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Biomarkers for Pathogenic *Clostridium perfringens* in Small Ruminants of Khyber Pakhtunkhwa, Pakistan

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Authors' Contribution
MA conducted research. AZD supervised the project. SBK, NUK and MAK helped in sample collection and processing. KP wrote the manuscript. MA, GR, KP and AUK helped in conducting research.

Key words
***C. perfringens*, Kids, Lambs, PCR, Toxin genes.**

ABSTRACT

Pathogenic isolates of *Clostridium perfringens* were identified in lambs and kids population from non-vaccinated dams of selected districts of Khyber Pakhtunkhwa province, Pakistan. Samples were obtained from non-vaccinated lambs and kids with the signs of enterotoxaemia. The isolates were initially identified by colony characteristics, Gram staining, biochemical tests and CFU/g. *Clostridium perfringens* was isolated from 43.45% of lambs and 50.59% of the kids. Obtained isolates were further analyzed for toxinotyping with conventional PCR. Sequence analysis of 73 strains from diseased lambs showed 13.10% type A, 9.52% type B and highest 20.83% proportion of *Clostridium perfringens* type D. Out of 108 strains from kids, 54.62% were type A, 9.25% were type B and 36.11% were type D. *Clostridium perfringens* type C and E were neither detected in lambs nor in kids. The association of various factors (*i.e.* area, housing, seasons, colostrum feeding, hygienic condition, body condition and animal sex) with each type of *Clostridium perfringens* infection were analyzed by Chi- square test of association (χ^2). The results indicated that Colostrums feeding, hygienic condition and body condition scores of animals are significantly ($P<0.05$) associated with *Clostridium perfringens* type A, B and D in both lambs and kids. Besides that, Season with *Clostridium perfringens* type A in kids, Area and Season with *Clostridium perfringens* type D in lambs are also significantly ($P<0.05$) associated.

INTRODUCTION

Goats and sheep are integral part of livestock enumerating 70.3 and 29.8 million, with 867 and 39 tons of milk production, respectively while mutton production from small ruminants has reached to 657 tones (Economic Survey, 2015-16).The surveys have reported 6.476 and 1.177 million of goat and sheep in Khyber Pakhtunkhwa (KPK) province while districts Mardan and Swat are well populated with small ruminants (Livestock Census, 2010). *Clostridium perfringens* causes enterotoxemia in small ruminants and other laboratory animals which cause huge economic losses in sheep and goat farming globally (Nillo, 1980). The host is vulnerable to *C. perfringens* infection at any age but younger age ismore susceptible (Uzal *et al*., 2004). *C. perfringens* is the normal GIT flora of various animals including sheep and goats (McClane *et al*., 2006).

Morphologically, the pathogen is anaerobic Gram-positive bacillus which produces spores in unfavourable environmental conditions in soil (Meer and Songer, 1997). *C. perfringens* infection is in endemic in goats and sheep in Pakistan (Khan *et al*., 2008; Tahir *et al*., 2013). This infection mostly occurs in acute form in young stock of sheep and goats when their mothers are not immunized (Javed *et al*., 2009; Nasir *et al*., 2013). The disease is classified into 5 types *i.e.* A-E on the production of 4 different toxins *viz*. α, β, ε and i (Petit *et al*., 1999). Polymerase chain reaction is being used as diagnostic tool for *C. perfringens* genotyping (Meer and Songer, 1997; Baums *et al*., 2004; Gkiourtzidis *et al*., 2001).

Keeping in view the mortality in young population of small ruminants the present study was planned to find out the common pathogenic types of the *C. perfringens* in lambs and kids not only to know the burden of this disease in young flock but also to develop effective toxoid to prevent and control future losses.

MATERIALS AND METHODS

The Animal Ethical Committee approved this study

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project via Ref. No. DAS/5121, dated March 9, 2016.

Selection of study area and population for sampling

This project was carried out in topographically different districts Swat and Mardan of Khyber Pakhtunkhwa, Pakistan. The samples were collected from lambs and kids brought to veterinary hospital from enterotoxaemia endemic areas.

