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ISOLATION AND MOLECULAR DETECTION OF *CLOSTRIDIUM CHAUVOEI* ALPHA TOXIN GENE FROM CLINICAL CASES OF BLACK QUARTER IN CATTLE

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ABSTRACT

The objective of the present study was to isolate and identify *Clostridium chauvoei* from swab (n=750) and tissue (n=50) samples from cattle suspected for black quarter in six districts belonging to different climatic zones (Punjab, Pakistan). Conventional culture methods showed 192(25.6%) and 19(38%) positive from swab and tissues respectively for this bacterium. These bacterial isolates were further processed and confirmed by Polymerase chain reaction (PCR) by amplifying and (Ccta) gene sequences. An amplicon of 836 bp corresponding to 16SrRNA genesequence was obtained. PCR confirmed 140 (72.9%) and 41 (82%) positive samples from swab and tissues respectively.It is concluded that PCR was found an efficient tool for precise detection of *Clostridium chauvoei*

Key words: Black quarter, Clostridium chauvoei, cattle, PCR.

INTRODUCTION

Clostridium chauvoei is a causative agent of blackleg in cattle, sheep, goats and other animal species (Radostits *et al.*, 2006; Quinn *et al*., 2004). The spores of *Clostridium chauvoei*are ingested and reach the muscular tissue through the blood stream. These spores may remain latent for years in the muscles until local hypoxia in tissues allows them to germinate and multiply, producing several potent toxins (Radostits*et al*., 2006). The alpha toxin is one of the major toxins involved in the pathogenicity of the disease (Useh *et al*., 2003; Hang'ombe*et al*., 2006). This toxin is reported as hemolytic, necrotic and histotoxic (Quinn *et al*., 2005). Although, blackleg has been recorded in cattle at any age but most of the cases occur in young stock between age of 6 to 24 months of age.Hind and forelimbs and tongue are reported as the predilection site of the organism with high fever occurs within 48 hours of infection. The involvement of limb muscles normally leads to stiffness and animal does not want to move (Van Vleet and Valentine, 2007: Langroudi *et al*., 2012).

Presumptive diagnosis of blackleg is achieved by clinical and pathological findings; confirmation is routinely performed by the identification of the microorganism involved, either by conventional microbiological isolation characterization methods, immune-fluorescence (Vannelli and Uzal, 1996) and molecular assay. The current study was carried out to isolate the organism from suspected cases of Black quarter from swab and tissues by conventional methods and also identify the gene encoding alpha toxin of *Clostridiumchauvoei.*

MATERIALS AND METHODS

Study area: A total of (n=750) swab and (n=50) tissue samples of cattle suspected for blackleg reported in six districts (please provide details) of Punjab, Pakistan were collected aseptically, placed in ice bags and transported to Department of Pathology, University of Veterinary and Animal Sciences, Lahore (Pakistan) for the isolation and identification of *Clostridium chauvoei.*

Isolation and identification of organism: The samples were inoculated in Reinforced Clostridial Medium (RCM) broth and incubated at $37C^{o}$ in anaerobic jar with oxyrase anaerobic sachet using gas pack anaerobic jar. Tubes containing growth were picked and streaked on RCM agar in petri plates and incubated. Suspected colonies were further streaked on blood agar (Quinn *et al.*, 2004).

Gram's staining and spore staining were used to observe microscopic characteristics of bacteria as described by *Gerhardt et al*. (1994). Morphological characteristics, biochemical tests, guinea pig inoculation intramuscularly with 1:1 of 10% $Cacl_2$ and were also performed from the culture as described by *Pires et al*., (2012)

Molecular detection of *Clostridiumchauvoei* by PCR

DNA extraction: DNA was extracted from swab samples by using Pure Extreme ^{TM,} DNA Purification kit(Invitrogen Life Sciences,Carlsbad,USA) using support protocol for gram negative bacteria following manufacturer's instructions. Boiling method was used for DNA extraction directly from muscle tissues with some modifications (Volkenandt *et al.*, 1993). Briefly, muscle tissues collected from affected area of thigh region was taken and about 200µg from each sample was cut,

minced and homogenized in test tube. The samples were then treated with a proteinase K buffer for lysis.

