

Short Communication

Toxin typing of *Clostridium perfringens* Associated with Enterotoxaemia in Sheep in Fars Province

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Abstract

Clostridium perfringens is implicated in the etiology of some diseases including fatal enterotoxaemia. Determining dominant toxin types of this microorganism can be helpful in epidemiologic surveys and the formulation of more proper vaccines. To understand the pathogenicity of this bacterium, it seems necessary to describe the toxin and virulence genes content of strains involved in enterotoxaemia and other associated diseases. The current study aimed to isolate and type the toxins of *C. perfringens* in sheep with suspected enterotoxaemia in Fars province by culture-PCR and ELISA methods and to compare them to isolates of a healthy group. Samples of intestinal contents were collected from enterotoxaemia cases and a healthy group of sheep. The presence of alpha, beta, and epsilon toxins were evaluated by ELISA method. After culture and isolation of *C. perfringens*, toxin typing and screening of isolates for the presence of beta-2 and enterotoxin were performed by PCR method. *C. perfringens* was isolated from 102 of 167 suspected enterotoxaemia cases of sheep and from 22 of 50 healthy sheep. The PCR results showed that type A was the most prevalent toxin type in both groups, but according to ELISA type D was the dominant toxin type in the clinical group. The enterotoxin gene was detected in 10% of all isolates from healthy and suspected group isolates of types A and D. The beta-2 gene was identified in 35% and 63.6% of enterotoxaemia-associated isolates and isolates not associated with disease, respectively. In conclusion, Type D of *C. perfringens* was the dominant causative organism of fatal enterotoxaemia in sheep in Fars province.

Keywords: *Clostridium perfringens*, toxin-typing, PCR, ELISA

Typage de Toxine de *Clostridium perfringens* Associé à l'Entérotaxémie chez les Moutons dans la Province de Fars

Résumé: *Clostridium perfringens* est impliqué dans l'étiologie de certaines maladies dont l'entérotaxémie mortelle. La détermination des types de toxines dominants de ce micro-organisme peut être utile dans les enquêtes épidémiologiques et la formulation de vaccins plus appropriés. Pour comprendre la pathogénicité de cette bactérie, il semble nécessaire de décrire le contenu en gènes de toxines et de virulence des souches impliquées dans l'entérotaxémie et d'autres maladies associées. La présente étude visait à isoler et typer les toxines de *C. perfringens* chez des moutons suspectés d'entérotaxémie dans la province de Fars par des méthodes de culture- RPC et ELISA et de les comparer aux isolats d'un groupe sain. Des échantillons de contenu intestinal ont été collectés à partir de cas d'entérotaxémie et d'un groupe sain de moutons. La présence de toxines alpha, bêta et epsilon a été évaluée par la méthode ELISA. Après la culture et l'isolement de *C. perfringens*, le typage des toxines et le criblage des isolats pour la présence de bêta-2 et d'entérotaxine ont été effectués par la méthode RPC. *C. perfringens* a été isolé de 102 des 167 cas suspects d'entérotaxémie de moutons et de 22

50 moutons sains. Les résultats de la RPC ont montré que le type A était le type de toxine le plus répandu dans les deux groupes, mais selon ELISA, le type D était le type de toxine dominant dans le groupe clinique. Le gène de l'entérotoxine a été détecté dans 10% de tous les isolats provenant d'isolats de groupe sains et suspects de types A et D. Le gène bêta-2 a été identifié dans 35% et 63.6% des isolats associés à l'entérotoxémie et des isolats non associés à la maladie, respectivement. En conclusion, le type D de *C. perfringens* était l'organisme causal dominant de l'entérotoxémie mortelle chez les moutons dans la province de Fars.

Mots-clés: *Clostridium perfringens*, typage des toxines, RPC, ELISA

1. Introduction

Enterotoxaemia is one of the most important and frequently occurring diseases of livestock in Iran and other countries (1). Its causative agent is *Clostridium perfringens*, an anaerobic gram-positive rod-shaped bacterium that produces many toxins, among which the four major lethal ones, i.e. alpha, beta, epsilon, and iota, are used for typing (2). Beta 2 and enterotoxin are two other toxins whose role in pathogenesis of enterotoxaemia remains ambiguous (2). To understand the pathogenicity of this bacterium, it seems necessary to describe the toxin and virulence gene contents of strains involved in enterotoxaemia and other associated diseases. Some techniques used for toxin typing include ELISA and PCR. As PCR is sensitive, specific, and rapid, it is widely used for toxin typing of *Clostridium* (3). ELISA is another helpful method which has been found to be 95% reliable for the detection of *C. perfringens* toxins in the intestinal contents of suspected cases of enterotoxaemia (4, 5). In contrast to PCR, in ELISA the expression of toxin genes, as the most accepted criterion, is evaluated, and positive results of this test confirm the diagnosis of enterotoxaemia (6).

