International Journal of Enteric Pathogens

Int J Enteric Pathog. 2016 August;4(3):e35249 Original Article

http://enterpathog.com

doi 10.15171/ijep.2016.02

Typing Toxigenic *Clostridium perfringens* Strains from the Ruminants of Yazd Province by Multiplex Polymerase Chain Reaction

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Published Online April 23, 2016

Keywords: Multiplex PCR, 16S rRNA, *Clostridium perfringens*, Ruminant, Yazd province

Abstract

Background: Anaerobic bacterial infections, especially enterotoxemia, are common ruminant disorders.

Objectives: The purpose of this study was to identify the toxigenic isolates of *Clostridium* perfringens in the ruminants of Yazd province.

Materials and Methods: In total, 485 fecal and intestinal samples were obtained and analyzed for typing *C. perfringens* toxovars by multiplex polymerase chain reaction (PCR). Only 179 bacterial strains passed the biochemical tests and 87 *C. perfringens* strains were confirmed by multiplex PCR.

Results: Interestingly, the predominant *C. perfringens* toxovar was type A (89.7%), while type D (9.2%) was also identified as a pathogen in the ruminants of Yazd province.

Conclusions: Detection of toxigenic *C. perfringens* isolates with multiplex PCR was performed for the first time in this field. The multiplex PCR used in this study provides a useful and reliable tool for *C. perfringens* genotyping in routine veterinary diagnostics, andepidemiological studies of the prevalent types of *C. perfringens* in Iran are possible by this technique. Genotyping of *C. perfringens* is recommended before starting vaccination programs.

Received December 5, 2015; **Revised** February 18, 2016; **Accepted** March 21, 2016

Background

Clostridium perfringens (*C. perfringens*) is a gram positive, sporulating bacterium that is extremely pathogenic and responsible for a wide spectrum of anaerobic diseases in animals and humans. This bacterium is widely spread in the soil and the gastrointestinal tract of animals and is classified into five toxinotypes (A, B, C, D, and E) based on the production of one or more of the four main toxins (α, β, ε, and ι)[1] which are responsible for specific enterotoxemia in animals.[2] Enterotoxaemia is one of the most widespread lethal diseases in ruminants. This disease is indicated when a high level of toxins produced in the gut contents may be absorbed into the general circulation.[3,4]

Typing *C. perfringens* strains is important, because different types of bacteria are associated with specific enteric diseases in animals.[5] Traditional methods have some limitations for bacterium typing. Serum neutralization test, enzyme-linked immunosorbent assay (ELISA),

and polymerase chain reaction (PCR) have been previously used for the classification of *C. perfringens*,[5] among which PCR is the best choice for typing and sub-typing. Various PCR protocols have been established for the typing of *C. perfringens* isolates.[6]

PCR has been applied in several studies and highlighted as a rapid and accurate method for the detection of low copy numbers of genes. This method is more accurate and faster than conventional methods.[7]

Objectives

To the best of our knowledge, there is a lack of data on the prevalence and typing of C. *perfringens* strains in Yazd ruminant farms. Therefore, we used PCR for typing toxigenic *C. perfringens* strains in this field.

Materials and Methods

Samples

In total, 485 fecal and intestinal samples were investigated from the following species: domestic sheep (n = 206), goat (n = 129), and cow (n = 150). Collection and preparation of the samples was performed as previously described by Alimolaei, et al.[8] Bacterial strains were isolated from the samples and the isolated colonies were analyzed according to the shape, color, type of hemolysis, and gram staining smears as described by MacFaddin.[9] Biochemical identification of the catalase negative colonies presenting *C. perfringens* characteristics was performed as described

previously.[8]

Reference Strains

Standard *C. perfringens* reference strains ($ATCC_{13124}$, CN_{228}, CN_{301}, and CN_{409}) were utilized as positive *C. perfringens* type controls (types A, B, C and D, respectively) as well as *C. septicum* strain CN_{913} as the negative control.

