DETECTION AND SEROTYPING OF *PASTEURELLA MULTOCIDA* BY POLYMERASE CHAIN REACTION

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Pasteurella multocida affects and causes heavy economic losses in a wide spectrum of hosts, including domestic ruminants like sheep and goat, besides cattle and buffaloes.

Although *Pasteurella multocida* are demonstrated *in situ* by direct microscopy, isolation of the organism is difficult, both from clinically infected and from apparently healthy animals. In case of live animals, the choice of clinical material for isolation is limited, when compared to dead animals. Moreover, contaminant flora often overgrows specific pathogens when samples are taken from a contaminated site of the animal such as nose or throat, making the isolation trials more difficult.

Limitations faced in the isolation and identification of bacteria can be overcome by employing nucleic acid based assays, which serve as alternate methods for bacterial identification. Polymerase chain reaction (PCR) has been practically useful in this regard, with the use of primer sequences designed to facilitate identification at any level of specificity, strain or species. Polymerase chain reaction based diagnosis developed by Townsend *et al.* (1998) was found to be very effective and sensitive, compared to conventional diagnostic systems.

In the present study, *P. multocida* species specific PCR (PM-PCR) and type-B specific PCR (HS-B PCR) were employed to detect the organism.

Materials and Methods

Seventy-seven samples (forty-nine blood samples and twenty eight blood smears) collected from animals suspected for haemorrhagic septicaemia (HS) in Thrissur and Palakkad districts and brought to the College of Veterinary and Animal Sciences, Mannuthy for disease diagnosis formed the biomaterials for this study. Blood samples were streaked onto blood agar plates and incubated at 37°C for 24 h in a candle jar, for isolation of *P. multocida*. Five isolates were obtained from forty-nine samples cultured for isolation and were designated as BP1 and BP2 from cattle, BuP1 and BuP2 from buffalo and GP1 from goat.

Polymerase chain reaction based detection of *P. multocida* was also attempted from these samples. The PM-PCR was used for initial detection of *P. multocida* from the samples. Samples positive by PM-PCR were subjected to HS-B PCR using HS-B specific primers and multiplex PCR using two sets (PM-Specific and HS-B Specific) of primers. The reference strain of *P. multocida* (P-52) obtained from IVRI, Izatnagar and two *P. multocida* serotype A isolates from ducks (DP1 & DP2) maintained in this laboratory were used as reference strains for comparison.

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Preparation of samples for PCR analysis

a) Blood samples and blood smears

Blood samples and blood smears obtained from suspected cases were processed to prepare DNA. Two hundred microlitres of blood was taken in Eppendorf tubes. Sterile triple distilled water was added to make up the volume to 1.5 ml and centrifuged at 3000 x g for 15 min. The supernatant was discarded and cell pellet was washed twice with sterile PBS (pH 7.4) and then resuspended in 100 ml sterile triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The samples were then thawed and again centrifuged at 3000 X g for 10 min and supernatant was stored at -20°C for further use as template for PCR reactions.

Blood smears were scraped with a blade into an Eppendorf tube. To this tube 1.5 ml of sterile triple distilled water was added and the mixture was kept at 37°C for 30 min. It was then processed as above and the final pellet was resuspended in 50 ml sterile triple distilled water. The mixture was boiled for 10 min and after chilling, thawing and centrifugation as above, the supernatant was stored at -20°C.

b) Reference strains and isolates of *P. multocida*

In case of reference strains and five isolates of *P. multocida,* the organisms were grown overnight in blood agar plates at 37°C, in a candle jar and a pure colony was inoculated into five millilitres of BHI broth and incubated at 37°C for 18 h. From this broth culture 1.5 ml was transferred to an Eppendorf tube and was processed as for blood samples, except that the samples after thawing was centrifuged for five min only at 3000 X g. The supernatant obtained was stored at -20°C for further use as template for PCR reactions.

