RESEARCH ARTICLE

Rapid Detection of *Pasteurella multocida* Infection by Direct Blood and Tissue Polymerase Chain Reaction in Animals and Poultry

Bharat B. Bhanderi*, Sneha J. Mistry, Ashish Roy

Abstract

The aim of the present study was rapid detection of *Pasteurella multocida* infection by direct blood and tissue PCR technique. A total 151 samples comprising of 112 blood samples, 37 tissues samples, one nasal swab, and one bone marrow from various ruminants and poultry were processed by cultural methods and confirmed by PM-PCR by amplifying a product of 460bp. Among them four *Pasteurella multocida* were obtained from tissue samples of buffaloes and two from poultry and, one from blood sample of cow. The clinical samples were processed for direct blood PCR (n=112 blood, 37 tissue) and direct tissue PCR (n=151) methods. Out of 112 blood samples processed, one blood sample of cow positive for *P. multocida* by cultural method was also found to be positive by direct blood PCR. Out of 151 samples processed for direct tissue PCR, six tissue samples positive for *P. multocida* by cultural methods were also found to be positive by direct tissue PCR. Conventional cultural method as well as PM-PCR for identification of *P. multocida* infection in animals and poultry requires minimum 24-48 hrs for identification, while direct blood and tissue PCR gives specific diagnosis within 3 hrs once the sample is received at laboratory. Thus direct application of PCR on blood and tissues will enable to give rapid diagnosis of *P. multocida* infection during the outbreaks in animals and poultry.

Keywords: *P. multocida,* Direct blood PCR, Tissue PCR, Diagnosis, Ruminants, Poultry.

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Introduction

Pasteurella multocida is a heterogeneous species of gram-negative bacteria and it has been recognized as an important veterinary pathogen for over a century. It causes different forms of disease in variety of animals and poultry resulting in heavy economic losses (Singh *et al.,* 2014). Direct blood PCR can reliably be used for rapid detection of *Theileria annulata* in conjunction with microscopic examination (Ganguly *et al.,* 2015). Laboratory diagnosis of *P. multocida* infection is based mainly on blood smear and tissue impression examination and/or cultural isolation and biochemical method of identification followed by PCR from culture sample. This procedure has its own limitation. The cultural isolation followed by PCR is a time consuming procedure and requires minimum 24-48 hrs. Direct application of PCR on blood and tissues will enable to give rapid diagnosis of *P. multocida* infection during the outbreaks in animals and poultry. Thus the aim of present study was to evaluate the rapid detection of *P. multocida* infection by direct blood and tissue PCR technique.

Materials and Methods

Sample Collection

Total 151 samples comprising of 112 blood samples, 37 tissues samples, one nasal swab, and one bone marrow from various animals, *viz.,* buffaloes (92) and cattle (29) suspected of haemorrhagic septicaemia, poultry (4) suspected of fowl cholera, and camel (23) and goat (3) suspected of pasteurellosis received at Department of Veterinary Microbiology were processed for cultural isolation of *P. multocida by* direct blood PCR and direct tissue PCR methods, P_{52} vaccine strain culture comprising of *P. multocida* was received from the Vaccine Institute, Gandhinagar, Gujarat for positive control to standardize the technique.

DNA Preparations

Bacterial DNA for PCR was extracted by boiling procedure. Isolates were cultured overnight in BHI broth at 37°C and 2 ml

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of the culture suspension was centrifuged and the pellet was suspended in 200 µl boiled doubled distilled water (ddH_2O) which was again boiled for 10 min in a water bath and then stored at -20°C for 10 min. After thawing, the suspension was once again centrifuged at 8,000 rpm for 10 minutes and 200 µl of the supernatant was taken as template DNA. The concentration of DNA was measured spectrophotometrically (Nanodrop 1000, Thermo scientific, USA) at 260 nm and the same DNA template was used to carry out PM-PCR.

P. multocida Specific PCR (PM-PCR)

P. multocida strains were subjected to PM-PCR to detect the species specific KMT gene (Townsend *et al.,* 1998). PCR based identification of *P. multocida* was done using species specific primers, forward primers: KMT1SP6 5'- GCT GTA AAC GAA CTC GCC AC - 3' and reverse primers KMT1T7 5'- ATC CGC TAT TTA CCC AGT GG – 3' as per Townsend *et al.* (1998). For PCR reactions 3 µl (~30 ng/µl) template DNA was added to the reaction mixture (22 µl) containing 1 µl of each primer pair (MWG, Biotech, Germany) in a 10 pmol primary concentration, 12.5 µl of 2x PCR Master Mix (Fermentas, USA) and 7.5 µl of molecular grade nuclease free water. The reaction mixture was subjected to amplification in a thermal cycler (Veriti, Applied Biosystem, USA) according to the following program: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension of 72°C for 6 min.

