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Prevalence of Clostridium perfringens β2-toxin in sheep and goat population in Punjab, Pakistan

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Short Communication

Prevalence of *Clostridium perfringens β2*-toxin in sheep and goat population in Punjab, Pakistan

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Abstract

Clostridium perfringens presents persistent threat to small animals in causing moderate to severe enterotoxemia. The pathogenicity of *C. perfringens* depends on the production of four major toxins. In addition to the major toxins, beta$_2$ (β_2) toxin, a minor toxin, is also produced by some strains of *C. perfringens.* In this study, a total of 107 fecal samples collected from healthy and diseased sheep and goats were cultured and processed for biochemical analysis. On the basis of biochemical characterization, 61 isolates were confirmed as *C. perfringens.* In order to detect *C. perfringens* types, multiplex PCR assay was carried out for the confirmed isolates. Results indicated that the gene encoding beta$_2$ (β_2) toxin was found in 73% of type A isolates and 67% of type D isolates. There was no significant difference in the presence of this gene in the sheep and goats. An association between beta$_2$ (β_2) gene and disease occurrence was also found non-significant in both sheep and goat species. The present study suggests the high prevalence of *C. perfringens* beta$_2$ gene (β_2) in fecal isolates of both sheep and goats. However, the role of beta$_2$ (β_2) toxin gene in pathogenesis of enteric diseases needs further investigations.

Keywords: beta$_2$ toxin gene, *Clostridium perfringens*, enteric diseases, multiplex PCR, prevalence, sheep and goats

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Introduction

Clostridium perfringens is associated with enteric diseases of both humans and many animal species (McClane et al., 2006; Uzal and Songer, 2008). It possesses an extremely wide toxin arsenal. It is able to produce at least 17 toxins (Alouf, 2005; McClane et al., 2006; Keyburn et al., 2008). In this way, it is unique not only in terms of the number of toxins produced, but also in terms of their toxicity and lethality. It has five types based on four major types of toxins (Petit et al., 1999; McClane et al., 2013). These are alpha (α), beta (β), epsilon (ε) and iota (ι) toxins (Rood, 1998; Uzal and Songer, 2008). Each toxinotype is associated with a particular disease due to the production of a different set of toxins. All types produce α type toxin (Sakurai et al., 2004), along with that type B produces β- and ε-toxin, type C produces β-toxin, type D produces ε-toxin and type E strains produce ι-toxin. Some minor toxins are also produced by *C. perfringens*. For example, *C. perfringens* type A produces CPA and it can also produce minor toxins, including CPE and CPB_2. CPB_2 is a unique toxin which has newly been known and its encoding gene has been characterized (Shimizu et al., 2002). The amino acid sequence of β_2-gene has no noteworthy similarity to β-toxin. However, both of them have similar lethal effect on mice and have cytotoxic effect on intestinal cells. In this way, they do have analogous activity (Gibert et al., 1997). Therefore, it may be assumed that β_2-toxin does have a role in causing disease. The CPB_2-producing type A strains of *C. perfringens* may cause enteric problems in different species of animals, including sheep and goats (Gkiourtzidis et al., 2001; Bueschel et al., 2003; Waters et al., 2003; Dray, 2004; Vilei et al., 2005). However, most evidences associating CPB_2 in pathogenesis of enteric diseases in ovine and caprine species are based on identification of CPB_2-positive *C. perfringens* from sick animals. Similarly, the presence of CPB_2-positive type A strains were also reported in sick pigs (Bueschel et al., 2003; Waters et al., 2003). Preliminary studies also suggested the role of β_2-toxin producing strains of *C. perfringens* with typhlocolitis and enterocolitis in horses (Herholz et al., 1999; Waters et al., 2003). Since β_2-toxin does have a high prevalence in different hosts, it is vital to include it in the typing of *C. perfringens*.