DNA was extracted with a phenol–chloroform solution and precipitated with a sodium acetate solution. The DNA was resuspended in distilled water and frozen at -20 ℃ until used as a template in the PCR reactions.

Amplification of universal oligonucleotide sequence (CC-193-F, CC-16S-R) and alpha toxin gene (ccta): PCR conditions were set according to primers used in amplification. For primer (CC-193-F, CC-16S-R) condition consisted of initial DNA denaturation at 94 ℃ for 5 minutes followed by 35 cycles of denaturation at 94℃ for 45 seconds, annealing at 51-55℃ (gradient) for 45 seconds, extension at 72 ℃ for 1 minute and a final extension of 72 ℃ for 10 minutes (OIE, 2010).

Purified genomic DNA isolated from tissue samples were subjected to PCR by targeting 16SrRNA gene of *Clostridium chauvoei*. In case of (ccta) primer, PCR conditions were consisted of initial denaturation at 94℃ for 4 minutes followed by 35 cycles of denaturation at 94℃ for 45 seconds annealing at 47-52℃ (gradient) for 45 seconds and extension at 72℃ for 45 seconds and a final extension of 72℃ for 10 minutes.

For the optimization of *ccta* gene annealing temperature was set in the range 47-52℃. The amplified product was achieved best at 51 ℃. For (CC-193-F, CC-16S-R) PCR conditions were kept variable. Annealing temperature was adjusted between 51-55℃ in a gradient PCR.

A 50 ml PCR reaction mixture was prepared containing PCR Master Mix DreamTaq™ (MBI Fermentas, USA) forward and reverse primers, Template DNA and water.

Table-1.Composition of PCR Reaction mixture.

S. No	Reagent	Qty.
1	DreamTaq™ Green PCR Master Mix (2X)	25 µl
2	Forward primer	1.2 µl
3	Reverse prime	1.2 µl
4	Template DNA	2 µl
5	Water, nuclease-free	20.6 µl

The amlified DNA product was analyzed by agarose gel (1.2%) electrophoresis(Hamoka*et al.,* 1993)

Guinea Pig Inoculation: A 0.3 ml whole culture of C. *chauvoei* with equal volume of $Cacl_2$ was injected into healthy guinea pigs each weighing 400-450 grams and waited for 24-48 hours for pathogenicity testing. $Cacl_2$ was injected to control negative group only.

Table 2. Primers used for the identification of *Clostridium chauvoei*

Sr.#	Target gene	Primer sequence (5'- 3')	Ampliconsize	Tm(C)	Reference
1	CC-193F	AGCTTCGCATGGAGCAGTA	836 b.p	57.6	Uzal*et al*.
	CC-16SR	TCTTCGGAGACAGGATGA		52.9	2003
2	CCTO2AL	AGTGAAGGAGTAAAGACTTTTATTAATAT	1400 b.p	48.3	Frey *et al*.
	CCTO2AR	CCTGCATGCTCAACAG		53.2	2010

RESULTS AND DISCUSSION

In cattle, *C. chauvoei* causes black quarter, which is considered one of the most devastating diseasesof livestock with significant economic impact around the globe (Quinn *et al.*, 2004; Radostitis *et al*., 2006). The *C. chauvoei* is a strict anaerobe microorganismand it survives in the environment and in host muscles for decades. Occasionally, *C. chauvoei*can infect animals grazing on contaminated pastures ("champs maudits"), leaving a need to establish diagnostic facilities at local settings for efficient monitoring of the disease. To this end, investigations made during this study indicated that microorganism was Gram positive motile rodsalong withdouble zone of hemolysis on blood agar.Moreover, biochemical analysis has shownthat isolated *C. chauvoei* was essentially glucose, maltose and lactose positive whereas Indoland urease negative. All these properties are characteristics for *C. chauvoei*.

