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Prevalence and Antibiogram of *Clostridium perfringens* in Small Ruminants of District Kalat, Balochistan

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ABSTRACT

Background: Among life-threatening infections of small ruminants (sheep/goat), enterotoxaemia has vital importance. This disease is caused by certain serotype of *Clostridium perfringens* and commonly known as pulpy kidney or overeating infection. The current study was carried out to determine the prevalence of *C. perfringens* in small ruminants in different zones (Kalat, Mangochar, Johan, and Gazg) of district Kalat, Balochistan.

Methods: Fecal samples (n=100) were collected aseptically, from rectum of enterotoxaemia suspected animals. The samples were cultured in Reinforced Clostridial Media (RCM). Biochemically identified isolates were further confirmed through polymerase chain reaction (PCR).

Result: Overall prevalence of *C. perfringens* was noticed 48%, with higher occurrence rate ($p < 0.01$) in sheep (60%) than goat (36%). Among fifty samples of each specie, 30 and 18 isolates recovered from sheep and goat respectively. The area wise analysis showed that, in goats, the highest prevalence was found in Kalat (50%) followed by the Mangochar (38.46%), Johan (30.77%) and Gazg (25%). The statistical analysis revealed the significant differences ($p<0.05$; 95% confidence interval (CI) 18.80-68.91) in *C. perfringens* prevalence in various areas of Kalat district. The results of the gender-wise prevalence of *C. perfringens* exhibited a numerically higher ($p>0.05$; 95% CI 9.23-49.02) prevalence in female goats (40%) than male goats (32%). Whereas, the age-wise analysis indicated that, in < 1 year age group (44.44%), the prevalence of *C. perfringens* was higher ($p<0.05$; 95% CI 8.00-57.89) than >1 but <2 years (29.41%) and ≥ 2-year (33.33%) age groups. Similarly, in sheep, the highest prevalence ($p<0.05$) was found in Kalat (76.92%) followed by Mangochar (66.67%), Johan (58.33%) and Gazg (38.46%). The age wise and gender-wise differences ($p<0.05$) were also observed in various age and gender groups of sheep similar to goat. Among 30 sheep isolates of *C. perfringens*, a highest number (28 isolates) showed resistance against amikacin, followed by amoxicillin/penicillin (25 isolates) and colistin sulphate (22 isolates); while among 18 isolates of goat 17 were found resistant against colistin sulphate and 16 were resistant against amoxicillin and amikacin. In conclusion enterotoxaemia is prevailing in small ruminants of district Kalat, Balochistan thus mass scale vaccination campaign should be carried out to prevent losses.

Key words: Antimicrobial susceptibility, Balochistan, Enterotoxaemia, Goat, Sheep.

INTRODUCTION

In human nutrition, sheep and goat products (milk and meat) have great importance and consumers' acceptability. Small ruminants (sheep/goat) are common livestock animals all over the globe that play significant role in human nutrition. In most of the developing countries small ruminants are considered the greatest resource of income. However, occurrence of various diseases in these animals is a serious issue as it causes extensive losses to small ruminants and their guardians (Singh *et al*., 2018). Among life-threatening infections, enterotoxaemia has vital importance. The disease usually occurs due to the ingestion of more grain or luxurious pasture (Taj *et al*., 2018). It has been estimated that defense of small ruminants from infectious diseases particularly from enterotoxaemia can help to get good production and profit. Production of the livestock and farmers' profit are mainly affected by enterotoxaemia (Chandran *et al*., 2010; Wang *et al*., 2011). Investigators from various parts of the world stated dissimilar incidence percentage (24.13 to 100 per cent) of enterotoxaemia in small ruminants (Fayez *et al*., 2013). Enterotoxaemia which is also known as pulpy kidney or overeating disease is caused by certain serotype of

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C. perfringens in small ruminants, which can even devastate the whole farm (Taj *et al.*, 2018; Uzal *et al.*, 2016).

Clostridium perfringens may be present in atmosphere, soil, water and decomposing animals as well as gut of animals and humans. It is an anaerobic, rod shaped, spore forming bacteria (Talukdar *et al.*, 2017). *C. perfringens* produces about 17 dissimilar toxins that belongs to four main types, named as iota, epsilon, beta and alpha *which prior to onset of disease* formed in gut and absorbed in overall circulation *(*Park *et al.,* 2019). Enterotoxaemia disease in small ruminants is produced by *C. perfringens t*ype D that rarely found in other livestock species. Clinical and pathological findings of the disease are relatively changed in sheep and goats. Type D of *C. perfringens* produce two types of toxins *i.e.,* epsilon and alpha toxins. Definite toxin types produce intestinal disease in numerous species of animals (Nazki *et al.*, 2017). A study reported the 14.5% occurrence of *C. perfringens* in food samples and recommended that it is important to ensure product protection mainly when it is to be consumed by newborns and elderly people (El-Bayomi *et al.*, 2020). A previous study conducted in Punjab, Pakistan showed 64 and 37% prevalence of *C. perfringens* in diseased and healthy animals respectively (Mohiuddin *et al.,* 2020). The high distribution of disease also reported in the neighboring countries including India, Bangladesh and China (Chai *et al.,* 2001; Milton *et al.,* 2017).