Sampling

Fecal or intestinal samples were obtained by purposive convenient sampling technique from lambs and kids suffering from the clinical signs of enterotoxaemia or sudden dead animals with the history of the above signs from 168 lambs and 170 kids up to 4 months age irrespective of breed and sex. These animals or their mothers had not been previously vaccinated with *C. perfringens*. The ingesta or fecal samples collected with swab and sent to the laboratory in sterile bottles under refrigeration temperature (Nayel *et al*., 2013).

Identification of C. perfringens

All the samples were cultured on prepared plates containing Perfringens Agar Base TSC (HiMedia Laboratories India) and TSC Supplement (FD014, HiMedia). The cultures were incubated for 24 h in anaerobic jar with Anaerogen (Oxoid Ltd, Basingstoke, Hants) at 37°C. The colonies were identified by morphology and Gram staining followed by biochemical tests (remel Rapid ANA 2 kit, USA). Being a normal inhabitant of intestinal tract in animals, *Clostridium perfringens* produce bacterial concentration 104-107 CFU/g as normal range in fecal samples while the positive samples produces elevated level of CFU/g (>107) for the *C. perfringens*. Colony count was performed on blood agar and only positive ones were included in this study (Kalender *et al*., 2005).

Molecular identification of C. perfringens

The identified isolates were cultured in Robertson cooked meat brothunder incubation at37°Cin shaking incubator for twelve hours.

DNA extraction kits (GeneAll Bldg, South Korea) was used for DNA extraction. For this purpose the cultured broth was centrifuged at full speed (1300-20000×g) in 1.5 mL micro centrifuge tube. The pellets of all samples were treated with 180µL Lysozyme 30mg/mL mixture (LYS702, Bioshop, Canada) and incubated 30 min at 37°C. 20µL proteinase K solution (20mg/mL) and 200uL Buffer BL were mixed. These samples were incubated for half an hour at 56°C and then for a further 30 min at 70°C after vertexing. 200µL absolute Ethanol was added and vertexed for proper mixing of the samples. The mixture was transferred the mixture into the SV column and centrifuged at (6000×g) for 60 sec. Washed with 600µL buffer BW and then 700µL buffer TW and centrifuged similarly after the addition of each buffer. At the end centrifuged at full speed for 60 sec to remove buffers and changed 1.5 mL micro centrifuge tube and added 200µl buffer AE to elute DNA.DNA's quantity and quality was calculated through NanoDrop (NanoDrop 2000, USA). For long term use DNA was stored at -20°C.

PCR assays were performed in thermocycler (BIO RAD T 100) with described primers in Table I. Their actions were performed in micro amplification tubes in 25µl volumes containing 10µL 2X Ampmaster$^{TM\ Taq}$ (Gene All Biotechnology Co., Ltd), 1.5µL forward and reverse primers (10 pmol/uL) alpha, beta, epsilon, and iota toxin primers, 5uL DNA and 7µL DNA free distilled water.

DNAs were amplified under initial denaturation at 95°C for five min, 35 cycles of denaturation at 94°C for 1 min, annealing at α=60°C, β=64°C, ε=53.4°C for 1/2 min and extension at 72°C for 1 min while final extension at 72.0°C for five min. PCR products were run on 1.50% agrose gel and stained with ethidium bromide. Optimized positive DNA, negative control product and 1 Kb DNA ladder of (Genesta^{TM}) as a marker were used. Visualized bands were compared and photographed in UV light (Greco *et al.,* 2005; Khan *et al*., 2017).

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

Table II.- Percentage of *C. perfringens* type A, type B and type D in *C. perfringens* infected lambs and kids in two districts of Khyber Pakhtunkhwa, Pakistan.