The *Pasteurella multocida* specific PCR reaction was carried out with the PM-PCR primer pair, KMT1T7 and KMT1SP6 and Type B specific PCR reaction with HS-B specific primer pair, KTSP61 and KTT72 developed by Townsend *et al.* (1998). For multiplex PCR both the primer sets were used in the reaction mixture. Boiled lysates prepared from blood samples, blood smears, isolates and reference strains (P-52, DP1and DP2) were used as template DNA for PCR reaction. The PCR was performed in a 25ml reaction mixture containing 20 picomoles of each primer, 200 mM of each dNTP, 1X PCR buffer containing 1.5mM $MgCl_2$ and IU *Taq* DNA polymerase. The programme of amplification for PM-PCR and HS-B PCR were as per the methods described by Townsend *et al.* (1998). For multiplex PCR, the programme of amplification same as that for HS-B PCR was used.

Products from the PCR assays were detected by electrophoresis in 1.5 per cent agarose gel in TBE buffer (IX) and 0.5 mg/ml of ethidium bromide. The gel was visualized and photographed under UV transillumination.

Results and Discussion

Out of the forty-nine blood samples and twenty-eight blood smears tested, only eight blood samples (including the five isolates obtained) and five blood smears were found to be positive by PM-PCR (Table). Presence of *P. multocida* DNA in sample was observed by amplification of a 460 bp fragment (Figs. 1, 2). Blood samples, which were found to be negative by isolation trials, could give positive amplification in PM-PCR. Thus, PCR was found to be more sensitive, when compared to isolation and identification, in the detection of *P. multocida* from clinical samples. Townsend *et al.* (1998) reported PCR as a rapid and sensitive method for identification of *P. multocida.* The results of present study are in agreement with the above finding.

The entire samples tested positive by PM-PCR were confirmed as type-B *P. multocida* by HS-B PCR, which gave an amplified product of size 590 bp (Figs. 3, 4). The reference strain P-52 used for comparison also gave amplified products of 460 bp and 590 bp in PM-PCR and HS-B PCR, respectively.

The present study indicated that the PM-PCR along with HS-B PCR could be helpful for the rapid and confirmative diagnosis of HS where disease remains endemic. These results are in accordance with those of (Townsend *et al.*, 1998; Towns end *et al.,* 2000; Shivshankara *et al.*, 2001; and Dutta *et al.*, 2004). Performing PCR on template DNA prepared from blood smears greatly reduces the time required for a specific diagnosis. *Pasteurella multocida* specific PCR (PM-PCR) and HS-B PCR assay using

template from blood smears gave clear-cut results, although the band intensity was less compared to those achieved by the use of boiled culture lysates prepared from the isolates and reference strain P-52 as template DNA (Figs. 2, 4). This technique was more useful in many cases where blood smears were the only material sent by field veterinarians to the Department of Microbiology. Moreover, the presence of artifacts resembling bipolar organisms frequently affected the accuracy of diagnosis based on microscopic examination of stained blood smears. Thus, PCR assay conducted on template DNA prepared from blood smears represents a novel and practical means of detection of animal pasteurellosis.

Multiplex PCR of the PM-PCR and HS-B PCR positive samples revealed two bands corresponding to 460 bp and 590 bp, respectively. Serotype-A isolates (duck isolates-DP1 & DP2) of *P. multocida,* which were used for comparison in multiplex PCR gave only single band corresponding to 460 bp (Figs. 5, 6). Only serotype B *P. multocida* generated two bands in multiplex PCR. These results are in accordance with those of Townsend *et al.* (2000) and Dutta *et al.* (2004).

From the present study it can be inferred that multiplex PCR using both the sets of primers could clearly differentiate between serotype B and serotype A isolates and that this could provide an alternative method to conventional capsular serotyping which is time consuming and laborious and can be carried out only in highly equipped laboratories. Moreover, this PCR assay could be helpful for rapid and confirmative diagnosis of HS in conditions where the disease is endemic.

