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HEMATOLOGICAL AND SOME BIOCHEMICAL ALTERATIONS IN SHEEP EXPERIMENTALLY INFECTED WITH *CLOSTRIDIUM PERFRINGENS* TYPE D INFECTION

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ABSTRACT

The study was designed to determine the hematological and biochemical changes in sheep following the experimental *Clostridium perfringens* type D infection. Mean leukocyte count was significantly higher ($p < 0.05$) between control and infected groups at 10 hours (11.2±0.3 vs 19.7±3.5), 20 hours (10.6±0.3 vs 16.9±0.2) and then started to decrease in infected group. A statistically significant increase ($p < 0.05$) was observed between the means within the infected for PCV at 30 hours. Mean blood glucose (74±6.7 vs 141±13.2), urea (26.3±3.5 vs 92.3±6.7) and serum creatinine 1.6±0.2 vs 7.5±1.4) levels were elevated significantly at 30 hours in group of experimentally infected animals as compared to control groups. The results of our study demonstrated that hematological values fluctuated after post experimental infection but almost remained within the normal range however, blood glucose, urea and serum creatinine levels increased significantly as the disease progressed and could be diagnostic under field conditions in animals suspected for type D enterotoxaemia.

Key words: *Clostridium perfringens* type D, Sheep, RBC, WBC, Platelets, PCV, blood glucose, urea, serum creatinine.

INTRODUCTION

Clostridium perfringens in particular is a leading microorganism involved in different diseases such as Pulpy kidney, Black quarter, Tetnus, necrotic enteritis in broiler chickens either by invading and damaging the tissues or by secreting different harmful substances (Akram *et al*., 2010). This organism is an anaerobe, Gram positive, non motile, bacterium having the ability to form heat resistant endospore. *C. perfringens* is grouped into five types (A-E) based on the production of four major lethal toxin alpha, beta, epsilon and iota (Sawires and Songer, 2006; Zerbini and Ossiprandi, 2009; Sayeed *et al.,* 2010)

Enterotoxaemia in sheep is a fatal enteric disease and is attributed to a toxigenic type of *Clostridium perfringens* type D worldwide (Lyerly *et al*., 2004) and is probably the significant cause of mortality in these animals of all ages (Blackwell and Buttler, 1992). This organism secretes lethal alpha and epsilon toxins which play major role in the production of the disease but epsilon toxin has a significant role in the pathogenesis of enterotoxaemia in ruminants (Popoff, 2011). Normally, epsilon toxin is produced in very minute amounts in the intestine of animals carrying *C. Perfringens* type D without any deleterious effects on the host but provoke an immune response for the formation of antibodies. Sudden dietary changes such as from green fodder to grains and reduction in intestinal motility are the predisposing factors for the enterotoxaemia (Smith and Sherman, 1994). As this disease has a wide range of clinical signs affecting almost all vital organs, so there are sufficient chances that particular disease can be confused with others having similar signs and symptoms such as *Cryptosporidium parvum, Corona virus, Enterotoxigenic E. coli* (Efuntoye and Adetosoye, 2003).

Presently, the diagnosis of enterotoxaemia is achieved by clinical and pathological findings, bacterial culture and enzyme linked immune-sorbent assay. These methods are time consuming and expensive. Mice inoculation and serum neutralization tests are also in practice but become increasingly undesirable due to high cost, complexity and inhuman (Garmory *et al*., 2000; Babe *et al*., 2012; Hadimli *et al.,* 2012). The analysis of hematology and serum biochemistry in small ruminants is not a normal practice and literature regarding this subject is very scanty. So, the present study was under taken in sheep by experimentally administration of *Clostridium perfringens* type D to determine the haemotological and biochemical changes occur with respect to the progress of the *C. perfringens* type D infection.

MATERIALS AND METHODS

Experimental Animals: Ten Kajli sheep having weight 8±2 Kg were allocated into two groups, six in group A (infected) while four in group B (control). These animals were not vaccinated nor their dams against enterotoxaemia. The animals in both groups were dewormed with Oxfendazole at the recommended dose

rate (Oxafax, Glaxowellcom) orally. The animals were observed for ten days before being used for experiments. Conventionally reared Albino Swiss mice (20-25g body weight) were used for mouse tests.

