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Short Communication

Detection and Characterization of *Clostridium perfringens* Serotype D from Small Ruminants of Balochistan

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Authors' Contribution
IT, FA and ZA conceived and designed the study. IT, MKT, DA and ZA collected the data. IT, AH and GM analyzed and interpreted the data. IT, SA and FS wrote the article. IT, FA and MKT statistically analyzed the data.

Key words
***Clostridium perfringens* Type D, Small ruminants.**

ABSTRACT

The current study was carried out in all administrative divisions of the province to explore the prevalence of *Clostridium perfringens* type D in small ruminants. A total of 204 fecal samples were collected from sheep and goats suffering from seasonal diarrhea. *C. perfringens* type D was identified through different biochemical tests and PCR. Antibiograms and lab animal trials were also carried out on local isolates of *C. perfringens* type D. Out of 204 analyzed samples, 89.18 % were found positive while 10.82 % were negative for *C. perfringens* type D. The division wise distribution showed that Quetta, Zhob, Sibi and Kalat were more effected zones of the Balochistan province. Sheep were more effected (54.78 %) than goats (34.4 %). *C. perfringens* was identified through different biochemical tests and PCR. Based on length of synthesized fragments by PCR, toxin gens of alpha and epsilon types were detected. *C. perfringens* type D was sensitive to tetracycline, ciprofloxacin, vancomycin, kanamycin, streptomycin, gentamicin, sulfamethoxazole and erythromycin, while showed resistance against carbenicillin, amoxicillin, penicillin g, colistin sulphate, bacitracin and metronidazole. In lab animal trial *C. perfringens* type D affected the fore stomach, intestine, liver, kidney, pancreas and gall bladder of mice.

In nature the *Clostridium perfringens* is found as gram-positive bacterium that survives in anaerobic and facultative conditions. It is worldwide present in the all-natural environmental conditions involving the ecological units with their functions in a natural system (Piet and Deperz, 2015). In small ruminants it is commonly found in the gastrointestinal tract (Hadimli *et al*., 2012).

The disease enterotoxaemia is caused by *C. perfringens* type D which is a pathogenic toxin disease in sheep and goats (Carlos *et al*., 2016; Nasir *et al*., 2006; Piet and Deperz, 2015). It is an obligate parasite of the intestinal tract (Linden *et al*., 2015) but under certain conditions it produces toxins Alpha (α) and Epsilon (ε) and which can be fatal for livestock (Uzal *et al*., 2016; Jamshid *et al*., 2014; Li *et al*., 2010). Heavy grain intake or lush pastures have been found to be favorable for the propagation of the bacteria and occurrence of the animal enterotoxaemia (Nagwa *et al*., 2013). Disease commonly affects well fed animals especially in intensive feed lot practices. Factors which result in intestinal stability or slow the passage of the ingest through the intestines such as heavy tape worm infestations favor to build up toxin accumulation to produce disease condition. Young animals, lambs (6-12 months) and kids (3-12 weeks) old have been found to be the most susceptible groups for the enterotoxaemia disease (Jones *et al*., 2015; Naz *et al*., 2012).

Endothelial cell damage by toxins is almost exclusively responsible for the host pathology (Linden *et al*., 2015). The toxins are produced in the gut by abundantly growing bacterial cells and activated by some feeding factors and absorbed in the general circulation (Zhenyu *et al*., 2017). The toxins are resistant to digestive enzymes; in fact, these enzymes convert the freshly secreted less active protoxin into the fully toxic form. Its absorption in to the general circulation increases capillary permeability in many organs and tissues, including intestinal mucosa. This increases it absorption rate and consequently the systemic effects leading to excessive renal damage, hyperglycemia, hypertension and edema in various organs, including the

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brain (Li *et al*., 2010).

Enterotoxaemia is one of the most frequently occurring diseases of sheep and goats in Balochistan, causing severe economic losses. Therefore, the present study was designed to determine the occurrence of *C. perfringens* type D in sheep and goats of Balochistan.

