Original Article

Determination of Type and Molecular Identity of *Clostridium perfringens* Isolated from Patients with Multiple Sclerosis (MS)

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Abstract

Background & Objectives: Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS). *Clostridium perfringens* ε-toxin (ETX) can cause emerging complications of MS due to its tendency to the blood-brain barrier. This study aimed to determine the presence of toxin-producing genes in intestinal *C.perfringens* in patients with multiple sclerosis.

Materials & Methods: Stool samples were taken from 70 MS patients and 70 without MS individuals. The samples were enriched in cooked meat media, and cultural and biochemical methods separated *Clostridium* isolates. PCR tested these isolates to identify *C. perfringens* species. In addition, the presence of alpha, beta, epsilon and iota toxin-producing genes was evaluated in all isolates by Multiplex PCR.

Results: Isolates containing the *etx* gene were observed in 10 patients, while no *itxA* gene was identified in any isolates. The results showed that isolates in 8 patients were type D. Also, the gene encoding toxin type D was identified in 2 isolates obtained from the control group.

Conclusion: Our findings indicated the high frequency of *C. Perfringens* in MS patients. In the studied samples with clinical presentations, most of these organisms were type D bacteria that produce ε-toxin.

Keywords: *Clostridium perfringence,* ε-toxin, multiple sclerosis, toxin-producing genes

Introduction

Multiple sclerosis (MS) is an autoimmune disease in which part of the immune system targets the brain and cells of the spinal cord and leads to demyelination and nerve cell degeneration (1). This degeneration occurs when some white blood cells (T cells, B cells and plasma cells) cross the blood-brain barrier, enter the central nervous system, then invade the myelin sheath of nerve cells (instead of foreign agents) and cause damage and hard spots in them (2). Previously, Iran was considered a region with low rates of prevalence of MS, but recent research shows that MS has increased significantly. The prevalence of MS is moderate in Iran, but the disease is increasing rapidly that its peak prevalence has reached 80 per 100,000 people (3). The primary causal agent has not yet

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been fully identified; however, several recent studies have shown that intestinal microbes, as one of the factors, may play a role in disease onset with a mechanism that is not yet fully understood (4). Research has shown that in all clinical forms of MS (i.e., clinically isolated syndromes (CIS), relapsing-remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS)), an increase in certain bacteria, in particular *Clostridium*, is evident. Several species of *Clostridium* may be suggested for MS, and *Clostridium* botulinum and *C.perfringens* have the foremost role (5). *Clostridium* is a anaerobic, gram-positive, spore-producing, immobile, coarse, round-ended bacilli (6). Approximately 40 to 50 species of this genus are involved in actual pathogenesis in humans and animals. *C.perfringens* is one of the species that cause severe pathogenicity. This bacterium is a causative agent of many animal diseases (7). This species produces several types of toxins. Diseases associated with *C.perfringens* are caused by food, and it is the bacteria that produce toxins in the intestines and lead to many diseases (8). *C.perfringens* has four main toxins (alpha, beta, epsilon and iota), which form the basis for classification of five types A through E. Since *C.perfringens i*s the natural flora of ruminants, toxin production varies under different nutritional conditions and causes different levels of pathogenicity in ruminants (cattle and sheep). In one study, results broadly support the previous findings and the role of Etx in the etiology of MS (9). Evaluation of the toxigenicity of this bacterium can be a good measure of its pathogenicity (8). In a study conducted by Leski *et al.* (2011) in Iraq and Kuwait, researchers have succeeded in separating *Clostridium* from the sand and dust of several sites of these two countries by molecular method and have proposed a respiratory way for the transmission of these pathogenic bacteria (9). Type A of this bacterium is only capable of producing alpha-toxin. In contrast, type B is capable of producing alpha, beta and epsilon toxins, type C can produce alpha and beta toxins, type D can produce alpha and epsilon toxins, and type E is capable of producing alpha and iota toxins (10). *C.perfringens* types B and D carry the *etx* gene, which encodes a protoxin with a molecular weight of 33 kDa. Protoxin is secreted during the growth phase. It is then broken down by trypsin and chymotrypsin in the gastrointestinal tract, producing an active toxin that is approximately 100 times more potent than protoxin (11). In a study conducted by Alshammari et al. (2020) on patients with autism, these bacteria showed a significant increase in children with autism compared with the control group (12). Another study by Nelson et al. (2009) on US military personnel stationed in the Middle East with Guillain-Barre Syndrome (a disease with neurological symptoms similar to MS) suggested the role of intestinal infections in the development of the disease (13). The present study aimed to determine the molecular identity of *C.perfringens* isolated from the stools of MS patients based on the εtoxin-producing genes.