Polymerase Chain Reaction Amplification and Assay

DNA extraction from the isolated and reference *C. perfringens* strains was performed as previously described by the authors.[10] *C. perfringens* typing was performed with the total reaction volume of 50 µL and the following reagents: 5 µL of the extracted DNA with 25 µL of ready-to-use PCR master mix (PR901638, Sinaclon, Iran), 1.25 µL (20 pmole/µL) of each primer, and dH_2O to reach a volume of 50 µL.

For the PCR amplification of 16S rRNA gene and α, β, ε, and ι toxin genes, specific primers (Sinaclon, Iran) were used (Table 1). Amplicons were obtained with 35 cycles following an initial denaturating step at 95°C for 5 minutes. Each cycle involved denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, synthesis at 72°C for 90 seconds, and final extension step at 72°C for 5 minutes.

Amplicons were evaluated by electrophoresis on 1.7% agarose gel. The 100 bp DNA ladder (PR911653 and PR901644, Sinaclon, Iran) was used as the molecular marker to indicate the size of the amplicons. The amplified bands were visualized and photographed under UV illumination.

Results

Out of 485 investigated samples, 849 bacterial isolates were morphologically selected for microbiological examination. From these isolates, only 179 strains passed the biochemical tests and were examined by PCR. In this study, we isolated and confirmed 87 *C. perfringens* strains by multiplex PCR (Table 2). Seventy-eight strains were classified as type A (possessing *cpa* gene), one strain belonged to type C (possessing *cpa* and *cpb* genes), and eight strains belonged to type D (possessing *cpa* and *etx* genes) (Figure 1). Other types of *C. perfringens* (B and E) were not detected.

Table 1. Sequence of Specific Primers Used for *C. perfringens* Typing in This Study

Toxin	Gene	Primers Sequence (5′–3′)	Amplicon Size (bp)	Reference
-	16S rRNA	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279	[11]
α	*cpa*	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	[5]
β	*cpb*	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	[5]
ε	*etx*	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655	[5]
ι	*iap*	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	[5]

