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Variation in Physiological Biomarkers with Different *Clostridium perfringens* Isolate Infections in Balkhi Sheep

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Authors' Contribution
MK conducted the project under the supervision of SBK. SA, IA, GR and MA helped in sample collection. KP IUK, AU, NU MU and SSAS helped in Lab work, statistical analysis and manuscript writing.

Key words
Bilirubin, Blood urea, *Clostridium perfringens*, Platelets, Serum Creatinine

ABSTRACT

The objective of this study was to determine variations of different physiological biomarkers in *Clostridium perfringens* (CP) infections in Balkhi breed of sheep in Khyber Pakhtunkhwa, Pakistan. Study was conducted on 184 sheep suspected of having enterotoxemia and 107 sheep were identified to be infected with *Clostridium perfringens*. Genotypic Analysis of all isolates from infected sheep was performed. Results showed, 53.27% isolates showed infection of *CP* type A, 10.28% of type B and 36.44% of type D. Animal infected with different serotypes (A, B, and C) were categorized into two groups healthy and diseased to compare the hematological and biochemical parameters. Hematobiochemical analysis indicated that mean erythrocyte count (RBC) and hemoglobin levels decreased while the mean leukocytes (WBC), platelets, packet cell volume (PCV) and total bilirubin levels increased significantly (P<0.05) in sheep infected with *Clostridium perfringens* type A. Sheep infected with *Clostridium perfringens* type B and type D showed significant (P<0.05) decreases in erythrocytes counts (RBC) and hemoglobin levels while the mean WBC, platelets, packet cell volume, serum creatinine, bilirubin, liver enzymes, total, glucose and urea in blood significantly (P<0.05) increased. Fluctuations in mean erythrocyte counts (RBC), hemoglobin, PCV and total bilirubin were beyond the limits while others were within normal ranges in sheep infected with *Clostridium perfringens* type A. The total bilirubin, Liver enzymes, creatinine level, glucose and urea levels in blood were abnormal while others were within normal ranges in sheep infected with *CP* type B and type D. by observing these changes it will be possible to diagnose disease at initial stages.

INTRODUCTION

Small ruminants face various health challenges including *Clostridium perfringens* (CP) induced enterotoxemia. The sickness is primarily grouped into five types (A to E) based on distribution of four major toxins i.e. alpha, beta, epsilon and iota (Uzal *et al*., 2014). Type A causes yellow lamb disease, type B lamb dysentery; type C struck, type D pulpy kidney and type E enterotoxemia respectively (Uzal and Songer., 2008). There are three types of enterotoxemia per acute, acute and chronic forms (Fernandez and Uzal., 2003). The syndrome is a common and endemic illness in goats and sheep in Pakistan (Khan *et al*., 2008; Tahir *et al*., 2013). It is rod shape, spore forming, gram positive anaerobic microbe that causes disease in animals and humans, primarily through the production of one or any combination of the four toxin types listed above. Certain factors are thought to contribute to be critical in stimulating toxin gene expression, for example, sudden change in feed, over ingestion of grains and rich green feed (Javed *et al*., 2009). During rainy season, access to rich green fields and high levels grain feeding regimens are considered as critical factor for this malady in small ruminants (Lewis, 2000). As of late polymerase chain response (PCR) is favored over other diagnosing strategies for the diagnosing of CP (Gkiourtzidis *et al*., 2001; Baums *et al*., 2004). CP toxins are absorbed by the small intestine, once in circulation; these toxins are disseminated throughout the host to target cells of numerous tissues, including liver, kidney, pulmonary, neurological, and intestinal epithelium

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(Ma *et al*., 2011). CP type D disease is characterized by changes in hematobiochemical parameters i.e. RBC, PCV, WBC, platelets, serum creatinine, blood glucose and urea levels (Nasir *et al*., 2013).

MATERIALS AND METHODS

Geographical area

This project was carried out in Khyber Pakhtunkhwa province, Pakistan from January to December 2016 in two different topographic regions; Swat (hilly) and Mardan (plain) districts. The Animal Ethical Committee approved this project via Ref. No. DAS/5121, dated: March 9, (2016).

Sample collection

The samples were obtained from cases brought to veterinary hospitals through convenient sampling. A total of 184 fecal samples were obtained from enterotoxaemia suspected Balkhi sheep. From which seven blood samples were collected from infected with each type of CP and twenty on from healthy as reference for biomarkers through convenient purposive sampling technique. These samples were collected from 6-12 months age Balkhi sheep having similar size and area. Healthy were only included which produced no growth on tryptose sulfite cycloserine agar media.