Materials & Methods

Sampling and sample preparation

In this descriptive cross-sectional study, the studied population included all patients with MS who were classified into four groups based on the physician's diagnosis and their clinical symptoms. Purposive sampling was performed: 70 samples from the stools of patients with multiple sclerosis and 70 samples without MS. Patients were divided into four categories: 8 patients with the clinically isolated syndrome, 48 patients with relapsing-remitting MS, 12 patients with primary progressive MS and two patients with secondary progressive MS. Secondary progressive MS is a continuation of relapsing-remitting MS, and both were evaluated in the same group (14). *Clostridium* strains were isolated from the stools of case and control groups in the laboratory of Islamic Azad University, Kerman Branch. First, 1 g of stool samples was placed in a culture medium containing cooked meat (Himedia-India). Then, incubation was performed at 37 °C for 24 hours for all samples. Finally, samples were examined for their growth (turbidity and gas production). Tubes

with growth symptoms were used for streaking on the surface of the SPS medium (Sulfite Polymyxin Sulfadiazine) (Himedia-India). Then, the samples were incubated under anaerobic conditions at 37 °C for 24 hours. Suspicious colonies were selected for confirmatory tests and the presence of *Clostridium*. Next purification was performed on the surface of another SPS medium. To maintain samples and to work on them at the same time, TSB (Tryptic Soy Broth) medium (Himedia-India) containing 30% glycerol was used first, after culturing the bacteria for 18 hours at 37 °C on this medium and incubation at 35 °C for 2 hours, the vials were transferred to a freezer at -70 °C (4).

Samples of these isolates, stored at -70 °C, were removed from the microtube near the flame and cultured on Blood Agar (Himedia-India). First, the cultured media were placed in a jar; then, the jar medium was anaerobized by an anoxomat (a device that creates anaerobic conditions by sucking the air out of the jar and replacing it with nitrogen, carbon dioxide and hydrogen gases). Next, the jar containing the plates was incubated at 37 °C for 24 hours. Suspected colonies were then biochemically evaluated.

Phenotypic and biochemical evaluation

After gram staining, a microscopic examination was performed. For biochemical evaluation, a catalase test was performed first. Next, a drop of hydrogen peroxide 3% was placed on the slide, and then a grown bacterial colony was carefully removed by a sterile loop and mixed with hydrogen peroxide on the slide. For the lecithin hydrolysis test, streaking of bacterial colonies was performed on Egg Yolk Agar medium (Himedia-India). Cultured plates were anaerobized by an anaerobic jar and were kept in an incubator at 37 °C for 24 hours. Colonies were inoculated in three culture media containing glucose, lactose and maltose, then placed in anaerobic conditions and incubated at 37 °C for 24 hours to observe the fermentation of sugars. These bacteria produce acid if they ferment sugar during the incubation period. Another test was the litmus milk reaction. For this evaluation, pure bacterial colonies were inoculated into Litmus Milk Media (Himedia-India), then placed in anaerobic conditions and incubated at 37 °C for 24 hours (6).

After evaluating test results and phenotypic confirmation, the genotype of *Clostridium* isolates was examined by PCR. Then, they were assessed for *C. perfringens* species and bacterial type. *C.perfringens* standard (VI: 62517) and *Clostridium septicum* standard (VI: 1504) were used as a positive control and negative control, respectively to ensure the accuracy of the tests.

DNA extraction

DNA was extracted from all isolates using the boiling method (8,14). In this method, all isolates stored at -70 °C in cryovial were incubated in cooked meat media at 37 °C for 24 hours under anaerobic conditions (sterile liquid paraffin was poured on the culture medium inside the tube). Next, 1 ml of each enriched medium was transferred to a microtube. All microtubes were centrifuged for 12 minutes at 12,000 RPM. The resulting precipitates were remixed with 200 µL of sterile distilled water, shaken thoroughly and heated at 95 °C for 20 min. They were then centrifuged at 12,000 RPM for 3 minutes and cooled on ice. Finally, 10 mL of this solution was used for PCR reaction (14). A BioPhotometer (Bio-Rad, USA) was used to determine the purity of the extracted DNA. The best light absorption by the nucleic acid is at 260 nm (4).

