Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/zvd1gb30

PREVALENCE, MOLECULAR IDENTIFICATION, AND CONTROL MEASURES OF CLOSTRIDIUM PERFRINGENS ON CALF DIARRHEA IN PAKISTAN

Sehrish Khan[1], Jawaria Ali Khan[*1], Muhammad Avais[1] and Masood Rabbani[2]

[1]Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan.
[2]Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.
Email: subtle.vet.doctor@gmail.com, jawaria.khan@uvas.edu.pk, mavais@uvas.edu.pk and mrabbani@uvas.edu.pk.

***Corresponding Author:** Dr. Jawaria Ali Khan,
Associate Professor/Chairperson at Department of Veterinary Medicine, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore 54000, Pakistan., Phone: + 92 300 4104696, ***Email Address:** jawaria.khan@uvas.edu.pk

ABSTRACT:
This study surveyed the frequency, identification, and antibiotic resistance patterns of *Clostridium perfringens* in calves with diarrhea in Jhelum District, Pakistan. *Clostridium perfringens*, a gram-positive, spore-forming bacterium, causes enteric diseases in livestock, leading to significant losses. To assess this pathogen's impact, 200 fecal samples from suspected diarrheic calves (n=100) and buffalo (n=100) were collected. Preliminary screening using "FASTest® *Cl. perfringens* toxin" diagnostic kits confirmed 90% of the samples as positive. Subsequent culturing at MML and UVAS Lahore showed an overall positivity rate of 75%, with a higher prevalence in cow calves (82%) compared to buffalo calves (68%). Molecular identification using PCR detected specific toxin genes in sources feeding on ovine animals, revealing *Cl. perfringens* type A as the predominant toxinotype in both cow and buffalo calves. The alpha toxin gene (*cpa* and *etx*) was identified in 120 isolates, indicating its significant role in infection morbidity. Antibiotic resistance was assessed using the Kirby–Bauer disc diffusion method, showing high resistance among isolates. Notably, *Cl. perfringens* exhibited significant resistance to tetracycline and clindamycin but was sensitive to ampicillin, vancomycin, and metronidazole. These findings underscore the importance of selecting appropriate antibiotics and addressing antibiotic resistance. Effective management and control strategies are crucial to reducing economic losses from *Cl. perfringens* in livestock. Special vaccination schedules and improved parlour management are recommended to minimize disease occurrence. Future research should explore additional control measures and investigate the pathogenicity and resistance mechanisms of *Cl. perfringens*.

INTRODUCTION:

Livestock is vital subsector of Pakistan; livestock share 62.68 % in agriculture sector with the growth rate of 3.78 percent in last year. Livestock contribute 14.36 percent in national GDP. Pakistan boasts a considerable population of livestock and is renowned for its favorable environmental conditions. The country is home to a substantial number of cattle, with approximately 55.5 million head, and a substantial number of buffalos, with approximately 45 million head. Additionally, there are

approximately 84.7 million goats, 32.3 million sheep, and 1.1 million camels (1). Livestock play a critical role in the food production industry, as they are capable of converting grasses into high-quality food products such as meat and milk (2).

Cl. perfringens is a spore-forming anaerobic Gram-positive bacterium that causes a variety of diseases in mammals and birds, as well as a normal inhabitant of human and animal intestines (3). *Cl. perfringens* is classified into five categories based on toxin production: A, B, C, D, E, and toxins alpha, beta, epsilon, and iota. Diagnosis of enterotoxemia caused by *Cl. perfringens* is based on clinical signs or post mortem lesion as confirmation cannot be made without laboratory confirmation (4, 5).

The prevention of enterotoxaemia requires regular vaccination and nutritional strategies of all animals in the herd (6). Effective immunization plans with high immunogenic powered vaccines are capable of protecting calves against *Cl. perfringens* infections (7). Vaccination is recommended in the prophylaxis against *Cl. perfringens* infections. Diarrhea caused by *Cl. perfringens* can be prevented by good management and control instead of treating sick animals. Take careful care of the farm to prevent the spread of pathogens (3). To get a clear diagnosis and manage the risk factors linked with diarrhoea in current industrial production systems, it is essential to seek out regular consultations and advice from qualified doctors and nutritionists (8, 9).

The current immunization strategy for these diseases does not reduce these losses to a minimum. The lack of information about the specific forms of *Cl. perfringens* that cause these disorders is one factor contributing to this failure (10, 11). The objective of the study was to ascertain the incidence of distinct *Cl. perfringens* toxinotypes, the profile of antibiotic resistance, and the risk factors associated with diarrheal cow and buffalo calves. In order to detect different *Cl. perfringens* toxinotypes and evaluate the in vitro effectiveness of antibiotics against known *Cl. perfringens* toxinotypes from diarrheal samples of cow and buffalo calves in District Jehlum, Pakistan, the following study was designed. This molecular identification may provide important information that will aid in the management and treatment of clostridial disease(s) in the community.

