AJAB

Asian J Agric & Biol. 2020;8(4):519-523.
DOI: 10.35495/ajab.2020.02.120

Short Communication

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

Azam Ali Nasir[1*], Muhammad Usman Ashraf[1], Asma Kausar[1], Nofil Mustafa[1], Zain-ul Fatima[1], Mobeen Sarwar[2], Rabia Riaz[1], Waseem Shahzad[1], Abdul Khaliq[2], Riaz Hussain[3]

[1]Veterinary Research Institute, Lahore Cantt, Pakistan
[2]Provincial Disease Diagnostic Laboratory, Cooper Road, Lahore, Pakistan
[3]College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan

Received:
February 18, 2020
Accepted:
September 21, 2020
Published:
October 31, 2020

**Corresponding author email:*
azamalinasir@gmail.com

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 black leg. 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:

Nasir AA, Ashraf MU, Kausar A, Mustafa N, Fatima Z, Sarwar M, Riaz R, Shahzad W, Khaliq A and Hussain R, 2020. Detection of *Clostridium perfringens* Alpha, Epsilon and *Clostridium chauvoei* A Toxin Genes in Blackleg. Asian J. Agric. Biol. 8(4): 519-523. DOI: https://doi.org/10.35495/ajab.2020.02.120

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 (Batool 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

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

Toxin gene	Primers	Sequence (5′-3′)	Fragment Length	Reference
cpa	PL3 PL7	AAGTTACCTTTGC TGCATAATCCC ATAGATACTCCA TATCATCCTGCT	283bp	(Fach and Popoff, 1997)
etx	1 2	GCGGTGATATCC ATCTATTC CCACTTACTTGTC CTACTAAC	655bp	(Marina et al., 2008)
C. chauvoei CctA	CCTO2AL CCTO2AR	AGTGAAGGAGTA AAGACTTTTATTA ATAT CCTGCATGCTCA ACAG	1400bp	(Idrees et al., 2014)
C. septicum	Hemolysin gene	AATTCAGTGTGC GGCAGTAG CCTGCCCCAACTT CTCTTTT	270bp	(Takeuchi et al., 1997)

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).

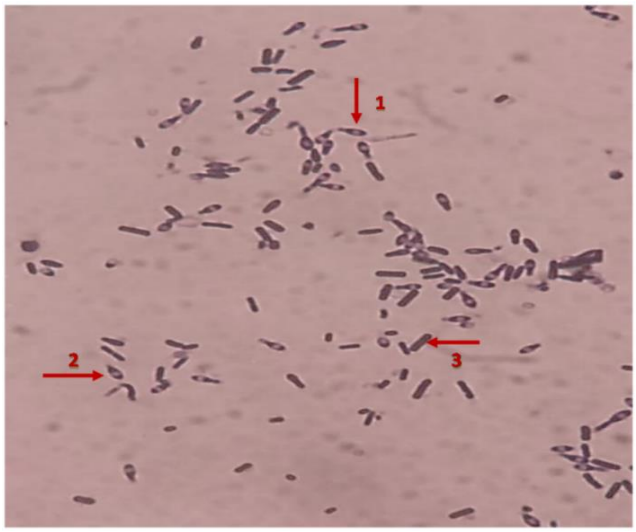

Figure-1. Gram's staining (x100) 1: sub-terminal spore, 2: central spore 3: thick cylindrical Gram-positive rod

Table-2. Sample No. with age in months and amplification of different toxin genes

Sample No.	Age in Months	*cpa*	*etx*	*CctA*	*C.septicum* Hemolysin gene
1	10	+	+	+	-
2	12	-	-	+	-
3	12	+	+	+	-
4	26	+	+	+	-
5	26	-	-	+	-

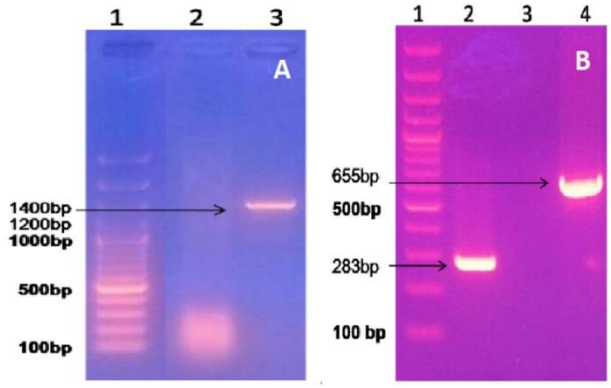

Figure-2. (A) PCR amplification of CctA encoding gene 1400bp can be visualized on agarose gel (B) PCR amplification of cpa encoding gene 283 bp and etx encoding gene 655 bp can be visualized on agarose gel.