Bacterial strains	Animals	District Swat		District Mardan		Overall	
		Infected/total	Distribution (%)	Infected/total	Distribution (%)	Infected/total	Distribution (%)
Type A	Lambs	12/73	16.44	10/73	13.70	22/73	30.14
	Kids	21/108	19.44	38/108	35.18	59/108	54.62
Type B	Lambs	10/73	13.70	6/73	8.00	16/73	21.92
	Kids	4/108	3.70	6/108	5.55	10/108	9.25
Type D	Lambs	24/73	32.88	11/73	15.07	35/73	47.95
	Kids	17/108	15.74	22/108	20.37	39/108	36.11

Risk factor analysis

Association of various risk factors *i.e.* area, season, housing for animal, colostrum feeding to animals, body condition of animal, hygienic condition and sex were studied by interviewing each owner at the retail shop selected for sampling. Each factor like area (Swat, Mardan), housing (farm, house hold, pastoral flock), season (summer, autumn, winter, spring), colostrum fed (least, moderate, excessive), hygienic condition (hygienic, moderate, poor), body condition (fatty, moderate, lean), and sex (female and male) was categorized accordingly.

Statistical analysis

Chi-square test (χ^2) was used for comparison of associations between risk factors to the prevalence of the disease at 95% confidence interval ($P<0.05$) while prevalence of each type of *C. perfringens* was analyzed through descriptive statistics.

RESULTS

Of 168 samples obtained from lambs, 95 (56.54%) were identified as *C. perfringens* by colony characteristics, Gram staining and biochemical tests. 73 isolates (76.84%) were found pathogenic by CFU/g out of 95 isolates. Genotyping of 73 pathogenic isolates from diseased lambs indicated 22 (30.14%) type A, 16 (21.92%) type B and 35 (47.95%) were type D. In total 22 (30.14%). Out of *C. perfringens* type A pathogenic isolates, 16.44% was obtained from district Swat and 13.70% from district Mardan. Of 16 (21.92%) *C. perfringens* type B pathogenic isolates 13.70% was obtained from district Swat and 8.00% from district Mardan. Of 35 (47.95%) *C. perfringens* type D pathogenic isolates, 32.88% were obtained from district Swat and 15.07% were obtained from district Mardan.

Of 170 samples obtained from kids, 130 (76.47%) were initially identified by colony morphology, Gram staining, and with biochemical tests. Total, 108 isolates (83.07%) were found pathogenic by CFU/g. Genotyping of 108 pathogenic isolates showed 59 (54.62%) type A, 10 (9.25%) type B and 39 (36.11%) type D. Types C and E were not identified in lambs and kids. Out of total 59 (54.62%) *C. perfringens* type A pathogenic isolates, 19.44% were obtained from district Swat and 35.18% from district Mardan, Out of 10 (9.25%) *C. perfringens* type B pathogenic isolates, 3.70% were obtained from district Swat and 5.55% from district Mardan and of 39 (36.11%) *C. perfringens* type D pathogenic isolates, 15.74% were obtained from district Swat and 20.37% from district Mardan.

Prevalence of C. perfringens *infection in relation to risk factors in lambs and kids*

In lambs the highest prevalence (23.81%) of *C. perfringens* type A was recorded during the spring followed by winter (14.29%) and autumn (11.90%) while the lowest prevalence (4.76%) was recorded during summer. The statistical differences were found nonsignificant (P=0.085). In kids the highest prevalence (52.38%) of *C. perfringens* type A was recorded during the spring followed by winter (35.71%) while the lowest prevalence (30.95%) was recorded both in summer and autumn. The statistical differences were found nonsignificant (P=0.134) among seasons and significant difference (P=0.000) between districts. Farm animals indicated highest prevalence (5.95%) in lambs and (14.70%) kids followed by house hold animals in lambs (4.16%) and kids (11.76) while the lowest prevalence (2.97%) in lambs (2.97%) and kids (8.23%) was recorded in pastoral animals. Nonsignificant difference in lambs (P=0.405) and kids (P=0.174) were observed among different groups of housing. Least colostrum fed animals indicated highest prevalence in lambs (7.73%) and kids (16.46%) followed by colostrum fed *ad libitum* animals in lambs (3.57%) and kids (11.76%) while the lowest prevalence in lambs (1.78%) and kids (6.47%) was recorded in moderate colostrum

