oxidase and sugar fermentation tests. Due to time consuming procedure and limitations of these methods, molecular techniques like polymerase chain reaction (PCR) were adapted nowadays. PCR has advantages over the conventional techniques in rapidity, sensitivity and specificity to identify the *P. multocida*. The present study was conducted to assess the efficiency of PCR in the identification of *P. multocida* from poultry and ruminants and to evaluate the different methods in PCR assay viz. PCR using genomic DNA, PCR using culture lysate and PCR by colony touch method.

MATERIALS AND METHODS

Isolation and Identification of *P. multocida*

Fifty two samples were collected from various geographical areas of Tamil Nadu, India. Specimens such as heart blood swab, liver, spleen and long bones collected from various animals, were streaked directly onto 5% sheep blood agar and *Pasteurella multocida* selective agar as reported earlier (Moore *et al.*, 1974) and incubated at 37°C with 5-10 % CO_2 for 24-48 h. Plates were examined for colonies, the suspected colonies were subjected to grams staining, and biochemical test as per standard techniques. Standard vaccine strain of *P. multocida* P_{52} (B:2) was taken as reference strain. Pathogenicity test in mice were carried out for all the fifteen isolates and PCR was performed for all the isolates.

Isolation and Purification of Genomic DNA

A 900 µl cell suspension of each sample were resuspended in 100 µl of 10x Tris-EDTA (TE) buffer (pH 8.3) with 10 mg of lysozyme and were incubated at 37°C for 1.5 h. Bacterial cultures were treated with 10 µl of proteinase K (10 mg/ml) and incubated at 50°C for 1 h. The nucleic acid was extracted with phenol-chloroform-isoamyl alcohol followed by ethanol precipitation as per the method of Sambrook *et al.*, (1989) and Sachithanandam *et al*., (2011).

PCR Using Culture Lysate

One Milliliter of 18 h broth culture or take few freshly grown pure colonies from blood agar plate and suspend in 500 µl sterile distilled water and centrifuge at 4000 g for 1 minute and collect the pellet. The pellet was washed with sterile distilled water, resuspended in 100 µl sterile distilled water and boiled for 10 min. The samples were centrifuged to sediment cell debris and 10 µl of the supernatant was used in the PCR reaction.

PCR Using Colony Touch Method

A single pure colony grown on agar plates was used to perform PCR. A pipette tip was lightly touched onto a colony and then suspend in PCR amplification mixture.

PCR Technique

The species-specific primers KMT1SP6 and KMT1T7 designed by Townsend *et al.*, (1998) were used in this study to amplify the gene sequences in *P. multocida.*

Primers 1 KMT1SP6 5'-GCT GTA AAC GAA CTC GCC AC- 3'

Primers 2 KMT1T7 5'- ATC CGC TAT TTA CCC AGT GG-3'

PCR mixture was prepared using PCR kit obtained from FINNZYME, Finland. The 50 µl of reaction mixture was prepared with 10 µl template DNA, 10ng of each primers, 200 µM concentration of each dNTPs, 10x PCR buffer and 1 unit Taq DNA polymerase. PCR amplification was carried out in an automated thermal cycler (Perkin Elmer Gene AMP PCR system 2400) with the following thermal programme. Initial denaturising at 95°C for 4 min followed by 30 cycles of denaturising at 95°C for 1 min., annealing at 55°C for 1 min., extension at 72°C for 1 min. and final extension at 72°C for 9 min, were carried out. After amplification, PCR products were checked in 1.5% agarose gel electrophoresis along with the standard molecular weight marker (Lambda DNA Hind III digest and ϕ X 174 DNA Hae III digest; FINNZYME, Finland).

The biochemical tests were carried out as per the standard procedure followed in Arun kumar *et al*., (2012)

RESULTS

Out of total collection of 52 suspected samples, procured from cattle sheep, goat and poultry, 15 samples were confirmed as *P. multocida* based on biochemical tests (Table 1) and PCR. All the *P. multocida* isolates were pathogenic to mice and dies within 24 h. PCR was performed for all the 15 isolates by 3 methods viz., colony touch method, culture lysate and with genomic DNA. P_{52} strain of *P. multocida*, obtained from the Institute of veterinary preventive medicine (IVPM) Ranipet, Tamil Nadu, taken as a positive control and the following bacteria *Escherichia coli*, *Clostridium chauvoei*, *Salmonella enteritidis, Salmonella typhimurium*, *Bacillus anthracis,*

Table 1. Biochemical Profiles for the Identification of *Pasteurella multocida* Isolates

Tests	Name of the Isolates														
	D_1P	D_2P	FP	GP	HP	KP	LP	NP	OP	AS	CS	TS	YS	BG	MC
Hemolysis on Blood agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on MacConkey agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin Liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl Red Test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S (Hydrogen sulphide)	+	+	-	-	+	+	+	-	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysine Decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine Decarboxylase	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyrase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin Hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VT (Voges Proskaeur	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Galactosidase (ONPG)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Glucuronidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-Xylosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl β-D-glucosaminedase	-	-	+	+	+	+	-	+	-	-	+	+	+	+	+