Direct Blood PCR

Direct blood PCR was performed using Phusion Blood Direct PCR Kit developed by FINNZYMES (Thermo Scientific). The composition of PCR mixture and PCR condition used are given in Tables 1.

Direct Tissue PCR

KAPA Express Extract (KK7100, KAPPA Biosystems, Massachusetts, United States) kit was used for extraction of DNA from blood, tissues, bone marrow and nasal swab as per the manufacturer's protocol (Table 2). KAPA2G Robust Hot Start Ready Mix PCR kit (KAPA2G Robust Hot Start Ready Mix PCR Kit KR0381 – v5.14, Boston, Kappa Bio systems, Massachusetts, United States) was used for PCR amplification. The composition of PCR mixtures used for two different methods (A&B) and PCR conditions are given in Table 3. In method 'B' 3 µl/reaction of forward and reverse primer and 5.50 µl DNAse free water/reaction was taken in comparison to method 'A'. Amplification products were analysed by gel electrophoresis on a 2.0 % agarose gel stained with ethidium bromide, and photographed at UV exposure (Syngene, Germany).

Results and Discussion

Cultural Isolation and Conventional PM-PCR

Out of 151 samples processed, seven *P. multocida* isolates were obtained by cultural methods, which comprised of four tissue samples of buffalo, two tissue samples of poultry and one blood sample of cow. All the seven isolates were confirmed by conventional PM-PCR (Figure 1). The isolated bacterial colonies on blood agar plates were small, glistening, mucoid and dew drop like, and appeared as Gram-negative coccobacilli when stained with Gram's stain. The isolates failed to grow on MacConkey agar, were found to be non-haemolytic on blood agar and positive for catalase, oxidase and indole tests. Similar cultural and biochemical results were reported by Bhimani *et al.* (2014).

Table 1: Composition, steps and condition for Direct Blood PCR

Components		*Quantity*	*Final concentration*
2x Blood PCR Buffer		10.00 µl	1x
DNA Polymerase Enzyme		0.40 µl	--
Primers	Forward	1.00 µl	10 pmole
	Reverse	1.00 µl	10 pmole
Dnase free water		6.60 µl	--
Suspected Whole Blood Sample		1.00 µl	--
Total		20.0 µl	--
Steps and conditions			
Cycle step		*Temp.*	*Time*	*Cycles*
Initial denaturation (Lysis of cells)		98°C	5 min	1
Denaturation		98°C	1 s	40
Annealing		55°C	10 s	
Extension		72°C	20 s	
Final Extension		72°C	1 min	1
Hold		4°C	hold	-

Table 2: Extraction of DNA for Tissue PCR

Step 1 – Reaction setup	
10X KAPA Express Extract Buffer	10 µl
1 U/µl KAPA Express Extract Enzyme	2.0 µl (2 U)
DNase free water	88 µl in tissue sample or 86 µl in blood sample
Sample	Blood - 2 µl or Tissue – 2 mm^3
Step 2 – Lysis in PCR	
Incubated in a thermo cycler	75°C for 30 min
Step 3 - Heat-inactivation in PCR	
Incubated for to inactivate the thermo stable KAPA Express Extract protease	95°C for 5 min
Step 4 - Sample recovery and use	
Reaction was vortex for 5 sec and Centrifuged at high speed for 1 min to settle pellet debris. DNA-containing supernatant was transferred to a fresh tube and 1 µl of DNA extracted was used directly in a 25µl PCR reaction. DNA was diluted in TE Buffer for long-term storage at -20°C.	

Table 3: Composition of PCR reaction mixture and steps of direct Tissue PCR

Components	*Quantity*	*Final concentration*	
2X KAPA2G Robust HotStartReadyMix	12.50 µl	1x	
Reverse primer	1.25 µl	15 pmole	
Reverse primer	1.25 µl	15 pmole	
Dnase free water	9.00 µl	--	
Template DNA	1.00 µl	--	
Total	25.00 µl	--	
Steps and conditions			
Cycle step	*Temp.*	*Time*	*Cycles*
Initial denaturation	95°C	3 min	1
Denaturation	95°C	10 s	40
Annealing	60°C	15 s	
Extension	72°C	15 s	
Final Extension	72°C	10 min	1
Hold	4°C	hold	-

Direct Blood PCR and Tissue PCR

Out of 112 blood samples processed, one blood sample of cow positive for *P. multocida* by cultural methods was also found to be positive by direct blood PCR (Figure 2), while rest of 111 blood samples negative by conventional cultural and PM-PCR methods were also found to be negative by direct blood PCR. Total 151 samples comprising of 112 blood samples, 37 tissues samples, one nasal swab, and one bone marrow were processed for direct tissue PCR. Among the samples processed, four tissue samples of buffalo, two tissue samples of poultry and one blood sample of cow positive for *P. multocida* by cultural methods were also found to be positive by direct tissue PCR by methods A and B by amplifying a product of 460bp (Figure 3), while rest of 144 samples negative by conventional cultural isolation and Conventional PM-PCR methods were also found to be negative by direct tissue PCR.