The project and animals used for the experimental purposes were approved by "The Advance Study and Research Board" of Unversity of Veterinary & Animal Sciencs, Lahore Pakistan.

Preparation of inoculums: The sample was collected in sterile, tightly closed container from intestinal scrapings on postmortem examination of sheep suspected for enterotoxaemia from Veterinary Research Institute, Lahore. The sample was placed in ice bags and immediately transported to Department of Pathology, University of veterinary and animal sciences, Lahore for the isolation and identification of *Clostridium perfringens* type D. Morphological characteristics, biochemical tests, mice inoculation and ELISA were also performed from this culture for identification of *C. perfringens* type D (Babe *et al.,* 2012; Effat *et al.,* 2007; Koc and Gokce, 2007). The culture was maintained in Reininforced *Clostridium* Medium (RCM) by periodical subcultures. About 2000 ml bulk flask culture was prepared in RCM for experimental infection.

Experimental inoculation and sample collection: The experimental dose was calculated as colony forming units (CFU) per milliliter in a standard spread technique on blood agar after incubation anaerobically for 24 hours (Tortora *et al.,* 2010). Approximately 150 ml inoculums with 4.6×10^{8}-5.7×10^{8} CFU /ml was administered per animal of infected group intaduodenally. For the inoculation of *C. perfringens* whole culture into the duodenum, a new experimental approach was adopted via a para-mid line. Brielfy, the animals were kept fasted a day before experimentally infection. The operation was performed under general anesthesia by intramuscular injection of xylazine hydrochloride (Xylase, Farvet Laboratory Netherland) @ 0.08 mg/kg body weight. The animals was restrained in dorsal recumbency. A right side para midline incision was given about two inches from xiphoid cartilage and about two inches from midline. The pyloric area of the abomasum and the first portion of the duodenum were exteriorized. At first about 200 ml of 20% solution of corn flour in 0.85% saline was injected in the abomasum of all animals (A, B). Then, 150 ml of the whole culture of *Clostridium perfringens* type D was inoculated into the duodenum through a drip in each animal of infected group. The abdominal incision was closed by separate muscle layers and skin sutures.

Hematological studies: Approximately 3 ml of blood was collected from the jugular vein of each animal at 0, 10, 20, 30 hours post infection with the help of sterile needle and poured in ethylenediaminetetraacetic acid (EDTA) mixed vaccutaners. Hematological parameters studied were total erythrocyte count (TEC), total leukocyte count (TLC), platelet count, and packed cell volume (PCV). All these parameters were simultaneously performed in an automated hematology analyzer (Backman Coulter, USA).

Serum Profile: Serum samples were collected from animals at 0, 10, 20, 30 hours post infection. Blood glucose level was measured at different time intervals on the spot before pouring blood in EDTA mixed vaccutaner by taking a drop of blood from each animal on the codefree^{TM} strip and reading was noted by Glucometer (codefree^{TM}, Korea). Blood urea (Reference #10017) and serum creatinine (Reference #10016) was measured by using a commercially avaialbe kit (Human, Germany, according to manufacturer instructions. Before the analysis of test samples standards were run.

Statistical analysis: The data obtained from the experiments was analyzed using analysis of variance (ANOVA, Duncan multiple range test) through SAS 9.1 (SAS System, SAS Institute, Cary, North Carolina) and the means ± standard error were compared so, that values of $P<0.05$ were considered to be statistically significant.

RESULTS

Isolation, identification and confirmation of *Clostridium perfringens* type D: Microscopic examination revealed gram positive and non-motile rods on Reinforced Clostridium Medium (RCM) (fig. 1).There was a double zone of hemolysis on blood agar (fig. 2). Turbidity along with abundant gas production was observed when *C. perfringens* type D cultured on RCM. Colony count was about 4.6×10^{8}-5.7×10^{8} CFU/ml on blood agar. Biochemical identification showed catalase positive. Gas and acid from glucose, fructose, lactose, sucrose and mannitol were observed. No growth was observed in the aerobic culture.