Bacteriological examination

Samples were collected with rectal swabs; these were placed in sterile air tight bottles and sent to the laboratory under 4°C (Nayel *et al*., 2013). The fecal samples were inoculated on tryptose sulfite cycloserine agar media (HiMedia Laboratories pvt Ltd. India) and incubated for 24 hours at 37°C in anaerobic jar with CO_2 packs (Oxoid Ltd). Initially the isolates were identified through colony morphology and Gram staining; followed by biochemical tests (Remel RapID ANA II test kit, USA). The isolates were quantified on blood agar to sort out pathogenic isolates from field cases of *CP* related enterotoxaemia (Philippeau *et al*., 2003).

DNA extraction

Before DNA extraction, isolates were inoculated in Robertson cooked meat medium and incubated at 37°C for 12 h in shaking incubator. DNA was extracted according to the protocol of DNA extraction kit (GeneAll, South Korea). Obtained DNA was quantified using NanoDrop (Nano Drop, 2000, Thermo-Scientifics, Wilmington, DE 19810 USA) and stored at -20 °C.

Confirmation of Clostridium perfringens *by PCR*

Genotyping of CP was performed through PCR, targeting four major toxins (α, β, ε, and ι), as described by Greco *et al*., (2005) in Table I. Briefly, the reaction was performed in a thermocycler (BIO RAD T 100) in 25µL volume, in micro amplification tubes (PCR tubes) containing 2XAmpmaster^{TMTaq} (Gene All Biotechnology CO. Ltd) 10µL, forward and reverse primers 1.5µL each, template DNA 5µL and DNA free distilled water 7µL. The amplification reactions consisted of 35 cycles, 5 minutes initial denaturation followed by 35 cycles; consisting of denaturation at 94^{0}C for 60 seconds, annealing at (α=60^{0}C, β=64^{0}C, ε=53.4^{0}C, ί=61^{0}C) for 30 seconds, extension at 72^{0}C for 60 seconds and final extension at 72^{0}C 5 for minutes. The PCR products (5 µL) along with DNA ladder of 1Kb (Genesta^{TM}) and optimized positive and negative control were run on 1.5% agarose gel for electrophoresis. Ethidium bromide (1.5µg/mL) was used for staining the gel before being photographed under UV light.

Hematobiochemical analysis

Six milli Liter blood samples each from infected and healthy Balkhi sheep were collected from the jugular vein using disposable syringes. The blood was transferred to two sterile vacutainers, one containing heparin. Total RBC, WBC, platelets count, hemoglobin levels, and PCV were analyzed through hematology analyzer (Beckman Coulter, USA) from heparin added blood. Heparin free coagulated blood was centrifuged at 1500 rpm for 20 minutes to collect serum for biochemical analysis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with spectrophotometer by using their test kits (Bio-Diagnostics, Cairo, Egypt) (Reitman and Frankel, 1975). Serum creatinine and urea were also measured by using their appropriate diagnostic kits (Human, Germany). Blood glucose level was measured directly on glucose strip (codefree^{TM}, Korea) and read with a Glucometer before the addition of anticoagulant.

Statistical analysis

Descriptive analysis was used to represent all parameters as mean ± Standard Error. Two tail *t*-test at the level of 95% confidence interval ($P<0.05$) was used for hematobiochemical comparisons between diseased and healthy animals through SPSS 21.0 version statistical program.

RESULTS

Clinical parameters

Clinically infected sheep showed the signs of abdominal pain, mild to severe (blood-tinged to bloody) yellow pasty diarrhea, frothy salivation and varying degree of nervous signs. *Clostridium perfringens* infected sheep

Table I. Primers used for PCR amplification of gene for *C. perfringens* toxins α, β, ε and i.

Toxin/gene	Oligonucleotide sequence	Fragment length (bp)	
α/cpa	5-TGC TAA TGTTAC TGC CGT TGA TAG-3 5-TGC TAA TGTTAC TGC CGT TGA TAG-3	247	Daube *et al*. (1994)
β/cpb	5-AAC TTA ACT GGA TTT ATG TCT TCA-3 5-ATA GTA GAA AAA TCA GGT TGG ACA-3	317	Kadra *et al*. (1999)
ε/etx	5-ATT AAA ATC ACA ATC ATT CAC TTG-3 5-CTT GTG AAG GGA CAT TAT GAG TAA-3	206	Daube *et al*. (1994)
i/iap	5 -TTT TAA CTA GTT CAT TTC CTA GTT A-3 5-TTT TTG TAT TCT TTT TCT CTA GGA TT-3	298	Daube *et al*. (1994)

Table II. Distributions of *Clostridium perfringens* types on the basis of the toxin genes from enterotoxemia infected Balkhi sheep.