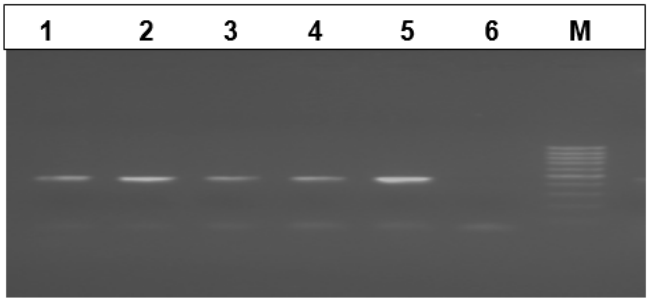

Figure 1: PM-PCR showing 460bp amplified product specific for *P. multocida* in PM-PCR
Lane 1 : Lane 1-5: *P. multocida* isolates
Lane 6 : Negative sample
Lane M: Marker, 100bp DNA ladder

Comparison of Different Methods

All the seven samples found to be positive for *P. multocida* by cultural isolation and PM-PCR method were also found positive

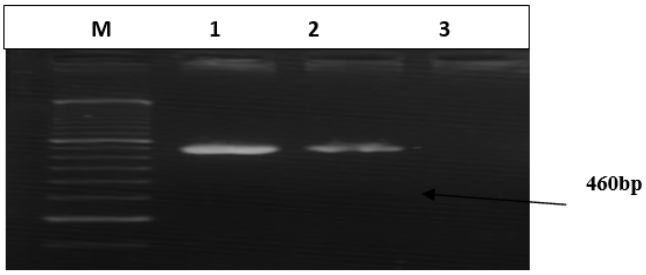

Figure 2: PM-PCR showing 460bp amplified product specific for *P. multocida* from blood sample
Lane 1 : *P. multocida* from blood sample
Lane 2 : P_{52} culture vaccine strain as positive control
Lane 3 : Negative sample
Lane M: Marker, 100bp DNA ladder

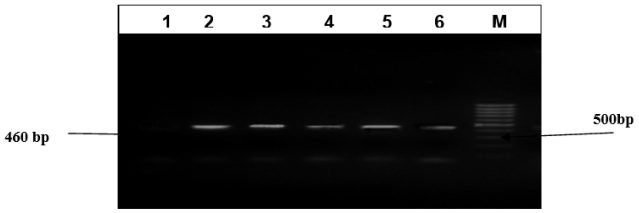

Figure 3: Direct tissue PCR showing 460 bp amplified product specific for *P. multocida* from tissue samples
Lane 1 : Negative samples
Lane 2,3, 4, 5, 6: *P. multocida* tissue samples
Lane M : Marker, 100bp DNA ladder

in tissue PCR technique, while one blood sample found to be positive by culture and PM-PCR method was also found positive in direct blood PCR technique. All the 144 samples found negative for *P. multocida* by cultural isolation method were also found negative in tissue PCR technique and all the 111 blood samples found negative for *P. multocida* by cultural isolation method were also found negative in direct blood technique.

Cultural isolation method as well as Conventional PM-PCR for identification of *P. multocida* from the cases of haemorrhagic septicaemia in cattle and buffaloes, fowl cholera in poultry and pasteurellosis in goat and camel requires minimum 24-48 hrs for identification, while direct blood and tissue PCR gives specific diagnosis within 3 hrs, once the sample is received in laboratory. This method would be extremely useful for rapid diagnosis of important diseases like hemorrhagic septicaemia, pasteurellosis and fowl cholera outbreaks, and infections in animals and poultry where the rapid diagnosis is extremely important.

Direct PCR does not require DNA isolation, is sensitive, quick, cost-effective and produces confident/clear results and identifies all the species of Pasteurella. The literature on direct PCR based detection of *P. multocida* in blood or tissue samples is scarce. However, it is an alternative method for accurate detection of *Plasmodium* microscopic and submicroscopic infections in humans, especially when a large number of samples require screening. Direct PCRs has been used to diagnose infectious diseases (Fuehrer *et al.,* 2011; Silbermayr *et al.,* 2014) and malaria using the 18s-rRNA following a nested PCR approach (Taylor *et al.,* 2011, Echeverry *et al.* 2016).

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