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Association between bacterial strain type and host biomarkers in *Clostridium perfringens* infected goats

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ABSTRACT

The study project was designed to determine the effects of *Clostridium perfringens* type D infection on hematological and biochemical parameters in goats. Purposive blood samples were collected from 6 healthy and 12 diseased goats positive for *C. perfringens* infection. Neither the animals nor their mother were vaccinated against *Clostridium perfringens* from whom samples were obtained. Study was carried out in two different topographic areas; hilly (district Swat) and plain (district Mardan) of Khyber Pakhtunkhwa, Pakistan but nonsignificant ($P > 0.05$) statistical difference was recorded between the prevalence of *Clostridium perfringens* infected goats. Mean erythrocytes count (RBC) and hemoglobin level decreased significantly ($P < 0.05$) while the white blood cells (WBC) increased significantly ($P < 0.05$) in diseased animals compared to the healthy animals. However non-significant differences ($P > 0.05$) were observed in packet cell volume (PCV) and platelets count in healthy and diseased animals. According to biochemical analysis, a significant increase ($P < 0.05$) in liver enzymes, total bilirubin, serum creatinine, blood urea and glucose was recorded in diseased goats. . The results demonstrated that fluctuation in most of the mean hematological values remained within the normal range however the mean liver enzymes, total bilirubin, serum creatinine, blood urea and glucose levels gone beyond the normal levels which demonstrated severe damages to liver and kidneys.

1. Introduction

The small ruminants face various health challenges salient of which is enteric *Clostridium perfringens* infections known as enterotoxaemia [29]. *Clostridium perfringens* is rod shape, gram positive, spore forming and anaerobic bacteria causing infection in domestic and wild animals [13]. *C. perfringens* type D is mostly prevalent in sheep and goats Enterotoxemia [1,24]. In Pakistan, enterotoxaemia is considered as the major endemic diseases in small ruminants such as sheep and goats and rarely occurs in large ruminants [28]. Least work has done on bacterial diarrhea in small ruminants in Pakistan and no record is available on its mortality rate. Normally it is considered that *C. perfringens* is the part of microbial flora reported in different animals including goats and sheep [15]. It mainly occurs in per acute form in young when the adult goats and sheep are not vaccinated [12,18]. *Clostridium* are mainly classified into five types known as A, B, C, D, & E which release four toxins such as alpha (α), beta(β), epsilon(ε) and iota [22]. In sheep clinically enterotoxaemia is characterized by sudden onset of death while in goats enterotoxaemia occurs in per acute, acute and chronic forms [7]. (see Table 3)

As pathogenesis concerned in enterotoxaemia, toxins are absorbed from the small intestine, comes in blood circulation, and reaches to the internal organs where severe damages occur in liver, kidneys, respiratory, gastrointestinal and neurological systems [14] Presumptive diagnosis can be made easy on the basis of clinical signs, history of the disease and gross pathological lesions on the conduction of post mortem examinations [29]. The indication of infection in body should be considered if larger microbial colony count is observed. The most accepted criterion in establishing

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definitive diagnosis of enterotoxaemia by *C. perfringens* is the detection of its toxins in intestinal contents [30].

It is the intensive need of the area to find out an easy and fast diagnostic way for the *Clostridium perfringens* in the field conditions where an average veterinarian is able to diagnose the disease.

Keeping view the scenario of goats enterotoxaemia, this study was designed to determine the hemotological and biochemical changes occurs in *C. perfringens* type D infected goats.

2. Materials and methods

The present study was carried out in two different topographic areas; hilly (district Swat) and plain (district Mardan) of Khyber Pakhtunkhwa, Pakistan during 2016. These areas are major small ruminants raising areas due to small agriculture land and poor comunities. The study protocol was approved by the Animal Ethical Committee (Reference No. 5121, dated 09.03.2016). Purposive blood samples were collected from 6 healthy and 12 *C. perfringens* type D infected goats in our study project where 336 samples were collected from *C. perfringens* suspected goats. Samples from healthy goats were collected at the time samples collected from diseased animals. The area, breed and size were kept similar to the disease animals. However healthy goats were confirmed when no growth obtained on TSC media. The samples were obtained before the treatment at veterinary hospitals where animals were brought for treatment or from the endemic area at the time of outbreak. History during sampling revealed that neither the animals nor their mother were vaccinated against *Clostridium perfringens* from whom samples were collected as vaccination against *Clostridium perfringens* is not common in KPK.