Multiplex PCR reaction to confirm *C.perfringens* isolates

In this study, PCR reaction with a final volume of 20 µL (Table 1) using a gradient thermal cycler (Eppendorf, Germany) for 35 cycles (Table 2) and using specific primers capable of amplifying a fragment of the 16s rRNA gene (Table 3) was performed to isolate and confirmed *C.perfringens* isolates.

Table 1. Characteristics of PCR reaction mixture (10)

Material and Required Concentration	Required Volume (µl)
PCR buffer 10x	5
$MgCl_2$ (50 Mm)	3
dNTPs (0.4 Mm)	0.4
16s rRNA primer (forward and reverse primers 0.8 Pm)	1*2
Double distilled water	5
Taq DNA Polymerase (5 U/µL)	0.6
DNA template	4
Total	20 µL

Table2. Thermal cycle program for identification of 16s rRNA gene and determination of *C.perfringens* (8)

Phase		Cycle	Time (s)	Temperature (°C)
Program 1	Initial denaturation	1	60	94
Program 2	Denaturation Annealing Elongation	33	30 40 60	95 55 72
Program 3	Final elongation	1	600	72

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Table 3. Sequences and characteristics of primers used in PCR to determine species and genes producing bacterial toxin (8, 10)

Purpose	Gene	Primer Sequence (5′→3′)	Reaction Type	Fragment Length (bp)
Bacterial species	*16s rRNA*	F:5/-AGAGTTTGATCCTGGCTCA-3/ R:5/-GGTTACCTTGTTACGACT-3/	PCR	147
Alpha	*plc(cpa)*	F: 5/-GCTAATGTTACTGCCGTTGA-3/ R: 5/-CCTCTGATACATCGTGTAAG-3/	M. PCR	324
Beta	*Cpb*	F: 5/-GCGAATATGCTGAATCATCTA-3/ R:5/-GCAGGAACATTAGTATATCTTC-3/	M. PCR	196
Epsilon	*Etx*	F:5/-GCGGTGATATCCATCTATTC-3/ R:5/-CCACTTACTTGTCCTACTAAC-3/	M. PCR	655
Iota	*itxA*	F:5/-ACTACTCTCAGACAAGACAG-3/ R:5/-CTTTCCTTCTATTACTATACG-3/	M. PCR	446

Multiplex PCR to determine the type of *Clostridium perfringens*

This study determined the type of bacterial isolates by using specific primers capable of amplifying a fragment of *plc, cpb, etx* and *itxA* genes that produce toxins (Table 3). This table provides sequences and characteristics of the pairs of forward and reverse primers. In addition, the characteristics of the Multiplex PCR mixture are also presented (Table 4).

Table 4. Characteristics of PCR reaction mixture (10)

Material and Required Concentration	Required Volume (µL)
PCR buffer 10x	5
$MgCl_2$ (50 Mm)	2
dNTPs (10 Mm)	0.6
Cpa primer (forward and reverse 10 Pm)	2.5*2

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Cpb primer (forward and reverse 10 Pm)	1.8*2
Etx primer (forward and reverse 10 Pm)	2.2*2
ItxA primer (forward and reverse 10 Pm)	2.6*2
Double distilled water	13.2
Taq DNA Polymerase (5 U/µL)	1
DNA template	10
Total	50 µL

The PCR temperature program for determining the bacterial type is shown in Table 5. The PCR product was then stored at 4°C. Afterwards, 1 mL of PCR product was mixed with 2 mL of loading buffer (6x). The product was moved through agarose gel 1% stained with CyberGreen and evaluated via electrophoresis. Sterile distilled water was used as a negative control. The bands formed after transfer to the UV Transilluminator were observed, and the resulting images were recorded.

