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RESEARCH ARTICLE

Characterization and Alpha Toxinotyping of *Clostridium chauvoei* from Cattle and Buffaloes in Punjab, Pakistan

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ABSTRACT

Blackleg, also known as clostridial myositis caused by *Clostridium chauvoei*, is an acute and often fatal disease primarily affecting cattle and buffalo posing a threat to livestock health and productivity worldwide. Despite routine vaccination against Blackleg in Pakistan, recurrent outbreaks continue to occur, highlighting the need for improved understanding of circulating strains and their toxigenic potential. In the present study, tissue samples were collected from 15 clinically suspected blackleg cases, from which five *C. chauvoei* isolates were confirmed based on morphological, biochemical, and molecular characterization. Initial identification was performed using culture-based methods under anaerobic conditions, followed by Gram and spore staining along with biochemical profiling. Molecular confirmation was achieved through amplification of the 16S rRNA gene, yielding ~1500bp product. Toxinotyping was conducted using gene-specific PCR assays targeting genes including a specie specific gene (*CCF516*) yielding an amplicon of 516bp while *cctA*, and *CCTO2AL* genes, yielded amplicons of 1120bp, and 1400bp, respectively, confirming the alpha toxin associated genes. Optimization of alpha toxin production was evaluated under different physical conditions, including variations in pH, temperature, and incubation time. Toxin activity was quantified using hemolytic assays with sheep red blood cells, revealing maximum activity at 37°C and neutral pH after 24 hours, while increased temperature and prolonged incubation resulted in a marked decline in toxin activity. These findings highlight the variability in toxin production among local *C. chauvoei* isolates, underscoring the need to characterize circulating strains to enhance diagnostics and inform better control strategies for Blackleg disease.

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INTRODUCTION

Blackleg (black quarter), caused by *Clostridium chauvoei*, remains a major constraint to livestock productivity worldwide (Tyasningsih *et al*., 2025). In Pakistan, where cattle (30 million) and buffalo (27 million) contribute ~60% of national milk/meat production, blackleg being an acute disease, inflicts annual economic losses exceeding PKR 13.5 billion through mortality (5-10% herd incidence) and treatment costs (Shahzad, 2022; Sher *et al*., 2025). Smallholder dairy systems are seriously affected by the high fatality (>90%) and the rapid spread of the disease in young stock (6-24 months) in Punjab and Sindh provinces (Khan *et al*., 2022).

Clostridium chauvoei is a gram-positive, sporulating, obligate anaerobic persister in soil and ruminant GI tracts (Sousa *et al*., 2024). The germination of spores in traumatized muscle occurs under hypoxic conditions, and it releases virulence factors such as the alpha toxin (CctA) that causes myonecrosis, gas gangrene, and toxaemia (Guyassa, 2022; Nicholson *et al*., 2019). Clinical features involve fever, lameness, crepitant swelling and abrupt death (Cáceres *et al*., 2022). This acute disease manifestation along with the environmental inertness of *C. chauvoei* spores makes blackleg especially difficult to control in endemic regions and highlights the significance of the disease as a significant challenge to veterinary and economic care (Guyassa, 2022).

The toxin repertoire of *C. chauvoei* is also a major factor in its pathogenicity, with the potent cytotoxin (*CctA*), also known as alpha toxin, taking the centre stage (Nicholson *et al*., 2019). The *CctA* is a haemolytic and cytotoxic protein, which plays a direct role in tissue destruction, vascular damage, and systemic reactions (Gupta *et al*., 2020). Disruption of the *cctA* gene has also proven to be a major determinant of pathogenicity by experimental studies that have shown that removal of this gene considerably decreases virulence. The *CctA* is highly immunogenic and has been shown to be a major antigen associated with severity of the disease, hence *CctA* can be highly useful in the development of vaccine and in molecular epidemiology (Frey *et al*., 2012). Therefore, alpha toxin-based characterization and toxinotyping of *C. chauvoei* isolates is a scientifically reasonable method to find diversity in strains, virulence capacity, and host-pathogen interactions. Blackleg may have mortality rates as high as 100% in clinical cases (Mussoyev *et al*., 2025). The disease spreads so fast and there is no time to do any clinical intervention. The animals that are usually vulnerable to outbreaks are young (6 months to 2 years old) and fast-growing cattle and buffaloes (Disasa *et al*., 2020). Such animals die and thus lead to the economic losses to the livestock industry. In addition to the immediate loss of lives, blackleg causes other financial losses in the form of decreased productivity of the herd, emergency treatment costs, higher costs of vaccination campaigns, and the long-term consequences of the disease on herd replacement strategies (Mussoyev *et al*., 2025). The losses are tremendous, especially in low- and middle-income countries, with mixed farming systems; thus, impacting food security and livelihood resilience (Guizelini *et al*., 2020).

Traditionally, most countries have used multivalent clostridial bacterin-toxoid vaccines as a method of prevention (Rossi *et al*., 2025). The vaccination is the most effective control measure because the decontamination of the environment is unrealistic and therapeutic intervention is seldom successful (Guizelini *et al*., 2020). Pakistan employs the locally-produced Black Quarter Vaccine (BQV) containing aluminum hydroxide-adsorbed toxoid from the reference strain *C. chauvoei* (OVI strain, originally imported from Onderstepoort Veterinary Institute, South Africa), administered biannually to calves (Livestock and Dairy Development Department Punjab, 2023). Despite vaccination, outbreaks persist, suggesting antigenic mismatch, cold-chain failures, or strain diversity (Baldwin and Black, 2021).

