Journal of Pharmaceutical and Scientific Innovation
www.jpsionline.com (ISSN: 2277–4572)
Research Article

Evaluation of Contamination level With *Clostridium Perfringens* Type B in Multiple Sclerosis Diseases

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DOI: 10.37532/2277-4572.2022.12(1).240

Received on: : 12/01/2023, Manuscript No.jpsi-22-60401; Revised on: 18/01/2022, Manuscript No. jpsi -22-60401(R); Published: 20/01/2022

ABSTRACT

Introduction: Multiple Sclerosis (MS) is a chronic disease of central nervous system which causes inflammation in the brain and the spinal cord, resulting in demyelination and damage to axons and nerve fibers. The cause of this disease is still unknown, and many neuroscientists are suspected that the disease is due to genetic, environmental and even infectious agents. The purpose of this study is to investigate the relationship between MS and Clostridium perfringens type B infection.

Method: A total of 39 stool and blood samples were collected from MS patients and 40 stool and serum samples from non-MS patients. Stool specimens were evaluated for the presence of epsilon toxin by rapid test″ Strips for detection of Clostridium Perfringens Epsilon Toxin″. Then stool samples were cultured. Positive colonies were evaluated by biochemical and PCR tests . Polymerase chain reaction (PCR) was performed for colonies for the presence of alpha, beta and Epislone toxins and serum samples were examined for the presence of anti- Epsilon toxin using″ Epsilon Toxin Serological Elisa Kit ″.

Results: Of the 79 stool samples tested in MS and non-MS patients, type B of Clostridium perfringens were not isolated. From the total of 39 stool specimens′ MS patients, 5 Clostridium perfringens were isolated which 80% of the total were belonged to type A and 20% of them were type C. Of the total of 40 stool samples of non-MS patients, only 4 samples of Clostridium perfringens were isolated which all of them were belonged to type A. Results also showed that anti-epilecin toxins shown in none of serum samples in the range of the disease.

Keywords: *Clostridium;* Anti epsilon toxi; Epsilon toxin; Perfringen, Outcomes

INTRODUCTION

The natural hosts for C. perfringens toxinotypes B and D are ruminant animals in whom ETX-mediated neurologic symptoms occur when carbohydrate rich feed or over grazing favors exponential growth of the bacilli. ETX is absorbed via the intestine enters the blood stream and permeabilizes the BBB, resulting in MS like symptoms (e.g. visual dysfunction, incoordination and spastic paralysis). Murrell and colleagues, because of these effects on the CNS, first suggested ETX as a potential MS trigger although humans are not natural hosts for C. perfringens types B or D. ETX binds to an unknown receptor present both in the brain vasculature and myelinated brain regions e.g. corpus callosum. Once bound to its receptor, ETX integrates into the plasma membrane as a heptameric pore, leading to osmolysis. When ETX is administered to rodents, BBB disruption occurs and white matter vasculature is especially vulnerable. Interestingly, intraperitoneal administration of protoxin in rats results in the formation of focal ovoid lesions within the corpus callosum, in which the long axis of the ovoid is oriented perpendicular to the surface of the lateral ventricle. Dawson first described this specific lesion morphology and the radiographic equivalent is all but pathognomonic for clinically definite relapsingremitting multiple sclerosis. We postulate that Clostridium perfringens epsilon toxin may be a candidate causative toxin for nascent lesion formation in MS worthy of further investigation.

A 21-year old woman (patient 73F) developed left lower extremity dyscoordination, and imbalance that evolved to its maximum deficit over three days. Two weeks after onset she was referred to a neurologist due to persistent symptoms and neuroimaging of the brain revealed multiple foci of increased T2/flair signal in the deep and subcortical white matter, with several ovoid lesions within the corpus callosum characteristic of MS. Following administration of IV gadolinium, several lesi-ons enhanced. CSF analysis revealed five IgG bands on isoelectric focusing that were not present in the corresponding serum sample. She met revised criteria for clinically definite relapsing remitting MS at the earliest clinical presentation termed a Clinically Isolated Syndrome (CIS). She received five days of IV methylprednisolone, 1 gram per day, and her symptoms resolved to normal neurological function within three weeks. She was referred to the Weill Cornell MS Center for confirmation of diagnosis and treatment planning. Repeat neuroimaging at Weill Cornell revealed lesions characteristic in morphology and location for Multiple Sclerosis. Approximately three months after onset of symptoms, she was enrolled in the HITMS (Harboring the Initial Trigger of Multiple Sclerosis) study, IRB protocol no. 1003010940, and a self-collected stool sample was obtained. Disease modifying treatment was initiated. Eight months after initiation of treatment she remained asymptomatic and her first treatment assessment MRI was performed which revealed several new contrast enhancing lesions .Three months after onset of her first symptoms, patient 73F was found to harbor C. perfringens type B in her GI tract. PCR analysis revealed carriage of genes encoding a, b, and e toxins. This represents the first human known to carry type B and the first MS patient found to carry an ETX producing C. perfringens . To exclude a possible laboratory-derived contaminant, we performed a lysogenic bacteriophage footprint analysis of the laboratory (ATCC 3626) and patient-derived C. perfringens strains. Three lysogenic bacteriophage insertions were identified in the laboratory strain, which matched the known whole genome sequence. The patient's strain contained just two lysogenic bacteriophage insertions, thus confirming that the patient-derived ETX amplicon was not a laboratory contaminant, Since a combination of toxinotypes C and D would also result in identification of a, b, and e toxin genes, we sequenced the patientderived ETX gene confirming that it was derived from a type B ETX plasmid.

