Detection of Clostridium perfringens Alpha, Epsilon and Clostridium chauvoei A toxin genes in Blackleg

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Abstract
Polymerase Chain Reaction (PCR) detected concurrent infection of Clostridium (C.) perfringens type D and C. chauvoei in samples of three cattle out of five which were submitted to Veterinary Research Institute (VRI) for confirmation of C. chauvoei. The animals had a history of fever, lameness and crepitating sound with death occurring within 48 hours after the onset of clinical signs and seemed to be typical cases of blackleg. Furthermore, the traditional methods including clinical examination, necropsy findings, cultural and biological methods are not solely enough for the confirmation of disease and are not sufficient to determine the number of pathogens involved in such cases.

Keywords: C. chauvoei, C. perfringens type D, Cattle, PCR, Concurrent infection

How to cite this:

Introduction
In Pakistan, the livestock animals such as buffaloes, cattle, camels, sheep and goats are known as the cornerstones of livelihood of people of rural areas and usually reared for milk and meat purpose (Ali et al., 2016; Ali et al., 2017; Hussain et al., 2018; Hussain et al., 2020). The dairy animals in Pakistan are mainly kept in tropical and subtropical conditions which contribute substantially (11.8%) in gross domestic product (GDP) of the country with 39.7 million cattle (Rehman et al., 2017). However, various infectious problems are the major issues of dairy animals in Pakistan and affect the productivity and food security (Batoel et al., 2019; Rashid et al., 2019). The dairy animals in Pakistan suffer from different bacterial (Mahmood et al., 2014a; Mahmood et al., 2017; Hussain et al., 2017), infections (Mahmood et al., 2014b; Hussain et al., 2016; Hussain et al., 2017b; Hussain et al., 2018; Zafar et al., 2019) and viral diseases (Khan et al., 2018; Hussain et al., 2020). Among different bacterial diseases, clostridial infections are the main threats to dairy animals (Hussain et al., 2019). Blackleg disease in cattle and buffalo whereas enterotoxaemia (Pulpy kidney disease) in sheep and goats are of considerable economic importance and caused by C. chauvoei (Hussain et al., 2019) and C. perfringens type D respectively (Falquet et al., 2013). Clostridia produce a number of potent toxins and enzymes which...
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are responsible for the development of various diseases (Rychener et al., 2017). The affected animal with Black leg may harbor the organism in the liver and represents the main epidemiological hazard for the infection and considered as non-traumatic endogenous infection (Casagrande et al., 2015). The affected animal usually shows signs of anorexia, fever, depression, hot painful swelling on affected area, crepitating sound, lameness and death within 12 to 48 hours (Aiello and Moses, 2016). The C. chauvoei have tropism for larger muscles especially thigh, heart and diaphragm which look spongy at necropsy while C. perfringens type D, an etiological agent of Enterotoxaemia is a normal inhabitant of intestine but under favorable conditions produce toxins which cause various gastrointestinal infections in most mammalian species. (Gacem et al., 2015). The diagnosis of black leg is established traditionally by history, clinical signs and gross lesions coupled with cultural and biological method.

The previous studies showed that the C. chauvoei is a usual etiological agent of Black leg in cattle and in certain cases combined with C. septicum whereas, the current findings revealed presence of C. perfringens type D alpha (cpa) and epsilon (etx) toxin genes which is unusual in gluteal muscles along with C. chauvoei toxin A (CctA) gene by PCR in three cases which were submitted only for the confirmation of black leg disease.

Material and Methods

The study included (n=5) samples of gluteal muscle pieces from cattle suspected for Black leg disease and were submitted immediately after death to avoid any contamination for its confirmation at VRI, Lahore Pakistan during 2016-2018. The animals were between 10-26 months of age and suspected for Black leg only. The death occurred within 48 hours after the onset of clinical signs. The impression smears from muscle pieces were prepared and simultaneously cultured in Cooked Meat Broth Medium (CMBM) for morphological examination. The blood agar plates were inoculated with growth taken from CMBM and kept under anaerobic conditions at 37ºC for 48 hours (Abreu et al., 2017). The colonies from blood agar plates were picked and used for molecular identification. The bacterial DNA was extracted by phenol chloroform isoamyl alcohol method (Eslami et al., 2017) and the polymerase chain reaction (PCR) was performed in 25ul volume and the molecular detection of C. perfringens type D, C. chauvoei and C. septicum was performed by amplification of the genes (Table 1). Briefly, the amplification was carried out in a thermal cycler (Biorad) using 30 cycles of 95ºC, 55ºC and 72 ºC for 45 seconds each for cpa and etx gene, 94ºC, 46ºC and 72ºC for one minute each for C. chauvoei toxin gene A (CctA) and 94ºC, 55ºC and 72 ºC for one minute each for C. septicum gene. The initial denaturation at 95ºC for 5 minutes and final extension at 72 ºC for 10 minutes was carried for amplification of all the genes. The amplified DNA fragments were examined by electrophoresis in a 1.5% agarose gel and visualized by UV transillumination.