Table 5. PCR temperature program for determining the bacterial type (8)

Phase		Cycle	Time (s)	Temperature (°C)
Program 1	Initial denaturation	1	6	94
Program 2	Denaturation Annealing Elongation	33	60 60 60	94 55 70
Program 3	Final elongation	1	420	70

Results

The genus *Clostridium* was identified by cultural and biochemical methods (Table 6). Thirteen *Clostridium* isolates were isolated from 70 stool samples of patients by cultural and biochemical evaluations. In contrast, only five strains of this genus were isolated from 70 samples without MS individuals. Among all isolates obtained from culture media, 11 and 2 isolates of *C.perfringens* were confirmed by PCR based on the target 16s rRNA gene for patients and without MS individuals, respectively. The number and frequency of each isolated strain are shown in Table 7.

Table 6. Results of phenotypic and biochemical evaluations of isolates

Test Name	Observed Results	Interpretation
Microscopic examination	Gram-positive, coarse and large bacilli with two round ends that looked like railroad cars (dumbbell-shaped) and arranged in parallel; they can have a terminal or subterminal oval spore.	*Clostridium genus*
Catalase test	No bubble formation or boiling was observed.	Negative
Lecithin hydrolysis test	White to opaque halo production was observed in the culture medium around the colony.	Positive
Fermentation of glucose, lactose and maltose	The culture medium turned yellow.	Positive
litmus milk reaction	The color of the milk culture medium changed from purple to pink; finally, stormy fermentation was observed.	Positive

Table 7. Number and frequency of isolates in cultural, biochemical and PCR experiments

	without MS	Patient		
		clinically isolated syndrome	relapsing-remitting MS (secondary progressive MS)	primary progressive MS
Cultural and biochemical tests to determine the genus *Clostridium* based on phenotypic methods	(7.1%) 5	(0%) 0	(17.1%) 12	(1.4%) 1
PCR to determine *C.perfringensbased* on the molecular identification	(2.8%) 2	(0%) 0	(15.7%) 11	(0%) 0

The experiments determined the type of *C.perfringens* isolates (whose identities were identified). It was observed that isolates *C.perfringens* without MS individuals were included 1 type A and 1 type D. On the other hand, in patients, the isolates were as follows: one type A, eight type D and two type B isolates. Both type A and D isolates were isolated from two patients (Figure 1, 2).

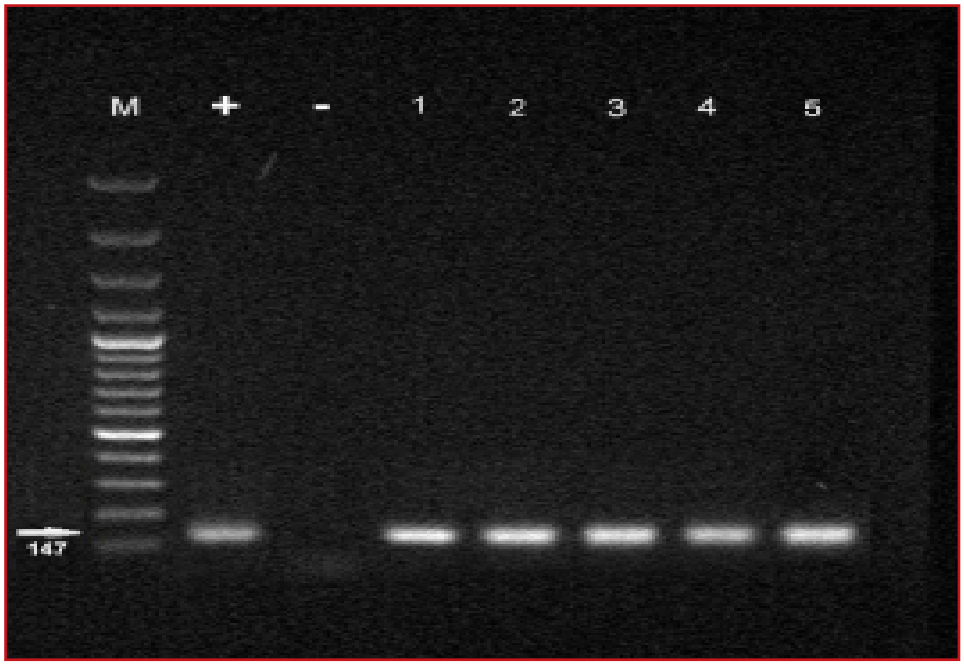

Figure 1. A PCR gel electrophoresis image of *C.perfringens* isolates on agarose gel 1% using a specific primer. M: marker, +: positive control, -: negative control