Pathogenicity test in Swiss Albino mice showed the death of animals within 3 days and by indirect ELISA, the optical density (OD) of the samples for alpha and epsilon toxin showed a difference greater than 0.150 hence, declared positive. The samples were found negative for the beta toxin.

Hematological and biochemical alternations in experimentally inoculated *Clostridium perfringens* type D in sheep

Hematological alternations: It has been observed in the study that at zero hour, the mean total number of red blood cells (RBC_S) were 11.7 ± 0.4 $10^{6}/\mu l$ but with the passage of time i.e. 10, 20 and 30 hour, as the disease progress in the experimentally infected sheep, the said blood parameter was started to decrease. The total number of white blood cells (WBCs) was considerable

increased at 10 and 20 hours post infection but at 30 hour, the total number of WBCs start to decrease while the percentage of packed cell volume continually increased within the specified time (Table 1). No significant changes were observed in animals of control group at different time intervals. Similarly, no mean significant change was found in animals of both groups for platelets.

Biochemical alternations: Blood samples for biochemical profile was collected and analyzed at different time intervals. It has been found that glucose, urea and serum creatinine concentrations consistently increased in the blood of the experimentally infected sheep with *Clostridium perfringens* type D while in controlled group, all biochemical values were in almost normal range (Table 2).

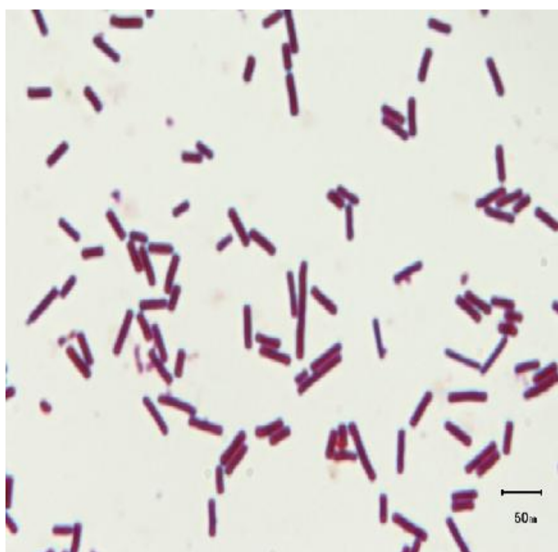

Fig.1(a) Growth on reinforced culture medium showed Gram-positive, rod shaped *clostridium perfringens* (b) *Clostridium perfringens* culture showed an inner zone of complete hemolysis on blood agar

Table. 1 Mean RBC, WBC, Platelets counts and PCV percentage in sheep of group A and B at different time intervals.

Time interval*	RBC $10^6/\mu l$		WBC $10^3/\mu l$		PLATELETS $10^5/\mu l$		PCV %	
	Control	Infected	Control	Infected	Control	Infected	Control	Infected
0	11.7±0.5^a	11.7±0.4^a	9.3±0.5^b	10.1±0.6^b	5.1±0.4^a	5.7±0.3^a	35.6±1.8^b	35.5±2.2^b
10	11.7±0.5^a	10.8±0.5^{ab}	11.2±0.3^b	19.7±3.5^a	4.8±0.4^a	5.4±0.5^a	37.3±1.3^b	38.8±1.6^b
20	11.3±0.3^{ab}	10.2±0.7^{ab}	10.6±0.3^b	16.9±2^a	4.9±0.2^a	4.6±0.3^a	37.6±1.1^b	40±2^{ab}
30	10±1.1^{ab}	9.5±0.3^b	10.8±0.8^b	14.7±0.9^{ab}	4.7±0.5^a	4.3±0.4^a	39.6±1.4^{ab}	45±1.7^a

RBC= Red Blood Cells **WBC** = White Blood Cell **PCV**= Packed Cell Volume
Means with the different letter in the columns (control and infected) for each parameter are significantly different (P< 0.05). *Time interval in hours

Table 2. Blood glucose, urea and serum creatinine levels in animals of control and infected groups at different time intervals (Means ± SE)