2.1. Haematological analysis

Blood samples were collected from infected and non infected goats in dry, clean and labeled EDTA test tubes. In hematology test total erythrocytes count (TEC), hemoglobin, total leukocyte count (TLC), platelets count and packed cell volume (PCV) were determined using hematology analyzer (Beckman Coulter, USA). Blood obtained from diseased animals were all positive for *C. perfringens* type D and confirmed through PCR.

2.2. Biochemical analysis

Blood samples were collected from infected and non infected goats in dry, clean and labeled test tubes and kept at 45° angles at room temperature to clot and then centrifuged at 1500 rpm for 20 min. The sera was separated and kept in a sterile labeled test tube at -20 °C. Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were measured using spectrophotometer with the appropriate test kits (Bio-Diagnostics, Cairo, Egypt) while the Blood Urea and Serum Creatinine was measured by commercially available kits (Human, Germany) according to manufacturer instructions. Blood glucose level was measured before addition of EDTA in blood directly on strip (codefreeTM, Korea) for Glucometer reading.

2.3. Bacteriological examination

Two grams of fecal samples were obtained with sterile cotton swab, in plastic zip bags and transported to the laboratory on ice [19].

2.4. Culture

Samples were spread on already prepared plates containing tryptose sulfite cycloserine agar media (TSC) M 837-500G (HiMedia Laboratories pvt Ltd. India) on arrival to the laboratory. The plates were incubatied at 37 °C for 24 h anaerobically using Thermo scientific AnaeroGenTM 2.5 L (Oxoid Ltd, Wade Road, Basingstoke, Hants). Growth in culture plates after 24 h were identified by colony characters, Gram staining and biochemical tests (remel RapID ANA II system test kit; 12076 Santa Fe Drive, Lenexa, KS66215, USA). Colony count more than 104–107 CFU/g considered, as pathogenic isolates of *C. perfringens*.

2.5. Optimization and preservation of bacterial isolates

The isolates from the culture plates were streaked in Robertson cooked meat medium (Oxoid UK) for optimization and preservation. These tubes were kept for 12 h in shaking incubator for maximum bacterial growth before DNA extraction.

2.6. Extraction of DNA for PCR reaction

2.6.1. DNA extraction

DNA was extracted by using commercially available DNA extraction kits (GeneAll Bldg, 303-7 Dongnam-ro, Songpa-gu, Seoul, South Korea) according to manufacturer's instructions. The quantity and quality of extracted DNA's was calculated by whole absorption spectrum (220-750 nm) using NanoDrop (NanoDrop 2000, Thermo-Scientifics, NanoDrop products, 3411 Silverside Rd, Bancroft Building, Wilmington, DE 19810 USA). DNA was measured in ng/μL at absorbance level of 260/280 and 230/260 nm reflected quality of the extracted DNA. Extracted DNA stored at -20 °C for long term use.

2.7. PCR reactions

PCR reactions were performed using primers described in Table 1 in 25 μL volumes in micro amplification tubes (PCR tubes) (see Table 2).

- 2× Ampmaster™ ^{Taq}: 10 μL (GeneAll Biotechnology CO. Ltd)
- Forward Primer: 1.5 μL
- Reverse Primer: 1.5 μL
- DNA free distilled water: 7 μL
- DNA: 5 μL

For ease and better results, master mix, forward primer, reverse primer and distilled water were taken together in pool as total quantity for each toxin gene primer to be used in PCR reaction in Eppendorf tube. These tubes were named as alpha MM pool, beta MM pool, epsilon MM pool and iota MM pool. 20 μL from each pool were taken in each PCR tube after vertex, and added 5 μL of DNA sample in these PCR tubes making 25 μL total PCR mixtures volume for reaction in thermocycler. Amplification was obtained with initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of 60 s of denaturation at 94 °C, 30 s of annealing at ($\alpha = 60C^0$, $\beta = 64C^0$, $\varepsilon = 53.4C^0$), 60 s of extension at 72 °C and 5 min of final extension at 72 °C. PCR products were run on 1.5% agrose gel with ethidium bromide along DNA ladder of 1 Kb (Genesta™) as a Marker and optimized positive and negative control product at 120 V charge of 500 mA current for 40 min. The DNA bands visualized photographed in UV light.

2.8. Statistical design

Results were represented as mean ± Standard Error for all parameters. Hematobiochemical comparison between diseased and healthy animals was analyzed through Student's *t*-test at the level