Epsilon toxin produced by Clostridium perfringens may be a main factor for development of multiple sclerosis symptoms

Maryam Sheikhzadeh Asadi[1], Babak Kheirkhah*[2], Nima Bahador[1]

[1]Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran

[2]Department of Veterinary Medicine, Baft Branch, Islamic Azad University, Baft, Iran.

*Corresponding author: Babak Kheirkhah, [2]Department of Veterinary Medicine, Baft Branch, Islamic Azad University, Baft, Iran.

Email: babakkheirkhah@yahoo.com

Tel: 00989133454787

Running title: Clostridium perfringens and multiple sclerosis.

Abstract

Introduction: Multiple sclerosis (MS) is a prevalent chronic autoimmune disease of the central nervous system. Clostridium perfringens epsilon toxin might be a cause of emerging MS complications due to its tendency to cross the blood-brain barrier. The aim of this study was to determine the molecular identity and toxigenicity of Clostridium perfringens isolated from multiple sclerosis patients and compare it with isolates obtained from other clinical and environmental sources.

Material and methods: In this cross-sectional descriptive study, 350 samples from clinical and environmental sources, such as feces of MS patients and healthy control individuals, soil, poultry carcasses, and sheep feces, were enriched and bacterial isolates were isolated by culture and biochemical methods. These isolates were tested by polymerase chain reaction method to identify Clostridium perfringens species and for the presence of alpha, beta, epsilon and iota toxin producing genes. Then, the level of toxicity and minimum lethality for each was evaluated.

Results: There was a high abundance of this type of bacteria in the intestines of patients with MS compared to non-afflicted people, on the other hand, epsilon toxin-producing toxinotypes (especially toxinotype D bacteria) also had a higher frequency. The lethality of toxins from the standard strain was significantly higher than the toxins from Clostridium perfringens from MS patients, soil, poultry carcasses, and sheep feces.

Conclusion: Due to the fact that the main causes of MS onset and its pathogenicity are yet to be clarified, it appears that more infections with epsilon toxin-producing toxinotypes and more lethality of the strains may be considered as a plausible factor for development of MS in the Iranian population.

Keywords: Clostridium perfringens; Multiple sclerosis; Epsilon toxin

Tob Regul Sci. ™ 2023;9(1): 375-384

DOI: doi.org/10.18001/TRS.9.1.27

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system, which is prevalent world widely (Asadzadeh Manjili et al. , 2020). The main causes of MS development and its related pathogenesis are yet to be clarified completely (Asadzadeh Manjili et al., 2020). Several genetics and environmental factors may be involved in the development of the disorder (Arababadi et al. , 2012, Asadzadeh Manjili et al., 2020). Accordingly, it has been hypothesized that microbial infections and their-related toxins may be considered as the potential factors for development of MS in people containing the genetic talents (Ochoa-Repáraz et al. , 2018). Accordingly, several investigations have been designed to explore the roles played by human-related bacteria and their products in development of MS and its-related pathogenicity (Malinova et al. , 2018, Mowry and Glenn, 2018, Ochoa-Repáraz et al., 2018).

Clostridium perfringens is a rod-shaped, gram-positive, immobile and anaerobic bacterium that causes some disorders in humans and animals (Aronoff and Marrazzo, 2022). This bacterium is an organism that is present everywhere and has a wider environmental distribution than any other pathogenic microorganism (Aronoff and Marrazzo, 2022). And it can be separated from soil, sewage and digestive organs of mammals. Hence, it has a high chance to infecting humans in various ethnics (Karimabadizadeh and Shamsaddini Bafti, 2021). This bacterium produces a large number of external toxins, which is responsible for its severity, and it is classified into five toxin types, including A, B, C, D, and E, based on the maximum production of each of the toxins (Poursoltani et al. , 2014). Recent studies have shown that seventeen types of toxins are secreted by this bacterium and four of which are the main toxins, including alpha, beta, epsilon and iota toxins (Leski et al. , 2011). It has been reported that Epsilon toxin enters the blood circulation through the absorbed intestine and by connecting to the brain-blood barriers, it causes symptoms similar to MS (Leski et al., 2011). Due to these effects on the central nervous system, it has been hypothesized that epsilon toxin may be a potential cause of exacerbation of MS symptoms in humans.