Table-1. Specific primers along with their target genes

<table>
<thead>
<tr>
<th>Toxin gene</th>
<th>Primers</th>
<th>Sequence (5′-3′)</th>
<th>Fragment Length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa</td>
<td>PL3</td>
<td>AAGTTACCTTTTGC</td>
<td>283bp</td>
<td>(Fach and Popoff, 1997)</td>
</tr>
<tr>
<td></td>
<td>PL7</td>
<td>TGCATAAATCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATAGATACTCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TATCATCTGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>etx</td>
<td>1</td>
<td>CCGGTGATATCC</td>
<td>655bp</td>
<td>(Marina et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ATCTATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCATTAATGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTACTAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. chauvoei</td>
<td>CCTO2AR</td>
<td>ATGAAAGGAGTA</td>
<td>1400bp</td>
<td>(Idrees et al., 2014)</td>
</tr>
<tr>
<td>C. chauvoei</td>
<td>CCTO2AL</td>
<td>AGAACAGTTATTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCATCCTGCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. septicum</td>
<td>Hemolysin</td>
<td>AATTCAAGTTGC</td>
<td>270bp</td>
<td>(Takeuchi et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCAGTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCTGCCCAACATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCTTIT</td>
<td></td>
<td></td>
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</tbody>
</table>

Results and Discussion

The history and clinical findings with death of animals lead to a presumptive diagnosis of black leg disease. The microscopic examination from CMBM culture and impression smear from muscle pieces revealed gram positive rods of variable size with central and sub terminal spores (Figure 1). The colonies of grayish white appearance with zone of hemolysis on blood agar were seen.

The PCR detected both C. perfringens type D cpa and etx encoding gene of 283 bp and 655 bp respectively while CctA encoding gene for C. chauvoei of 1400 bp on agarose gel in three samples (Figure 2). However, Hemolysin gene of C. septicum was not detected from any sample (Table 2).
Hyaluronidase and Neuraminidase in disease progression is less well described. C. chauvoei DNAse is responsible for the degradation of DNA while Hyaluronidase cleaves hyalurone which is essential component of extracellular matrix, resulting in loosening of tissue and facilitate the spread of pathogen while Neuraminidase acts on host cell surface and decreases the rigidity of cell membrane and degrade the tight junction by cleaving the sialic acid (Frey and Falquet, 2015). The circulation of hemolysins in blood stream results in cytolysis and hemolysis causing typical lesions of Black Leg (Popoff, 2016; Abreu et al., 2017). A mixed infection of C. chauvoei with C. perfringens type A, C. septicum, C. novyi and C. sordelli have been reported during a retrospective study for the diagnosis of clostridial myonecrosis in ruminants (Pires et al., 2017) whereas only C. perfringens was detected instead of C. chauvoei in two calves with typical signs of Black Leg (Askari et al., 2016). The amplification of cpa and etx genes which are specific for C. perfringens type D from muscle pieces was certainly surprising during present study as this bacterium inhabits the large intestine of sheep and goats but not muscles. It seems that the spores of C. perfringens type D were ingested, absorbed, transported and lodged in various tissues including the gluteal muscles. The clinico-pathological findings in different tissues have been recorded in goats experimentally infected with C. perfringens type D along with molecular detection of cpa and etx genes (Nasir et al., 2015). The previous studies revealed that C. perfringens type D has affinity for gastrointestinal tract while C. chauvoei target especially heavy muscles. However, the finding of the current study suggests that C. perfringens type D not only targets the alimentary tract but can invade muscle tissues as well. It would have not been possible to detect C. perfringens type D toxin genes if only traditional approach for the diagnosis of Black leg was followed.

**Conclusion**

The identification of C. perfringens specifically type D cpa and etx along with C. chauvoei CctA gene from blackleg case in cattle is an unusual finding. However, further experimental study is needed to find out the pathogenic role of C. perfringens type D alone and in combination with C. chauvoei for the progression of disease.
Disclaimer: None.
Conflict of Interest: None.
Source of Funding: None.

References


**Contribution of Authors**

Nasir AA: Conceived idea, designed research methodology, collected and analysed data and wrote manuscript

Ashraf MU: Designed research methodology, collected and analysed data and wrote manuscript

Kausar A: Designed research methodology, collected and analysed data and wrote manuscript

Mustafa N: Designed research methodology, collected and analysed data and wrote manuscript

Fatima Z: Designed research methodology, collected and analysed data and wrote manuscript

Sarwar M: Data collection, analysis and interpretation

Riaz R: Data collection, analysis and interpretation

Shahzad W: Literature review, edited and gave final approval of manuscript

Khalil A: Designed research methodology, collected and analysed data and wrote manuscript

Hussain R: Literature review, edited and gave final approval of manuscript