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RESEARCH ARTICLE

Isolation and detection of *Pasteurella multocida* from clinical hemorrhagic septicaemia cases of buffaloes

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ABSTRACT

Hemorrhagic septicaemia (HS) is an economically important disease of livestock affecting various species of animals. The present investigation was conducted to detect and isolate the causative organism from the HS suspected cases of the buffaloes in two different outbreaks. Total 24 samples (14 nasal swabs, 6 lung swabs and 4 blood samples) from the suspected HS cases were processed for the bacterial isolation and DNA was extracted for PCR detection. Culture and biochemical assays from two samples showed characteristics of the *Pasteurella multocida* organism. The KMT1 gene was targeted as the marker gene for *P. multocida* for the PCR assay confirmation. Out of 24 samples, four were confirmed to be positive for *P. multocida*. In conclusions, *P. multocida* associated respiratory illness was common in buffaloes during stressful climatic conditions and to minimize the spread, the animal owners should ensure appropriate medication, vaccination programs and good hygienic practices in crowded areas.

Keywords: Buffaloes, isolation, *Pasteurella multocida*, PCR

INTRODUCTION

Hemorrhagic septicaemia (HS) is a per acute and devastating disease of buffaloes caused by *P. multocida*, a commensal bacterium of upper respiratory tracts of several livestock, poultry and domestic pet animal species resulting into high morbidity and mortality[1]. Under the influence of stress factors such as poor nutrition, unfavorable weather, long distance travelling, dehydration, nasopharyngeal settlement and concurrent infections of other bacteria, viruses and parasites, the organism leads to more profuse destruction to the host animal[2]. *P. multocida* is a small, gram-negative, non-spore-forming, coccobacillus bacterium with bipolar staining characters[3].

In Himachal Pradesh, HS is being reported regularly despite vaccination and even considered as one of the major bacterial killer disease among buffaloes. However, a number of studies were carried out in the state for the identification of *P. multocida* from clinical affected cattle, buffaloes, sheep, goats, rabbits and poultry[4-6]. For the detection and characterization of P. multocida, cultural and serological procedures are adopted and in recent times PCR is merely being preffered[7]. This study was carried out to detect the presence of P. multocida from clinically infected buffaloes showing symptoms of HS.

MATERIAL AND METHODS

Outbreak history

Fifteen buffaloes of age group (1 to 5 years) were presented with history of high fever (105-106^{O}F) followed by respiratory distress, septicaemia, mucopurulent nasal discharge, inappetance, oedematous swelling of the brisket region and recumbency leading to death of six animals. During post-mortem examination, subcutaneous edema in the brisket and mandibular regions and petechial-to-echymotic hemorrhages along with congestion of lungs, fibrinous pneumonia and pericarditis were recorded. The outbreaks have been reported during the period of heavy rainfall between Mid-July to September.

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Sample collection

A total of 24 samples (14 nasal swabs, 6 lung swabs and 4 blood samples) were collected randomly from clinical cases following outbreaks of HS in buffaloes. The samples were transported to Department of Veterinary Microbiology using transport medium (Normal Saline Solution on ice) and then processed for bacterial isolation.

Isolation of bacterial organisms

The clinical samples were put in tryptic soy broth (Himedia, Mumbai) and incubated at 37^{O}C for 18-24 hrs. Then swabs were plated onto 5% sheep blood agar (Himedia, Mumbai) and incubated at 37^{O}C overnight[8]. The isolates were identified by cultural and morphological

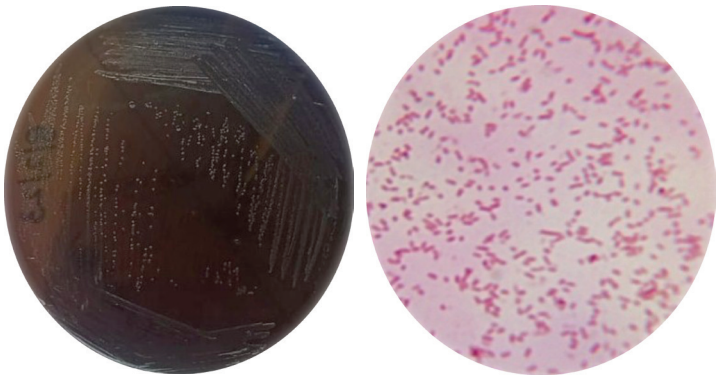

Fig. 1. Non-hemolytic colonies of *Pasteurella multocida* on 5% sheep blood agar; **Fig. 2.** Gram's stained *Pasteurella multocida* organisms.

features. Further a smear was prepared from single isolated colony and grams' staining was performed to demonstrate its morphological characteristics[9].