MATERIAL AND METHOD:

1. Sample Collection:

A total of N=200 fecal samples were taken from cow (n=100) and buffalo (n=100) calves in district Jhelum after each calf was checked for the predetermined criteria.

I. Collection Process:

Aseptic procedures were followed in the collection of 5 grams of diarrheal feces from the rectum, which were then transferred into a sterile container to preserve the cold chain. "FASTest® *Cl. perfringens* Toxin" commercial kits were used to screen the feces samples. Following the first screening, samples that tested positive for *Cl. perfringens* were sent for further confirmation to the University of Veterinary and Animal Sciences, Lahore's Microbiology Laboratory.

II. Screening of diarrheic calves for *Clostridium perfringens*:

The calves with the above said signs were further screened using “FASTest® *Cl. perfringens* Toxin” commercially available kit. There was a buffer diluent in the sample tube. To create a homogenous solution, a standard sample volume—compact: one level spoon, pulpy: two level spoons, fluid-watery: three level spoons of feces—was collected and gradually mixed into the buffer diluent using a vortex. A pink-purple test line with varied intensities (from very weak to highly intense) and a pink-purple control line appear. Only a pink-purple control line is visible. This line shows that the test was carried out correctly, regardless of its intensity. There is no visible control line. The test should be redone with a different dipstick (12, 13).

III. Identification and Isolation of the Samples:

The positive samples after screening were regional to the Microbiology laboratory at the University of Veterinary and Animal Sciences at Lahore. Under a microscope, Gram's staining is a helpful

method for examining the characteristics of bacteria. After incubation, reagents A and B were pipetted in the tubes and the contents were mixed thoroughly by shaking. The test was wrong at the instance that the colour changed from Pink to Red (14).

2. Molecular Characterisation of *Cl. perfringens* isolates:

To check the kind of *Cl. perfringens* that produces toxins, *Cl. perfringens* isolates underwent further processing for molecular confirmation, optimisation and preservation. To provide the best possible long-term preservation, the culture plate isolates were spread out in Robertson cooked meat medium (Oxoid UK) (15, 16).

I. DNA Extraction

The bacteria in these tubes grew as much as possible before DNA extraction by being kept in a shaking incubator for twenty-four hours. Following the manufacturer's instructions, a genomic DNA purification kit (Thermo Fisher Scientific, Lithuania) was used to extract DNA. Using a Nano-drop 260/280 nm wavelength, the extracted DNA's amount and purity were assessed, and the material was preserved at -20°C (17).

II. PCR and Gel Electrophoresis:

The ability of each of the six distinct primer sets for each *Cl. perfringens* toxin to differentiate between various bacterial strains was examined as shows in Table 1. The 96-well thermal cycler (Applied Biosystems, Singapore) was used to carry out the PCR described above. The thermal processes, stages, and cycles used. To transfer DNA across the agarose gel, a 400-mA current at 120 volts was supplied for 40 minutes (18). To make the gel visible, a UV light illuminator was put on it. The size of each sample product was ascertained by comparing the bands of the samples with the bands of the ladder (19).

Table 1: *Showing toxin genes with respective primer sequences.*

Gene	**Primers**	**Nucleotide Sequences 5'-3'**	**Size(bp)**	**Types of *Cl. perfringens***
Cpa (*α* toxin)	CPAlphaF CPAlphaR	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324 pb	A, B, C, D and E
Cpb (*β* toxin)	CPBetaF CPBetaR	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196 pb	B and C
Etx (epsilon toxin)	ETXnteroF ETXnteroR	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233 pb	B and D
iA (iota toxin)	CPIotaF CPIotaR	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446 pb	E
Etx (enterotoxin)	ETXpsilonF ETXpsilonR	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655 pb	Untyped
Cpb2 (beta2 toxin)	CPBeta2F CPBeta2R	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	567 pb	Untyped

III. Nucleotide Sequencing:

The alpha genes of *Cl. perfringens* strain A were sequenced using genomic DNA. The epsilon toxin gene was sequenced using *Cl. perfringens* strain D. These three toxin genes were sequenced using the Sanger dideoxy technique. For sequencing analysis of the alpha and epsilon toxin gene, PCR was performed with same set of primers that used for PCR analysis. Sequencing was performed at the CAMB sequencing facility at the University of Lahore, Lahore, Pakistan. The NCBI database was searched for related or comparable sequences using the Basic Local Alignment Search Tool (BLAST) (http: //www.ncbi.nlm.nih.gov/BLAST/). To match the submitted sequences with those in the

database, and identify the degree of similarity between the sequences, the analysis involved the use of ClustalW tool (20, 21).