Therefore, this project was designed to explore the molecular identity and toxigenicity of Clostridium perfringens isolated from multiple sclerosis patients in comparison to the isolates obtained from other clinical and environmental sources.

Material and methods

Subjects

In this descriptive-cross-sectional study, 350 samples were isolated from several clinical and environmental sources. Accordingly, they were isolated from feces of MS patients (70 samples) and non-infected individuals (70 samples), soil (70 samples), poultry carcasses (70 samples), and

sheep feces (70 samples). The samples were collected in sterile conditions and transferred to the laboratory at 4 °C. MS patients have been selected and based on the Guidelines for MS Diagnosis: McDonald Criteria, which is derived from Magnetic Resonance Imaging (MRI) technique and clinical symptoms by an expert physician in neurology. The Islamic Azad University Ethical Committee, Shiraz Branch, approved the project protocol (Ethical code: IR.KMU.REC.1399.69), and the participants filled out a written informed consent.

Isolation of Clostridium perfringens from different samples

The poultry carcasses samples were transferred to Nutrient broth as transport medium and then inoculated onto Sulfite Polymyxin Sulfadiazine agar (HiMedia, India) in an anaerobic chamber at 37°C for 48 h. The feces of sheep, MS patients, healthy control individuals and soil samples were cultured in cooked meat (Merck, Germany) for pre-enrichment and then and sub-cultured onto Sulfite Polymyxin Sulfadiazine agar (HiMedia, India) in an anaerobic chamber at 37°C for 48 h. The bacterial cultures were identified with Gram staining and cultured onto blood agar (Merck, Germany) plates containing 7% sheep blood agar for showing dual hemolytic zones. Different biochemical tests carried such as, catalase, fermentation reaction with different sugars and Litmus milk reaction were done too.

Molecular detection of Clostridium perfringens

Genomic DNA was extracted using a DNA extraction kit (Cinnagen, Iran) based on according to the manufacturer's instructions. DNA concentration and quality were explored by determination at 260/280 nm absorption and agarose gel electrophoresis, respectively. The polymerase chain reaction mixture contained the universal primers 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) in a total volume of 25 microliters. Accordingly, 10 µL master mix, 1 µL primer mix (10 µm), 1 µL DNA (1 µg), and 8 µL RNase/DNase free distilled water were added to a PCR microtube and placed in a thermal cycler (T100, Bio-Rad, USA). The following program was run: 95 ºC for 1 minute, and then 45 cycles 95 ºC for 30 seconds, 55 ºC for 40 seconds, and 72 ºC for 60 seconds.

Determination of toxin-encoding genes of C. perfringens isolates

To detection of Clostridium perfringens-related toxins, a PCR was performed using specific primers for each toxin in a PCR master mix (Cinnagen, Iran).

Accordingly, 10 µL master mix, 1 µL of each primer mix (10 µm), 1 µL DNA (1 µg), and 5 µL RNase/DNase free distilled water were added to a PCR microtube and placed in a thermal cycler (T100, Bio-Rad, USA). The following program was run: 95 ºC for 1 minute, and then 45 cycles 95 ºC for 60 seconds, 55 ºC for 60 seconds, and 72 ºC for 60 seconds.

Evaluation of toxin production

Mouse survival

For evaluation of the bacteria to produce toxins, the isolated bacteria were cultured in three media, including nutrient broth culture medium containing liver extract, nutrient broth culture medium containing liver pieces, and Clostridium perfringens toxinotype D culture medium. Then the supernatants were centrifuged at 2500 RPM for 25 minutes, and then the supernatants were moved to the tubes containing 0.04 g trypsin and incubated at 37 °C for 45 minutes. The plausible toxins were injected, 1 mL, to a mouse model to evaluate its survival. The mice were kept in standard conditions for lights and food/water accessibility.