Multiplex polymerase chain reaction (PCR) is now being widely used for the typing of *C. perfringens* (Albini et al., 2008; Goldstein et al., 2012). PCR genotyping provides a useful alternative to *in vivo* toxin neutralization tests for the typing of *C. perfringens* isolates (Meer and Songer, 1997; Miyashiro et al., 2007). It should be noted that up to this moment, no research on the prevalence of β_2-toxin has been carried out in Pakistan. Therefore, the present study was proposed to identify the occurrence of β_2-toxin in sheep and goats.

Materials and Methods

Sample site and isolated source: Fecal samples (n=107) of sheep and goats were randomly collected from selected districts (Bhakkar, Dera Ghazi khan, Faisalabad, Layyah, Muzaffargarh, Okara) of Punjab, Pakistan. The sheep and goats belonged to both genders and included healthy and diseased animals (diarrheal or animals having enteric problems). The samples were collected aseptically from the rectum of the animals in sterile plastic bags and were transported to the laboratory over ice for storage under refrigerated conditions.

Sample processing: The samples were diluted in Phosphate Buffered Saline (1:10) and placed in a water bath for 10 minutes. The bath temperature was maintained at 80°C in order to eliminate non-spore-forming bacteria. The processed samples were then inoculated on 5% blood agar and tryptose sulfite cycloserine agar (TSC) plates. The inoculated plates were kept in anaerobic jars at 37°C for 24 to 36 hours. Anaerobiasis was created by using anaerogen sachet. Identity of the isolates was confirmed by characteristic colony morphology, Gram's staining and biochemical tests. All culture media and additives used in this study were obtained from Oxoid (UK).

DNA extraction and multiplex polymerase chain reaction (PCR): To extract bacterial DNA, a few colonies of each *C. perfringens* isolate grown anaerobically overnight on tryptose sulfite cycloserine agar plate at 37°C were suspended in 1 ml distilled water in clean 1.5 ml Eppendorf tubes (about 10^6 cells per ml). Centrifugation was done at 6,000xg for 5 minutes at room temperature. Supernatant was removed completely and cells were resuspended in 200 µl cold Tris-EDTA (TE Buffer). DNA was extracted using a commercial DNA extraction kit (Bio-Basic, Canada). To detect all variants of cpb_2 gene, primers were used corresponding to each toxin of *C. perfringens*.

Multiplex PCR was carried out for the samples in a final volume of 25 µl. Each reaction mixture contained 1 µl of template DNA, 1X PCR buffer, 4 mM $MgCl_2$, 250 µM dNTPs, 0.05 U Taq DNA polymerase (Fermentas, Lithuania), 0.12 µM of alpha, beta, epsilon and iota primers, and 0.16 µM of $beta_2$ primer as listed in Table 1. The thermal cycling conditions included initial denaturation for 10 minutes at 95°C, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 1 minute 30 seconds and synthesis at 72°C for 1 minute 30 seconds. The final extension step was conducted at 72°C for 10 minutes. Positive control samples of individual toxin primers were run separately while negative control without DNA was run parallel with the test. PCR products were resolved by electrophoresis on 1.5% agarose gel with ethidium bromide stain. Amplified bands were observed under UV illumination.

Results and Discussion

Out of the 107 fecal samples, 68 were found culture positive and exhibited typical black colonies on the tryptose sulfite cycloserine agar plates. Most isolates showed presence of Gram positive rods and possessed subterminal spores. All culture positive isolates were further resolved through biochemical differential tests and sugar fermentation reactions. It

was found that 61 culture isolates belonged to *C. perfringens*.

All 61 isolates of *C. perfringens* were examined for the presence of alpha, beta, epsilon, iota and beta$_2$ gene by the multiplex PCR technique. The alpha toxin gene (*cpa*), which is a characteristic of all the *C. perfringens* strains regardless of their toxin type, was indicated by a band of 324 base pair. Moreover, a 548 base pair sequence representing beta$_2$ toxin gene (*cpb$_2$*) was confirmed in 72% of the isolates. The epsilon toxin gene (*etx*) indicated by a band of 376 base pair was confirmed in 15% of the isolates.