Table. Results of clinical samples tested by polymerase chain reaction

Sample	Polymerase chain reaction				
	Number tested	Number positive			Per cent positive
		By isolation and PCR	PCR only	total	
Blood samples	49	5	3	8	16.32
Blood smears	28	5			17.86
Total number of samples	77	13			16.88

Summary

A total of forty-nine blood samples and twenty-eight blood smears received for diagnosis of haemorrhagic septicaemia (HS) were processed for detection of *Pasteurella multocida* DNA by polymerase chain reaction (PCR), using species specific (PM-PCR) and type-B specific (HS-B PCR) primers. Culturing of the forty-nine blood samples yielded only five isolates of *P. multocida*. Thirteen samples (eight blood samples and five blood smears) found to be positive by PM-PCR, generated an amplified product of 460 bp. The entire samples tested positive by PM-PCR were confirmed as type-B *P. multocida* by HS-B specific PCR, which generated an amplified product of 590 bp. Multiplex PCR of these samples with two sets of primers gave two bands of size 460 bp and 590 bp, respectively. Five isolates obtained could be confirmed as serotype-B *P. multocida* using multiplex PCR and HS-B PCR.

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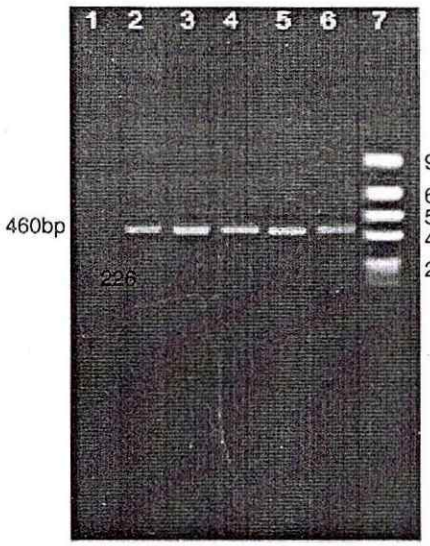

Fig. 1. PM- PCR of *Pasteurella multocida* isolates

Lane 1 Negative control
Lane 2 BP1
Lane 3 BP2
Lane 4 BuP 1
Lane 5 GP1
Lane 6 P52
Lane 7 pBR 322 DNA *Alu 1* / Digest

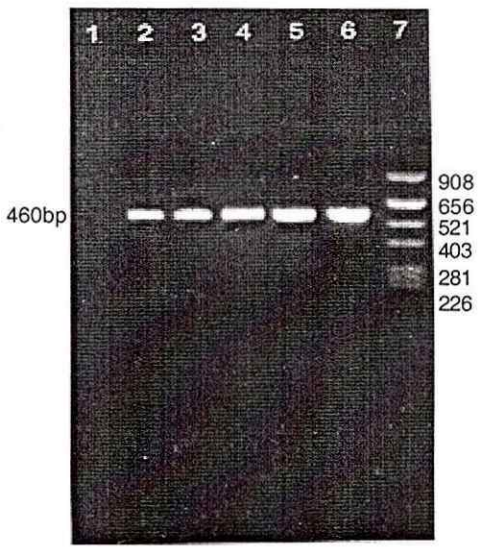

Fig. 2. Detection of *Pasteurella multocida* by PM - PCR of blood samples and blood smears

Lane 1 Negative control
Lane 2 to 4 Blood samples
Lane 5 to 6 Blood smears
Lane 7 pBR 322 DNA *Alu 1* / Digest

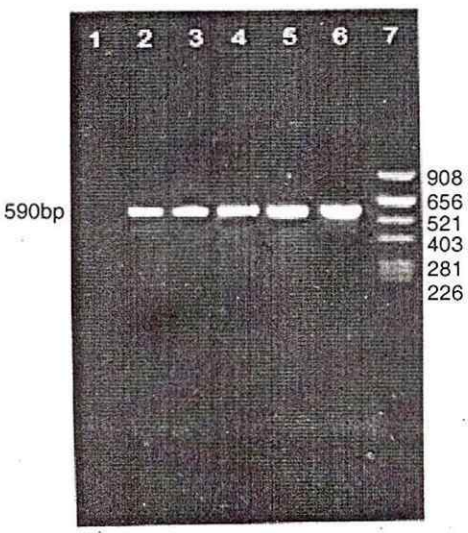

Fig. 3. HS - B PCR of *Pasteurella multocida* Isolates

Lane 1Negative control
Lane 2 BP1
Lane 3 BP2
Lane 4 BuP1
Lane 5 BuP2
Lane 6 p52
Lane 7 pBR 322 DNA *Alu 1* / Digest