Biochemical methods

Biochemical tests catalase, oxidase, indole, VP, urea, citrate and motility were used as described by[9] with some modifications. The result found were recorded and compared for verification of the isolates.

Detection of *P. multocida* by PCR

All the clinical samples and bacterial isolates were subjected to DNA isolation using DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's instructions. Conventional PCR was carried out to detect *P. multocida* in GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, California, USA) targeting the KMT1 gene amplified using species specific primers[10] (Forward: KMT1SP6, 5′-GCTGTAAACGAACTCGCCAC-3′; Reverse: KMT1T7, 5′-ATCCGCTATTTACCCAGTGG-3′). The components for the DNA amplification master mix (25 µl) for *P. multocida* comprised of nuclease-free water 8.7 µl, 5 x PCR buffer 5 µl, 10 mM dNTPs 2 µl, 10 µM forward primer 1µl, 10 µM reverse primer 1 µl, 3 mM $MgCl_2$ 2 µl and Taq DNA polymerase 3 units 0.3 µl. The PCR was performed with thermal conditions of initial denaturation at 95^{O}C for 2 min followed by 30 cycles of denaturation at 95^{O}C for 1 min, annealing at 45^{O}C for 1 min, extension at 72^{O}C for 30 sec, final extension at 72^{O}C for 5 min[10].

Gel electrophoresis

A 5 µl aliquot of the PCR product was resolved on a 1.5% agarose gel and observed under UV in a Gel Documentation system (Alphaimager, BioRad, USA). Using PM-PCR assay, *P. multocida* isolates were identified based on the amplification of an approximately 460 bp fragment.

RESULTS

Cultural characteristics of *P. multocida*

Culturing of total 24 samples (blood and swab), it was observed that only two (8.3%) isolates were showing the cultural characteristics of *P. multocida* (small, glistening, mucoid with no haemolytic effect) on blood agar (Fig. 1). The *P. multocida* isolates were coccobacilli with rounded ends, stained negative by Gram staining (Fig. 2).

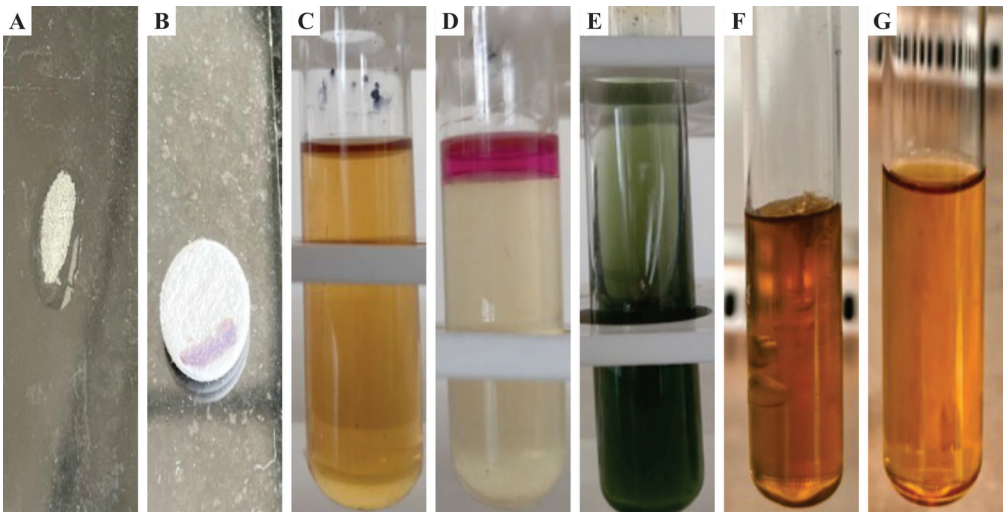

Fig. 3. Biochemical identification of *Pateurella multocida.* **A:** Catalase Positive, **B:** Oxidase Positive, **C:** Urea Negative, **D:** Indole Positive, **E:** Citrate Negative, **F:** Non-Motile (SIM), **G:** VP Negative.