METHODS BASICALLY USED

His tagged protoxin was procured from BEI Resources and 1 mg was fluorescently labeled using Alexa Fluor 594 Protein labeling Kit (Invitrogen) as per manufacturer's instructions. Retina. Fresh frozen tissue sections were incubated with BSLI (Vector Labs) 1:200, and Alexa 594 labeled His-tagged protoxin (50 nM) for 1 hr at RT. After three 5 minute washes in PBS, stained sections were post fixed in 4% PFA for 10 mins at RT. The stained tissue was washed 3X in PBS, mounted and imaged. Brain. Fixed frozen coronal brain sections were permeabilized in a 1% sodium cholate, 1% BSA, 10% donkey serum, PBST solution overnight at 4°C. Sections were then incubated with rabbit anti-PLP (ThermoScientific) at 1:1000 overnight at 4°C. Following three washes with PBS, sections were then incubated with Donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch) at 1:1000, and Alexa 594 labeled His-tagged protoxin (50 nM) for 2 hrs at RT. The stained tissue was washed 3X in PBS and prepared for microscopy at the Rockefeller Bio-Imaging facility.

Operative technique

Stool specimens were self-collected by patients and healthy controls in a clean single use vessel and stored at 220°C until returned to the MS Center. Approximately one gram of stool was collected and stored in a fecal collection tube (Sarstedt) containing 9 ml of buffered glycerin-salt solution (10% glycerin, 71.2 mM K2HPO4, 29.4 mM KH2PO4, 71.9 mM NaCl made in distilled water, adjusted to pH 7.2 and autoclaved) under IRB protocol no. 1003010940. Upon receipt, samples were resuspended in 40 ml of modified Rapid Perfringens Media (RPM); D-cycloserine (400 mg/L) was substituted for neomycin/polymyxin B and litmus milk was omitted to improve DNA extraction. The resuspended samples were cultured in 50 ml falcon tubes with tightly closed caps at 47°C.

We find that people with MS are less likely to harbor C. perfringens type A when compared to controls. Soil studies have identified that the presence of C. perfringens type A is coincident with the absence of other toxinotypes, suggesting that toxinotype A may compete with other C. perfringens toxinotypes for resources. While the type A toxinotype may outcompete C. perfringens types B and D within an ecological niche, there are other factors that could contribute to, or account for the observed difference in C. perfringens carriage. Important considerations are host genetics, diet, use of probiotics, medications, gut microbiota, and use of antibiotics. In this study, none of the subjects received cytotoxic or immune suppressing agents. Furthermore, none of the subjects had undergone antibiotic treatment of any kind in the six months prior to sample collection, or greater than two weeks of antibiotics in the two years prior to sample collection. Host genetics and diet were not assessed in this study. The absence of C. perfringens type A may open a theoretical ecological niche for C. perfringens types B or D, but its absence is not tantamount to the presence of these toxinotypes. We identified one case in which a newly diagnosed patient harbored C. perfringens type B. Eight months after initially testing positive for C. perfringens type B, she remained positive for toxinotype B upon repeat analysis (data not shown). However, we expect that identification of C. perfringens types B or D in humans will be difficult, as C. perfringens forms endospores that are resistant to standard DNA extraction methods. Additionally, the organism is likely to exist in low abundance in the upper GI tract, only rarely entering growth phases that render it detectable. Although ETX binds to Peripheral Nervous System (PNS) myelin, as it does CNS myelin, autoradiograph studies show that ETX only targets the CNS and not the PNS. We propose that ETX fails to bind to PNS endothelial cells that comprise the blood-nerve barrier; therefore PNS myelin is not exposed to the toxin. Finally, binding of ETX to retinal veins that form the bloodretinal barrier (BRB), a CNS barrier analogous to the BBB, may explain the enigmatic observation of periphlebitis retinae in people with MS. The human retina is typically devoid of myelin, yet vascular scarring occurs. Primary ETX action on the BRB may result in retinal phlebitis that is independent of oligodendrocytes or myelin. Furthermore, serum protein leakage and the accumulation of perivenular monocytes in the absence of oligodendrocyte apoptosis or demyelination are often observed in pathologic MS brain specimens. These observations may similarly be due to subtle insult of the endothelium and a secondary innate immune response.

Conclusion

Of the 79 fecal specimens tested, patients with MS and non-affected Clostridium perfringens type B were not isolated. of the 39 fecal specimens in patients with MS, 5 samples of Clostridium perfringens were isolated. Of the 40 stool samples of non MS patients, only 4 samples of Clostridium perfringens were isolated . All bacteria isolated after biochemical tests, were used for final confirmation of PCR. Which was determined by PCR after toxinotyping, samples of MS patients were 80% Clostridium Perfringens type A and 20% Clostridium Perfringens type C, whereas isolated bacteria from non MS patients were 100% Clostridium Perfringens type A. In the serum samples, none of the anti-epislon toxin was detected. Therefore, based on the results of this study, there is no correlation between the infection of patients with Clostridium perfringens type B and MS disease.

How to cite this article:
R. Ali-askarian. Evaluation of Contamination level With Clostridium Perfringens Type B in Multiple Sclerosis Diseases J Pharm Sci Innov. 2023;12(1): 1-2.
http://dx.doi.org/10.7897/2277-4572.114233

Source of support: Nil, Conflict of interest: None Declared