Polymerase chain reaction-based temporal detection of *Pasteurella multocida* B:2 in the blood of experimentally infected mice

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Hemorrhagic septicemia (HS) is a major disease of cattle and buffaloes characterized by an acute, highly fatal septicemia with high morbidity and mortality. Sporadic cases are more difficult to diagnose clinically and hence diagnosis at an early stage is required for control of the disease. The present study was conducted to compare the temporal sensitivity of multiplex PCR and bacterial isolation to diagnose hemorrhagic septicemia due to *Pasteurella multocida* at an early stage i.e. before the appearance of clinical signs in mice. Multiplex-PCR (mPCR) was evaluated for simultaneous as well as temporal detection and identification of *P. multocida* at type level for early and accurate diagnosis of hemorrhagic septicemia (HS). Swiss albino mice were experimentally infected with 10⁰ colony forming units (CFU) of the bacteria *P. Multocida* Type B:2 Strain P52. Heart blood samples were collected, 2, 4, 8, 12 and 24 h post infection for bacterial isolation as well as detection and identification by mPCR. The *P. multocida* was isolated from the heart blood sample collected as early as 8 h post infection while mPCR detected the bacterial DNA as early as 4 h post infection before the appearance of clinical signs hence mPCR for *P. multocida* Type B:2 was found to be more sensitive than the bacterial isolation for diagnosis of HS.

**Keywords**: Hemorrhagic septicemia, PCR, Multiplex PCR, Early diagnosis of HS, *P. multocida* B:2, *P. multocida* B:2 specific PCR, Molecular diagnosis of HS

*Pasteurella multocida* B:2 causes hemorrhagic septicemia (HS) in cattle and buffaloes in India. The disease causes heavy economic losses due to death of diseased animals. Young buffaloes are more susceptible to the disease³. The disease is endemic in India and the outbreaks continue to occur despite of vaccination.

The diseased animals die of endotoxic shock due to bacterial lipopolysaccharides (LPS). The bacteria grow very fast in the blood of diseased animals and are killed. The killed bacteria releases LPS⁵,¹¹. It is general practice to treat the diseased animals with antibiotics during the outbreaks. However, the antibiotics therapy is effective during early stages of the disease and the antibiotic therapy may exacerbate the condition of animals if antibiotic therapy is started at a later stage of the disease. The incubation period of the disease could be very short, few hours only, in per-acute disease condition¹. It is, therefore, important to diagnose the disease at an early stage so that antibiotic therapy could be started at an early stage of the disease and life of the animal is saved⁹.

Conventionally the disease is diagnosed on the basis of clinical symptoms and confirmed by bacterial isolation and identification¹⁶. Bacterial isolation is a time-consuming process and precious time of immediate start of therapy is lost, resulting in death of the animal. Presently, polymerase chain reaction (PCR) protocols are available for direct detection of the bacterial DNA in the blood of infected animals⁸,¹⁶. The PCR protocols are available for detection of *P. multocida*, *P. multocida* type B, and *P. multocida* B:2²,¹⁵.

PCR is reported to be a highly sensitive molecular method of diagnosis¹³. However, the scientific literature is not available on the capacity of the test as a tool for early diagnosis of the disease, before the onset of clinical symptoms and comparative performance of the PCR protocols described for *P. multocida*, *P. multocida* type B, and *P. multocida* B:2.

In the study, all three protocols of PCR were evaluated in a multiplex format for their use as a diagnostic tool for early diagnosis of HS. Since, Swiss Albino mouse is laboratory model for HS of cattle and buffaloes thus for developing a methodology for mPCR based early diagnosis of HS, all three type of PCR protocols (*P. multocida*, *P. multocida* B and *P. multocida* B:2) were performed simultaneously on blood samples of mice experimentally infected with *P. multocida* B:2 at various time post infection.

**Material and Methods**

**Bacterial Isolate**

Vaccine strain of *P. multocida* i.e. *P. multocida* B:2 strain P⁵² was used for inoculation in mice. The purity
HAZARI et al.: PCR BASED TEMPORAL DETECTION OF P. MULTOCIDA B:2

Experimental infection of mice and bacterial isolation from heart blood of infected mice

Approximately 5 mL of brain heart infusion broth was inoculated with a single colony P. multocida B:2 grown on blood agar. The number of viable bacteria was determined by serial dilution plating method. Serial tenfold dilution of stock of bacteria was prepared in brain heart infusion (BHI) broth (10^{-1} to 10^{-12}). A volume of 50 µL of a dilution was dropped and then spread on one 35 mm 5% sheep blood agar plate. This way, three plates were seeded for each dilution. Average numbers of colonies were counted on the blood agar plate of a dilution where the colonies were countable in number and were approximately 30-40 in number. A total of ten male Swiss albino mice of 8 weeks of age (Animal experimentation IAEC/88-108 dated 19/04/2014) were infected by inoculating 100 cfu (colony forming unit) of the bacteria. The stock of bacteria was suitably diluted to contain 100 cfu in 0.5 mL of the BHI broth. Each mouse received 0.5 mL of the broth subcutaneously. The heart blood samples were collected at 2, 4, 8, 12 and 24 h post-infection and subjected for both PCR analysis and isolation on 5% sheep blood agar plate. Two mice were used for each collection.

