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Original Research

Molecular identification of drug resistant mutations to tetracycline in *Mycoplasma spp.* isolated from patients with multiple sclerosis

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Abstract: Bacterial infections play a significant role in causing or intensifying the attacks in MS and there are reports based on the interference of Mycoplasma with a global distribution. Mycoplasma causes autoimmune attacks by imitating the host cell membrane, which is a way of resistance to antibiotics. The purpose of this study was to evaluate the molecular identification of mutations causing resistance to tetracycline in Mycoplasma isolated from MS patients. A total number of 32 cerebrospinal fluid samples and 48 urinal fluid samples were collected from MS patients. The samples were enriched in 7 PPLO broth for one night and continuous cultivation in agar PPLO and PPLO broth for one week. DNA was extracted, and then nested PCR and Doublex PCR were used for bacteria genus identification and the presence of potential tetracycline-resistant alleles (rrs_4 and rrs_3), respectively. A total number of 12 samples created colonies. However, only 5 samples (1 cerebrospinal fluid and 4 urinal samples) were detected to be Mycoplasma. The urinal samples showed the desired alleles and were tetracycline-resistant. By sequencing the PCR products, it was shown that these alleles have mutated in various points. Based on the results it seems that the resistant mutated Mycoplasma can be detected in MS patients in our population and may be considered as a risk factor for the disease.

Key words: Mycoplasma; Multiple Sclerosis; Antibiotic resistance; Tetracycline.

Introduction

Mycoplasmas are the smallest bacteria that live independently and have accepted parasitic life due to the low volume of genomes (1). Plasma membrane and the surface proteins specific to Mycoplasma act as covers and imitate the host cell membrane which may lead to activation of host's immune responses, causing inflammation in different tissues as has been observed in rheumatoid, meningitis, encephalitis, poly radio colitis, cerebral ataxia, inflammation of spinal cord, psychosis, epilepsy, Guillain-Barre syndrome, Multiple sclerosis (2-4). Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease in which the central nervous system is demyelinated (5, 6). The clinical results are in the form of reducing or losing the neurological performance of the related section, depending on the size and the location of the wounds (scleronic plaques) which will cause minor to major disabilities (7). As mentioned previously, Mycoplasma can be considered as a risk factor for MS and has spread throughout the world and neglecting it with the common misconception that it is powerless has led to inattention to its antibiotic resistant property (8). This resistance is often due to the mutations in 23S rRNA gene which have captured macrolide interaction with the great subunit of ribosome (8). Now substituting antibiotics for macrolide, tetracycline and quinolones have been introduced (9). Based on the fact that the exact etiology of MS is not known as yet, it has been hypothesized that infection with antibiotic resistant *Mycoplasma* may be considered as a risk factor for development of MS and its complications. It has been reported that rrs_3 and rrs_4 alleles in 16SrRNA ribosome gene are considered as the initial adhering parts of tetracycline to the ribosome, and accordingly mutation in the alleles results in disconnection of tetracycline and resistance formation (2). Therefore, the purpose of this study was to evaluate the genetic mutations causing medicinal resistance to Tetracycline in Mycoplasma of cerebrospinal fluid and urine of patients with MS in an Iranian population, Kerman, Iran, to determine the plausible risk of the antibiotic resistance for MS development.

Materials and Methods

Subjects and Mycoplasma isolation

This cross-sectional descriptive study was carried out on 80 samples including 32 cerebrospinal fluid samples and 48 urinal samples of patients with MS in Kerman Province (MS Society of Kerman) that were provided purposively and nonrandomly. MS was diagnosed according to Revised McDonald criteria (10) and based on clinical and physician neurologist observations, radiology and laboratory analyses.

At first, 0.5 ml from each sample was transferred to sterile PPLO broth medium and was incubated at 37 °C for 24 h. PPLO broth medium was filtered by the special PVDF filters. Using sterile syringes, 2ml of the

broth solution was separated and was transferred to the second cultivation medium including the PPLO broth with a 7.6 pH and incubated for 3 to 5 days in CO_2 up to 10 percent. Two passages were taken from each medium to PPLO broth and PPLO agar medium. The PPLO agar medium was incubated in CO_2 incubator at 37 ºC for 7 days and PPLO broth was incubated for 2 to 3 days. After this period, 2 passages were taken: one to the broth medium and the other to the PPLO agar medium. This process continued for 7 weeks. At the end of the seventh week, colonies were formed on some PPLO agar mediums. After that, molecular tests were conducted to confirm the genus Mycoplasma. The protocol of the study has been approved by the Ethical Committee of Islamic Azad University and participants have filled out and signed the informed consent form.

DNA extraction Nested PCR and Doublex PCR

Mycoplasma DNA was extracted using a DNA extraction kit from Kiagene Company, USA. Nested PCR was used to confirm diagnosis of Mycoplasma genus. Accordingly, the specific primers for 16SrRNA were used in the first and second round to proliferate the 927bp and 163bp, respectively. The standard genus Mycoplasma was used for positive control and standard E.coli as well as sterile distilled water was used as negative control. Doublex PCR was applied to identify rrs_3 and rrs_4 alleles in 16SrRNA gene to identify Tetracycline resistance. Accordingly, primers MB-282-F and MB-tet 3/4R were used to identify rrs_3 alleles to proliferate in 1857bp area and primers MB-rrs-3F and MB-287-R were used to identify rrs_4 alleles to proliferate in 5294bp area.