Measurement of total proteins

Total protein in the supernatants were explored using a commercial kit from Nanond-Salamat Company, Tehran, Iran.

Statistical analysis

The normality of data distribution was explored using Kolmogorov Smirnov test, under SPSS software version 16. Accordingly, One-Way ANOVA and Chi-square ($\chi 2$) test was used to analyze the differences between the groups. The data are presented as percent and p value less than 0.05 was considered significant.

Results

The results showed that from a total of 350 examined samples (70 samples from each clinical and environmental source), a total of 52 suspected bacteria were isolated as follows:

There were 5 isolates in poultry carcass samples, 17 isolates from sheep feces, 12 isolates from soil samples, 5 isolates from the feces of healthy control, and 13 bacterial isolates from the feces of patients with MS, based on Gram reaction (Gram positive bacilli), observation of black colonies on the SPS medium, dual hemolytic zones on Blood agar medium, fermentation of glucose, lactose, sucrose and observation of pink to red color fermentation of lactose, and stormy clot with gas formation in coagulated casein curd in Litmus medium.

PCR results for confirmation of Clostridium perfringens

Out of 52 isolates of Clostridium perfringens in the molecular evaluation of polymerase chain reaction based on 16s rRNA target gene (Figure 1), 11 isolates were found in MS people and 2 isolates in non-MS people, 10 isolates in soil samples, 17 isolates from sheep feces and 4 isolates of Clostridium perfringens species were confirmed from poultry carcasses.

PCR results for evaluation of genes encoding toxin

It was found that in non-MS people, one isolate had toxinotype A and one isolate had toxinotype B (Figure 2). In MS patients, there was one isolate of toxinotype A, eight isolates of toxinotype D and two isolates of toxinotype B. Both toxinotype A and toxinotype D were isolated in two patients (Figure 3). There were 5 isolates of toxin type B and 12 isolates of toxin type D in the feces of animals. In the soil samples, 1 toxin type A, 3 toxin type B and 6 isolates were toxin type D. Regarding the poultry carcass, one toxin type A and 3 isolates of toxin type C were identified.

Data analysis for production of total proteins

Considering that the equality of the variance of the investigated protein in the three studied groups is also one of the assumptions of the analysis of variance, this hypothesis was also evaluated. The results of Leven's test also showed that the assumption of equality of variances in three groups is maintained (P= 0.995). Therefore, due to the normality of the data distribution and the equality of variances in the three groups, One-Way ANOVA was used to compare the average protein in the three groups, and the results of this test showed that there was not a significance difference between the amount of total protein in the three groups (P= 0.701).

Minimum lethality of Clostridium perfringens toxinotype D isolates

After 72 hours of the minimum lethality test, the minimum lethality of the samples was determined based on the dead mice. Examining the lethality of epsilon toxin produced by isolates of Clostridium perfringens toxotype D shows that the minimum lethality of the samples varied from 5 to 400.

Comparison of lethality ratio of epsilon toxin produced by toxinotype D isolate by different groups

The lethality ratio in the groups of MS patients and, soil, and sheep feces revealed that there are no statistically significant differences among the groups with various dilutions (Table 1).

Comparison of the lethality of epsilon toxin produced by isolates of Clostridium perfringens and standard strain

The results demonstrated that the lethality of epsilon toxin produced by isolates of Clostridium perfringens from MS patients, soil, and sheep feces were 1/400, however, it was 1/12000 for standard strain. Statistical analysis revealed there were a significant difference between the lethality of epsilon toxin produced by isolates of Clostridium perfringens from standard strain when compared to the examined sources (P< 0.001).