Because of the high fatality rate associated with enterotoxaemia, which consequently affects the farming industry, this disease and studies related to it are of great importance. There are few published reports from Iran of toxin typing of *C. perfringens* involved in enterotoxaemia in the field (1). Furthermore, there has been no research on this issue in Fars province in the south of Iran, although there are

many reports of this disease annually in this province. Notably, formulation of a proper and efficient polyvalent vaccine depends on the knowledge of prevalence of *C. perfringens* toxin types in a region.

The current study aimed to determine the toxin types of *C. perfringens* isolates of sheep with suspected enterotoxaemia in Fars province by culture-PCR and ELISA methods and to compare them to isolates from healthy sheep.

2. Material and Methods

2.1. Sample Collection

A total of 167 samples of intestinal contents of sheep with sudden death suspected of enterotoxaemia from different parts of Fars province were obtained from a Shiraz veterinary office, veterinary networks, and the private sectors in different parts of Fars province. Indications for enterotoxaemia are usually based on either clinical suspicion of animals showing symptoms of severe and profuse diarrhea or, as in most fatal cases, sudden death.

Samples of intestinal contents for the control group were collected from 50 healthy sheep in slaughterhouses. The samples were transferred on ice to the microbiology laboratory. A portion of the samples was inoculated onto blood agar supplemented with 40 µg/ml neomycin and tryptose sulphite cycloserine agar (Oxoid, Germany) containing 400 mg/l cycloserine (SR88, Oxoid). The plates were incubated anaerobically at 35 °C. The suspected colonies of *C. perfringens* which were gray, smooth, sometimes rhizoid with a double zone of hemolysis on blood agar, and black colored on TSC agar, were picked, gram stained, purified, and subjected

to biochemical tests (7). Further confirmation and toxin typing were done by PCR.

2.2. PCR

PCR tests were carried out using primer pairs for each toxin gene, including α , β , ϵ , ι , β_2 , enterotoxin and the species-specific primer of 16SrRNA. The primer sequences are listed in Table 1. Suitable reference strains for toxin types were included as positive controls in the PCR tests. The PCR program was set up and conducted for 30 cycles of 95 °C, 60 °C, and 72 °C for 1 min each. The exception was annealing temperature, which decreased to 55 °C for CPE and

CPB2 primers.

2.3. ELISA

Intestinal contents and body fluids were screened for the presence of *C. perfringens* toxins using the related ELISA kits (Cypress Diagnostics Co., Belgium). Alpha, beta, and epsilon toxin *C. perfringens* ELISA kits were used to evaluate α , β , and ϵ toxin, respectively. Each kit contains 96-well microtitration plates sensitized by specific monoclonal antibodies to allow a specific capture of the corresponding antigen (toxin) present in the samples. The tests were performed according to the kit protocol.

Table1. List of primers used in this study

Primer name	Gene specificity	Sequence	Product size (bp)	Reference
16SrRNA	Species specific	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279	(18)
CPA	α toxin	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	(3)
CPB	β toxin	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	(3)
ETX	ϵ toxin	GCGGTGATATCCATCTATTC CCACTTACTTGTCTACTAAC	655	(3)
Ia	ι toxin	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	(3)
CPE	enterotoxin	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233	(3)
CPB2	β_2 toxin	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	567	(19)

3. Results

C. perfringens was isolated from 102 of 167 suspected enterotoxemic cases of sheep and from 22 of 50 healthy sheep and confirmed by biochemical tests. The isolates were non-motile, catalase negative, sucrose and lactose fermentation positive, urease and indole negative, gelatinase and lecithinase positive, and lipase negative.

The PCR results showed that all isolates were positive for the 279 bp segment of the 16SrRNA gene, confirming these isolates as *C. perfringens* strains. PCR products of toxin typing for alpha, beta, and epsilon toxins were 324 bp, 196 bp, 655 bp, respectively (Figure 1a).

Typing of *C. perfringens* isolates by PCR showed that 46, 18, 6, and 32 of isolates from the enterotoxemic group were type A, B, C, and D, respectively. In the control group, 16 isolates were type A and 6 were type

D. There was no type E in the isolated strains of either group (Table 2).

The results of ELISA showed that all intestinal content samples from the healthy group were negative for the presence of 3 major toxins, except one sample which was positive for the alpha toxin. In suspected cases, 68 samples were positive for at least one alpha, beta, or epsilon toxins. The number of different toxin types based on ELISA method are listed in Table 2.

The screening of isolates for the presence of enterotoxin and beta-2 toxin genes by PCR (Figure 1b and c) revealed that nine isolates out of 102 from the suspected group (8.8%) and four out of 22 isolates from the healthy group (18.1%) were positive for enterotoxin. A total of 36 (35.2 %) and 14 (63.6%) isolates were identified as positive for the beta-2 gene from the suspected and healthy group, respectively. Toxin types of these isolates are listed in Table 2.