Table 1 illustrates the primer sequences used in this project and Table 2 and 3 show the details of materials and PCR protocols, respectively.

After that, the products were presented by Ethidium bromide coloring and electrophoresis in agarose gel. Having made sure that PCR products had proliferated and bands had formed, DNA purification was done using the protocol kit for DNA purification (Vivantis Company). The obtained products were sent to the Pioneer Company (representative of Bioneer in South Korea) for sequence detection and then the data were analyzed by BioEdit and MEGA software (Version 5.04).

Results

Of the 80 available samples, 12 samples grew in agar PPLO medium including one cerebrospinal fluid sample and 11 urinal samples of patients with MS.

During the first round of the Nested PCR reaction, all 12 samples (1 cerebrospinal fluid and 11 urinal samples) showed a 927bp amplification band (Fig.1).

During the second round of the Nested PCR reaction, only 5 samples including 1 cerebrospinal fluid and 4 urinal samples showed 163bp amplification bands (Fig. 2).

In the final PCR reaction (Doublex PCR) only 4 urinal samples had 1857bp and 5294bp bands and the cerebrospinal fluid sample showed no bands (Fig. 3). The results in detail are presented in Table 4.

Table 1. Primers used in Nested and Doublex PCR.

Nested PCR 1	AB ABO	16s rRNA	F: 5′GCAGTGAAGAACGAGGGG3′ R: 5′GTCCTCGCTTCGGTCCTCTCG3′
Nested PCR2	GSO MGSO	16s rRNA	F:5′GGGAGCAAACAGGATTAGATACCCT 3′ R:5′TGCACCATCTGTCACTCTGTTAACCTC 3′
Multiplex PCR	MB-282-F MB-tet ¾-R	*rrs3*	F:5′GGATATCTAACGCCGTGTCT3′ R:5′CGTTCTCGTAGGGATACCT3′
	MB-rrs-3F MB-287-R	rrs_4	F:5′CGAGTTTGACCTCCTGGCTC3′ R:5′CTAATTCCAAGTGCCACTAGCG3′

Table 2. Reaction mixture of Nested PCR and Doublex PCR.

Reagent	**Volume**		
D.W	6/7µl	10µl	2/7µl
Buffer	2µl	2µl	2µl
MgCl2	1µl	1/5µl	1µl
dNTPs	1µl	1/5µl	1µl
Primer F	2µl	1µl	2µl
Primer R	2µl	1µl	2µl
Taq	0/3 µl	0/3µl	0/3µl
DNA	5µl (extracted)	3µl (first round product)	5µl (second round product)

Table 3. Protocols of Nested and Doublex PCR reactions.

		Denaturation1	**Denaturation 2**	**Annealing**	**Extension 1**	**Extension 2**	**Cycle**
Nested PCR	**First round**	94ºC/1′	94ºC/1′	60ºC/1′	70ºC/1.5 ′	72ºC/1.5 ′	35
	Second round	94ºC/6′	94ºC/1′	55ºC/1′	70ºC/1′	70ºC/1 ′	33
Doublex PCR		98ºC/5′	98ºC/45′′	62ºC/1′	72ºC/5′	72ºC/5′	35

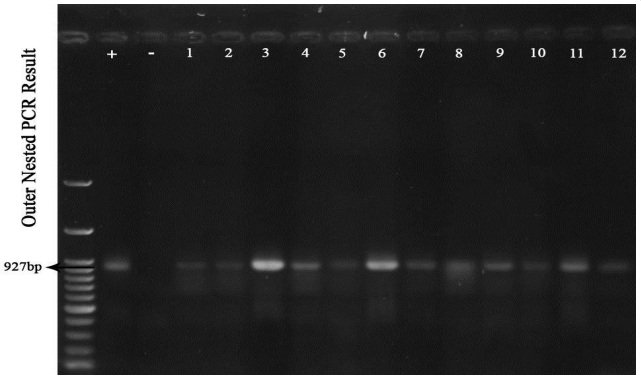

Figure 1. The first round of Nested PCR product electrophoresis. The figure illustrates that all 12 of the positive cultures were amplified by the specific primers. M: Marker 100bp; + : Positive Control; - : Negative Control; 1-12 Positive Samples.