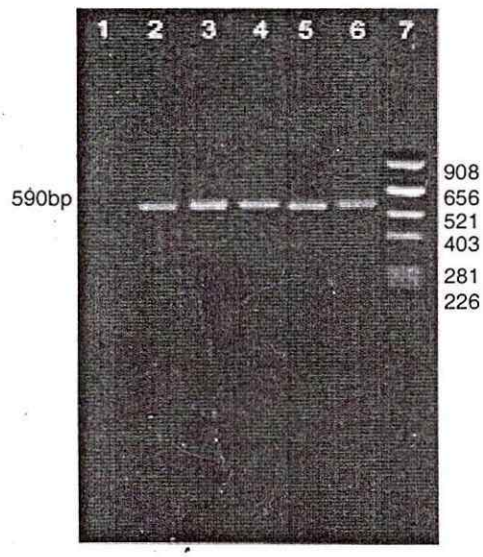

Fig. 4. Detection of *Pasteurella multocida* by HSB-PCR of blood samples and blood smears

Lane 1 Negative control
Lane 2 to 3 Blood samples
Lane 4 to 6 Blood smears
Lane 7 pBR 322 DNA *Alu 1* / Digest

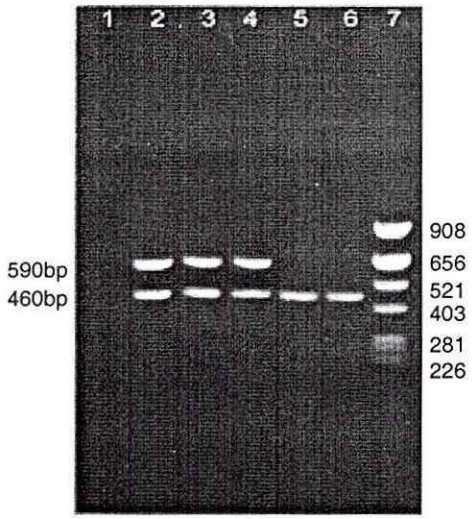

Fig. 5. Multiplex PCR of *Pasteurella multocida* isolates

Lane 1 Negative control
Lane 2 to 4 Serotype B isolates of Pasterurella multocida (BP1, P52 and GP1)
Lane 5 to 6 Serotype A isolates of Pasterurella multocida (DP1 and DP2)
Lane 7 pBR 322 DNA AluI/Digest

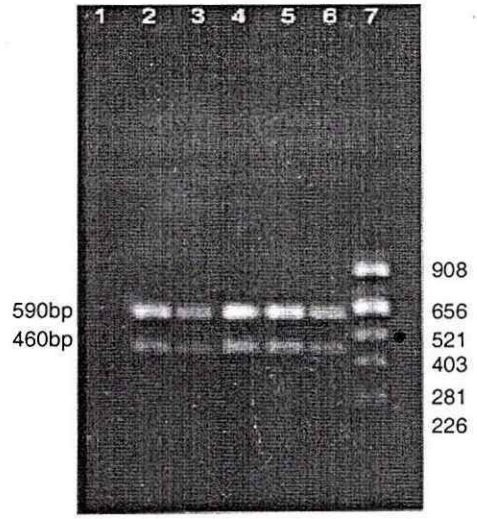

Fig. 6. Detection of *Pasteurella multocida by Multiplex* PCR of blood samples and blood smear

Lane 1 Negative control
Lane 5 to 6 Blood smears
Lane 2 to 4 Blood samples
Lane 7 pBR 322 DNA *Alu 1* / Digest

References

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