Multiplex polymerase chain reaction (mPCR)

P. multocida, serogroups B and type B:2 specific mPCR was performed on blood samples without isolation of DNA for detection and identification. The set of primer and reaction mixture used for mPCR are listed in Tables 1 & 2.

The PCR was carried out in a thermocycler (Veriti, Invitrogen) with a total reaction volume of 25 µL using KAPA blood PCR kit (KK7003). Reaction components and concentrations were mentioned in the (Table 2). Since the very little volume of blood collected from mice thus we have selected direct blood PCR kit for amplification of the gene of interest. Using direct blood PCR kit there is no processing required for template preparation, blood collected in EDTA containing vial is directly applicable for PCR reaction as template. Blood from negative control mouse was also included in every run as a negative control. Amplification was performed with 35 cycles following an initial denaturation step at 95°C for 5 min. Each cycle involved denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 5 min.

Results and Discussion

Experimental infection of mice and bacterial isolation from heart blood of infected mice

The viable bacterial count was 27 × 10^{-12} cfu/mL in the stock of the bacteria in BHI broth. There was no apparent symptom of the disease recorded in the mice sacrificed up to 12 h post infection. The mice were not noticed moribund or dull. However, there was some indication of huddling together at 8 h post-infection onwards and the mice were found dead at the time of 24 h post infection.

The bacterial colony was successfully isolated from the heart blood of the mice sacrificed at 8, 12 and 24 h post infection (Fig. 1). An average number of colonies was 20 at 8 h sample and 45 colonies at 12 h sample. The number of bacterial colonies was countable at 8 and 12 h post infection heart blood samples but the bacterial growth was very heavy on the plates.
The P. Multocida specific mPCR was performed on direct heart blood samples and showed positive amplification in the samples collected at 4, 8 and 12 h post infection (Fig. 2) but the lane for samples collected at 24 h post-infection showed only smearing however after 10 fold dilution same was found positive (Fig. 2, Lane 8-10) for targeted DNA. The smearing in the lane having samples of 24 h post infection might be due to the high amount of bacterial DNA as a template. The high amount of template DNA is one of the reasons for smearing in agarose gel electrophoresis after DNA amplification in PCR.7,12. Faint band in the lane for a sample of 4 h post infection indicated poor amplification might be due to the low concentration of template DNA because of the low level of bacteremia. The band intensity of P. multocida B:2 specific amplicons was very high as compared to other two bands, which revealed the high amplification efficiency of B:2 specific primers and hence comparatively more sensitive than other two primers of the multiplex. In multiplex PCR, shorter size of amplicons is generally preferred over larger sized amplicons during amplification. On the basis of mPCR result, the P. multocida appeared in the heart blood as early as 4 h post infection. However, the amount of bacteria was low. Therefore, it could not be isolated from heart blood of mice sacrificed 4 h post infection.

Accurate early diagnosis of HS may permit treatment of diseased animals successfully with appropriate antibiotics. Molecular diagnosis with PCR is generally used to confirm and type the isolated P. multocida4,6. Though, it is, described that PCR can now be performed on infected blood10, however, perusal of scientific literature could not find any report on PCR performed directly on infected blood collected from diseased animals in HS outbreaks. With advancement in PCR technology, commercial kits are available to allow PCR directly on blood samples. The infected blood can be used directly in PCR without interference by inhibitors of PCR present in the blood. However, amount of template DNA may vary depending upon the collection of the sample. In this study, it was also observed that the sample collected at an early stage of the disease showed poor amplification because of the low amount of template DNA and the samples collected from the heart blood of dead animals (24 h post infection) may contain a high amount of template DNA. The study also demonstrated the adverse effect of the high amount of bacterial DNA on the performance of PCR. This necessitates the testing of few dilutions of the samples to overcome false negativity in PCR.

The constraint of time for diagnosis may not permit isolation, purification, and quantification of bacterial DNA in the blood sample. Direct testing of infected blood without extraction, purification, and quantification of template DNA may not permit use of optimum template DNA amount in PCR. In both situations of a low amount of template DNA in the sample collected at an early stage of the disease and in the diluted samples collected from dead animals, molecular diagnosis of the disease can be successfully done with most sensitive PCR.

For early and accurate molecular diagnosis of HS with infected blood, it is suggested that mPCR should be performed and three to four dilutions of samples likely to contain high template DNA, be tested. The study indicated that HS could be diagnosed early before the appearance of an appreciable amount of bacteria in the blood of an infected animal. The lower amount of bacteria in such early stage of the disease may permit
antibiotic therapy with no or least adverse reaction of LPS and the animal can be successfully treated.

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