Figure 2. A PCR gel electrophoresis image of *C.perfringens* types on agarose gel 1% using specific primers. M: marker, -: negative control

Discussion

In this study, the frequency of *C.perfringens* infection was higher in patients with MS than in those without MS. The predominant bacterial types in patients were B and D which can produce ε-toxin. According to previous research, ε-toxin can cause various neurological symptoms, including MS. The ε-toxin, which is produced in types B and D, has been considered in most studies (5, 11, 12). In this study, the mentioned types were isolated from MS patients. In addition, type A has been isolated from two patients with type D. Type A causes food poisoning and does not produce ε-toxin (15). Type D was observed only in one case without MS. Furthermore, only one of the two isolated *C.perfringens* bacteria without individuals with MS was type A. The genus *Clostridium* includes a variety of pathogenic species found mainly in soil. Some species are minor components of the natural flora of the gastrointestinal tract in humans or animals. There have been many studies on the abundance of these bacteria in the intestines of animals. In a study by Hatam et al., 42 cases of *Clostridium* infections were isolated from 50 intestinal samples of animals suspected of having enterotoxemia. At the same time, 10 *Clostridium* bacteria were isolated from 50 soil samples. The results were based on identifying *C.perfringens* using PCR to detect positive cases and illustrate a high frequency of *C.perfringens* compared to other species (8). In a study of *C.perfringens* in the intestinal content of ostrich, Zandi et al. shows that *Clostridium* is isolated from about 36% of samples. This is less than the frequency reported by Hatam et al. for this bacterium in the intestines of sheep. The frequency of *C.perfringens* was 68%, which was more than the abundance of this genus in the intestinal contents of animals (16). Other *C.perfringens* genes have also been investigated in the some studies. Ghorchian et al. studied the enterotoxin gene in bulk and packed dehydrated vegetables in Iran. The results showed that more than 9% of the samples were infected with this bacterium and all isolates had the enterotoxin gene (17). The abundance of these bacteria in soil has always been considered. According to studies conducted in Iran, this bacterium has been isolated from the soils of areas with a high prevalence of MS (8). In a study by Leski et al., *C.perfringens* has been identified in Iraq and Kuwait *C.perfringens* is a pathogen that is carried by dust and sand particles and is transmitted to new hosts (9). With regards to the variable frequency of this bacterium in the gastrointestinal tract of different animals, its transmission through soil as well as the importance of distance to latitude on the prevalence and incidence MS (18), a remarkable consensus could be reached on the introduction of MS risk factors by studying the present study and evaluating the abundance of bacteria in the gastrointestinal tract of human populations in different geographical areas.

The species of *Clostridium* are generally not invasive, but they produce several toxins and enzymes responsible for their pathogenic effects. Most pathogenic species in this family produce one or more lethal toxins. As a result, many clostridial infections quickly lead to death. However, depending on the host, the lethality of these toxins can be variable and complex (14). Van Asten et al. reported that differences in the pathogenicity of *C.perfringens* strains are closely related to bacterial toxin production. Toxin-producing genes can be studied by molecular methods, especially PCR, to identify the type of bacteria based on their toxin-producing capacity (19, 20). In this study, the molecular PCR method and evaluation of toxin-producing genes were used to identify the type of bacteria. However, despite the findings of this study, which showed a higher frequency of epsilon toxin-producing genes in MS patients, as well as other similar findings mentioned above, it should be noted that reproductive causes of toxins in the intestines of infected individuals cannot be only the presence of toxin-producing genes. However, certain conditions must also be provided for the gene to be expressed and pathogenic toxins to be produced and secreted.

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Previous studies have shown that gender and age are two important factors in the prevalence and incidence of MS (18). However, the aim of this study was not to determine the prevalence and incidence of this disease. In addition, the number of samples available was limited, so epidemiological studies and statistical calculations on the incidence and prevalence of MS were not possible.

Conclusion

Our findings showed that *C.perfringens* could be present in the intestines of all individuals, but MS patients had a higher frequency of this bacterium in their intestines than without MS individuals. Epsilon toxin-producing types also had a higher frequency in MS patients. Type A was also found in individuals with and without MS. This type does not produce the ε-toxin but can cause food poisoning.

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Ethical approval

The ethics committee of Kerman University of Medical Science (IR.KMU.REC.1399.69)

Conflict of interest

None declared.

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