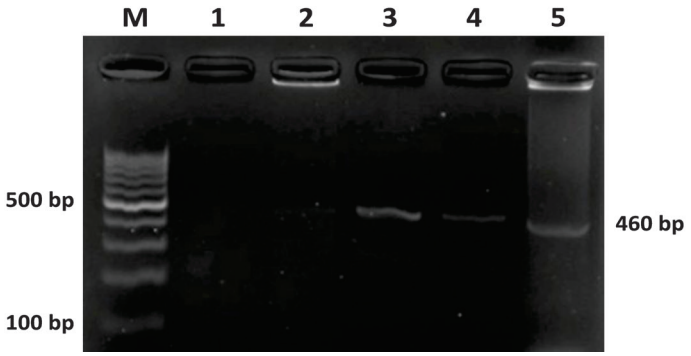

Fig. 4. PCR products (460 bp) in 1.5% agarose gel. Lane M: DNA marker, Lane 1: Negative Control, Lanes 2, 3: Clinical samples amplified using primer KTT1T7 & KMT1SP6, Lane 4: *P. multocida* isolate amplified using primer KTT1T7 & KMT1SP6, Lane 5: Positive Control.

Biochemical characteristics of *P. multocida*

The isolates of *P. multocida* were found oxidase, indole and catalase positive. Further, isolates did not produce hydrogen sulfide, urease and motility, did not have hemolytic effect and were negative in VP reaction (Fig. 3).

Molecular identification

Overall, 16.7% (4/24) of the samples from the two outbreaks were positive by PM-PCR (Fig. 4). Based on the sample type the highest positivity of *P. multocida* was recorded in the nasal samples with 20.0% (3/15) and only 16.7% (1/6) for the lung swabs. The bacterial isolates from both outbreaks were also confirmed as *P. multocida* by species specific PM-PCR. As expected, buffaloes having a HS-like disease were found to be infected with *P. multocida*.

DISCUSSION

P. multocida is an opportunistic pathogen causing a number of diseases in various animal species. In buffaloes, HS is one of the most important disease causing heavy economic losses to the farmers. This study was under taken on the samples collected from two outbreaks aimed to isolate and detect *P. multocida* resulting to HS by conventional methods and PCR. In the present study, two *P. multocida* isolates were showing characteristic features of the organism. In the previous studies, similar results were recorded for the identification of *P. multocida* as gram-negative, coccobacillus, with no haemolysis on blood agar[11,12]. Also, number of studies have stated similar results for biochemical characteristics such as positive reaction for oxidase, catalase, indole tests and negative for H_2S, urea and motility[13-15]. In the study, the overall isolation rate of *P. multocida* from the total number of clinical samples was recorded as 8.3%. Shuka *et al.*[16] reported 3.39% isolation rate of *P. multocida* from blood and swab samples of cattle which stands less in comparison to our findings. In contrast, a study carried out on calves in the region of Zambia, a higher percentage (17.5%) of *P. multocida* bacterial isolates were reported[17]. In HP, Kapoor *et al.*[18] screened 470 samples from different animal species from different districts to assess the prevalence of HS. According to the study, they were also able to isolate fewer number of *P. multocida* isolates and found isolation of *P. multocida*, a tedious and cumbersome process. This kind of ambiguity in the isolation rate of organism might be due to the uneven number of animals screened for the diagnosis for the HS like disease.

For confirmation of *P. multocida* as the principal cause of HS the PCR test was done based on Townsend *et al.* by using universal and specific primers[10]. Although, in the previous studies the organism has been found as causative agent of HS in HP. Likely, in this study 16.7% of the samples were found positive for *P. multocida* by PM-PCR using the amplification of an approximately 460 bp fragments which agree with the findings of the previous studies. The two *P. multocida* isolates procured from positive nasal swabs were also confirmed by PM-PCR. In a previous study, eighteen isolates of *P. multocida* were isolated from 138 bovine samples (nasal swabs, heart blood, spleen, lung and liver tissues) and confirmed by species specific PM-PCR[6]. Similarly, 23 *P. multocida* isolates from 335 clinically healthy and diseased cattle were confirmed by PM-PCR and further screened for the presence of virulence associated genes[5]. In HP, pasteurellosis is enzootic and has been an extended standing delinquent occurring mostly on the seasonal basis. In a comprehensive study carried out by Chaudhary *et al*., HS is the major bacterial disease with highest mortality rate in animals in HP[19]. Also in another study, HP along with other states of northern India, showed irregular occurrence of HS outbreaks between the periods 1995-2000 and 2006-2008 with overall 5.6% proportion of outbreaks[20]. Nevertheless, the vaccination is followed regularly in the state but despite of that emergence of new strains for ineffectiveness of the vaccines and increase in outbreak rates of HS cannot be ignored. The study has confirmed that the buffaloes in the outbreak were infected by *P. multocida*. Further research on *P. multocida* genetic diversity and the molecular epidemiology is required to development improved immunoprophylactics.

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