Blackleg is highly fatal for cattle commonly affecting animals younger than 24 months of age and caused by *C. chauvoei* and is linked to virulence factors especially Hemolysins which includes *Cct*A and chauveolysin (Popoff, 2016) but the role of DNAse, Hyaluronidase and Neuramindase 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 Neuraminadase 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. sordellii* 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

Abreu CC, Edwards EE, Edwards JF, Gibbon PM, Leal-de-Araujo J, Rech RR and Uzal FA, 2017. Blackleg in cattle: a case report of fetal infection and a literature review. J. Vet. Diag. Invest. 29: 612-621.

Aiello SE and Moses MA, 2016. The Merck Veterinary Manual, 11th ed. Wiley, USA.

Ali F, Hussain R, Qayyum A, Gul ST, Iqbal Z and Hassan MF, 2016. Milk somatic cell counts and some hemato-biochemical changes in sub-clinical mastitic dromedary she-camels (Camelus dromedarius). Pak. Vet. J., 36: 405-408.

Ali HM, Qureshi AS, Hussain R, Urbinati G, Mustafa MZ, Ali F, Manan A and Massaad-Massade L, 2017. Effects of natural environment on reproductive histo-morphometric dynamics of female dromedary camel. Anim. Reprod. Sci. 181: 30–40.

Askari N, Ghanbarpour R, Kheirandesh R, Tajik J and Alimolaei M, 2016. Detection of *Clostridium perfringens* bacterium in the clinical specimens from blackleg calves: a report of two cases. Comp. Clin. Pathol. 26: 255-259.

Batool M, Nasir S, Rafiq A, Yousaf I and Yousaf M, 2019. Prevalence of tick infestation in farm animals from Punjab, Pakistan. Pak. Vet. J. 39: 406-410.

Casagrande RA, Pires PS, Silva ROS, Sonne L, Borges JBS, Neves MS, Rolim VM, Desouza SO, Driemeier D and Lobato FCF, 2015. Histopathological, immunohistochemical and biomolecular diagnosis of myocarditis due to *Clostridium chauvoei* in bovine. Ciencia Rural. 45:1472-1475.

Eslami G, Khalatbari-Limaki S, Ehrampoush MH, Gholamrezeal M, Hajimohammadi B and Oryan A, 2017. Comparison of three different DNA Extraction Methods of Linguatula serrate as a food borne Pathogen. Iraq J. Parasitol. 12: 236-243.

Fach P and Popoff MR, 1997. Detection of enterotoxigenic *Clostridium perfringens* in food and fecal samples with a duplex PCR and the slide latex agglutination test. Appl. Environ. Microbiol. 63: 4232-4236.

Falquet L, Calderon-Copete and Frey J, 2013. Draft genome sequence of the virulent Clostridium chauvoei reference strain JF4335. Genome Announc. 01: 001.

Frey J and Falquet L, 2015. Patho-genetics of *Clostridium chauvoei*. Res. Microbiol. 166: 384-392.

Gacem F, Madadi MA, Khecha N and Bakour R, 2015. Study of vaccinal properties of *Clostridium chauvoei* strains isolated during a black leg outbreak in cattle in Algeria. Kafkas Univ. Vet. Fak. Derg. 21: 825-829.

Hussain R, Javed MT,, Khan I, Siddique AB, Aslam B, Ghaffar A, Tariq N, Qayyum A and Wareth G, 2019. Pathological and clinical investigations of an outbreak of Blackleg disease due to C. chauvoei in cattle in Punjab, Pakistan. J. Infect. Dev. Count. 13: 786-793.

Hussain R, Khan A, Abbas RZ, Ghaffar A, Abbas G, Rahman T and Ali F, 2016. Clinico-Hematological and Biochemical Studies on Naturally Infected Camels with Trypanosomiasis. Pak. J. Zool. 48: 311-316.

Hussain R, Khan A, Jahanzaib, Qayyum A, Abbas T, Ahmad M, Mohiuddin M, and Mehmood K, 2018. Clinico-hematological and oxidative stress status in Nili Ravi buffaloes infected with *Trypanosoma evansi*. Microb. Pathog. 123: 126–131.

Hussain R, Mahmood F, Ali HM and Siddique AB, 2017. Bacterial, PCR and clinico-pathological diagnosis of naturally occurring pneumonic pasturellosis (mannheimiosis) during subtropical climate in sheep. Microb. Pathog. 112: 176e181.