fed animals. Significant differences in 'colostrum feeding in lambs (P=0.023) and kids (P=0.015) were observed among different groups. Highest prevalence in lambs (7.73%) and kids (16.46%) was recorded followed by lean animals in lambs (3.57%) and kids (11.17%) while the lowest prevalence in lambs (1.78%) and in kids (7.05%) was recorded in moderate animals. Significant differences in lambs (P=0.023) and kids (P=0.024) were recorded in various groups of body condition. Highest prevalence (7.73%) in lambs and (15.88%) in kids was observed in poor hygienic housed animals followed by animals housed with moderate hygienic conditions in lambs (3.57%) and kids (11.76%) while the lowest prevalence in lambs (1.78%) and kids (7.05%) were observed in hygienic housed animals. Significant difference in hygienic condition in lambs (P=0.023) and kids (P=0.039) were noted among various groups. Male indicated higher prevalence (15.62%) in lambs and (35.71%) kids than the female in lambs (14.91%) and kids (34.50%). Nonsignificant differences in lambs (P=0.931) and kids (P=0.902) were indicated in both groups of sex in lambs and kids infected with *C. perfringens* type A.

In lambs the highest prevalence of *C. perfringens* type B was recorded during the winter (21.43%) followed by autumn (11.90%) and spring (7.14%) while the lowest prevalence (4.76%) was recorded in summer season. The statistical differences were found nonsignificant among seasons (P=0.077) and between districts (P=0.223) in lambs. In kids the highest prevalence of *C. perfringens* type B was recorded during the winter (11.90%) followed by autumn (7.14%) and summer (4.76%) while the no prevalence was recorded in spring. The statistical differences were found nonsignificant among seasons (P=0.136) and between districts (P=0.514) in kids. The highest prevalence of *C. perfringens* type B was recorded in House hold animals in lambs (5.35%) and kids (3.52%) followed by farm animals in lambs (2.38%) and kids (1.76%) while the lowest prevalence in lambs (1.78%) and in kids (0.58%) was recorded in pastoral animals. The statistical differences were found non-significant in lambs (P=0.135) and in kids (P=0.143) in various groups of housing. Highest prevalence in lambs (5.95%) and kids (4.11%) were noted in animal fed on colostrum *ad-libitum* followed by animals fed on least colostrum (2.38%) in lambs and (1.17%) in kids while the animals fed on moderate colostrum fed animals indicated lowest prevalence in lambs (1.19%) and in kids (0.58%). The statistical differences were found significant in lambs (P=0.034) and in kids (P=0.042) in various groups of colostrum feeding. Highest prevalence in lambs (5.95%) and in kids (4.11%) were noted in fatty animals followed by lean animals in lambs (2.38%) and kids (1.17%) while the moderate animals indicated lowest prevalence in lambs (1.19%) and in kids (0.58%). The statistical differences were found significant in lambs (P=0.034) and in kids (P=0.042) in various groups of body condition. Highest prevalence in lambs (5.95%) and in kids (4.11%) were noted in animals housed in poor hygienic condition followed by animals housed in moderate hygienic conditions in lambs (2.38%) and in kids (1.17%) while the lowest prevalence in lambs (1.19%) and in kids (0.58%) were noted in animals housed in hygienic condition. The statistical differences were found significant in lambs (P=0.034) and in kids (P=0.042) with various groups of 'hygienic condition. Higher prevalence was noted in the female (9.56%) than in the male (9.09%) in lambs while in kids higher prevalence was noted males (6.16%) than in female (5.7%). The statistical differences were found nonsignificant in lambs (P=0.701) and in kids (P=0.899) in various groups of sex.