Molecular diagnostic and typing have enhanced the identification and verification of *C. chauvoei* with polymerase chain reaction (PCR) assays which target species-specific genes and toxin-coding loci including cctA that is replacing or complementing traditional anaerobic culture method (Uzal *et al*., 2003). The understanding of genomics has showed that despite *C. chauvoei* having a low genetic diversity at the species levels, there is variation between isolates that belong to various geographic setting and ecological conditions (Bagge *et al*., 2009). Nevertheless, a sizeable portion of the available molecular data has been obtained using reference strains or isolates from a small number of countries, while the data from buffalos are particularly underrepresented. Because buffalo plays an important role in the livestock economy of south Asia, southeast Asia and some areas of Africa, this absence of host-inclusive information is a gap in knowledge.

In Pakistan, prior studies confirmed *C. chauvoei* via culture/isolation (Rais *et al*., 2016; Hussain *et al*., 2019) and amplified the cctA gene from clinical samples (Idrees *et al*., 2023). However, no studies have performed systematic alpha toxinotyping to assess cctA sequence diversity, expression potential, or correlation with clinical severity across cattle/buffalo hosts in Punjab. This represents a critical gap, as toxinotyping directly informs vaccine strain selection and measures regional virulence evolution. This study fills this gap by characterizing indigenous *C. chauvoei* isolates from Punjab outbreaks using classical microbiology, 16S rRNA phylogeny, and alpha toxin (cctA) toxinotyping. By linking toxin profiles to host species and geography, the study provides baseline data for vaccine optimization and surveillance in Pakistan's major dairy region.

MATERIALS AND METHODS

Sample collection and processing: Tissue samples (skeletal muscle from necrotic limbs) were collected from 15 suspected clinical cases of blackleg disease each in cattle and buffaloes. Samples originated from outbreaks reported by livestock owners across two districts (Bahawalpur and Lodhran) in Punjab, Pakistan, over a 3-month period through the field surveillance network of the Institute of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore. Following owner notifications of acute cases with characteristic crepitus and swelling, samples were aseptically collected as per the protocol of Alam *et al*. (2019). In affected herds, remaining animals received immediate preventive vaccination with blackleg polyvalent antiserum and improved biosecurity measures (e.g., wound management and deep burial of carcasses) to mitigate further losses, given the disease's high mortality.

Properly labelled samples were transported to anaerobe biology laboratory, Institute of Microbiology, University of Veterinary and Animals Sciences, Lahore for further processing. Culture media like Reinforced Clostridial Medium (RCM) (Oxoid Ltd., Basingstoke, UK; catalog no. CM0151), Reinforced Clostridia Agar (RCA) (TM Media, Zanair Nagar, India; catalog no. M1084) and fluid thioglycolate broth (FTB) (Titan Biotech Ltd., Delhi, India; catalog no. MLT001) were prepared according to the manufacturer's guidelines. Approximately 5g of each sample was added in 10ml of PBS (phosphate buffer saline) and was grinded properly in tissue homogenizer. Samples were then enriched in FTB and incubated anaerobically for 48 hours at 37°C (Sathish and Swaminathan 2008; Alam *et al*., 2019).

Bacterial isolation and preliminary identification: The enrichment culture was streaked on Reinforced clostridial Media (RCM) with glucose and incubated anaerobically at 37°C for 48 hours as described by Oyeniran *et al*. (2020). Suspected colonies were purified through multiple streaking in anaerobic conditions, followed by inoculation on blood agar. Colonies displaying double zone

haemolysis underwent gram staining and endospore staining. Additionally, various biochemical tests including catalase, oxidase, triple sugar iron, carbohydrate fermentation, methyl red, Voges proskauer, indole, and double zone hemolysis tests were conducted for identification (Songer and Post, 2005; Quinn *et al*., 2011).

Molecular Confirmation and Characterization: According to the manufacturer guidance, deoxyribose nucleic acid (DNA) was extracted by DNA extraction kit (Luoyang Aisen Biotechnology Co., Ltd. Luoyang, China; catalog no. AS-1921). For molecular confirmation, PCR was performed using 16SrRNA gene primers Forward: 8FLP: 5`- AGTTTGATCCTGGCTCAG-3` Reverse: XB4: 5`-GTGTGTACAAGGCCCGGGAAC-3` (Kuhnert *et al*., 1997; Asghar *et al*., 2016; Tariq *et al*., 2022). For PCR, reaction mixture was prepared by adding 1µl forward primer, 1µl reverse primer, 2µl DNA templates, 12.5µl master mix and 8.5µl nuclease free water. PCR was carried out in thermocycler at 94ºC for 10min, 35 cycles 1min at 94°C, 1 min at 55°C, 2min at 72°C and final extension at 72ºC for 10min. The samples were run on agarose gel (1.5% gel). PCR products were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany; catalog no. 28104) according to the manufacturer's instructions. The purified amplicons were then submitted for sequencing. The obtained sequences were confirmed by NCBI nBLAST. Finally, the phylogenetic analysis was performed using MEGA-12 software.