Time interval*	Blood glucose (mg/dl)		Blood urea(mg/dl)		Ser. creatinine (mg/dl)	
	Control	Infected	Control	Infected	Control	Infected
0	58±3.3^c	58±3.4^c	22.3±1.7^c	21±1.8^c	1.1±0.1^d	1.2±0.1^d
10	60±2.2^c	75±5.9^c	28.8±3.2^c	39±4.3^c	1.2±0.1^d	2.5±0.2^c
20	71±2.2^c	114±11.7^b	29.5±2.8^c	66.6±10^b	1.5±0.1^{cd}	5.4±0.4^b
30	74±6.7^c	141±13.2^a	26.3±3.5^c	92.3±6.7^a	1.6±0.2^{cd}	7.5±1.4^a

Means with the different letter in the columns (control and infected) for each parameter are significantly different (P < 0.05). *Time interval in hours.

DISCUSSION

Alpha and epsilon toxins of the *Clostridium perfringens* type D are considered to be the major toxins, involved in the disease pathogensis in sheep. The activity of alpha toxin is lethal, necrotizing and hemolytic where as epsilon is considered as lethal and necrotizing (Quinn *et al.,* 2004; Fatmawati *et al*., 2013). It has been observed from the study (Smith, 1975) that there is increase concentration of acid-soluble phosphate, when red cells exposed to the low concentration of alpha toxin. This caused the hydrolysis of phospholipid in the cell membrane, may result directly in the release of hemoglobin. In the present study as the disease progressed, total number of red cells started to decrease with individual value declined to 8.4 $x10^6$/µl. The hemolytic activity may be due to binding of apha toxin on the red cells receptor, which activate the signaling pathway in the cell, resulted in the hemolysis. Our finding is supported by Ombe *et al.* (2006) who conducted studied on *C.chauvoei* hemolysin and reported that the existence of toxin binding receptors on the surface of erythrocytes of various species are associated with hemolysis. It is also speculated that the sphingomycin / phospholipids ratio of red cells may be the factor (Ochi *et al*., 2003). Similarly, in the present, alpha toxin also causes the lysis of platelet and leukocytes. This might be due to same mechanism, as in the case of red cells hemosysis. There was an increase in total white blood cells at the early hours with individual value reaches upto 30.1 $x10^3$/µl at 10 hours PI in experimentally inoculated animals indicated the activation of immune cells but at later hours, the decreased in white cells might be due to cytotoxic activity of apha toxin (Dennis and Bryant, 2002). Smith and Sherman (1994) also found the mean white cell count of 16000/mm^3 with individual value reaching up to 47000/mm^3 and considered as an aid in diagnosing the enterotoxaemia in living animal. An increase in mean PCV % age during present studies seems to be associated with dehydration along with the hemolysin activity produced by *clostridium perfringens.* Similarly, a significant increase in PCV % age was also observed (Useh *et al.,* 2008) in an out break of blackleg caused by *C. chauvoei* hemolysin along with steady drop in thrombocytes after infection to the time of death of the animals.

Epsilon toxin results in the increased vascular permeability and absorbed through the intestinal mucosa and then spread to all organs by blood circulation causing accumulation of fluid in different organs (Khan *et al.,* 2008; Popoff, 2011). It has been reported that *Clostridium* phospholispase C unmasked the insulin receptors in the cell membrans and stimulated the glucose uptake (Mollby *et al.,* 1973). Simultaneiously, phospholipase C, at the high concentration causes the lysis of cells (Martin, 1966). This might be resulted the loss of glucose in the extraxellular matrix, caused the hyperglycemia. During present study, an increase in blood glucose was recorded with individual value ranged from 51 to 159 mg/dl at different time intervals. Hyperglycemia is supportive diagnosis of enterotoxemia (Radostitis *et al*., 2006; Filho *et al*., 2009; Javed *et al.,* 2009) which support our findings.