IV. Antibiotic Susceptibility Testing:

Twelve Clostridium perfringens isolates were tested for antibiotic sensitivity to penicillin, ampicillin, tetracycline, vancomycin, clindamycin, and metronidazole using the Kirby Bauer Disc Diffusion method, following CLSI 2019 guidelines. A 0.5-McFarland standard was prepared by mixing 0.05 mL of 1.175% BaCl2.2H2O solution with 9.95 mL of 1% H2SO4. Mueller Hinton agar (MHA), a non-differential and non-selective medium with a pH of 7.3±0.1, was used for the antibiotic susceptibility test. Variations in pH can affect the potency of antibiotics like tetracycline (increased at pH < 7.2) and aminoglycosides (decreased at pH < 7.2) (22). The Kirby Bauer disk diffusion technique by the Clinical Laboratory Standards Institute (CLSI) was employed for determining bacterial sensitivity or resistance. Antimicrobial testing was conducted on MHA using 0.5 McFarland standards, an antibiotic dispenser, and caliper measurements of the inhibition zones (23).

V. Haemato-biochemical Parameters:

Targeted blood samples were taken from 10 healthy animals (n = 5 cow calves and n = 5 buffalo calves) as well as 10 infected animals (n = 5 cow calves and n = 5 buffalo calves) that tested positive for *Cl. perfringens* during initial screening. In the haematological examination, TEC was done by using Beckman Coulter (USA) haematological analyzer, while TLC, platelet count and PCV was estimated using photometer. Only those animals with confirmed *Cl. perfringens* type D through PCR in sample of their blood were included in the sampling if it was from a sick animal (24).

VI. Biochemical Analysis:

To encourage coagulation, both the infected and uninfitted blood samples collected from the calves were preserved in dry clean test tubes labelled accordingly and were placed at 450-degree room temperature for a period of 20 minutes. The separated serum was then aliquoted and stored at -20°C on a clean test tube with appropriate label. A spectrophotometer and the relevant kits (Bio-diagonestic, Cairo, Egypt) were utilized to determine ALT and AST. Conforming to the guidelines of the manufacturer, a commerially available kit (Human, Germany) was employed for the determination of serum blood urea and creatinine.

VII.Risk factor analysis:

A developed questionnaire (dichotomous type) was used to research the disease's risk variables. Seasons, age, sex, area and essential components were all the subjects of data collection (25). Using this method and these findings, the percentage occurrence of *Cl. perfringens* was computed and is given below:

$$Prevalence\ (\%) = \frac{No.of\ samples\ positive}{no.of\ samples\ tested}$$

3. Statistical analysis:

Data analysis was done using the Chi-square test statistical tool for social science (SPSS). The haemato-biochemical data were analysed using the Student's t-test; a p-value of less than 0.05 was considered significant. A p-value of less than 0.05 was considered significant when the data on risk factor relationships were analyzed using the odd's ratio (OR) (26).

RESULTS

1. Initial Screening and Identification:

Initially, "FASTest® *Cl. perfringens* Toxin" commercial kits were used to screen the fecal samples. On the TSC media plates, colonies of *Cl. perfringens* appeared to be small blackish in color as usual hence thought to have confirmed the species. Since the bacteria being studied causes botulism, egg yolk was incorporated into the medium to assess the lecithinase activity. The activity of the black

colonies of *Cl. perfringens* was indicated by an opaque white zone around the bacterial colonies (Figure 1).

Figure 1: Showing black colonies of *Cl. perfringens* on TSC media

The results showed 90% (180/200) and 75% (150/200) occurrence of *Cl. perfringens* in study area through kit and culturing method respectively. After culturing, occurrence of *Cl. perfringens* in cow and buffalo calves was 82% (82/100) and 68% (68/100), respectively as shown in Table 1.

Species	**No of samples**	**Kit positive samples**	**Culturing positive samples**	**Odd Ratio (OR)**	**95% C.I**	**Chi Square**	**P-value**
Cow calves	100	90	82	1.09	0.72-1.63	0.155	0.694
Buffalo calves	100	90	68	1.10	0.72-1.69	0.200	0.655

Table 1: Showing Occurrence of Clostridium perfringens through diagnostic screening Kits test and culturing method.

2. Gram's staining:

The blackish colony of *Cl. perfringens* was streaked on fresh blood agar plate to make a thin smear and the slide was allowed to dry. Having sampled *Cl. perfringens*, the next method done was the Gram's staining procedure. During the Gram staining, *Cl. perfringens* retained the purple colour and on examination through 100X oil immersed light microscope, the bacterium was observed as violet coloured and rod-shaped bacilli as confirmed in Figure 2.

Figure 2: Showing rod shape colonies of *Cl. perfringens* under 100X microscopic lens

3. Nitrate Reduction Test:

By converting nitrate into nitrite, *Cl. perfringens* ability to reduce nitrate was tested. Thus, a nitrate reduction test was run to determine the presence of *Cl. perfringens*. Reagents A and B were introduced to the test tube culture after an overnight cultural growth in nitrate broth. Test tubes that tested positive for *Cl. perfringens* are red, but test tubes that were negative do not change colour (Figure 3).

Figure 3: Showing *Cl. perfringens* appearing as a red colour after adding reagents.