In lambs the highest prevalence of *C. perfringens* type D was recorded during spring (35.17%) followed by winter (23.71%) and summer (21.41%) while the lowest prevalence was recorded in autumn (11.9%) season. Statistically these differences were non-significant among seasons (P=0.000) and between districts (P=0.017) in lambs. In kids the highest prevalence of *C. perfringens* type D was recorded during spring (16.66%) followed by summer and winter (9.52%) each while the lowest prevalence was recorded in autumn season (4.76%). These statistical differences were found nonsignificant among seasons (P=0.342) and between districts (P=0.442) in kids. Farm animals indicated highest prevalence in lambs (6.54%) and in kids (11.17%) followed by house hold animals in lambs (4.76%) and in kids (7.05%) while the lowest prevalence in lambs (3.57%) and in kids (4.70%) were observed in pastoral animals. The statistical differences were found nonsignificant in lambs (P=0.222) and in kids (P=0.075) in various groups of housing. Highest prevalence in lambs (11.31%) and in kids (11.76%) was noted in animals fed with *ad-libitum* colostrum followed by animal in lambs (5.35%) and in kids (7.05%) fed with least colostrum while the lowest prevalence in lambs (4.16%) and in kids (4.11%) was noted in animals fed with moderate colostrum. The statistical differences were found significant in lambs (P=0.022) and in kids (P=0.027) in various groups of colostrum feeding. Highest prevalence in lambs (11.30%) and in kids (11.17%) was noted in fatty animals followed by lean animals in lambs (5.95%) and in kids (8.23%) while the lowest prevalence in lambs (7.73%) and in kids (3.52%) was observed in animals with moderate body score. The statistically significant differences were observed in lambs (P=0.016) and in kids (0.027) in various groups of body condition scores. Highest prevalence (10.31%) in lambs and (11.76%) in kids was

noted in animals in poor hygienic condition followed in lambs (7.73%) and in kids (7.05%) in moderate hygienic condition while the lowest prevalence in lambs (6.54%) and in kids (4.11%) was observed in hygienically housed animals. The statistically significant differences were observed in lambs (P=0.035) and in kids (P=0.027) were observed in various groups of hygienic condition. Higher prevalence in lambs (28.84%) and in kids (29.33%) was observed in the males as compared to the females in lambs (17.84%) and in kids (17.89%). Nonsignificant differences in lambs (P=0.086) and kids (P=0.078) were note in various groups of sex.

DISCUSSION

Clostridium perfringens is the causative agent of enterotoxaemia. It is an important disease in sheep and goats causes great economic losses in these animals throughout the world due to high prevalence and fatality rates (Uzal and Songer, 2008). Our results of indicated that *C. perfringens* type A, type B and type Din lambs and kids are the most predominant causes of enterotoxaemia in Khyber Pakhtunkhwa, Pakistan. Overall *C. perfringens* infection in present study is in line with the finding of Raana (2007) who reported about 12% *C. perfringens* infection in sheep and about 57% *C. perfringens* infection in goats from Punjab area of Pakistan. The results of our study are in close accordance with the findings of indicated by Kalender *et al*. (2005) from Turkey who reported 50% prevalence of *C. perfringens* infection in sheep with Enterotoxaemia signs; Aschfalk *et al*. (2002) from West Africa who reported *C. perfringens* prevalence 71% healthy sheep; Greco *et al*. (2005) from Italy who reported *C. perfringens* prevalence 25% in healthy lambs; and kids and Uzal and Songer (2008) from California who reported prevalence of *C. perfringens* in goats. Some discrepancy might be because of different geographical area, climate, breed, and system of rearing. The percentages of bacterial isolates of present study remained agreed with the outcomes of researchers.