Similarily, It has been reported (Buxton and Morgan, 1976) that epsilon toxin reacts with specific receptor on the cells, caused the degenerative changes in the vascular endothelial cells, resulted alternations in fluid dynamic. In *Clostridium perfringens* infection, the primary abnormalities evident on the serum biochemistry were associated with renal pathology, resulted an increase in serum creatinine and blood urea. The action of *C. perfringes* toxins on kidneys especially the epsilon toxin has also been demonstrated by other researchers (Miyakawa *et al.,* 2007; Heba *et al*., 2009). Higher blood urea concentration with individual level upto 101 mg/dl in infected animal during study lend support to the work of above authors. As blood urea is directly proportional to serum creatinine so the mean serum creatinine levels were also increased significantly. The increase in osmolality was thought to be a product of the increase in urea and glucose concentration which resulted the vascular damage by epsilon toxin led to loss of fluid into tissues and cavities (Zerbini and Ossiprandi, 2009). It seems that increase in blood glucose, urea and creatinine are associated with enterotoxaemia but its level may vary with individual variation and the time elapsed after infection.

In conclusion, the hematological values including RBC, WBC and platelets fluculated but almost remained within the range while an increase of PCV % age was observed. Blood glucose, urea and serum creatinine levels increased significantly as the disease progressed. However, further study is needed to report the cell signaling mechanism when the *Clostridium perfringens* type D toxins react with their receptors in different cells of body.

REFERENCES

Akram, F. S. F., K. F. Mohamed, A. Sameer, M. H. Ashgan, A. M. H. Azab and S.A. Selim (2010). Preparation of a combined vaccine for clostridial diseases in sheep. Global Vet. 4:463-473.

Babe, T., B. Jafari and S. Bahmanpour (2012). Typing of toxigenic isolates of *C. perfringens* by ELISA in ostrich. Afr. J.Microbiology Res. .8:1766-1769.

Blackwell, T. E and D. G. Buttler (1992). Clinical signs, treatment and postmortem lesions in dairy goats with enterotoxaemia in 13 cases (1979-1982). J. American Vet. Medical Association. 200:214-217.

Buxton, T and K. T. Morgan (1976). Studies on lesions produced in the brains of colostrums deprived lambs by *clostridium welchii* (*Clostridium perfringens*) type D toxin. J Com Pathol. 86. 435-447.

Dennis, L. S and B. Bryant (2002). The Role of Clostridial toxins in the pathogenesis of gas gangrene. Clinical Infectious Diseases. 35: 93–100.

Effat, M. M., Y. A. Abdalla, M. F. Soheir., M. M. Rady (2007). Characterization of *Clostridium perfringens* field isolates, implicated in necrotic enteritis outbreaks on private broiler farms in Cairo, by multiplex PCR. African J. Microbiology Research pp 29-32.

Efuntoye, M. O and A. I. Adetosoye (2003). Clostridial diarrhea in food animals in Ibadan, Nigeria Israel Vet. Med. Assoc.58:1-4.

Fatmawati, N. N., Y. Sakaguchi, T. Suzuki, M. Oda, K. Shimizu, Y. Yamamoto, J. Sakurai, O. Matsushita and K. Oguma. (2013). Phospholipase C produced by *Clostridium botulinum* types C and D: comparison of gene, enzymatic, and biological activities with those of *Clostridium perfringens* alpha-toxin. Acta Med Okayama. 67:9-18.

Filho, E. J. F., A. U. Carvalho, A. R. Assis, F. F. Lobato, M. A. Rachid, A. A. Carvaho, P. M. Ferreira, R. A. Nascimen, A. A. Fernandes, J. E. Vidal and F. A. Uzal (2009). Clinicopathologic features of experimental *Clostridium perfringens* type D enterotoxaemia in cattle. Vet. Pathol. 46: 1213–1220.

Garmory, H. S., N. Chanter, N. P. French, D. Bueschel, J. G. Songer and R. W. Titball (2000). Occurrence of *Clostridium perfringenes* 2-toxin amongst animals, determined using genotyping and Sub typing PCR assays. Epidemiol. Infect. 124: 61-67.

Hadimli, H. H., O. Eganis, Z. Syin and Z. Aras (2012). Toxinotyping of *Clostridium perfringens* isolates by ELISA and PCR from lambs suspected of enterotoxemia Turk. J. Vet. Anim. Sci. 36(4): 409-415.