4. Molecular identification and confirmation of *Cl. perfringens*:

Randomly selected 120 isolates of *Cl. perfringens* type A out of 200 samples underwent PCR for cpa (alpha toxin) and all of them were positive (Supplementary Figure 1). All isolates of *Cl. perfringens* type D out of 100 samples were confirmed by etx (epsilon toxin) PCR (Supplementary Figure 2). Strain typing analysis of *Cl. perfringens* isolates showed that, 55% of cow calves and 45% of buffalo calves possessed *Cl. perfringens* type A, whereas 53.33 % cow calves and 46.66 % buffalo calves possessed *Cl. perfringens* type D. The toxinotypes of the *Cl. perfringens* isolates from the fecal samples collected from cow and buffalo calves are shown in Table 2. It came out that none of the *Cl. perfringens* isolates were positive for types B, C, and E.

Table 2: *Showing The distribution of different toxin genes of Cl. perfringens in cow and buffalo calves.*

***Cl. perfringens* Types**	**Toxin gene**	**No. of isolate**	**Isolates in cow calves (%)**	**Isolates in buffalo Calves (%)**	**Occurrence (%)**
A	*cpa (α)*	120	55	45	80.00
B	*cpa (α), cpb (β), etx*	0.00	0.00	0.00	0.00
C	*cpa (α), cpb (β)*	0.00	0.00	0.00	0.00
D	*cpa (α), etx (ε)*	30	53.33	46.66	20.00
E	*cpa (α), iap (ι)*	0.00	0.00	0.00	0.00
Total		**150**			**100**

5. Comparison of the toxinotypes of *Cl. perfringens* isolates among different species

On analyzing data in relation to calves and using statistics based on this data it has been revealed that the cow calves possess a higher potential of disease dynamics which was found to be 54/150 affected by *Cl. perfringens* infection. The odds ratio (OR=1. 22; 95% CI=0. 79-1. 89) showed the highest capability for inducing the disease among the subject type was Type A, then Type D as shows in Table 3.

Table 3: *Showing species wise occurrence of toxinotypes of Cl. perfringens isolates.*

Cl. perfringens types	**Toxin gene**	**Absolute frequency**	**Relative frequency**	**Cow calves**	**Buffalo calves**	**Odd Ratio (OR)**	**95% CI**	**Chi-Square**	**P-value**
A	*cpa* (α)	120	80 %	66 (55%)	54 (45 %)	1.22	0.79-1.89	0.801	0.371
D	*cpa* (α), *etx* (ε)	30	20 %	16 (53.3 %)	14 (46.6%)	1.14	0.48-2.72	0.089	0.766

6. Occurrence of *Cl. perfringens* Types A and D in Cow and Buffalo calves:

I. Tehsil wise occurrence:

Regarding *Cl. perfringens* type A, it was higher in Dina tehsil (34.84%) as compared to other tehsils such as Sohawa, Pind Dadan Khan and Jhelum. On the other hand, *Cl. perfringens* type D was high in Jhelum (50%) while it was low in Sohawa (31.25%%), Pind Dadan Khan (12. Cognitive comparison showed that there is no statically significant difference for the appearance of *Cl. perfringens* type A ($p > 0.05$) in four tehsils. Likewise, equal densities for *Cl. perfringens* type D counts also did not pose any significant variation among all these different tehsils ($P>0.05$). Enumeration of toxinotypes of *Cl. perfringens* isolates from cow calves in 4 tehsils has been summarised in Supplementary Table 1. The occurrence of *Cl. perfringens* type A in buffalo calves was recorded higher in Sohawa (38.88 %) as compared to other tehsils like Jehlum (22.22%), Pind Dadan Khan (20.37%) and Dina (18.5%) while the occurrence of *Cl. perfringens* type D was recorded higher in Jhelum (50%) as compared to other tehsils. Distribution of toxinotypes of *Cl. perfringens* isolates in four tehsils from cow calves has been documented in Supplementary Table 2.

II. Age-wise occurrence

Age-wise, the calves were split into three groups: 1D to 2 M, 2 to 4 M, and 4 to 6 M. Upon splitting the data into these three age groups, it was shown that calves between the ages of 4 and 6 months had a higher incidence of *Cl. perfringens* infections compared to the other two age groups. Occurrence of Type A (*cpa*) was 35.8% in age group 1 D to 2 M and 4 to 6 M and occurrence of Type D *(cpa*, *etx*) was 36.6% in age group 4 to 6 M that is comparatively higher than the other two age groups have been documented in Supplementary Table 3.

III. Sex wise occurrence

Cl. perfringens was found more frequently in the calves of female cows (55.5%) and buffaloes (44.44%) than in the calves of male cows (53.3%) and buffaloes (46.6%). Supplementary Table 4 shows the distribution of *Cl. perfringens* isolate toxinotypes in male and female calves.