Toxinotypes in our results were in accordance with findings of Gkiourtzidis*et al*. (2001), Bueschel *et al.* (2003) and Khan *et al*. (2017). However, in contrast Kalender *et al.* (2005) and Greco *et al.* (2005) did not identify *C. perfringens* type B in their studies in Turkey and Italy, respectively. Their studies are in agreement with our data where types A and D were the leading cause of *C. perfringens* infection. Similarly types C and E were not prevalent in lambs and kids. Type E of *C. perfringens* was not isolated in our study agreed with previous data of Songer (1996). However Kurtkaya and Alver (1969) and Ozcan and Gurcay (2000) in Turkey isolated *C. perfringens* type A, type B, type C and D from sheep with enterotoxaemia, although the role of type A in disease production was considered doubtful by some researchers (Nillo, 1980). From the results of our study, in lambs were the most prevalent type was *C. perfringens* type D than type A and type B. However, in kids the prevalence of pathogenic *C. perfringens* type A was maximum of as compared to type D and type A. Our results are in line with the findings of most conducted researches in various countries where highest infection was recorded in poor hygienic animals. The pathogens find their way to animal body through contaminated feed and water. *C. perfringens* also found dust, fomites, air and beddings which transfer the infection from diseased to healthy animals (Schultz *et al*., 2003; Kobayashi *et al*., 2009). The multiplication and survival of pathogens are triggered by poor digestion or absorption in GIT in animals thus results increased toxin production. Poor hygienic environment and unconscious feeding might be the reason of high *C. perfringens* type A and type D prevalence in farms animals. High proteins and carbohydrates amounts trigger the growth of *C. perfringens* in gut (Roberfroid *et al*., 2010; Riddell and Kong, 1992; Katyal *et al*., 1999). Highest ratio of enterotoxaemia recorded in *ad libitum* colostrum fed animals in our study was in line with that of Quinn *et al*. (2011) who described various risk factors like change in diet, low proteolytic efficiency in neonates, trypsin inhibitors of colostrum, malnutrition, inefficient pancreatic secretion, no established intestinal flora, carbohydrates engorgement and intestinal hypomotility in enterotoxaemia (Sato *et al*., 1978). Increased quantity of anti-trypsin factor or protease inhibitors like sweet potatoes, soybeans and colostrum are the risk factors for enterotoxaemia (Palliyeguru *et al*., 2010; Palliyeguru *et al*., 2011). Highest prevalence in fatty animals in our project might be due to greedy nature. Our results are in line with the results of many researchers (Van Immerseel *et al*., 2004; Beaugerie and Petit, 2004). *C. perfringens* is known to be the main proteolytic species in the gut and it requires regular supply of amino acids. Any deficiency in the amino acids reduces its number rapidly because it cannot synthesize some amino acids (Roberfroid *et al*., 2010; Shimizu *et al*., 2002).

Animals, like cattle, horses, goats and sheep, fed on greater amount of carbohydrates develop enteritis (Waggett *et al*., 2010). *C. perfringens* cannot produce some essential amino acids (Roberfroid *et al*., 2010; Shimizu *et al*., 2002). The production of alpha, beta, and epsilon toxins are increased by dextrin or glucose feeding *in vitro* (Sakurai and Duncan, 1979). Starch injected to the abomasum of sheep and goats and dextrin into the duodenum in cattle was used in the challenge of infection of type D of *C. perfringens* (Uzal *et al.*, 2004; Layana

et al., 2006). Carbohydrates overfeeding increase the *C. perfringens* intensity in the rumen and caecum in cow (Allison *et al.*, 1975).

Highest prevalence of *C. perfringens* infection was noted in animals on *ad libitum* colostrum feeding which is in line with the findings of Quinn *et al*. (2011), who described that the presence of trypsin inhibitors in colostrum is the main risk factor for enterotoxaemia. The activity of trypsin in the gut is also affected by malnutrition (Sato *et al*., 1978; Lawrence and Walker, 1976).

CONCLUSION

Large number of non-vaccinated lambs and kids were observed to have *C. perfringens* infection. *C. perfringens* type A, type B and type D were observed in many animals which emphasizes the role of α, β and ε-toxins in the pathogenesis. *C. perfringens* isolates were precisely and quickly characterized for toxinotyping with PCR technique. The identification of the prevalent *C. perfringens* types facilitated the development of adequate vaccines in epidemiological situation for protection and in control of the disease in small ruminants.

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Statement of conflict of interest

The authors declared no conflict of interest.

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