Heba, H., E. L. Ghareib and H. A. M. Amer (2009). Pathological and bacteriological studies on *Clostridium perfringens* infection in kidney of cattle, camel and sheep. Egypt J. Comp. and Clinic. Path. 22:88 – 108

Javed, T. M., M. Irfan, N. Mukhtar, S. Rahman and R. Hussain. (2009). An outbreak of enterotoxaemia at livestock farm during subtropical summer. Acta Tropica 112:225–227.

Khan, A., I. Ali., I. Hussain and N. Ahmad (2008). *Clostridium perfringens* type D Enterotoxaemia in the Chinkara Deer (*Gazella bennettii*). Turk. J. Vet. Anim. Sci. . 32 : 225-228.

Koc, R. and H. I. Gokce (2007). Determination of *C. perfringens* toxin types in sheep with suspected enterotoxemia in Kars Province, Turkey. Turk. Vet. Animal. Sci.31:355-360.

Lyerly, D. M. T., T. Willkins, F. A. Uzal, M. F. Miyakawa and B. A. McClane (2004). The enterotoxigenic clostridia In M. Dworkin and S. Falkow Edi., The prokaryotes, 3rd , Springer-Verlag, New York, NY.

Martin, R. (1966). Metabolism of isolated fat cells: The similar effects of phospholipase C (*Clostridium perfringens* alpha toxin) and of insulin on glucose and amino acid metabolism. The J. Biological Chemistry. 241(1): 130-139.

Miyakawa, M. E. F., J. F Derek, R. Poon, S. Sayeed, V. Adams, J. I Rood, B. A. McClane F. A. Uzal (2007). Both epsilon-toxin and beta-toxin are important for the lethal properties of *Clostridium perfringens* type B isolates in the mouse intravenous injection model. Infection and Immunity. 75: 1443–1452.

Mollby, R., C. E. Nord and T. Wardstrom. (1973). Biological activites contaminating preparations of phospholipase C (alpha toxin) from *Clostridium perfringens.*Toxicon. 11: 139-147.

Ombe, B. M. H., T. Kohda, M. Mukamoto and S. Kozaki. (2006). Purfication and sensitivity of *Clostridium chauvoei* hemolysin to various erythrocytes. Comparative Immunology, Microbiology and Infectious diseases. 29:263-268.

Ochi, S., M. Oda, M. Nagahama and J. Sakurai. (2003). *Clostridium perfringens* alpha toxin induced hemolysis of horse erythrocytes is dependent on calcium uptake. Biochimica *et* Biophysica Acta 1613:79-86.

Popoff, M. R (2011). Epsilon toxin: a fascinating pore-forming toxin. FEBS J. 278:4602-4615.

Quinn, P. J., M. A. Carter, B. Markey and G. R. Carter (2004). Clinical Veterinary Microbiology. pp 191-208.

Radostitis, O. M., C. C. Gay, K. W. Hinchcliff and P. D. Constable (2006). Diseases associated with *Clostridium* species in Veterinary Medicine.10 edi. WB. Saunders Company Ltd. London. pp 841-843.

Sayeed, S., J. Li and B.A. McClane (2010). Characterization of virulence plasmid diversity among *C. perfringens* type B isolate. Infection and immunity 78:495-504.

Sawires, Y. S. and J. G. Songer (2006). *Clostridium perfringens*:insight into virulence evolution and population structure. Anarobe. 12:23-43.

Smith, L. D. S (1975). The pathogenic anaerobic bacteria. (2nd Ed.) Charles C. Thomas. Springfield, IL.

Smith, M. C and D. M. Sherman (1994). Goat Medicine. Lippincott, 2n Edi. Williams and Wilkins, Baltimore: 298-302.

Tortora, G. J., B. R. Funke and C. l. Case (2010). Microbiology an introduction.10th edi. Pear sons, education, Inc. Sanfrancisco.

Useh, N M., A. J. Nok, N. D. G. Ibrahim, S. Adamu, K. A. N. Esievo (2008). Hematological and some noval biochemical changes in zebu cattle with black leg. Bulgarian J. Veterinay Medicine. 11:205-211.

Zerbini, L., M. C. Ossiprandi (2009). Molecular typing of *Clostridium perfringens* strains isolated from dog by toxin gene amplification. Ann. Fac. Medic. Vet di parma.29:115-128.