C. perfringens types	Toxin genes of *C. perfringens*	Number of isolates	Percentage of isolates
A	Cpa	57	53.27%
B	*cpa*, *cpb*, *etx*	11	10.28%
C	*cpa*, *cpb*	0	0%
D	*cpa*, *etx*	39	36.44%
E	*cpa*, *iap*	0	0%
Total		107	100%

were dehydrated with pale mucous membranes and their mean body temperature was 40.1 ± 0.21^{0}C.

Bacteriological parameters

Growth of CP was identified as small black colonies on tryptose sulfite cycloserine agar media (HiMedia Laboratories pvt Ltd. India); identity was confirmed by Gram staining and biochemical testing panel results with test kit (remel-RapID ANA II system test kit, USA). Colony counts were quantified on blood agar and colony countsmore than 104–107 CFU/g were considered, as pathogenic. Balkhi sheep were only considered healthy when no growth was obtained on tryptose sulfite cycloserine agar media (Hi Media Laboratories pvt. Ltd. India) after 24 hours incubation at 37°C in anaerobic jar with CO_2 packs. Out of 184 suspected sheep,107 (58.15%) were identified infected with CP. Genotyping of 107 strains from infected sheep indicated 57(53.27%) infected with CP type A, 11 (10.28%) with type B and 39 (36.44%) with type D by conventional PCR however no case of type C and type E were found (Table I).

Hematobiochemical parameters

Mean hemoglobin levels and erythrocyte counts (RBC) decreased while the white blood cells (WBC), packed cell volume (PCV), platelet counts and total bilirubin increased significantly (P<0.05) in CP type A infected sheep (Tables III and IV). In CP type B and type D infected sheep mean erythrocytes count (RBC) and hemoglobin levels decreased while white blood cells (WBC), packed cell volume (PCV), platelets count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, serum creatinine, blood glucose and urea increased significantly (P<0.05). Fluctuations in mean hemoglobin level, erythrocyte counts (RBC), packed cell volume (PCV) and total bilirubin were beyond the normal limits while others were within normal ranges in CP type A infected sheep. Alanine Aminotransferase (ALT), total bilirubin, serum creatinine, blood urea and glucose were beyond the normal limits while others were within normal ranges in CP type B and type D infected sheep (Tables III and IV).

DISCUSSION

Clostridium perfringens is the normal occupant of the gastrointestinal tract in animals and people but turns pathogenic when gastrointestinal tract condition disturbed by sudden changes in feed, eating regimen or stasis in digestive system. The number of CP increases exponencially and production of distinctive toxins is increased many folds (Uzal, 1996). The sickness is associated with elevated amounts of toxins in the digestive system, with death of the animal or acute form of disease (Vaikosen and Ikhatua, 2005). Present study demonstrated 58.15% pathogenic isolates of CP from enterotoxemia suspected sheep. Out of them, 57 (53.27%) were CP type A, no. (10.28%) type B and no. (36.44%) type D, types C and E were not diagnosed. Itodo *et al*. (1986) depicted the pervasiveness of CP writes A (22.05%), type B (4.0%) and type D (15.75%) in their examination in Nigeria.

Table III: Haematological parameters (Mean± SE) of *C. perfringens* type A, B and D infected and healthy Balkhi sheep.

Parameters	Type A		Type B		Type D	
	Normal	Infected	Normal	Infected	Normal	Infected
Hemoglobin (g/dL)	9.81+0.15	6.61+0.13 *	10.03+0.15	9.15+ 0.18	9.81+0.17	8.42+0.35*
RBC (10^6 /µL)	11.47+0.15	7.64+0.09*	11.45+0.19	9.72+0.25*	11.47+0.15	9.77+0.19*
WBC (10^3/µL)	17.40+0.43	9.25+0.06*	9.15+0.03	18.04+0.96*	9.39+0.09	17.99+0.63*
Platelets (10^5/µL)	5.33+0.09	4.70+0.06*	4.63+.08	5.35+0.10	4.76+0.04	5.32+0.09*
PCV%	45.89+0.29	37.22+0.32	37.89+0.45	42.04+1.43	37.21+0.37	42.44+0.98*

[1]Alanine Aminotransferase, [2]Aspartate Aminotransferase; * at the value indicates the statistically significant difference between healthy and infected groups

Table IV: Biochemical parameters (Mean± SE) of *C. perfringens* type A, B and D infected and healthy Balkhi sheep.

Parameters	Type A		Type B		Type D	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
ALT[1] (U/L)	33.77+87	17.51+0.45*	14.76+0.13	37.97+0.67*	14.51+0.18	38.52+0.59*
AST[2] (U/L)	58.28+1.76	30.35+1.51*	27.36+0.77	78.73+0.81*	27.21+0.87	78.83+1.50*
Total bilirubin (mg/dL)	4.94+0.18	0.37+0.03*	0.37+0.02	4.48+0.21*	0.31+.03	4.49+0.13*
Blood glucose (mg/dL)	70.03+1.97	67.7+0.69*	67.57+0.73	134.55+3.67*	67.70+0.69	130.51+4.26*
Urea (mg/dL)	20.71+0.88	19.6+0.59*	19.69+0.42	66.49+1.51*	19.60+0.50	70.11+3.11*
Creatinine (mg/dL)	1.66+0.07	1.57+0.08*	1.50+0.04	5.66+0.26*	1.50+0.05	5.80+0.23*

[1]Alanine Aminotransferase, [2]Aspartate Aminotransferase; * at the value indicates the statistically significant difference between healthy and infected groups.