IV. Season wise percentage occurrence

Summer had the highest frequency of *Cl. perfringens* type A in calves (28.33%), followed by spring (27.50%) and winter (25%); autumn had the lowest frequency (19.16%). *Cl. perfringens* type D was found in calves most frequently in the winter (30%), followed by the fall (26.66%) and summer (23.33%), with the lowest frequency (20%) seen in the spring have been documented in Supplementary Table 5.

7. Occurrence of toxinotypes of *Cl. perfringens* isolates:

I. Tehsil wise occurrence

Cl. perfringens type A was found more frequently in Dina (27.5%) compared to the other three tehsils (24.6%), whereas *Cl. perfringens* type D was found more frequently in Jhelum (40%) compared to other tehsils such as Sohawa (30%), Pind Dadan Khan (20%), and Dina (10%). Between the four tehsils, there was no discernible variation in the prevalence of *Cl. perfringens* type A ($p>0.05$). Comparably, there was no discernible variation in the prevalence of *Cl. perfringens* type D ($P>0.05$) among all tehsils. Table 4 shows the distribution of the toxinotypes of the *Cl. perfringens* isolates in two tehsils from cow and buffalo calves.

Table 4: *Tehsil wise occurrence of toxinotypes of Cl. perfringens isolates*

Cl. perfringens type	**Toxin gene**	**Absolute frequency**	**Relative frequency (%)**	**Jhelum**	**Pind dadan khan**	**Sohawa**	**Dina**	**Chi Square**	**P-value**
A	*cpa* (α)	120	80 %	24.16%	24.16%	24.16%	27.50%	0.32	0.9570
D	*cpa* (α), *etx* (ε)	30	20 %	40%	20%	30%	10%	4.86	0.1820

II. Sex wise occurrence

Comparing female calves (60%) to male calves (40%), the incidence of *Cl. perfringens* type A was found to be higher. *Cl. perfringens* type D was also found in higher concentrations in female calves (60%) compared to male calves (40%). Table 5 lists the toxinotype distribution of *Cl. perfringens* isolates in both male and female calves. Between male and female calves, there was no discernible difference in the prevalence of *Cl. perfringens* types A and D ($P>0.05$).

Table 5: *Sex wise percentage occurrence of toxino-types of Cl. perfringens isolates from cows and buffalo calves*

Cl. perfringens type	**Toxin gene**	**Absolute frequency**	**Relative frequency (%)**	**Male calves**	**Female calves**	**Odd Ratio (OR)**	**95% CI**	**Chi Square**	**P-value**
A	*cpa* (α)	120	80 %	40%	60%	1.5	0.96-2.34	3.214	0.073
D	*cpa* (α), *etx* (ε)	30	20 %	40%	60%	1.5	0.62-3.61	0.804	0.370

8. Sequencing Analysis of *cpa* and *etx* genes:

The cpa and etx genes amplified to produce PCR products that were around 324 bp and 233 bp, respectively. The study's isolates' nucleotide sequences were compared to *Cl. perfringens* reference sequences that were obtained from GenBank. The sequences obtained from this investigation have been entered into the GenBank database with accession codes OQ605871, OQ605872, and OQ605873 for cpa and OQ658825, OQ658826, and OQ658827 for etx. Comparison of one sequence with other sequences is shown in Supplementary Table 6 and 7.

9. Anti-bio gram of *Cl. perfringens* Against Different Antibiotics:

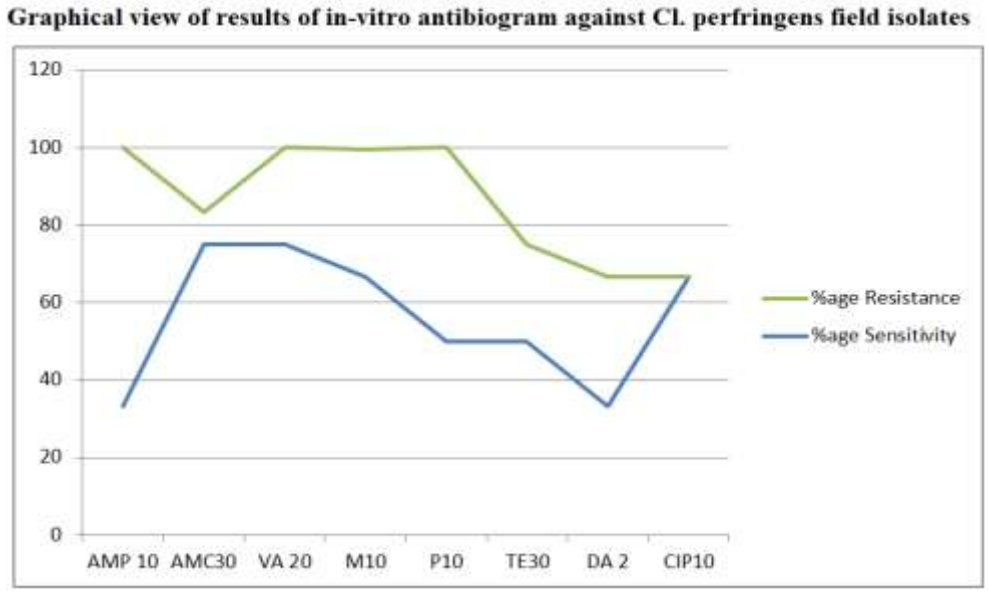
A total of eight antibiotics were examined for their sensitivity and resistance to twelve distinct isolates of *Cl. perfringens* that were obtained from the diarrheal cows and buffalo calves. A test for antibiotic susceptibility revealed that ampicillin had the highest level of resistance, 66.66%. Penicillin was the second antibiotic against which 50% isolates were resistant followed by metronidazole and clindamycin against which 33.33% isolates were resistant as stylized. A total number of 9 (75%)