Discussion and Conclusion

Epsilon toxin produced by *Clostridium perfringens* may be a main factor for development of multiple sclerosis symptoms

It has been reported that MS is associated with inflammation in the central nervous system (Asadikaram et al. , 2021). The main responsible mechanisms lead to activation of immune system against myelin, the protective coating around nerve fibers, in MS are yet to be clarified. It has been hypothesized that microbial infections can be considered as a plausible activator of immune system during MS (Zangeneh et al. , 2021). Accordingly, it may be hypothesized that Clostridium perfringens and its-related toxins may be a plausible inducer of immune system against myelin in the patients suffering from MS. Our results showed that the patients suffering from MS has significantly more Clostridium perfringens isolates when compared to non-MS controls, which were confirmed in the bacterium gene and also in its phonotype. Additionally, the isolated Clostridium perfringens from MS patients has more epsilon toxin-producing toxinotypes than non-MS patients and other sources. Therefore, it appears that Clostridium perfringens and its epsilon toxin-producing toxinotypes may be an important factor for onset or progression of demyelination of CNS neurons in MS patients. In parallel with our results, a study by Wagley and colleagues revealed that Clostridium perfringens isolates in MS patients contain epsilon toxin-producing gene more than the bacterium from non-MS individuals (Wagley et al. , 2019). Additionally, the investigations on the animal models showed that epsilon toxin from Clostridium perfringens isolates is a potent pore-forming toxin responsible for BBB breakdown and injury to myelin forming cells (Linden et al. , 2015, Rumah et al. , 2015). Another animal model investigation revealed that epsilon toxin from Clostridium perfringens may cause of demyelination by inhibition of potassium inward rectifier (Kir) channels (Bossu et al. , 2020). Freedman et al., reported that epsilon toxin from Clostridium perfringens affects vascular permeability and damages neurons (Freedman et al. , 2016). In agreement with the hypothesis Linden and colleagues demonstrated that Clostridium perfringens epsilon toxin induces BBB damages through caveolae-dependent transcytosis (Linden et al. , 2019). Therefore, due to our results that showed MS patients suffer from more Clostridium perfringens infections and also the bacterium was more epsilon toxin-producing than other sources, it appears that the toxin may a main inducer or factor for development of MS in Iranian population. The hypothesis also confirmed by another study that shows oral drugs inhibit the growth of epsilon toxin producing Clostridium perfringens isolates (Rumah et al. , 2017). However, the results revealed that the lethality of epsilon toxin produced by Clostridium perfringens isolated from various sources was not different. It means that, although the lethality was not different but its effects on the myelin of neurons may be different, which needs more investigations. However, low sample size and evaluation of the genetic form only, but not phonotype, are the main limitation of this project, which needs proved by further investigations on Iranian MS patients.

In addition, due to the fact that the lethality of standard strain was higher than the clinical sources, it may be hypothesized that the lethality of epsilon toxin produced by the clinical sources can be increased to be reached to standard strain in future. Hence, evaluation of the lethality of the toxins produced by toxinotypes needs to be followed by investigators.

Acknowledgements

The authors appreciate the MS and non-MS volunteers for their warmly corporations.

Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

References

1. Arababadi MK, Mosavi R, Ravari A, Teimori H, Hassanshahi G. Association of interleukin-4 polymorphisms with multiple sclerosis in southeastern Iranian patients. Ann Saudi Med. 2012;32:127-30.
2. Aronoff DM, Marrazzo JM. Infections caused by Clostridium perfringens and Paeniclostridium sordellii after unsafe abortion. Lancet Infect Dis. 2022.
3. Asadikaram G, Meimand HAE, Noroozi S, Sanjari M, Zainodini N, Arababadi MK. The effect of IFN-β 1a on expression of MDA5 and RIG-1 in multiple sclerosis patients. Biotechnology and applied biochemistry. 2021;68:267-71.
4. Asadzadeh Manjili F, Yousefi-Ahmadipour A, Kazemi Arababadi M. The roles played by TLR4 in the pathogenesis of multiple sclerosis; A systematic review article. Immunology letters. 2020;220:63-70.
5. Bossu JL, Wioland L, Doussau F, Isope P, Popoff MR, Poulain B. Epsilon Toxin from Clostridium perfringens Causes Inhibition of Potassium inward Rectifier (Kir) Channels in Oligodendrocytes. Toxins. 2020;12.
6. Freedman JC, McClane BA, Uzal FA. New insights into Clostridium perfringens epsilon toxin activation and action on the brain during enterotoxemia. Anaerobe. 2016;41:27-31.
7. Karimabadizadeh A, Shamsaddini Bafti M. Evaluation of toxinogenesis of Clostridium perfringens type D isolates in three kinds of culture media. Veterinary Res Biol Product. 2021;34:2-12.
8. Leski TA, Malanoski AP, Gregory MJ, Lin B, Stenger DA. Application of a broad-range resequencing array for detection of pathogens in desert dust samples from Kuwait and Iraq. App Environment Microbiol. 2011;77:4285-92.
9. Linden JR, Flores C, Schmidt EF, Uzal FA, Michel AO, Valenzuela M, et al. Clostridium perfringens epsilon toxin induces blood brain barrier permeability via caveolae-dependent transcytosis and requires expression of MAL. PLoS pathogens. 2019;15:e1008014.
10. Linden JR, Ma Y, Zhao B, Harris JM, Rumah KR, Schaeren-Wiemers N, et al. Clostridium perfringens Epsilon Toxin Causes Selective Death of Mature Oligodendrocytes and Central Nervous System Demyelination. mBio. 2015;6:e02513.

11. Malinova TS, Dijkstra CD, de Vries HE. Serotonin: A mediator of the gut-brain axis in multiple sclerosis. Mult Scler. 2018;24:1144-50.
12. Mowry EM, Glenn JD. The Dynamics of the Gut Microbiome in Multiple Sclerosis in Relation to Disease. Neurol Clin. 2018;36:185-96.
13. Ochoa-Repáraz J, Kirby TO, Kasper LH. The Gut Microbiome and Multiple Sclerosis. Cold Spring Harb Perspect Med. 2018;8.
14. Poursoltani M, Mohsenzadeh M, Razmyar J. Toxinotyping of Clostridium perfringens strains isolated from packed chicken portions. Iran J Med Microbiol. 2014;8:9-17.
15. Rumah KR, Ma Y, Linden JR, Oo ML, Anrather J, Schaeren-Wiemers N, et al. The Myelin and Lymphocyte Protein MAL Is Required for Binding and Activity of Clostridium perfringens ε-Toxin. PLoS pathogens. 2015;11:e1004896.
16. Rumah KR, Vartanian TK, Fischetti VA. Oral Multiple Sclerosis Drugs Inhibit the In vitro Growth of Epsilon Toxin Producing Gut Bacterium, Clostridium perfringens. Frontiers in cellular and infection microbiology. 2017;7:11.
17. Wagley S, Bokori-Brown M, Morcrette H, Malaspina A, D'Arcy C, Gnanapavan S, et al. Evidence of Clostridium perfringens epsilon toxin associated with multiple sclerosis. Multiple sclerosis (Houndmills, Basingstoke, England). 2019;25:653-60.
18. Zangeneh Z, Abdi-Ali A, Khamooshian K, Alvandi A, Abiri R. Bacterial variation in the oral microbiota in multiple sclerosis patients. PloS one. 2021;16:e0260384.

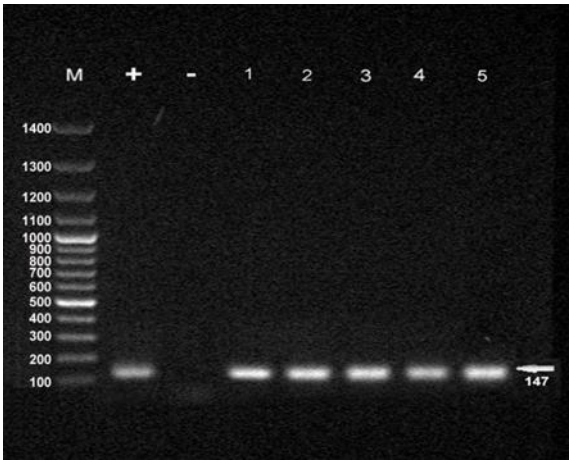

Figure 1. Gel electrophoresis image of PCR product of Clostridium perfringens isolates on 1% agarose gel using specific primer. M: 100 bp marker, +: positive control, -: negative control, 1 to 5 species positive samples