Materials and methods

A total 204 fecal samples of enterotoxaemia-suspected sheep and goats suffering from seasonal diarrhea were collected from all divisions of Balochistan. The samples were collected aseptically in polyethylene sachets with UV treated contamination free with double volume of phosphate buffer solution (PBS) (Haq *et al*., 2016).

The samples were inoculated deep into reinforced clostridial medium RCM tubes (Oxoid-England). The tubes were placed in water bath for a period of 10-15 min at 80°C to eliminate the non-spore forming bacteria. Finally, the tubes were incubated in anaerobic jar at 37°C for 24-48 h. For pure colony isolation the culture was streaked on reinforced clostridial medium plate. The bacterial culture was identified with Gram staining. Different biochemical tests carried such as, fermentation reaction with different sugars, IMViC, H_2S, Litmus milk reaction, Gelatin hydrolysis, Motility test, Egg yolk agar (Lecithinase and lipase) (Rahaman *et al*., 2013).

For detection of enterotoxin gene for *C. perfringens* type D, PCR was performed by using specific primers. Alpha toxin gene primers forward: 5'-GCTAATGTTACTGCCGTTGA-3', reverse: 5'-CCTCTGATACATCGTGTAAG-3' – Beta toxin gene primers forward: 5'-GCGAATATGCTGAATCATCTA-3', revers: 5'-GCAGGAACATTAGTATATCTTC-3' Epsilon toxin gene primers forward: 5'-GCGGTGATATCCATCTATTC-3', reverse: 5'-CCACTTACTTGTCCTACTAAC-3'and Iota toxin gene primer forward: 5'-ACTACTCTCAGACAAGACAG-3', reverse: 5'-CTTTCCTTCTATTACTATACG-3' (Rahaman *et al*., 2013; Ahsani *et al*., 2010; Yuan *et al*., 2012). Genomic DNA purificatation kit (HiMedia-India) was used for extraction of genomic DNA. The DNA template was (elute) stored at -20°C for further process. The amplifications alpha and epsilon gene of *C. perfringens* type D was done by using thermo cycler. The 30µl master mix contains 15µl 2X *AmpMaster*^{TM} *Tag* (GeneAll-Korea), 2µl primers, 30ng template DNA and distilled water up to 30µl. All amplification reaction consisted of an initial denature 94°C for 5 min prior to 35 cycles of 94°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 30 sec, followed by a final 72°C extension for 7 min. The PCR products were then analyzed by electrophoresis on 2.0% agarose gel.

Standardized antibiotic sensitivity test was performed on Mueller Hinton agar using disc diffusion bauer technique and McFarland turbidity standard method 0.5 following CLSI protocols. Isolates were considered as sensitive and resistant to a particular antimicrobial agent on the basis of inhibitory zone (Haq *et al*., 2016).

To test the pathogenicity of strain, lab animal (mice) has been selected and 0.5mL growth suspension having $1x10^9$cfu/mL of each sample was injected intra peritoneally. The post-mortem of the dead animal was performed (Haq *et al*., 2016). The data was analyzed through SPSS software version 6.

Results

C. perfringens type D was Gram-positive, non- motile, thick single or paired rods measuring 4-6×1µm. Total 204 fecal sample were collected from which 89.18 % were positive and 10.82 % were negative for *C. perfringens* type D. Figure 1 shows division wise distribution Zhob (16.17 %), Sibi (15.68 %), Quetta (15.19 %) and Kalat (14.7 %) divisions were highly infected with enterotoxaemia compared to other divisions of Balochistan.

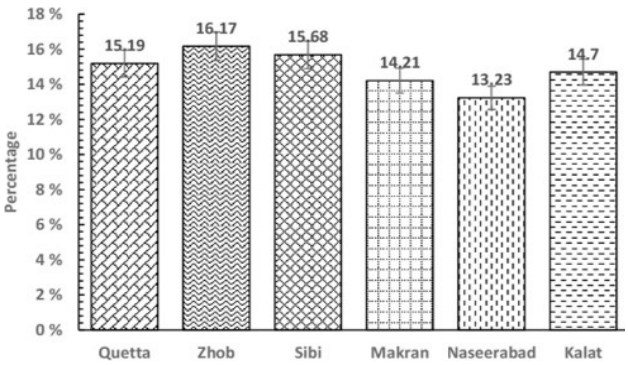

Fig. 1. Divisional situation of *C. perfringens* type D in Balochistan at provincial level.