isolates showed highest sensitivity to amoxicillin/clavulanic acid and vancomycin while 8 (66.66%) isolates showed sensitivity to both metronidazole and ciprofloxacin. Only 4 (33.33%) isolates showed susceptibility against penicillin and tetracycline as shown in Figure 4.

Table 6: *Showing Results of in-vitro antibiogram against Cl. perfringens field isolates*

Antibiotics	**%age Sensitivity**	**%age Intermediate**	**%age Resistance**
Ampicillin 10	33.33	-	66.66
Amoxicillin/Clavulanic Acid 30	75.00	16.66	8.30
Vancomycin 20	75.00	-	25.00
Metronidazole 10	66.66	-	33.00
Penicillin 10	50.00	-	50.00
Tetracycline 30	50.00	25.00	25.00
Clindamycin 20	33.33	33.33	33.33
Ciprofloxacin 10	66.66	33.33	-

Figure 4: Anti-biotic disc diffusion plates of Clostridium perfringens showing zones of inhibition



Graph 1: Showing The graphical presentation of the results of in-vitro antibiogram against *Cl. perfringens* field isolates

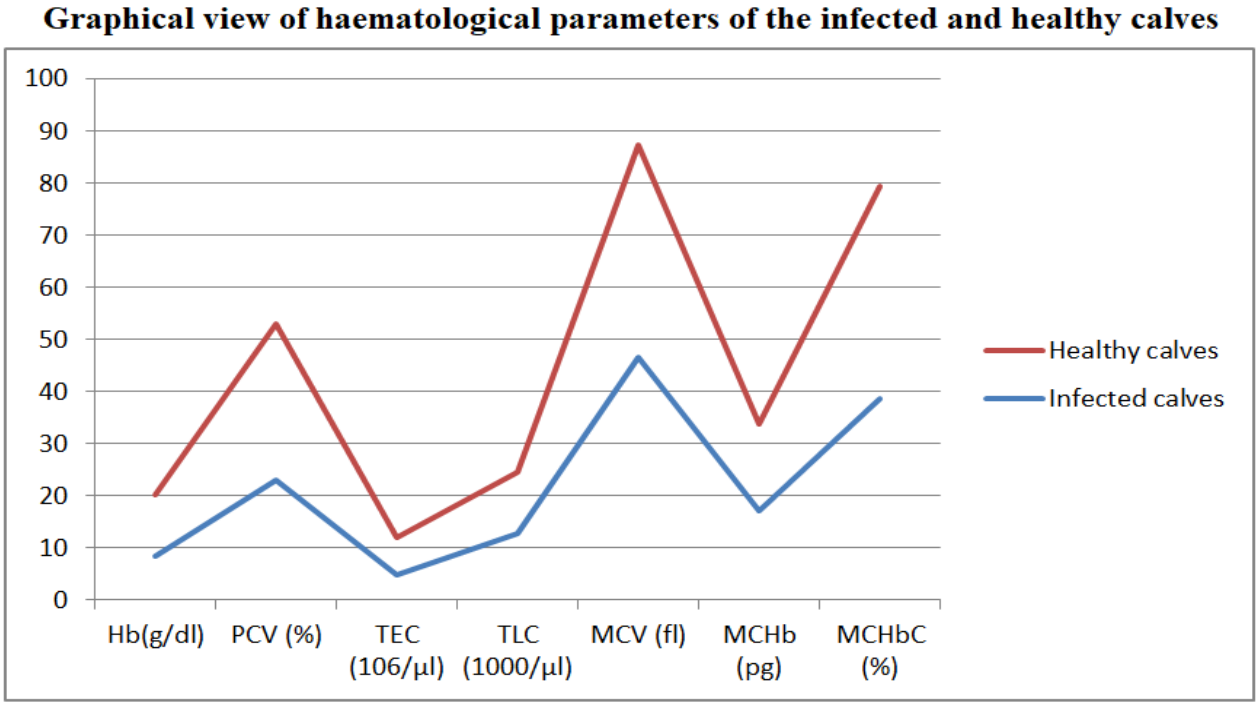
4.6: Haemato-biochemical Parameters of Cow and Buffalo Calves:

Among the blood tests conducted, Hb, PCV, TEC, TLC, LFT, and RFT were some of the tested blood biomarkers. In order to ensure that the group of calves that fell under the category of infected does not possess healthy values for haematology and serum biochemistry, data obtained from the infected and healthy calves was compared. As for the Haemoglobin (g/dL) in healthy calves, it was relatively high at 11. 670±0. 567 while in infected calves it was much lower, at 08. 44±0. 824 (Graph 1). Therefore, mean Hb concentrations between both the healthy and infected calves were revealed to have significant differences ($p<0.05$). Thus, the percentage of carrier state as PCV (%) in healthy calves was 29. 75±0. 134 while in infected calves was 23. 12±0. 374 and statistically differed ($p<0.05$) as depicted in Table 7.