Lethality test indicated that inoculated guinea pig died after 72 hours of infection. The *C. chauvoei* was cultured on different media including RCM, thioglycolate, terrozi and cooked meat with result typical to *C. chauvoei*, as were previously reported by Moosawai *et al*. (1999), Naz *et al*. (2005) and Bagge*et al.*(2009). These culture properties andability to cause death in guinea pig, laboratory animal-of-choice for the identification of *C. chauvoei*, are suggesting that isolate belong to Clostridium genus. Furthermore, 25.6% swab and 38.0% tissue samples from inoculated guinea pig were positive for *C. chauvoei* which were determined by biochemical tests (Table 3).The simplest explanation of higher percentage of positivity in tissues is related to affected area and due to the presence of the said bacterium in a particular site.Corresponding findings have also been reported by the Pires *et al., (2012*) and Frey *et al., (2010)*.

For molecular detection and characterization, PCR assay was performedon kit extracted purified DNA

from swab and tissue samples. The universal primer set (CC 193-F/CC 16S/R) has targeted and hybridized the 16SrDNA sequenceof *C. chauvoei.* Prior to its application, the primer set was optimized at annealing temperature of 50.2 °Cfor the field strain. The amplified product was found to be 836 base pairs (bps) long on electrophoresis gel. This size of the PCR product was expected for positive samples for *C. chauvoei* (Fig.1).

Further attempts were made to confirm whether local field strain prevalent in the sampled areas was capable of producing alpha toxins or not on the basis of *ccta* gene. Out of two different sets of primers used in this study, only one of the set with sequence identity 3 (CCTO2AR and CCTO2AL) was capable to amplify the targeted portion of the bacterial genome. Similar amplification pattern was also observed by the Uzal *et al*. (2003).They succeeded in specific detection of *C. chauvoei* on the basis of 16-23SrDNA spacer region. However, owing to different location of primers, they amplified 509 bpsproduct corresponding to the spacer region of 16S-23S rDNA of the pure culture of isolated bacteria.

In other studies, Frey *et al.*, (2010, 2012) have reported the amplification of ccta gene sequence for identification of *C. chauvoei*using four sets of primers. Applying similar methodology, a PCR product of expected 1400 bp was amplified as shown in Fig.2. These results counter confirm the specificity of detection and presence of the isolate in the samples. However, failure of other set of primers might be due to the fact of not using restriction fragment sequence. Nevertheless, these two methods of molecular detection are sufficient for confirmation of positive samples.

An economically affordable method of DNA extraction,by boiling of the sample,was also investigated. However, in contrast to Nasir *et al*., (2012) report, this failed to provide sufficient purified DNA for PCR amplification. Therefore, purification with commercially available kit was applied with satisfactory results. A possible explaining for this inconsistency may be the difference in the fragility of cell wall of bacteria used in both studies.