Phylogenetic analysis: The 16S rRNA gene sequences (≈1,400 bp) from the five *Clostridium chauvoei* isolates were analysed phylogenetically. Reference sequences of *C. chauvoei* (n=5), closely related Clostridium species (n=21) were retrieved from NCBI GenBank (accession numbers provided in Table 3). Multiple sequence alignment was performed using BioEdit v7.7.1 (Hall, 1999) with the CLUSTAL W algorithm (Thompson *et al*., 1994), followed by manual editing to resolve gaps.

Phylogenetic trees were constructed using MEGA X v12.0 (Kumar *et al*., 2018) via the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993), selected as optimal by ModelTest implemented in MEGA. Tree topology robustness was evaluated with 1,000 bootstrap replicates. *Clostridium septicum* (GenBank: NR_121195) served as the outgroup. Final trees were visualized and edited in FigTree v1.4.4. The ML method is especially appropriate to analyse closely related sequences, because it considers the complexities of evolution and the small variations in sequences are resolved more accurately.

Toxin typing by PCR: *Clostridium chauvoei* toxin typing targeted CCF516, CCTO2A, and cctA genes using Table 1 primers. PCR mixtures (12.5µl master mix, 2µl DNA, 1µl each primer, 8.5µl nuclease-free water) were run at annealing temperatures. Bands were visualized on 1.2% agarose gel using an ImageGel-Doc system.

Toxinotyping of Alpha toxin: The haemolytic activity of the alpha toxin of *Clostridium chauvoei* was assessed following the method described by Gupta *et al*. (2020), with minor modifications. The haemolytic assay was performed using 96-well round-bottom microtitration plates. Briefly, 100µL of *C. chauvoei* culture was inoculated into 10mL of Fluid Thioglycolate Broth (FTB) adjusted to three different pH levels (6.5, 7.0, and 8.0). The inoculated tubes were incubated at three temperatures (37°C, 42°C, and 44°C). After incubation for 24, 48, and 72 hours, the cultures were centrifuged at 12,000rpm for 12min, and the supernatants were collected. For the haemolytic assay, 1% washed RBCs in PBS were mixed with serially diluted culture supernatant (100µL each) in 96-well plates (wells 1–11; well 12 as control). Plates were incubated at 37°C, and absorbance read at 540nm (negative control: RBCs in PBS). Haemolytic activity was also qualitatively assessed on 5% sheep blood agar plates incubated at 37°C (Gupta *et al*., 2020).

Table 1: Primer for toxinotyping of *Clostridium chauvoei*

Sr #	Target gene	Primer sequence (5'- 3')	Amplicon Size Bp	Temp (C)
1	CCTO2A-*L* CCTO2A-*R*	AGTGAAGGAGTAAAGACTTTTAT TAATAT CCTGCATGCTCAACAG	1400	53
2	*cctA-F* *cctA-R*	TGCTTGCTTTAGCAACAACAACT GGATGCGTCAACAATTTCTCA	1120	61
3	*CCF516-F* *CCR516-R*	ATCGGAAACATGAGTGCTGC AGTCTTTATGCTTCCGCTAG	516	54

RESULTS

All isolates were first inoculated in fluid thioglycolate broth (FTB); growth and gas production were noted after 48h of anaerobic incubation at 37°C. From the initial 15 tissue samples, pure colonies with distinct macroscopic morphologies were obtained by streak plating on reinforced clostridial medium (RCM). Five presumptive clostridium isolates (n=5) grew successfully on RCM and exhibited double-zone hemolysis on blood agar. Microscopic examination confirmed gram-positive rods with terminal to subterminal oval endospores, which were selected for biochemical evaluation. Biochemical tests (Table 2) confirmed these 5 isolates as *Clostridium chauvoei*. Representative broth cultures, agar plates, biochemical reactions, and microscopic characteristics are shown in Fig. 1 and 2.

Molecular confirmation: Biochemically characterized (n=5) isolates were confirmed by PCR targeting 16S rRNA gene sequence primer. A band of 1500bp was observed on agarose gel. With the help of nanodrop, purity of DNA of the isolates was determined and visualized on agarose gel as described in Table 3 and in Fig. 3.

Phylogenetic analysis: The resulting phylogenetic tree (Fig. 4) revealed evolutionary trends of *C. chauvoei* isolates in the world with reference to five query isolates, designated as PX450090 to PX450094. The five isolates comprise one clad with a bootstrap of 93 indicating with high confidence that they share a common ancestry. This clade also contained Indian and Swiss reference strains which suggest a moderate to high genetic similarity of these sequences. The Indian isolates were consistent with moderate to low bootstrap support, which means that there was low intraspecific diversity there. The distribution of

Table 2: Culture Characteristics, microscopic characteristics and biochemical activity of Clostridium *chauvoei*