Hussain R, Mahmood F, Aslam B, Siddique AB, Rafique A, Khaliq SA, Khan I, Imran S, Mubeen M, Jahanzaib and Nasir AA, 2020. Investigation of different serotypes of FMDV in vaccinated Buffaloes (*Bubalus bubalis*) in Southern Areas of Punjab Province, Pakistan. Pak. Vet. J. 40: 118-122.

Hussain R, Mahmood F, Khan A and Mehmood K, 2017b. Prevalence and pathology of bovine coccidiosis in Faisalabad district, Pakistan. Thai. J. Vet. Med. 47: 401-406.

Idrees A, Chaudary ZI, Younus M and Ashraf K, 2014. Isolation and molecular detection of *Clostridium chauvoei* alpha toxin gene from clinical cases of Black quarter in cattle. J. Anim. Plant Sci. 24: 755-759.

Khan A, Saleemi MK, Ali F, Abubakar M, Hussain R, Abbas RZ and Khan IA, 2018. Pathophysiology of peste des petits ruminants in sheep (Dorper & Kajli) and goats (Boer & Beetal). Microb. Pathog.

117: 139–147.

Mahmood F, Khan A, Hussain R and Anjum MS, 2014b. Prevalence and pathology of dictyocalus viviparous infection in cattle and buffaloes. J. Anim. Plant. Sci. 24: 743-748.

Mahmood F, Khan A, Hussain R and Khan IA, 2014a. Molecular based epidemiology of bovine pulmonary tuberculosis – a mortal foe. Pak. Vet. J. 34: 185-188.

Mahmood F, Khan A, Hussain R, Khan IA, Abbas RZ, Ali HM and Younus M, 2017. Patho-bacteriological investigation of an outbreak of Mycoplasma bovis infection in calves - Emerging stealth assault. Microb. Pathog. 107: 404-408.

Marina CF, Tereza CC and Iveraldo SD, 2008. Genotyping of *Clostridium perfringens* isolated from calves with neonatal diarrhea. Anaerobe. 24: 328-331.

Nasir AA, Younus M, Rashid A, Abdul Khaliq S, Khan E, Shah SH, Aslam A, Ghumman MA and Joiya MH, 2015. Clinico-pathological findings of *Clostridium perfringens* type D enterotoxemia in goats and its hemolytic activity in different erythrocytes. IJVR. 16: 94-99.

Pires PS, Ecco R, Silva ROS, Araujo MR, Salvarani FM, Heneine LGD, Junior CAO and Lobato FCF, 2017. A retrospective study on the diagnosis of clostridial myonecrosis in ruminants in Brazil. Cien. Rur. Santa Maria. 47: 1-5.

Popoff MR, 2016. Toxins of histotoxic clostridia: Clostridium chauvoei, Clostridium septicum, Clostridium novyi and Clostridium sordellii pp. 21-43 In John Willey & Sons (1st). Clostridial diseases of animals. John Willey & Sons Inc. Hoboken, New Jersey, USA.

Rashid I, Saqib M, Ahmad T and Sajid MS, 2019. Sero-prevalence and associated risk factors of Q fever in cattle and buffaloes managed at institutional dairy farms. Pak. Vet. J. 39: 221-225.

Rehman A, Jingdong L, Chandio AA and Hussain I, 2017. Livestock production and population census in Pakistan: Determining their relationship with agricultural GDP using econometric analysis Inform. Process Agricul, 4 (2): 168-177.

Rychener L, Inalbon S, Djorjevic SP, Chowdhury PR, Nicholson P, Ziech RE, Devargas AC, Frey J and Falquet L, 2017. *Clostridium chauvoei*, an evolutionary dead-end pathogen. Front. Microbiol. 8: 01-13.

Takeuchi S, Hashizume N, Kinoshita T, Kaidoh T and Tamura Y, 1997. Detection of *Clostridium septicum* Hemolysin gene by Polymerase Chain Reaction. J. Vet. Med. Sci. 59: 853-855.

Zafar A, Khan MK, Sindhu ZUD, Abbas RZ, Masood S, Abbas Z, Mahmood MS, Saleemi MK, Khan JA, Hussain R, Naseer MU, Iqbal Z, and Javed H, 2019. Seroprevalence of Fasciola hepatica in small ruminants of District Chakwal, Punjab, Pakistan. Pak. Vet. J. 39: 96-100.

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
Khaliq A: Designed research methodology, collected and analysed data and wrote manuscript
Hussain R: Literature review, edited and gave final approval of manuscript