Table 7: *Showing Haematological parameters of the infected and healthy calves*

Blood Parameters	**Infected calves (Mean ± SE)**	**Healthy calves (Mean ± SE)**	**P-value**
Hb (g/dl)	08.44±0.824	11.67±0.567	0.0210
PCV (%)	23.12±0.374	29.75±0.134	0.000*
TEC (10^6/µl)	04.79±0.216	07.25±0.078	0.0136
TLC (1000/µl)	12.87±0.18	11.71±0.06	0.000*
MCV (6)	46.55±1.836	40.75±0.445	0.0001*
MCH (pg)	17.19±0.490	16.47±0.169	0.0405
MCHC (%)	38.67±0.556	40.53±0.196	0.0123

Data is indicated as Haemoglobin concentration (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC). While * shows the significant value.



Graph 2: The graphical presentation of haematological parameters of the infected and healthy calves

The above graph showed that the PCV (%) was decreased significantly ($p<0.5$). The TLC (1000/µl) and MCV (6) were increased significantly ($p<0.5$) while other parameters were not significantly affected as shown in Graph 2.

4.7: Biochemical Parameters of Cow and Buffalo Calves:

Serum levels of bilirubin and LFTs AST and altitude of calves infected with the virus were also analyzed and compared to healthy calves. The ALP of the diseased calves (45. 35 ± 541861 U/L) was significantly higher (Sp <0. 05) compared to the healthy calves (30. 2 ± 1768867 U/L). As such, the AST of both healthy and infected calves was significantly different where healthy calves recorded 73. 3 ± 8. 97286 while the infected calves 130. 4 ± 9. 948981 p < 0. 05. The Bilirubin concentration of healthy and infected calves was not significantly different where healthy calves recorded 0. 812 ± 0. 112198 and infected calf 1. 23 ± 0. 259936 as shows in Table 8.

Out of the six renal function tests that have been researched into, both infected and healthy calves have been studied on BUN and creatinine levels. Similarly, in the creatinine level (mg/dL) the infected calve had the value of 1. 870 ± 256926, whereas the healthy calve had 1. 7 ± 0. 187972, the difference was not significant (p>0. 05) However, BUN value has highly significant difference observed between healthy and infected calves i. e 2. 3 ± 3. 051593 and 47. 1.

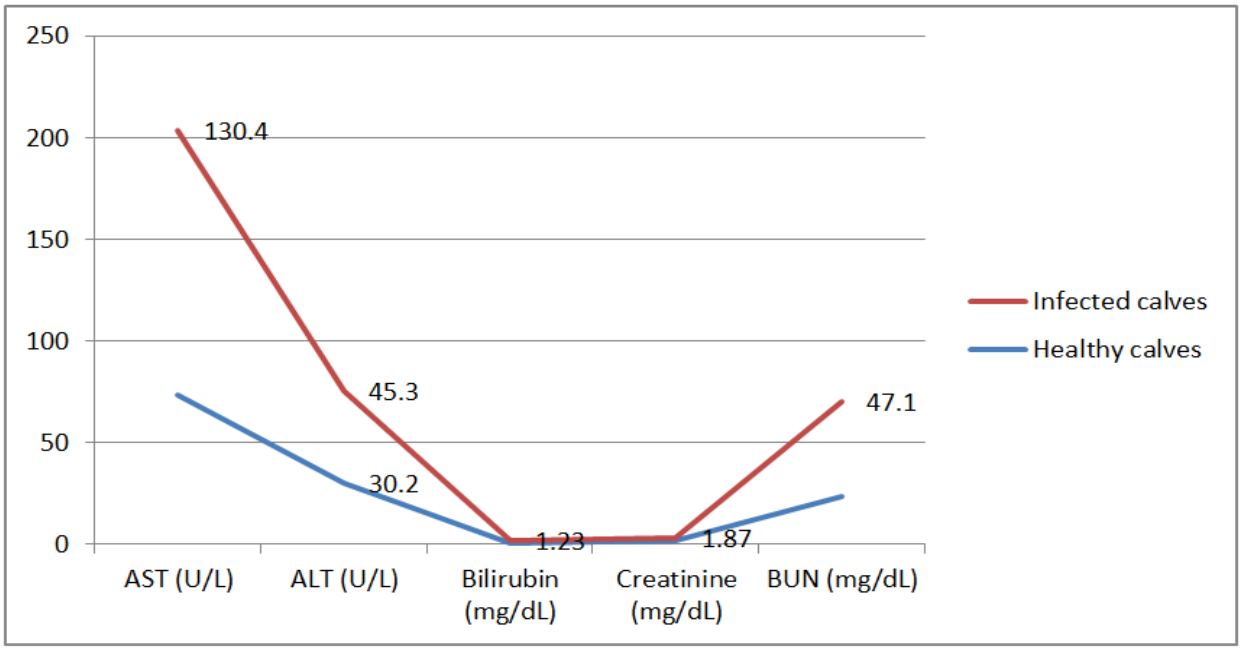
Table 8: *Biochemical parameters of the infected and healthy calves*