The study result revealed that sheep (54.78 %) were more affected with *C. perfringens* type D as compared to goats (34.4 %).

Biochemical identification showed the *C. perfringens* biochemically was saccharolytic because it formed acid when it utilized glucose, sucrose, maltose and lactose but did not utilized mannitol and dulcitol. The gas of fermentation was hydrogen sulphide. Biochemical tests result revealed that *C. perfringens* was positive for litmus milk test, lecithinase test and gelatin liquefaction test while negative for catalase, indole, methyl red and voges-proskauer tests.

Molecular detection of alpha and epsilon toxin genes was carried from broth culture. After the extraction of

DNA, alpha and epsilon toxin genes was amplified at annealing temperature 55˚C with amplicon size 324bp (alpha) and 655bp (epsilon) were observed as shown in Figure 2.

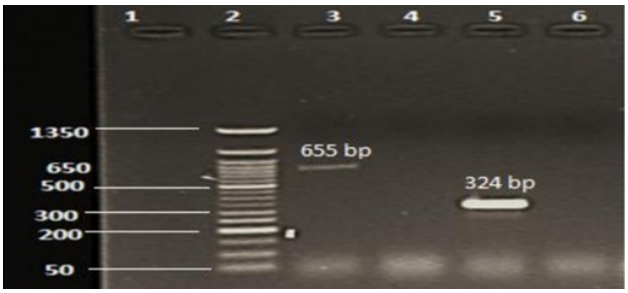

Fig. 2. Agarose gel electrophoresis of PCR product: Lane 1, negative control; Lane 2, DNA ladder (50 bp); Lane 3, epsilon toxin encoding gene, (655 bp); Lane 4, beta toxin encoding as negative; Lane 5, alpha toxin encoding gene as positive (324 bp), Lane 6, Iota toxin encoding as negative control.

C. perfringens type D was sensitive to tetracycline (32 mm), ciprofloxacin (27 mm), sulfamethoxazole (25 mm), gentamicin (20 mm), erythromycin (20 mm), kanamycin (20 mm), and streptomycin (13 mm) while carbenicillin, amoxicillin, penicillin g, colistin sulphate, bacitracin and metronidazole showed resistance against *C. perfringens* type D.

Total 204 fecal sample were collected from which 89.18 % were positive and 10.82 % were negative for *Clostridium perfringens* type D. The division wise distribution showed that Quetta, Zhob, Sibi and Kalat were more effected zones of the Balochistan province. Species wise result showed that sheep (54.78 %) were more affected than goats (34.4 %). Our findings were similar with Haq *et al*. (2016). *C. perfringens* type D was confirmed through different biochemical tests (IMViC, Sugar fermentation, Catalase reaction, Oxidase tests, Gelatin hydrolysis, Litmus milk reaction and Lecithinase) and PCR. Both toxins alpha and epsilon of *C. perfringens* type D are considered to be the lethal toxins involved in the enterotoxaemia disease in sheep and goats. Alpha and epsilon toxins gens were amplified with amplicon size about 324bp and 665bp, respectively (Kumar *et al*., 2016). Different drugs result showed that *C. perfringens* type D was sensitive to tetracycline, ciprofloxacin, vancomycin, kanamycin, streptomycin, gentamycin, sulphamethoxazole and erythromycin, while carbenicillin, amoxicillin, penicillin g, colistin sulphate, bacitracin and metronidazole showed resistance.

Conclusion

This study confirmed presence of *Clostridium perfringens* type D in small ruminants of the province. The polymerase chain reaction is good tool for identifying/ confirming the *clostridium perfringens* type D from fecal samples. The local isolates will help researcher to build a good vaccine against enterotoxaemia.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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