+ : Positive, - : Negative,

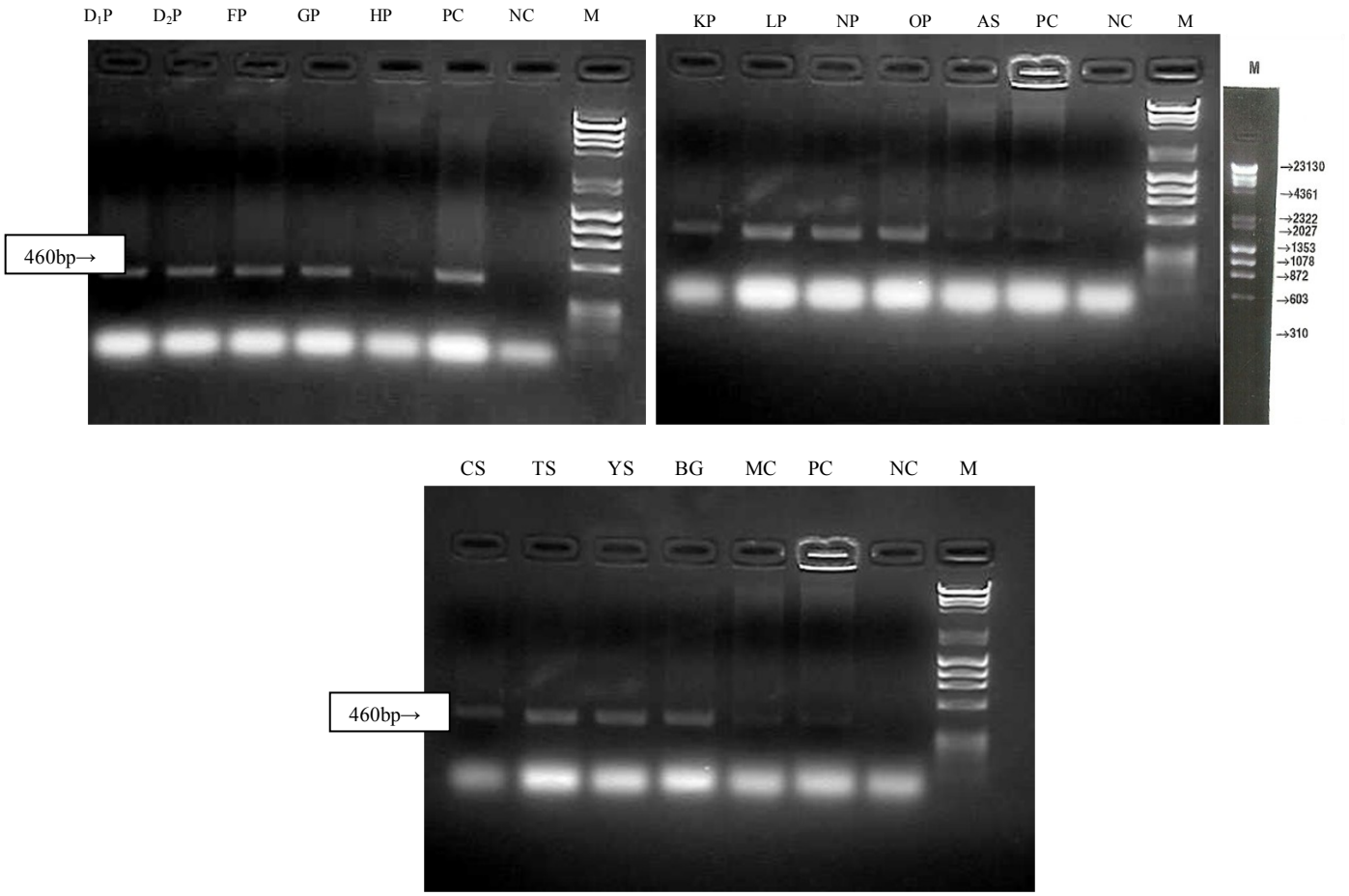

Figure 1: *Pasteurella multocida* – specific PCR (PM-PCR) assay

These figures illustrate fragments specifically amplified by PCR in all the *P. multocida* isolates by means of the primers KMT1SP6 and KMT1T7. Variation in the intensity of the amplified product was observed, due to variation in DNA concentration of each sample.
D_1P, D_2P, FP, GP, HP, KP, LP, NP, OP, AS, CS, TS, YS, BG, and MC are the names of *P. multocida* isolates.

Staphylococcus aureus and *Klebsiella* spp. used as negative controls. The expected amplification size of 460 bp was obtained in all the 15 isolates. PCR amplification was noticed at approximately 460 bp by all the three methods and in all the 15 isolates as like that of positive control (figure 1). No amplification product was observed in negative controls (figure 1). Molecular weight of PCR product was estimated based on the standard molecular weight marker.

DISCUSSION

The 15 isolates of *P.multocida* collected from different places and sources of origin produced approximately 460 bp amplified product as that of reference strain P_{52}, but no amplified product was noticed among the negative controls. It is concluded that the primers were highly specific to *P. multocida* isolated from various sources. The above result agrees with the previous reports of earlier workers (Townsend *et al*., 1998; Hunt *et al*., 2000; Miflin and Blackall, 2000; OIE manual, 2000; Dutta *et al*., 2001). In this study the amplified product of approximately 460 bp was observed using three different methods viz. colony touch method, culture lysate method and purified genomic DNA method (figure 1). The intensity of the amplified PCR product varies (figure 1), due to the variation in DNA concentrations. Townsend *et al*., (1998) reported that PCR using colony touch method produced amplification

product approximately at 460 bp and the intensity of the amplified product varied due to inconsistency of the DNA concentration. Dabo *et al*., (2000) reported that the boiled cell extract method has the advantages of simplicity and rapidity in the identification of *P. multocida* isolates. Since the PCR amplified product of 460 bp was noticed in all samples of poultry and ruminants, using oligos KMT1SP6 and KMT1T7, the oligos are considered as specific to *P. multocida* affecting all species of poultry and ruminants. Considering the cost and time involved in the preparation and purification of genomic DNA, the colony touch method has advantages of simplicity and rapidity for epidemiological surveys involving large number of *P. multocida* isolates. PCR using colony touch method would be an adaptable easy to perform method in regional laboratories for rapid diagnosis of HS and FC from field cases without the need to obtain pure culture and extensive biochemical and serological tests.

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