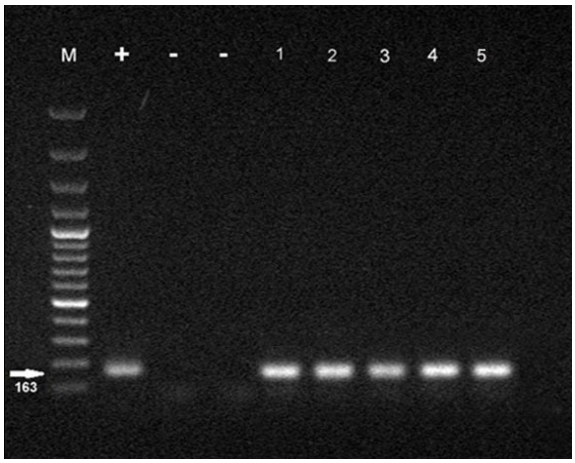

Figure 2. PCR products of the second round of Nested PCR. Figure shows that only 5 samples (1 cerebrospinal fluid and 4 urinal samples) were amplified using the specific primers for GSO and MGSO. M: Marker 100pb; + : Positive Control; - : PCR Control; - : Negative Control; 1-5 Positive Samples.

The only isolates with rrs_3 and rrs_4 were Mycoplasmas which were recorded in gene bank as *Mycoplasma arginini* (accession No. APO14657), *Mycoplasma canadense* (accession No. APO14631) and *Mycoplasma californicum* (accession No. APO13353).

Discussion

In this study, Mycoplasmas were separated from the cerebrospinal fluid and urinal samples and confirmed the roles played by the bacteria in the pathogenesis of MS. In parallel with our results, an investigation in the Faroe Islands showed a relationship between MS disease and pathogens during and after the Second World War (11). In the study conducted by Fujinami in 2014, the relationship between Mycoplasma and MS was addressed (12). In a study conducted in 2013, Tran et al., detected the relationship between *Mycoplasma pneumoniae* and demyelination (13). The relationship between *Mycoplasma pneumoniae* and the autoimmune disease MS

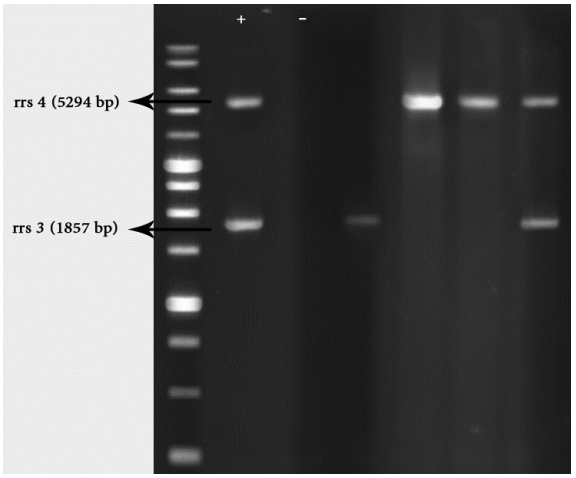

Figure 3. The electrophoresis gel of the multiplex PCR product using the specific primers of MB-282, MB-tet3/4 for rrs_3 and MB-ees-3 and MB-284 for rrs_4.M: Marker 500bp; +: Positive Control; -: Negative Control; 2-5: Positive Samples.

was pointed out in a study conducted by Booth in 2013 (11). Additionally, Bahar and colleagues by approving the serological presence of Mycoplasma in cerebrospinal fluid of patients with MS demonstrated the probable role of *Mycoplasma pneumoniae* in the pathogenesis of MS in an Iranian population (14). Therefore, it seems that Mycoplasma may play key roles in induction/stimulation of immune responses to demyelination of central nerve system. Our results also confirmed the presence of both mutated rrs_3 and rrs_4 alleles, as the markers of Tetracycline resistance in human Mycoplasma for the first time. Based on the plausible roles played by microbial infection in the pathogenesis of MS and according to the high prevalence of Mycoplasma in MS, it appears that the resistance strains may be considered as a risk factor for development of MS. As mentioned previously, Mycoplasma is treated by Tetracycline nowadays, hence, resistance to the antibiotic increases the risk of persistence of Mycoplasma infection, and may, consequently increase the chance of development of MS. Accordingly, it may be hypothesized that Mycoplasma achieve Tetracycline resistance to remain alive and it may be considered as a biomarker for development of MS which needs to be further investigated with more studies. Thus, antibiotic therapy of MS patients may be a plausible rout to overcome and ameliorate the disease.

Moreover, based on our results it seems that using Doublex PCR by the described primers may be a suitable method to diagnosis rrs_3 and rrs_4 mutated alleles, which are associated with Tetracycline resistance, and probably estimate MS development.

Furthermore, due to the presence of rrs_3 and rrs_4 alleles in 16SrRNA gene of urinal Mycoplasmas and their absence in cerebrospinal fluid of MS patients, it may be hypothesized that the anatomical location of Mycoplasma infection may be considered as an important factor

Table 4. General results obtained from Nested PCR and Doublex PCR reactions.

Result	Total	Outer +	Inner +	*rrs3* +	*rrs4* +	rrs3+rrs4 +
CSF	32	1	1	0	0	0
Urine	48	11	4	1	2	1
S	80	12	5	1	2	1

to the induction of mutation in the critical genes of Mycoplasma. However, based on the fact that one cerebrospinal fluid was infected by Mycoplasma, the hypothesis needs to be confirmed by additional investigations.

In order to determine Mycoplasma resistance to tetracycline in future research, it is suggested that a phenotype study via MIC be conducted to investigate the effect of neighboring genes of the aforementioned alleles, and tests on laboratory animals be carried out.

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