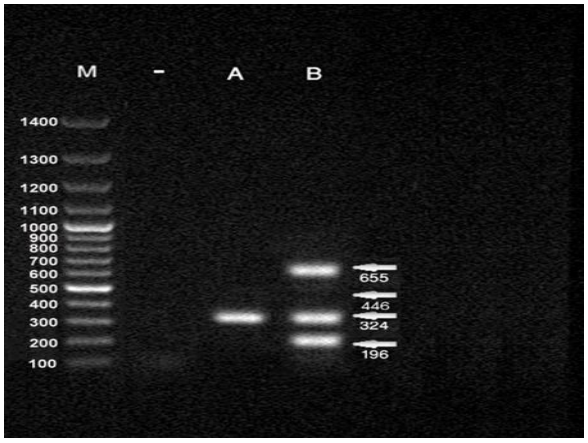

Figure 2. Gel electrophoresis image of multiplex PCR product-different toxin types of Clostridium perfringens on 1% agarose gel using specific primers in non-MS people. M: 100 bp marker, -: negative control, A and B toxin types

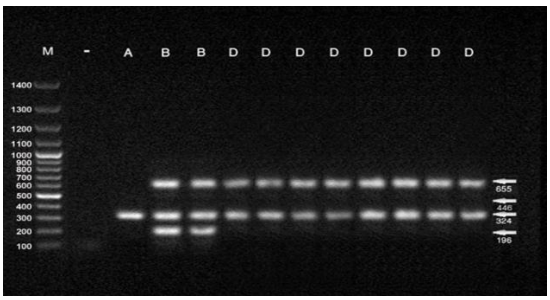

Figure 3. Gel electrophoresis image of multiplex PCR product-different toxin types of Clostridium perfringens on 1% agarose gel using specific primers in MS people M: 100 bp

Primer sequence	gene	Toxin
F: 5/-GCTAATGTTACTGCCGTTGA-3/ R: 5/-CCTCTGATACATCGTGTAAG-3/	plc(cpa)	Alpha
F: 5/-GCGAATATGCTGAATCATCTA-3/ R:5/-GCAGGAACATTAGTATATCTTC-3/	Cpb	Beta
F:5/-GCGGTGATATCCATCTATTC-3/ R:5/-CCACTTACTTGTCCTACTAAC-3/	Etx	Epsilon
F:5/-ACTACTCTCAGACAAGACAG-3/ R:5/-CTTTCCTTCTATTACTATACG-3/	itxA	Iota

marker, -: negative control, A, B, D Toxin types

Table 1. Primers used in this study to determine the presence of the studied genes

Table 2. Comparison of lethality ratio of epsilon toxin produced by isolates of Clostridium perfringens toxin type D derived from various sources.

Dilution			Lethality		P-value (Fisher exact)
			Yes	No	
1/10	Group	MS	8	4	0.755
		S	6	6	
		SH	8	4	
1/50	Group	MS	5	7	1.000
		S	4	8	
		SH	5	7	
1/100	Group	MS	3	9	1.000
		S	2	10	
		SH	3	9	
1/200	Group	MS	2	10	0.516
		S	0	12	
		SH	2	10	
1/400	Group	MS	1	11	1.000
		S	0	12	
		SH	0	12	

Data analysis revealed that there were no significant differences regarding the lethality of the epsilon toxin produced by Clostridium perfringens from various sources.MS: The patients with MS, S: Soil, and SH: Sheep feces.