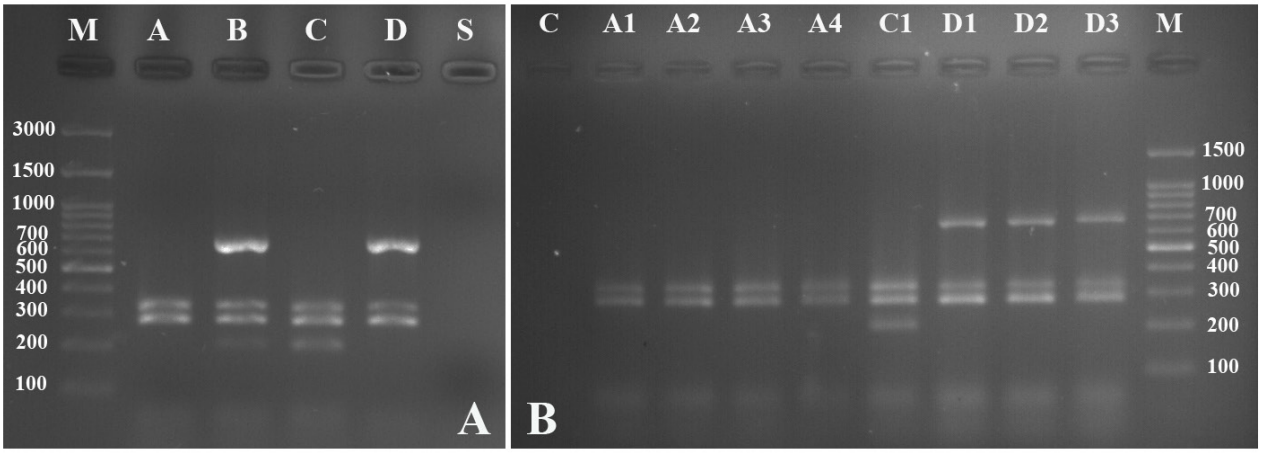

Figure 1. Typing of *C. perfringens* Isolates From The Ruminants of Yazd Province With *cpa* (324 bp), *cpb* (196 bp), *etx* (655 bp), *iap* (446 bp), and 16S rRNA (279 bp) Gene Primers by Multiplex PCR. A: Multiplex PCR on reference strains. M: 100 bp DNA ladder (PR911653, Sinaclon, Iran); Lane A: *C. perfringens* type A reference strain (ATCC® 13124™); Lane B: *C. perfringens* type B reference strain (CN228); Lane C: *C. perfringens* type C reference strain (CN301); Lane D: *C. perfringens* type D reference strain (CN409); Lane S: negative control, *C. septicum* reference strain (CN913). B: Multiplex PCR on isolated *C. perfringens*. Lane C: negative control, dH2O; Lanes A1-A4: *C. perfringens* type A field isolates; Lane C1: *C. perfringens* type C field isolates; Lanes D1-D3: *C. perfringens* type D field isolates; M: 100 bp DNA ladder (PR901644, Sinaclon, Iran)

Table 2. Genotyping of *C. perfringens* Isolates From the Ruminants of Yazd Province

C. perfringens Type	Positive Isolates	
	No.	%
A	78	89.65
B	-	-
C	1	1.15
D	8	9.20
E	-	-
Total	87	100

Discussion

C. perfringens infection in ruminants and other species is generically called enterotoxemia,[4] in which *C. perfringens* proliferates in large numbers and produces several toxins.[12] These toxins are responsible for enterotoxaemia in almost all the ruminants.[13] There are geographical differences in the prevalent types of the these bacteria and the type could be different depending on the animal species in the area.[7]

Toxigenic typing of *C. perfringens* isolates could be performed by different conventional and modern methods. Serum neutralization test and ELISA assay have been applied previously.[14-16]

PCR method for the typing of *C. perfringens* was developed by Daube et al,[15] and has been used in different conditions by other researchers.[5,7,8,10,13,17-22] The multiplex PCR presented here included all the important toxin genes in *C. perfringens* mediated enterotoxaemia.[8,21] Compared to the conventional techniques, PCR method was shown to be much more rapid, provided results in a few hours, and was much more reliable.[23]

In the present study, typing of *C. perfringens* isolates by multiplex PCR revealed that types A and D were the most important types of *C. perfringens* in the ruminants of Yazd province. Type A was the most frequent type of the bacteria which was isolated from the environment and digestive tract of the animals and humans.[1] Similar results were obtained by Greco et al,[17] who reported that, while *C. perfringens* types A and D were detected at the rates of 84% and 16% by PCR, respectively, type A was reported as the dominant type of *C. perfringens* in lambs and sheep worldwide.[7]

In conclusion, the detection of toxigenic *C. perfringens* isolates circulating in the ruminant fields of Yazd province is very important and was performed for the first time in this field. In contrast to other protocols, in this study, the used multiplex PCR was specific for *C. perfringens*, since we applied a specific primer (16S rRNA) for the diagnosis of *C. perfringens* species. This multiplex PCR provided a useful and reliable tool for *C. perfringens* genotyping in veterinary diagnostics and the type A of *C. perfringens* was more than other types in small ruminants.

Acknowledgments

The authors wish to thank the staff of Razi Vaccine and Serum Research Institute, Kerman.

Authors Contributions

Mehrdad Shamsaddini Bafti and Majid Ezatkhah: study concept and design; Mojtaba Alimolaei and Maryam Amini: acquisition, analysis and interpretation of data; Mojtaba Alimolaei and Mehrdad Shamsaddini Bafti: drafting of the manuscript; Majid Ezatkhah and Maryam Amini: administrative, technical, and material support; Majid Ezatkhah and Mehrdad Shamsaddini Baft: study supervision.

Conflict of Interest Disclosures

None.

Funding/Support

This study was supported by a grant (No. 0-85-18-90010.5) from the Razi Vaccine and Serum Research Institute,

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