Keeping in view the above situation the present study planned to study the prevalence of *C. perfringens* in goat and sheep of district Kalat, Balochistan using culture- and PCR (polymerase chain reaction)- based techniques. The study further investigated the associated risk factors as well as the antibiogram profile of *C. perfringens* isolates.

MATERIALS AND METHODS

Study area and sampling

Fecal samples (n=100) were collected from various zones (Kalat, Mangochar, Johan and Gazg) of Kalat district during October to December 2020. Rectal swabs were collected aseptically from sheep and goats (n=50 each) showing diarrhea or loose stool, stomach pain characterized by kicking their belly or repeated laying down and getting up, panting and crying. The samples collected in sterile polyethylene sachets and brought to Center for Advanced Studies and Vaccinology and Biotechnology, University of Balochistan, Quetta for isolation and identification of *Clostridium perfringens* using standard bacteriological techniques (Musawa *et al.*, 2021). In brief, PBS diluted (1:10) samples were cultured onto reinforced clostridial media (RCM) and incubated anaerobically at 37°C for 24 h. After primary identification based on colony characteristics and Gram staining, the isolates were confirmed by biochemical characterization as stated earlier (Mohiuddin *et al.,* 2020).

Genomic DNA extraction and polymerase chain reaction

Extraction of genomic DNA was done from *C. perfringens* isolates using commercially available extraction kit following the instruction of the manufacturer (GeneAll, Korea). Polymerase chain reaction was performed for recognition of target Cpe gene by using specific primers CpeF 5′ - GGAGATGGTTGGATATTAGG - 3′ cpeR5′ - GGACCAGCAG TTGTAGATA - 3′ according to the procedures of Taj *et al.* (2018). For performing PCR, the total volume of reaction mixture was 30 µl that comprises 12 µl of grade water, 6 µl of DNA template, 1 µl of each primer and 10 µl of master mix. Initial temperature provided for denaturation was 94°C for 5 minutes. Then 55°C for 30 seconds for annealing and 72°C for 7 minutes for extension was provided. Denaturation, annealing and extension was repeated for 25 cycles. A 324 bp amplification product was confirmed through 5% agarose gel, having 0.5 µl of ethidium bromide.

Antibiotic sensitivity test

The test was carried out to check susceptibility and resistance of *C. perfringens* to various antibiotics according to guidelines of CLSI (2017). Commercially available antibiotic discs (Oxoid, UK) used *via* disk diffusion method and all samples were analyzed in triplicate. Antibiotic discs [*viz.*, trimethoprim (5 µg), colistin sulphate (10 µg), amoxicillin (25 µg), clarithromycin (15 µg), tetracycline (10 µg), ciprofloxacin (5 mcg), ceftazidime (30 µg), amikacin (30 µg), penicillin g (10 units) and meropenem (10 µg)] were placed onto the surface of Muller Hinton agar with the help of disc dispenser and pressed slightly with sterile forceps for fixing. Then petri dishes incubated for 24 h at 37°C. After overnight incubation, diameter of zone of inhibition was measured.

Statistical analysis

All data was tabulated in Microsoft Excel spreadsheets and analyzed by JMP® Statistical Package Software. Chi-square test was applied to calculate the association of risk factors with prevalence of *C. perfringens*.

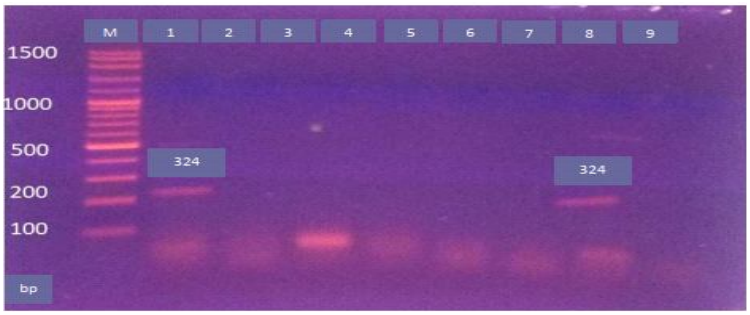
RESULTS AND DISCUSSION

Overall prevalence of *C. perfringens*

As shown in Table 1, from 100 feacal samples *C. perfringens* was identified in 48 samples, while 52 samples were found negative. The prevalence of *C. perfringens* was found significantly higher ($p<0.01$) in sheep (60%) as compared to goats (36%). The confirmation of positive samples was done by amplification of 324 bp fragment of *CPA* gene using PCR (Fig 1).