Their outcome demonstrated the presence of CP type A (40.62%), type B (3.125%), type C was (28.125%) and type D (28.125%). A few examinations conducted in different countries revealed the most predominant type A in sheep (Itodo *et al*., 1986; Efuntoye and Adetosoye, 2003). It is a typical view that CP types B, C, and D have major part of enterotoxaemia, while CP type A alone is not included or has extremely constrained impact. As of late, a few analysts have given an account of the significance of CP type A (Manteca *et al*., 2001). Greco *et al*. (2005) revealed that CP types A and D were recognized, 84% and 16% by PCR, in 87 sheep and 15 kids.

Results of our study have indicated pronounced anemia because of diminished erythrocyte check and decreased hemoglobin level while increase in leukocyte count, platelets count, and packed cell volume expanded in all CP type A, B and D infection in sheep when compared with sound sheep. Comparative discoveries were accounted for by Hassanein *et al*. (2017) in Egypt. In another study Nasir *et al*. (2013) reported similar findings following the experimental CP type D disease in sheep. Alpha toxin is phospholyphase having hemolytic and necrotizing while epsilon is lethal in nature (Quinn *et al*., 2004; Fatmawati *et al*., 2013). Comparable perceptions were noted by Smith (1975) in his investigation demonstrating the rise of acid soluble phosphate are because of alpha toxin. Alpha toxin hydrolyzes the phospholipids in the cell film of RBC's, causing its lysis. Our findings were supported by the findings of Ombe *et al*. (2006) who detailed that the presence of toxin restricting receptors on the surface of erythrocytes of different species is related with hemolysis. The alpha toxin likewise causes the lysis of leukocytes and platelets. This may be because of a similar mechanism, like RBC hemolysis. The increase in white platelets is considered as a guide in diagnosing the enterotoxemia in living organisms (Smith and Sherman, 1994; Dennis and Bryant, 2002), same results are obtained in present study. Clostridium chauvoei hemolysins receptors exist on RBSs surfaces in different species, examined by Ombe *et al*. (2006) additionally supported our findings. Increase in WBCs and platelets can help as supportive in the diagnosis of enterotoxaemia (Smith and Sherman, 1994; Dennis and Bryan, 2002). Increase in packed cell volume was because of lack of hydration which was noted in all infected sheep.

In our results mean AST, ALT and bilirubin increased significantly ($p<0.05$) in CP type A infected sheep while

the mean AST, ALT add up to bilirubin, serum creatinine, blood glucose and urea levels increased significantly ($p<0.05$) in both CP type B and D infected sheep. Our outcomes were strengthened by Radostitis *et al*. (2007), Hassanein *et al*. (2017) who have reported fluctuation in liver enzymes and bilirubin because of harm in hepatic tissue in enterotoxaemia. Hyperglycemia is steady analysis of enterotoxemia (Radostitis *et al*., 2007; Filho *et al*., 2009) which strengthened our findings. Alpha toxins are necrotizing in nature which are found in all CP type A, type B and type D infections causes damages of veins, liver and hemolysis (OIE, 2004). Mylashiro *et al*. (2007) showed that epsilon toxin produces renal and liver damages, gastroenteritis, and systemic hemorrhage in various organs upheld our results of expanded liver and kidneys enzymes are because of the harms created in these organs. Epsilon toxin of CP causes harms in the kidneys (Miyakawa *et al*., 2007; El-Ghareib and Amer, 2009; ElSify *et al*., 2016). *Clostridium perfringens* causes renal damage, which brings about serum creatinine and blood urea increase. The activity of CP toxin on kidneys particularly the epsilon toxin has likewise been exhibited by different researchers (Miyakawa *et al*., 2007; Heba *et al*., 2009).

CONCLUSION

All in all, the hematological and biochemical parameters varied significantly in all CP type A, B and D. however. the vast majority of these stayed within the normal range. Mean RBC counts, hemoglobin level, PCV and total bilirubin fluctuated beyond the normal limits in CP type A infected sheep while liver enzyme, total bilirubin, serum creatinine, blood urea and glucose levels fluctuated beyond the normal limits in CP type B and D infected sheep.

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Statement of conflict of interest

All authors declare no conflict of interest.

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