Parameters	Healthy calves (Mean ± SEM)	Infected calves (Mean ± SEM)	P-Value
AST (U/L)	73.3 ±8.97286	130.4±9.948981	0.000235*
ALT (U/L)	30.2±1.768867	45.3±5.541861	0.009132*
Bilirubin (mg/dL)	0.812±0.112198	1.23±0.259936	0.078556
Creatinine (mg/dL)	1.7±0.187972	1.87±0.256926	0.299934
BUN (mg/dL)	23.3±3.051593	47.1±5.400514	0.000604*

Data is indicated as AST stands for Aspartate Aminotransferase, ALT stands for Alanine Transaminase and BUN stands for Blood Urea Nitrogen. While * shows the significant value.

The graphical presentation of biochemical parameters of infected and healthy calves is shown in Graph 3.

Graphical view of biochemical parameters of the infected and healthy calves



Graph 3: The graphical presentation of biochemical parameters of the infected and healthy calves

The above graph demonstrated that whereas bilirubin (mg/dL) and creatinine (mg/dL) were not substantially (p>0.5) elevated, AST (U/L), ALT (U/L), and BUN (mg/dL) were.

DISCUSSION

It must be said that *Cl. perfringens* is a part of the normal gastrointestinal microbiota in both humans and animals, and the pathogenic properties manifest themselves in case of the disruption of the mucosa environment – for example, due to the intestinal stasis or rapidly changing feeding regime. This bacterium is on the increase and it forms several toxins, namely: Alpha toxin, Beta toxin, Epsilon toxin, and Iota toxin (27). Diarrhea with high levels of toxins in the intestine, deaths among animals, or an acute form of enterotoxemia are often characteristic of such a phenomenon. *Cl. perfringens* causes enterotoxaemia Enterotoxaemia is caused by Cl perfringens. Given the fact that the presently analyzed disease is common in small ruminants and associated with high patterns of mortality, the aforementioned essential disease leads to substantial losses in these animals all over the world (28).

To determine occurrence of *Cl. perfringens* in diarrheic calved, 200 fecal samples were samples were collected and analyzed using "FASTest® *Cl. perfringens* Toxin" kits. The level of bacterial quantity of C. perfringens is as such expected since the bacteria are normally located in the gut of the animal (29). Intestinal Clostridium perfringens (CFU/g) increases during illness and is higher in African children who have diarrhea (30). Fecal bacterial with cfu/g concentration of 10 7 have potential to differentiate the healthy Pale from the affected ones; nonetheless, it cannot be claimed that this is the ultimate criterion for identifying the sheep's illness (31, 32).

Clostridium perfingers is an anaerobic, Gram-positive, spore-forming bacillus that is normally carried in the intestine. In this state it is not incapacitating but becomes a causative agent of ailment only when it finds a favorable breeding ground to blossom as well as let out its poisons. Proliferation of this bacterium is another possible area of concern generated by the following risk factors (33, 34). The following is a list of other risk factors that this ongoing investigation looked into. High numbers of bacteria in the colon were found to be positively related to in male and female patients of all ages sampled at different times of the year and in different regions (35).

In Pakistan, *Clostridium perfringens* is developing resistance to antibiotics used in field (36). The only drugs identified in the current study as being very sensitive against *C. perfringens* were amoxicillin/clavulanic acid (75%) and Metronidazol (66.6%). The ampicillin and tetracycline were shown to be less effective (33.33% and 50%) against *Cl. perfringens*; penicillin and ciprofloxacin were found to be more effective (50% and 66.66%) against *Cl. perfringens.* In Pakistan, although amoxicillin was deemed an antibiotic resistant strain, *Cl. perfringens* was shown to be responsive to ciprofloxacin, chloramphenicol, metronidazole, and ceftriaxone.

CONCLUSIONS:

It was concluded that cow and buffalo calves in the district of Jhelum in the province of Punjab, Pakistan, had *Cl. perfringens* infections. Species, age, sex, season were all thought to be risk factors found to be less or more significantly associated with the development of *Cl. perfringens* infections. Moreover, it was determined that haematobiochemical changes could facilitate the identification of calves' Clostridium perfringens infections.

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