A total of 11 *C. perfringens* isolates from sheep with diarrhea or enteric disease and 21 without disease (healthy) were genotyped. Nine diseased and 18 healthy isolates were genotype A, and 2 diseased and 3 healthy isolates belonged to genotype D. In the case of goats, 7 isolates from diseased and 22 isolates from healthy animals were genotyped. Six diseased and 19 healthy isolates were type A, and 1 diseased and 3 healthy isolates belonged to type D (Table 2).

Table 1 Oligonucleotide primers

Toxin gene	Primer	Sequence (5′-3′)	Product	Reference
cpa (α-toxin)	CPAlphaF CPAlphaR	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324 bp	(Komoriya et al., 2007)
cpb (β-toxin)	CPBetaF CPBetaR	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	195 bp	(Komoriya et al., 2007)
cpb$_2$ (β$_2$-toxin)	CPBeta2F CPBeta2R	AAATATGATCCTAACCAACAA CCAAATACTCTAATCGATGC	548 bp	(Van Asten et al., 2008)
etx (ε-toxin)	CPEpsilonF CPEpsilonR	TGGGAACTTCGATACAAGCA AACTGCACTATAATTTCCTTTTCC	376 bp	(van Asten et al., 2009)
iap (ι-toxin)	CPIotaF CPIotaR	AATGGTCCTTTAAATAATCC TTAGCAAATGCACTCATATT	272 bp	(van Asten et al., 2009)

Table 2 Genotypes of *Clostridium perfringens* isolates

Sample origin	Health status	No. of samples collected	No. of isolates positive for *C. perfringens*	Genotype										% β$_2$-positive isolates
				A	A$^{\beta2}$	B	B$^{\beta2}$	C	C$^{\beta2}$	D	D$^{\beta2}$	E	E$^{\beta2}$	
Sheep	Diseased	19	11	4	5	-	-	-	-	-	2	-	-	64
	Healthy	33	21	5	13	-	-	-	-	2	1	-	-	67
Goat	Diseased	13	7	1	5	-	-	-	-	1	-	-	-	71
	Healthy	42	22	4	15	-	-	-	-	-	3	-	-	82

A, B, C, D, and E refer to genotype; b$_2$ refers to presence of the gene encoding b$_2$ toxin.

During the last two decades, numerous epidemiological studies have revealed extensive presence of *C. perfringens* β$_2$-toxigenic strains in diseased and healthy humans as well as animal species. On the basis of these epidemiological studies, *cpb$_2$* toxin gene and enteric disease were found strongly correlated in pigs and weakly correlated in case of horses. No association between β$_2$-toxin and gastrointestinal disease in humans or other animal species could be identified. However, *C. perfringens* types possessing *cpb$_2$* gene have been found in cattle, horses, pigs, small ruminants, poultry, fish, carnivores, domestic wildlife species and humans (Schotte et al., 2004; van Asten et al., 2010). Therefore, the association between *cpb$_2$* gene and enteric disease is yet to be decided. Moreover, different studies indicated varied prevalence of *cpb$_2$* gene in different species of animals. This study showed the high prevalence, i.e. 72% of beta$_2$ gene in sheep and goat population. Bueschel et al. (2003) also found high prevalence of beta$_2$ gene, i.e. 85.8% in swine isolates of *C. perfringens*. Zerbini and Ossiprandi (2007), however, found that 23.1% of *C. perfringens* type A isolates obtained from dogs (diarrheal) were *cpb$_2$* positive. Dysenteric lambs also showed low frequency (7/117) of *cpb$_2$* gene (Gkiourtzidis et al., 2001). Another study conducted in Arizona, USA mentioned low prevalence of beta$_2$ toxin. They found 197/1537 (12.8%) of bovine isolates positive by PCR for *cpb$_2$* gene (Bueschel et al., 2003). Garmony et al. (2000) examined the presence of *cpb$_2$* gene in more than 50% of the genotyped isolates from piglet, lamb, foal and calf enteritis.