Prevalence of *C. perfringens* in goats

The results presented in Table 2 exhibited a highest prevalence in Kalat (50%) followed by the Mangochar (38.46%), Johan (30.77%) and Gazg (25%). The statistical analysis revealed the significant differences ($p<0.05$; 95% confidence interval (CI) 18.808-68.913) in *C. perfringens* prevalence in various areas of Kalat district. The results of the gender-wise prevalence of *C. perfringens* exhibited a numerically higher ($p>0.05$; 95% CI 9.228-49.023) prevalence in females' goats (40%) than male goats (32%). All the specimens were collected from dissimilar age groups



In the first and in the last veil ladder was loaded. *C. perfringens* isolates were loaded in veil no 1 to 5, all the isolates showing predicted size of 324-bp amplicon of *CPA* gene.

Fig 1: Molecular identification of *C. perfringens* isolates.

Table 1: The overall prevalence of *C. perfringens* in goats and sheep of district Kalat.

Species	Number of samples tested	Number of +ve samples	Number of -ve samples	%age of positive samples
Goats	50	18	32	36%
Sheep	50	30	20	60%*
Overall	100	48	52	48%

*Significantly higher at p<0.01.

Table 2: The prevalence of *C. perfringens* in goats in district Kalat.

Variable	Category	Number of samples tested	Number of +ve samples	Prevalence percentage	95% Confidence limits		*p*-value
					Lower	Upper	
Area	Kalat	12	6	50.00	18.808	68.913	0.013
	Mangochar	13	5	38.46			
	Johan	13	4	30.77			
	Gazg	12	3	25.00			
Sex	Male	25	8	32.00	9.228	49.023	0.901
	Female	25	10	40.00			
Age	< 1 year	18	8	44.44	8.002	57.888	0.037
	>1 but < 2 years	17	5	29.41			
	≥ 2 year	15	5	33.33			

of goats and categorized as <1 year, >1 but <2 years and ≥2 year. The age-wise analysis indicated that, in <1 year age group (44.44%), the prevalence of *C. perfringens* was higher (p<0.05; 95% CI 8.002-57.888) than >1 but < 2 years (29.41%) and ≥ 2-year (33.33%) age groups.

Prevalence of *C. perfringens* in sheep

As shown in Table 3, the highest prevalence was found in Kalat (76.92%) followed by Mangochar (66.67%), Johan (58.33%) and Gazg (38.46%). The statistical analysis revealed the significant difference (p<0.014; 95% CI 19.009-93.092) in *C. perfringens* prevalence in various areas of Kalat district. Similarly, the age wise analysis indicated that, in <1 year age group (68.18%), the prevalence of *C. perfringens* was higher (p<0.05; 95% CI 29.514-82.467) than >1 but <2 years (62.50%) and ≥2-year (41.67%) age groups. However, the results regarding gender-wise prevalence of *C. perfringens* exhibited a numerically higher (p>0.05; 95% CI 38.038-83.594) prevalence in female (72%) as compared to male (48%) sheep.

Antibiogram of *C. perfringens* isolates

As shown in Table 4, among 30 sheep isolates of *C. perfringens*, a highest number (28 isolates) showed resistance against amikacin, followed by amoxicillin/penicillin (25 isolates) and colistin sulphate (22 isolates). Whereas, among 18 isolates of goat a highest number (17 isolates) found resistant against colistin sulphate, followed by amoxicillin/ amikacin (16 isolates) and penicillin (13 isolates). All the isolates (100%) of sheep and goat origin found susceptible to ceftazidime.

In current investigation, the overall prevalence of *C. perfringens* was detected as 48%; with comparatively higher incidence in sheep (60%) as compared to goats (36%). A total of 48 isolates (30 sheep and 18 goat) were confirmed using biochemical characterization from 48 positive colonies. Similar results of *C. perfringens* prevalence have been reported by Taj *et al.* (2018), who reported 34.4% prevalence in goats and 54.78% in sheep

Table 3: The prevalence of *C. perfringens* in sheep in district Kalat.