Sr No.	Tests	Explanation	*C. chauvoei*
1	Primary culture in broth	Turbidity or without turbidity	Turbidity with gas production
2	Pure culture on RCM	Uniform growth of bacteria	Small irregular whitish pale color granular in center invisible toward periphery.
3	Gram staining	Gram negative pink rods or cocci, Gram positive purple rods or cocci.	Gram positive long and short rods single and with chain
4	hemolysis test on blood agar	No hemolysis (gamma) Partial hemolysis(alpha) Clear zone hemolysis(beta)	Clear zones were observed on blood agar around bacterial colonies.
5	Spore staining	Pink color vegetative cell Green color spore oval, round, terminal, sub terminal or middle	Red color vegetative cell and green color terminal and sub terminal oval spores.
6	Oxidase test	Purple color (positive) No Purple color (negative)	No color was developed (negative)
7	Catalase	Bubble formation (positive) No, bubble formation	Negative
8	Methyl red test	Pink color (positive) Yellow (negative)	Negative
9	Voges Prauskers test	Precipitate (positive) No, Precipitate(negative)	Negative
10	Indole	Red color (positive) Yellow color(negative)	Negative
11	Triple iron sugar test (TSI)	H_2S production (positive) No H_2S production (Negative)	Positive
12	Glucose	Fermentation pink (positive) No fermentation (negative)	Positive
13	Sucrose	Fermentation pink (positive) No fermentation (negative)	Positive
14	Mannitol	Fermentation pink (positive) No fermentation (negative)	Negative
15	Maltose	Fermentation pink (positive) No fermentation (negative)	Positive

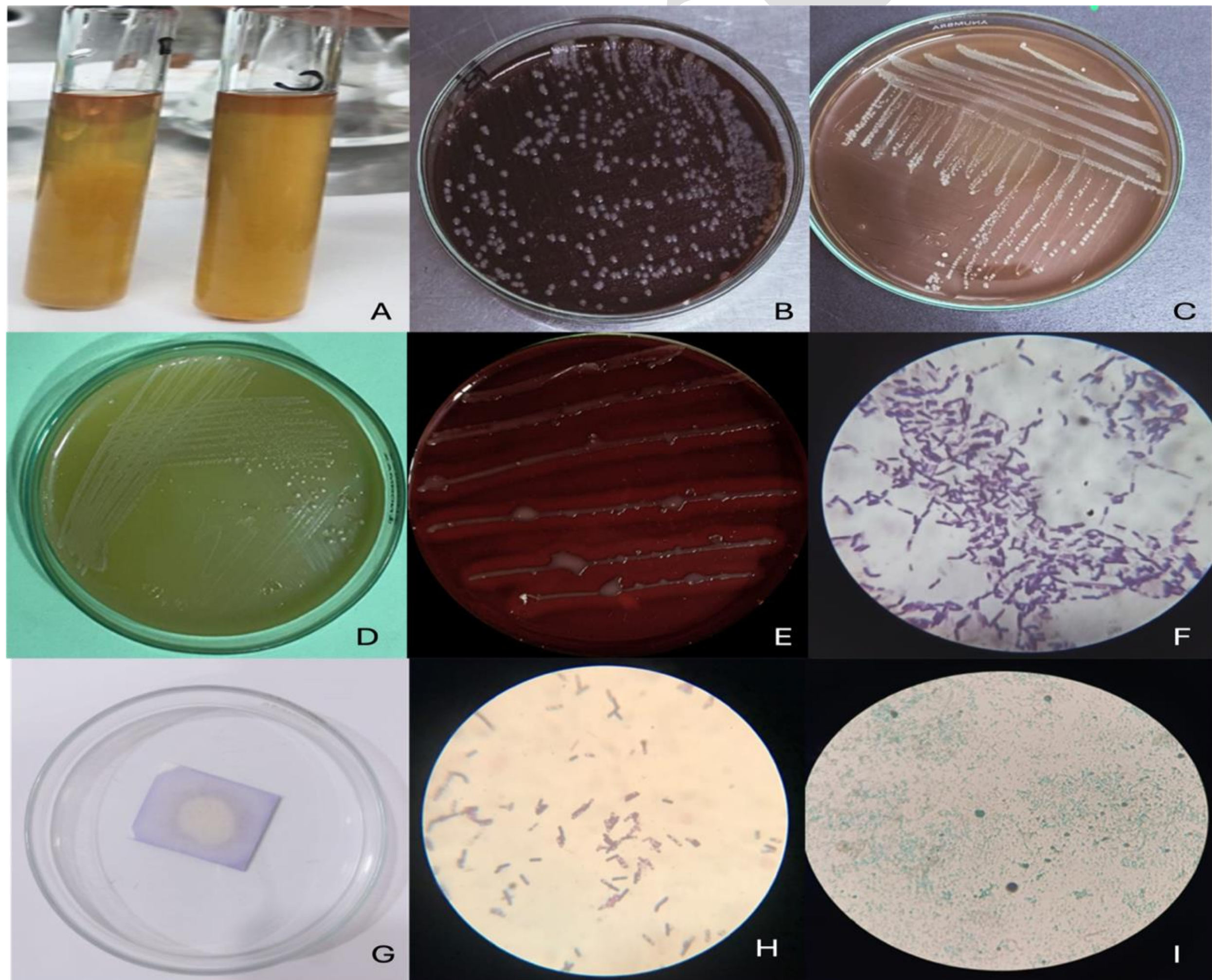

Fig. 1: Culture Characteristics, Microscopic characteristics and biochemical activity of Clostridium *chauvoei,* Growth in Fluid thioglycolate broth (A), Spreading on RCA (B), Pure culture (C), Pure Culture (D), Blood agar double zone haemolysis (E), Microscopic Morphology (F), Oxidase test (G), Visible spore of Clostridium chauvoei in gram staining (H), Spore staining (I).