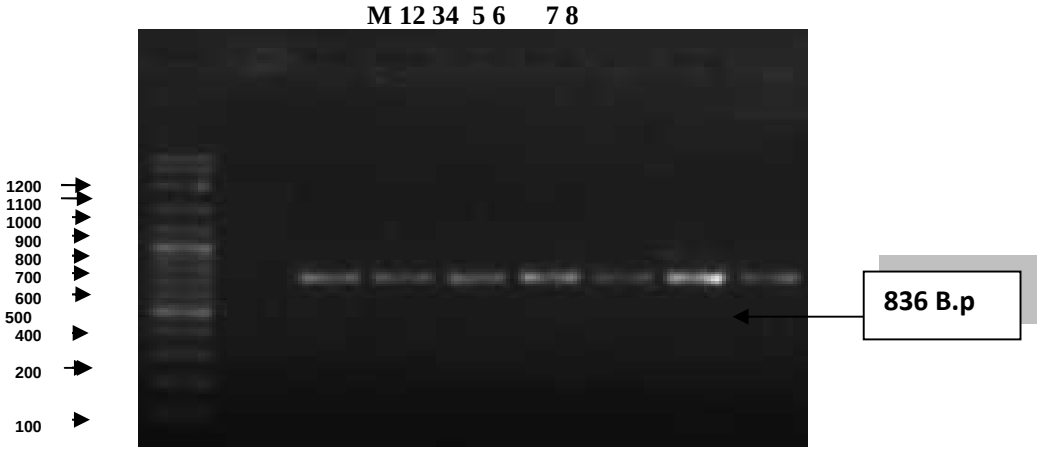

Figure 1: Agarose gel electrophoresis of 35-cycled PCR

1 Kb 1 2 3 4

Ladder

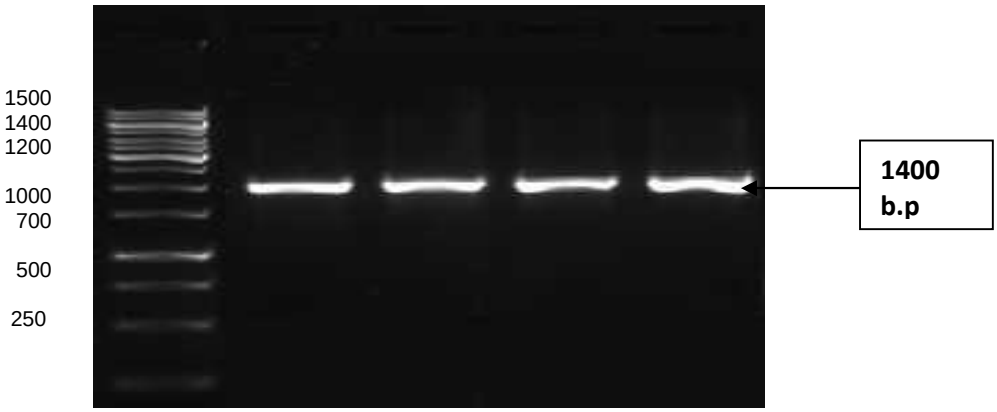

Figure 2: PCR amplification of *ccta* gene

Using optimized methods of detecting described above, the level of prevalence of *C. chouvoei* was determined at different sites of sampling. The highest percentage of BQ in district Muzaffargarh (36.8%) was followed by Bhakkar, Layyah, R.Y. Khan and Nankana Sahib. A high percentage of positive cases in Muzaffargarh and Bhakkar districts can possibly be associated with the sandy desert areas, which facilitates spore development and propagation through aerosol transmission. These findings are further supported by the investigations made by Khan (2010) who observed higher percentage of positive cases in another desert area of R.Y. Khan in a scanning surveillance programme. However, in this study, more positive cases were found in culture methods compared to PCR assay. This could possibly be due to either significant difference in the targets sites with in *C. chouvoei* DNA or lack in proper optimization of the assay in specific laboratory settings. Moreover, *C. septicum*has also been reportedin clinical cases, which shares 99% homology to *C. chouvoei*, however, differences may exist in the targets sites.

Out of 750 samples tested, 140 (72.9%) swab samples and 41 (82%) tissue samples were found positive as shown in the Table 3, respectively. It is clearly indicating that the ratio of positivity was significantlyhigher when tissue samples were taken directly fromdead animals. In contrast, Bagge *et al.*(2009)have shown higher percentage of *C. chauvoei* positive cases in tissue samples as compared to swab samples. However, this positivity was recorded usingconventional culture and biochemical methods.Application of molecular test such as PCR applied in this study may have noticeable affect on positivity of samples.Moreover, reason behind this difference couldalso be due to difference in organism abundance in the tissue compared to swab samples.

Table 3, Comparison of *Clostridiumchauvoei* detection through culture and PCR methods in cattle of different districts of Punjab

Properties of disease	MG	RYK	BKR	LYH	LHR	NS
No. of Animal	125	125	125	125	125	125
Culture +ve	46	40	45	43	2	16
%Prevalence	36.8	32	36	34.4	1.6	12.8
PCR +ve	32	29	37	31	nil	11
%Prevalence	25.6	23.3	29.6	24.8	0	8.8

MG= MuzaffarGarh; RYK=Rahim Yar Khan; BKR=Bhakkar; LYH=Layyah; LHR=Lahore; NS=Nankana Sahib

In conclusion, findings of this study indicate that*C. chauvoei* is prevalent in cattle throughout tested area of Punjab, Pakistan. Moreover, the PCR detection is significantly precise and effective method when compared to conventional culture and biochemical tests forthe diagnosis of BQ. Targetingalpha toxin gene, a major toxin of*C.chauvoei*, can be exploited effectively for both diagnostics and virulence purposes.These findings provide foundations to build future countrywide epidemiological studies, effective vaccine programs and design strategies for eradication of the disease with ultimate goal to minimize associated economic loss.

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