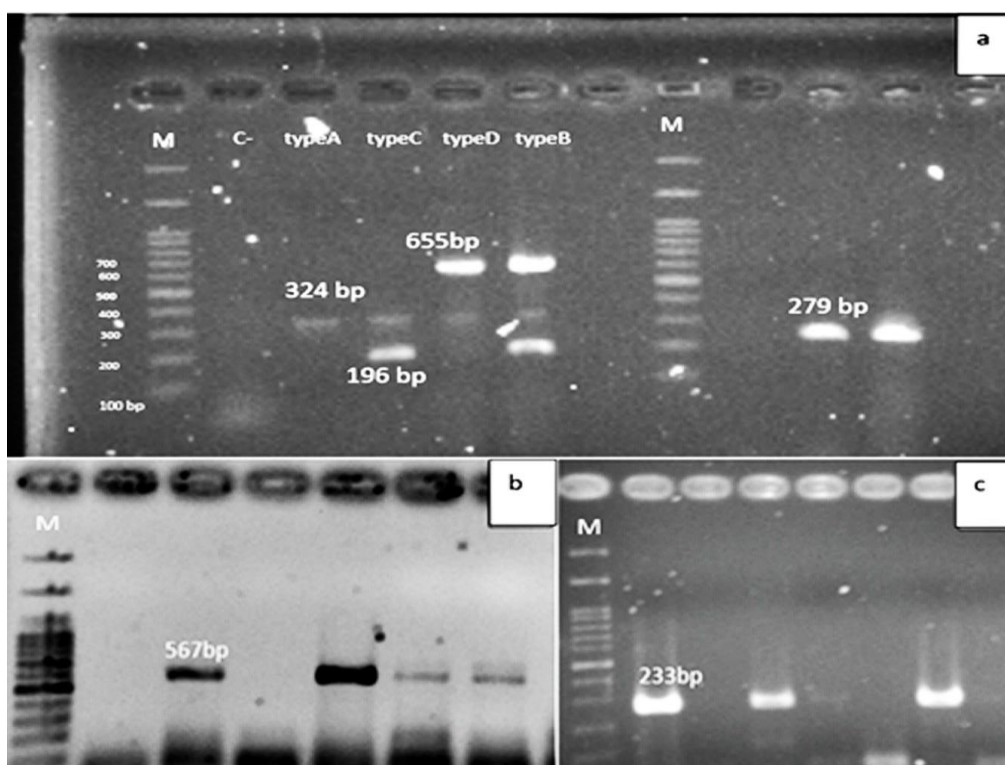


Figure 1. Agarose gel electrophoresis of PCR assays for detection of a: *C. Perfringens* 16srRNA, a band of 279 bp (right) and toxin typing of *C. perfringens* isolates (left), bands of 196 bp, 324 bp and 655 bp are amplicons of beta, alpha, and epsilon toxin genes, respectively. Lane2 is PCR negative control; lanes 3, 4, 5, and 6 are representative of types A, C, D, and B of *C. perfringens*, respectively. The marker (M) is a 100 bp gene ruler, RTU, cat No. PR911653 (Cinnagen, Iran);b: detection of beta-2 toxin gene (567bp); and c: detection of enterotoxin gene (233 bp).

Table 2. Number of different toxin types in suspected enterotoxemic and healthy groups

Number of Toxin types	Type A	Type B	Type C	Total	Type D
Number of isolates in suspected group(ELISA Method)	17	18	6	68 (40.71%)	27
Number of isolates in healthy group(ELISA Method)	1	-	-	-	1 (2%)
Number of isolates in suspected group (PCR Method)	46	18	6	102 (61.07%)	32
Number of isolates in healthy group (PCR Method)	16	-	-	22 (44%)	6
Number of enterotoxin positive isolates in suspected group	5	-	-	9 (8.82%)	4
Number of enterotoxin positive isolates in healthy group	2	-	-	4 (18.18%)	2
Number of beta-2 positive isolates in suspected group	7	10	1	36 (35.29%)	18
Number of beta-2 positive isolates in healthy group	5	-	-	14 (63.63%)	9

4. Discussion

Clostridium perfringens is an under-studied pathogen with the most prolific toxins, including alpha, beta, and epsilon as major ones (8). It has been well established that detection of these toxins in intestinal contents and body fluids is a definitive evidence of enterotoxaemia disease (6). The fatality rate of this disease in sheep is high, ranging between 58% and 100% and presenting mostly as sudden death (9).