Fig. 2: Culture Characteristics, Microscopic characteristics and biochemical activity of Clostridium *chauvoei,* Catalase test (A), Glucose fermentation (B), Methyl red (C), Vogues Proskauer's test (D), Indole test (E), Triple sugar iron test (TSI) (F), Mannitol fermentation (G), Sucrose fermentation (H), Maltose fermentation (I).

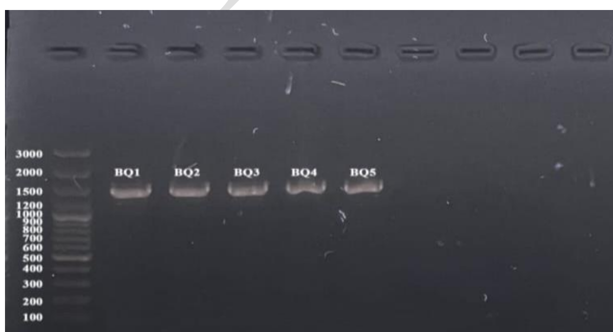

Fig. 3: Molecular Confirmation of *C. chauvoei* BQ1 (PX450090), BQ2 (PX450091), BQ3 (PX450092), BQ4(PX450093) and BQ(PX450094).

Table 3: Purity analysis of isolated DNA from biochemically characterized *C. chauvoei* by nano drop.

Samples	A-260 10 mm path	A-280 10 mm path	260/280	260/230	ng/µl
BQ1	0.188	0.101	1.84	1.78	9.5
BQ2	0.788	0.413	1.92	1.87	242.8
BQ3	0.677	0.436	1.54	1.04	15.1
BQ4	5.460	3.460	1.56	1.69	180.0
BQ5	5.666	4.875	1.15	0.18	32.5

the Japanese isolates (some of which also contained sequences in the intergenic spacer region and both the 16S and the 23S rRNA genes), on the other hand, was more diffused, perhaps due to a higher genetic variability. *Clostridium zeae*, like the rest of the tree, became an outgroup to provide the tree with a root. Interestingly, the

extreme divergence of the Blackleg isolates segregates other *C. chauvoei* sequences which could suggest the existence of a new strain or new geographic origin. This finding is epidemiologically significant, and it may reflect an evolutionary situation in the area and may be utilized in the disease surveillance and in developing vaccines and designing the region-specific diagnostic tool. Further molecular characterization is required to determine the uniqueness and potential clinical importance of these BQ isolates.

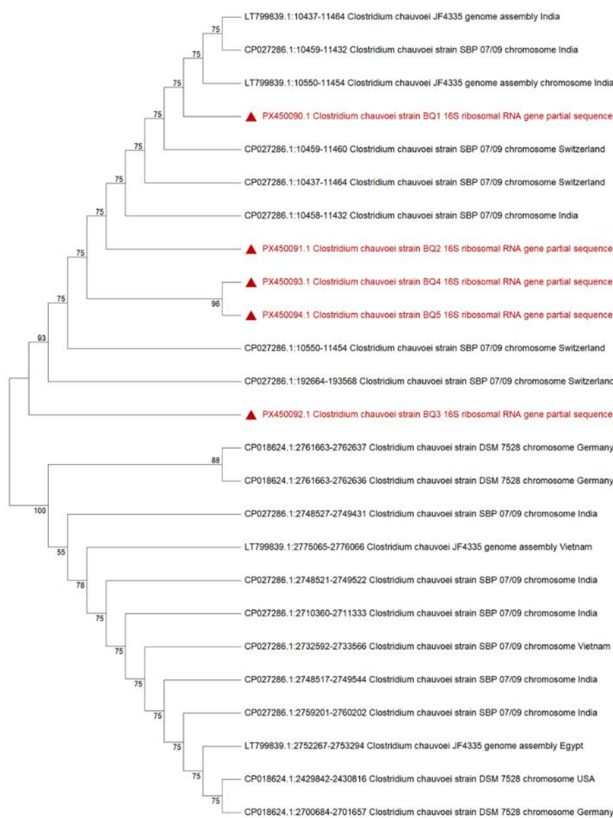

Fig. 4: Phylogenetic analysis of *Clostridium chauvoei.*

The physical conditions under which alpha toxin production by *C. chauvoei* occurred best were examined, including variations in temperature (37ºC, 42ºC and 44ºC), pH (6.5, 7.2 and 8.0) and incubation time (24, 48 and 72h). Following sequencing, accession numbers were assigned to the five confirmed isolates as BQ1 (PX450090), BQ2 (PX450091), BQ3 (PX450092), BQ4 (PX450093), and BQ5 (PX450094). Haemolytic activity analysis indicated that alpha toxin production depended on temperature, pH and incubation time. Overall, a significant decrease in haemolytic activity was observed in all isolates by increasing the temperature and prolonging the incubation.