The results of this study indicated that there was no significant difference in the presence of beta$_2$ gene in sheep and goats. Twenty-one of the 32 isolates (66%) from sheep and 23 of the 29 isolates (79%) from goats were positive for the *cpb2* gene. Moreover, the prevalence of beta$_2$ gene was found high in both healthy and diseased animals, indicating that there is no significant correlation between *cpb$_2$* toxin gene and enteric disease in these animals. One study conducted in 2011 also indicated that 34 out of 36 isolates from diseased flocks and 42 out of 43 isolates from healthy avian species were *cpb$_2$* positive (Tolooe et al., 2011). Some epidemiological studies carried out in 2003 advocated a strong association between *C. perfringens* isolates carrying the gene encoding beta$_2$ (*cpb$_2$*) and clostridial enteric diseases in domestic animals (Waters

et al., 2003). There is no local literature available on the prevalence of *C. perfringens* beta$_2$ toxin gene from sheep and goats in Pakistan. In our study, the prevalence of beta$_2$ gene was found high in both sheep and goats. The number of *cpb*$_2$-harboring isolates was also equally dispersed between the healthy and diseased animals.

The investigations carried out in healthy sheep and goat and those with diarrhea has shown a weak correlation between the occurrence of the disease and presence of *cpb*$_2$ gene. However, future research is essential to understand the role of beta$_2$ toxin in the induction of enteric disease, the capability of the *cpb*$_2$ gene to produce toxin and the regulatory mechanisms involved in the expression of beta$_2$ toxin.

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บทคัดย่อ

อุบัติการณ์ของ *Clostridium perfringens* ท็อกซิน $\beta 2$ ในแกะและแพะ ในรัฐปัญจาบ ประเทศปากีสถาน

มูดาซซา โมฮีดิน[1*] ซาฮีด อิคบาล[2] ซาจาด ยัว เราะห์มาน[3]

เชื้อ *Clostridium perfringens* มีความสำคัญเนื่องจากก่อให้เกิดโรคระบบทางเดินอาหารที่รุนแรง การก่อโรคของเชื้อ *C. perfringens* จะขึ้นกับการผลิตชีวพิษหลัก 4 ชนิด ส่วนชีวพิษย่อยชนิด $\beta 2$ จะสร้างโดยเชื้อ *C. perfringens* บางสายพันธุ์ การศึกษาครั้งนี้ได้เก็บตัวอย่างอุจจาระแกะและแพะจำนวน 107 ตัวอย่าง จากนั้นเพาะแยกเชื้อร่วมกับทดสอบทางชีวเคมี จากผลทดสอบทางชีวเคมีสามารถตรวจพบเชื้อ *C. perfringens* ได้ 61 ตัวอย่าง และตรวจยืนยันเชื้อด้วยวิธี multiplex PCR ผลการทดสอบพบยีน $\beta 2$ คิดเป็นร้อยละ 73 ของเชื้อ *C. perfringens* ชนิดเอ และร้อยละ 67 ของเชื้อ *C. perfringens* ชนิดดี แต่ไม่พบความแตกต่างของยีนที่พบในตัวอย่างแกะและแพะอย่างมีนัยสำคัญ และไม่พบความสัมพันธ์ระหว่างยีน $\beta 2$ กับการเกิดโรค ผลการศึกษาครั้งนี้แสดงให้เห็นถึงอุบัติการณ์สูงของเชื้อ *C. perfringens* ที่มียีน $\beta 2$ จากตัวอย่างอุจจาระของแกะและแพะ อย่างไรก็ตามควรมีการศึกษาบทบาทของยีน $\beta 2$ ต่อพยาธิกำเนิดของโรคระบบทางเดินอาหารต่อไป

คำสำคัญ: ยีนเบต้าสอง เชื้อ Clostridium perfringens โรคทางเดินอาหาร ปฏิกิริยาลูกโซ่โพลิเมอเรส อุบัติการณ์ แกะ แพะ

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