In this study, collected samples of suddenly death cases suspected of enterotoxaemia from different flocks and veterinary departments in various regions of Fars province in Iran were transferred to our lab for isolation of *C. perfringens* and confirmation of disease by ELISA method, typing of isolates, and comparison of these isolates with the isolates from healthy flocks. Results of this study showed that from 167 suspected cases, 61% were positive for isolation of *C. perfringens*, while only 40% were definitively demonstrated to be positive for the presence of at least one of either alpha, beta, or

epsilon toxins in their intestinal contents and body fluids. This result indicates that most suspected cases were not diagnosed as enterotoxaemia by ELISA, and evaluation for other diseases and agents should be considered. There are some reports of enterotoxaemia prevalence and typing of *C. perfringens* isolates from different parts of the world. In Iran, Ahsani, Mohammadabadi (1) reported the isolation and typing of *C. perfringens* from non-vaccinated sheep in Kerman province. According to Ahsani, Mohammadabadi (1) results, type C was the most and type A the least prevalent isolates in Kerman province. Conversely, the results of the current survey in Fars province showed that type A was the most prevalent isolate in two groups of clinical and non-clinical isolates by culture and PCR method. This result is consistent with the results of (3), Gökce, Genc (4), (5, 9, 10).

Gökce, Genc (4) reported that from 220 samples of suspected cases of enterotoxaemia from sheep in

Turkey, 58.6% and 84.6% were positive for the presence of typing toxins with type A as the dominant type by latex and ELISA method, respectively.

According to other research in Turkey by Hadimli, Erganis (5), the most common type of *C. perfringens* was type A, which was determined by both ELISA and PCR methods. They isolated this bacterium from 8.66% of lambs suspected of enterotoxaemia (5).

In this study the dominant type detected by ELISA method was type D in the suspected group, which reveals the important role of this type in the pathogenesis of the disease.

As it is obvious that isolation of clostridium is not enough for confirmation of the disease, the bacteria were isolated from about 20% of clinical cases with negative ELISA results and 44% of healthy ones. This can be indicative of the role of *C. perfringens* type A as a dominant normal flora in sheep intestine. Based on PCR results, the beta toxin gene was not detected in the healthy group, and the iota gene, representing the uncommon type E, was not present in any group.

According to published data, some toxin genes are located on conjugative plasmids (11). It can be suggested as previously postulated by others that types B, C, D, and E can be derived from type A by acquiring the toxin plasmids or vice versa (11, 12).

PCR experiments showed that the other two non-typing toxins, beta-2 and enterotoxin, were present in some isolates of both groups.

It has been reported before that enterotoxin is associated with food-borne and non-foodborne gastroenteritis and may be produced by some type A, C, D, or E, but not by any known type B isolates (12, 13). This chromosomally or plasmid encoded toxin is considered to be one of the pathogenesis factors which induces histological damages (6, 8, 12, 14).

According to previous publications, less than 5% of all *C. perfringens* isolates in the world carry the enterotoxin gene (15). In this study, the enterotoxin gene was detected in 10% of all isolates from healthy and suspected group isolates of types A and D. Based on new classification described by Rood et al. in 2018,

these nine isolates of type A which harbor the *cpe* gene can be reclassified as type F, meaning 5% of the isolates belonged to this type (16).

The other toxin, beta-2, was detected in 63.6% and 35.2% of healthy and clinical isolates, respectively. The presence of this toxin was seen in all isolates of types A, B, C, and D.

It has been stated in previous studies that the beta-2 toxin can be produced by all *C. perfringens* types. The exact role of beta-2 toxin in pathogenesis has not yet been determined, although its cytotoxicity for CHO cells and induction of hemorrhagic necrosis in Guinea pig intestine and enterocolitis in foals have been reported (2, 8, 12). Enterotoxin is another toxin which is associated with sporulation and has been implicated in food-borne and non-foodborne gastroenteritis (8, 17).

Results of the current study showed that not only was the presence of the two accessory enterotoxin and beta2 genes not higher in clinical isolates, but it was even less prevalent in this group in comparison to the healthy group.

Collectively, based on ELISA results, type D of *C. perfringens* was the dominant cause of fatal enterotoxaemia of sheep in Fars province. ELISA results for estimation of prevalence and typing of *C. perfringens* differ from those of culture and the PCR method in enterotoxaemia cases. The use of the ELISA method for reporting the disease in a region and evaluating other diseases in negative cases is recommended.

Authors' Contribution

Study concept and design: M. H.

Acquisition of data: M. H.

Analysis and interpretation of data: M. H.

Drafting of the manuscript: M. H.

Critical revision of the manuscript for important intellectual content: Y. T.

Statistical analysis: Y. T.

Administrative, technical, and material support: M. H. and Y. T.

Ethics

All procedures performed in studies involving animals were in accordance with the ethical standards of the Shiraz branch of Razi Vaccine and Serum Research Institute (grant No. 2-84-18-87028).

Conflict of Interest

The authors declare that they have no conflict of interest.

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