Optimal production of alpha toxin was found after 24h incubation at 37°C and pH 7.2 implying that these conditions are favourable to the production of alpha toxins (Fig. 5). In this case, BQ1 (PX450090) had the highest haemolytic activity (2048.19±0.01HU/mL), and BQ5 (PX450094) had the least (342.77±0.01HU/mL). Major drop of haemolytic units was after 48 and 72h of incubation at a similar temperature and pH which indicates degradation or loss of expression of the toxin at the long incubation times. At 48h, the highest producer was BQ1 with the BQ3 and BQ5 always showing less haemolytic activity. The results of all isolates after 72h showed minimal haemolytic activity. At incubation temperatures of 42°C, haemolytic activity was significantly reduced relative to 37°C regardless of pH conditions. Though the toxin activity could be measured after 24h, once more with BQ1 generating the greatest number of haemolytic units, there was still a noticeable decrease after 48 hours and little activity thereafter. Activity of alpha toxin decreased rapidly at 44°C and minimal haemolytic units were observed at 24 and 48h, while no activity at 72h, which means that high temperatures had a negative effect on the stability of toxins or their production.

Fig. 5: Toxinotyping of *Clostridium chauvoei* CCTO2A gen amplification 1400 bp (A), cctA gene amplification 1120 base pair (B), CCF516 gene amplification 516 bp (C). Optimization of alpha toxin.

The pH effect on haemolytic activity demonstrated that pH 7.2 was the most preferable to alpha toxin production, then pH 6.5, while pH 8.0 gave a lower haemolytic activity in all isolates. The moderate haemolytic activity was noted at pH 6.5 and temperature 37°C after 24h and gradually decreased at 48 and 72h. At the pH of 8.0, measurable activity was observed at 24 and 48h, but the production of toxin was significantly low in comparison to that at pH 7.2, and the activity was insignificant at the end of the long incubation period. In all experimental conditions, BQ1 (PX450090) had higher haemolytic activity compared to the other isolates, and BQ5 (PX450094) tended to have the lowest activity.

Statistically significant differences (P<0.05) in haemolytic units were observed among the isolates under most temperature, pH, and incubation time combinations, except at extended incubation times (72h) and higher temperatures (44°C), where haemolytic activity was minimal or absent and differences were not statistically significant (P>0.05). Overall, these findings indicated that alpha toxin production by *C. chauvoei* was optimal at 37°C, neutral pH, and shorter incubation periods, and that both elevated temperature and prolonged incubation time negatively impacted haemolytic activity. A detailed summary of haemolytic units recorded under all tested conditions is provided in Tables 4-6.

Table 4: An Optimization of alpha toxin of *Clostridium chauvoei* under different physical parameter with pH 7.2

Culture temperature	Samples	Culture Hours (time)		
		24 hours	48 hours	72 hours
37°C	BQ1(PX450090)	2048.19±0.01^{a}	604.67±0.01^{a}	22.20±0.10^{a}
	BQ2(PX450091)	1024.22±0.01^{b}	600.50±0.01^{b}	18.51±0.01^{b}
	BQ3(PX450092)	347.30±0.01^{d}	326.29±0.01^{e}	9.98±0.01^{c}
	BQ4(PX450093)	357.06±0.01^{c}	354.04±0.01^{c}	9.08±0.01^{d}
	BQ5 PX450094)	342.77±0.01^{e}	328.56±0.01^{d}	7.86±0.01^{e}
42°C	BQ1(PX450090)	596.34±0.01^{a}	149.08±0.01^{a}	20.26±0.01^{a}
	BQ2(PX450091)	590.20±0.10^{b}	133.05±0.02^{b}	15.37±0.01^{b}
	BQ3(PX450092)	160.24±0.01^{d}	52.30±0.10^{d}	10.26±0.01^{c}
	BQ4(PX450093)	196.44±0.01^{c}	65.20±0.10^{c}	7.52±0.02^{d}
	BQ5(PX450094)	101.07±0.01^{e}	41.70±0.01^{e}	3.86±0.01^{e}
44°C	BQ1(PX450090)	86.86±0.01^{b}	40.16±0.01^{a}	0±0^{a}
	BQ2(PX450091)	65.88±0.01^{c}	9.08±0.01^{e}	0±0^{a}
	BQ3(PX450092)	49.63±0.15^{a}	20.25±0.01^{c}	0±0^{a}
	BQ4(PX450093)	63.02±0.01^{d}	10.03±0.02^{d}	0±0^{a}
	BQ5(PX450094)	53.20±0.01^{e}	20.54±0.01^{b}	0±0^{a}

In this table the Means with different letters (a,b,c) in column are significantly different at P<0.05

Table 5: Optimization of alpha toxin of *Clostridium chauvoei* under *different* physical parameters with pH 6.5