Variable	Category	Number of samples tested	Number of +ve samples	Prevalence percentage	95% Confidence limits		*p*-value
					Lower	Upper	
Area	Kalat	13	10	76.92	19.009	93.092	0.014
	Mangochar	12	8	66.67			
	Johan	12	7	58.33			
	Gazg	13	5	38.46			
Sex	Male	25	12	48.00	38.038	83.594	0.028
	Female	25	18	72.00			
Age	< 1 year	22	15	68.18	29.514	82.467	0.035
	>1 but < 2 years	16	10	62.50			
	≥ 2 year	12	5	41.67			

Table 4: The antibiogram of *C. perfringens* isolated from goats and sheep of district Kalat.*

Antibiotic (Potency)	Sheep (Total no. of isolates=30)		Goat (Total no. of isolates=18)	
	Susceptible (No.)	Resistant (No.)	Susceptible (No.)	Resistant (No.)
Trimethoprim (5 µg)	27	3	18	0
Colistin sulphate (10 µg)	8	22	1	17
Amoxicillin (25 µg)	5	25	2	16
Clarithromycin (15 µg)	14	16	8	10
Tetracycline (10 µg)	13	17	9	9
Ciprofloxacin (5 mcg)	21	9	14	4
Ceftazidime (30 µg)	30	0	18	0
Amikacin (30 µg)	2	28	2	16
Penicillin G (10 units)	5	25	5	13
Meropenem (10 µg)	22	8	15	3

*Calculated according to CLSI, (2017) manual.

in different areas of Balochistan. In another study, Rahimoon *et al.*, (2020) examined 100 samples, collected from small ruminants, out of which 45 samples were found positive for *C. perfringens*. In contrast, Ajaz-ul-Haq *et al.* (2016) reported 66.5% incidence of *C. perfringens* in goats and sheep of district Khuzdar Balochistan. This high prevalence percentage of the organism may be due to environmental conditions, vaccination status and management (Shehzadi *et al.*, 2021). In current investigation, we also used polymerase chain reaction assay to confirm the isolates of *C. perfringens* by amplifying the 324 bp fragment of *CPA* (alpha toxin gene) as suggested in literature (Alsaab *et al.,* 2021).

Our study showed the area-wise differences (25 to 50% in goats and 38.46 to 76.92% in sheep) in the prevalence of *C. perfringens* in small ruminants in Kalat district. Similar results have been reported by Rahimoon *et al.* (2020) who reported the 11.11 to 44.44% prevalence of *C. perfringens* in goats of different areas of district Tharparkar. The authors postulated that occurrence rates of enterotoxaemia in different geographical locations might be due to improper vaccination schedule against enterotoxaemia, geographical differences and/or difference in breeds used in study. Earlier studies have reported the breed-wise differences in farm animals for susceptibility against infectious diseases (Mangi *et al.,* 2015).

Gender wise prevalence of *C. perfringens* in goats and sheep exhibited the high prevalence in females as compared to males. These results reveled that females are at high risk then males. Our results are in agreement with the results of Ajaz-ul-Haq *et al.* (2016) who observed that 38% females are positive with *C. perfringens*, as compared to males 28.5%. Previous published literature have also demonstrated that females are comparatively more susceptible to chronic as well as acute microbial infections as compared to male animals (Malhi *et al.,* 2020).

The current investigation demonstrated the high incidence of *C. perfringens* in small ruminants of less than 1 year age group, which are in aggrement with the results of Ajaz-ul-Haq *et al.* (2016), who reported 18.5% prevalence of *C. perfringens* in age group of 6 months to 1 year as compared to adult small ruminants (7.5%). In another study, Nazki *et al.* (2017), reported high prevalence of *C. perfringens* in lambs (56.16%) and kids (46.16%) than adult goat and sheep (3.84%). The authors described that high prevalence rate of *C. perfringens* in young animals is probably due to heavy feeding of lambs and kids on lush pastures in addition to milking by their mothers; they also

detected higher incidence in adult small ruminants grazed on luxurious pastures (Nazki *et al.*, 2017).

In this study antibiogram of the isolated organisms from small ruminants was conducted by disk diffusion method. The obtained results revealed that *C. perfringens* isolates of both sheep and goat origin exhibited 100% susceptibility against ceftazidime (a third generation cephalosporin) which is also in accordance with study of Mohiuddin *et al.* (2020) who reported 100% inhibition of *C. perfringens* isolates against a third generation cephalosporin *viz.*, ceftiofur. Our results revealed trimethoprim as a second most susceptible antibiotic against *C. perfringens* isolates. In contrary, the study of Haider *et al.* (2022) reported the little efficacy of trimethoprim against *C. perfringens* isolates of poultry. This might be due to the frequent use of this antibiotic in poultry production to get utmost production (Mohsin *et al.,* 2019). Our results are also in agreement with the study of Elariny *et al.*, (2021), who reported the resistant behavior of *C. perfringens* against oxytetracycline (87.5%), amoxicillin (83.4%), ampicillin and erythromycin (75%).

CONCLUSION

In conclusion, enterotoxaemia causing bacterium *viz.*, *C. perfringens* is prevalent in goats and sheep of district Kalat, Balochistan. Sheep were more affected than goats, the female animals were at high risk than males. Moreover, higher prevalence of *C. perfringens* was detected in <1 year age group. This study will be help full in designing the large-scale epidemiological studies and vaccination programs for control of the disease particularly in the study area.

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Conflict of interest

The authors declared no conflict of interest.

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