Culture Temperature °C	Samples	Culture Hours (time)		
		24 Hours	48 hours	72 hours
37°C	BQ1(PX450090)	451.95±0.01^{a}	178.53±0.01^{a}	10.40±0.01^{a}
	BQ2(PX450091)	442.65±0.01^{b}	177.30±0.01^{b}	10.13±0.10^{b}
	BQ3(PX450092)	96.15±0.01^{e}	40.52±0.01^{e}	5.09±0.09^{d}
	BQ4(PX450093)	214.86±0.01^{c}	87.65±0.01^{c}	5.75±0.01^{c}
	BQ5(PX450094)	148.08±0.01^{d}	86.83±0.01^{d}	4.79±0.12^{e}
42°C	BQ1(PX450090)	196.17±0.02^{a}	74.75±0.01^{a}	12.04±0.01^{a}
	BQ2(PX450091)	172.18±0.01^{b}	59.65±0.02^{b}	10.80±0.10^{b}
	BQ3(PX450092)	86.65±0.02^{c}	56.34±0.02^{c}	4.73±0.01^{d}
	BQ4(PX450093)	76.63±0.01^{d}	32.13±0.01^{d}	5.86±0.01^{c}
	BQ5(PX450093)	47.84±0.10^{e}	22.40±0.10^{e}	4.80±0.10^{d}
44°C	BQ1(PX450090)	53.13±0.01^{a}	13.14±0.02^{a}	0±0^{a}
	BQ2(PX450091)	42.13±0.01^{b}	12.07±0.02^{b}	0±0^{a}
	BQ3(PX450092)	23.12±0.01^{c}	9.92±0.03^{c}	0±0^{a}
	BQ4(PX450093)	23.03±0.03^{d}	9.82±0.01^{d,c}	0±0^{a}
	BQ5(PX450094)	20.11±0.01^{e}	9.62±0.03^{d}	0±0^{a}

In this table the Means with different letter (a,b,c) in column are significantly different at P<0.05

Table 6: Optimization of alpha toxin of *Clostridium chauvoei* under different physical parameters with pH 8.0

Culture temperature	Sample	Culture hours		
		24 hours	48 hours	72 hours
37°C	BQ1(PX450090)	445.73±0.01^{a}	165.43±0.01^{a}	5.77±0.01^{a}
	BQ2(PX450091)	358.24±0.01^{b}	163.15±0.01^{b}	4.73±0.01^{b}
	BQ3(PX450092)	87.11±0.01^{e}	39.95±0.01^{e}	3.40±0.10^{c}
	BQ4(PX450093)	202.02±0.01^{c}	84.96±0.01^{c}	4.79±0.01^{b}
	BQ5(PX450094)	105.08±0.01^{d}	84.93±0.01^{d}	3.07±0.01^{d}
42°C	BQ1(PX450090)	187.24±0.02^{a}	86.81±0.01^{a}	2.08±0.01^{b}
	BQ2(PX450091)	138.04±0.03^{b}	59.64±0.01^{b}	2.03±0.01^{c,b}
	BQ3(PX450092)	84.32±0.02^{c}	52.31±0.01^{c}	2.03±0.02^{c,b}
	BQ4(PX450093)	65.80±0.03^{d}	30.37±0.01^{d}	6.23±0.01^{a}
	BQ5(PX450094)	32.59±0.01^{e}	19.33±0.01^{e}	1.33±0.02^{d}
44° C	BQ1(PX450090)	52.03±0.01^{a}	9.06±0.03^{a}	0±0^{a}
	BQ2(PX450091)	40.60±0.10^{b}	8.93±0.06^{b}	0±0^{a}
	BQ3(PX450092)	19.43±0.15^{d}	8.84±0.01^{c}	0±0^{a}
	BQ4(PX450093)	19.95±0.01^{c}	9.02±0.01^{a}	0±0^{a}
	BQ5(PX450094)	19.80±0.10^{c}	8.90±0.02^{c,b}	0±0^{a}

In this table the Means with different letters (a,b,c) in column are significantly different at P<0.05.

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DISCUSSION

Blackleg, caused by *C. chauvoei* is one of clostridium's most pathogenic species—affects cattle/buffaloes with leg edema, myonecrosis, fever, and sudden death (Nicholson *et al*., 2019; Compiani, 2021). The pathogen produces key virulence factors including alpha toxin (CctA; 32 kDa pore-former), DNase, neuraminidase, hyaluronidase, delta toxin, and flagella, typically isolated from necrotic muscle. Commercial vaccines use toxoided supernatants and inactivated cultures for control (Frey and Falquet, 2015).

In the present study, the successful isolation of *C. chauvoei* from 33% (5/15) of skeletal muscle samples from clinically suspected blackleg cases in Punjab represents a vigorous field-to-laboratory workflow validated for Pakistani conditions. This recovery is consistent with previous reports of 25-40% success in mixed farming outbreaks (Rais *et al*., 2016; Hussain *et al*., 2019; Idrees *et al*., 2023). FTB enrichment was the best, in line with Bhatti (2005) who obtained 80% primary growth of Lahore field samples using the same pre-reduction procedures. The fact that FTB has remained consistently better than RCM/RCA in Punjab samples highlights the fact that FTB is the gold standard in resource-limited diagnostic laboratories working with polymicrobial clinical specimens.

Doubled-zone hemolysis, sub terminal oval endospores, and biochemical profiling gave 100 percent agreement with Pakistani reference strains. The typical hemolysis of blood on agar appears in two zones, as identified in Idrees *et al*. (2014) and Nasir *et al*. (2020), respectively, with gram morphology (long/short rods, singly/pairs/short chains) resembling those of Beigh *et al*. (2017) in Kashmir. Major biochemical consistency was negative (catalase/oxidase/VP/MR reaction) with positive glucose/sucrose/maltose/TSI fermentation, which agreed with Sathish and Swaminathan (2008). Nevertheless, intermittent catalase variability here reverberates Idrees *et al*. (2014), which is probably a strain polymorphism across South Asia, and not an artifact of methodology. This regional biochemical fingerprint affirms the usefulness of these markers in field diagnosis and differentiating *C. chauvoei* and the phenotypically similar *C. septicum* prevalent in Punjab abattoirs.

The 16S rRNA PCR (1500bp amplicons, A260/A280 1.8-2.0) and Sanger sequencing confirmed a definitive species identification, which stands based on prior Pakistani detection studies. Initially, Idris *et al*. (2023) amplified cctA (516bp) with the help of Punjab clinical samples without pure culture and the diagnostic sensitivity was obtained. It is upon this that we base our study, which combines the use of culture-based isolation with an in-depth toxinotyping that establishes baseline strain diversity that is not available in PCR-only surveys. Their suitability to monitor Pakistani is confirmed by the ability to use 8FLP/XB4 success with universal 16S rRNA primers and the presence of toxin-related genes, which are necessary in vaccine antigen development.

Phylogenetic analysis identified a monophyletic clade (93% bootstrap support) of five Punjab isolates (PX450090-94) and Indian (JF414043) and Swiss (NR_026104) references that diverged between Japanese strains. It is the first Punjab-specific phylogeny that seals the geographic gap identified by Nicholson *et al*. (2019) as missing Asian *C. chauvoei* genomic information. The narrow range (99.2-99.8% pairwise identity) suggests that there is low intraspecific diversity in Pakistan, whereas the scattered Japanese distribution is suggestive of higher East Asian diversity (Frey *et al*., 2012). This local ancestry also aids the evolution of vaccine strains unique to South Asia, because the antigenic drift of European/African reference strains could be the cause of the current BQV field failures.

The optimization of the alpha toxin is the main novelty of the study, which measures the interactive temperature, pH and incubation time effects on hemolytic activity of *C. chauvoei* for the first time in Pakistan. Optimal production occurred at 37°C, pH 7.2, 24h (BQ1: 2048.19 ± 0.01HU/mL), exceeding Negru *et al*. (2022; 1200HU/mL) and Cortinas *et al*. (1994; 1500HU/mL). This peak coincides with the growth optimum reported by Rais *et al*. (2016) but is innovative by setting the 24h production window preceding proteolytic degradation (>48h). The 6-fold increase in toxigenicity of BQ1 as compared to BQ5 represents a high-virulence candidate strain, whereas the uniformity of negative outcomes of hyperthermia (42-44 C) and alkalinity (pH 8.0) indicates physicochemical instability reported regionally.

These findings directly address Pakistan's blackleg vaccine crisis. Locally made Black Quarter Vaccine (BQV; OVI strain, Onderstepoort origin) has 70% protection in controlled trials but a <40% field efficacy despite 65% calf coverage (L&DD Punjab, 2023). The increased toxigenicity of BQ1 (2048HU/mL vs OVI baseline of about 1200HU/mL) offers mechanistic insight into breakthrough outbreaks, in which the prevailing antigens do not adequately represent local virulence potential. The optimal commercial production time is determined through the 24-hour harvest window, and the bioreactor conditions are optimized to produce autogenous vaccines against PKR 13.5 billion annual losses in Punjab (30M cattle + 27M buffalo × PKR 150,000 × 3-5% incidence) (Shahzad, 2022; Sher *et al*.,

Comparative toxinotyping goes beyond the PCR detection of Idrees *et al*. (2023) to quantify haemolytic units to clinical virulence and vaccine efficacy. As they determined cctA presence, our work quantifies expression potential in native conditions, defining hyperproducers that question current immunization. The baseline will allow longitudinal monitoring of the toxins development and testing the effects of cold chain on the stability of antigens and would allow precise surveillance, autogenous vaccine development, and targeted interventions to reduce the high economic burden of livestock in Pakistan.

Conclusions: This study presents the first complete characterization and alpha toxinotyping of local *C. chauvoei* isolates of blackleg outbreaks in Punjab, Pakistan. During survey pathological cases with characteristic muscle necrosis, crepitant edema and hemorrhages, five high-purity isolates were obtained, confirmed using 16S rRNA phylogeny and toxinotyped due to haemolytic activity. Isolate BQ1 was found to be extraordinarily virulent (2048HU/mL at 37ºC, pH 7.2, 24h) and a hyperproducer that challenges existing BQV

efficacy. These results provide the baseline strain diversity of PKR 13.5 billion yearly losses. Future directions: Develop BQ1-based autogenous vaccines, introduce 24h antigen harvest protocols, and create UVAS toxinotyping to use national surveillance to improve immunization against regional strains.

Authors Contribution: NT and AG conceived and designed the study. NT, TY, and MI executed the experiments and analysed the sera and tissue samples. MI analysed the data. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

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