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MOLECULAR DIAGNOSIS OF NOCARDIA (SPP) IN MULTIPLE SCLEROSIS PATIENTS BY PCR

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ABSTRACT

Multiple sclerosis (MS) is one of the common diseases of the central nervous system.

The myelin disease in the transmission of nerve impulses along nerve fibers, is damaged, this disease has an immune nature and the cause is still unknown and some factors such as genetic and infectioncan be named.

The infectious agent that may be involved in causing the disease is the bacterium Nocardia. The aim of this study is to diagnose Nocardia in serum samples from patients with (MS) and healthy controls samples by PCR technique.

Method In this study, 50 serum samples from patients with (MS) and 50 control subjects were collected as well. DNA samples were extracted through DNG-PLUS Kit. NG1 and NG2, and 16s rRNA target gene primers were used as the suitable primer, and the PCR test was optimized PCR product was cloned.

Results**:** PCR test for diagnosis of Nocardia was optimized and product 595 bp was amplified. The sensitivity and specificity were analyzed and PCR product was cloned by plasmid PTZ57 R E.Coli-JM107 in host. Among 50patients who suffered MS, 3samples (6%) were positive by PCR test. While among 50 samples of the control group only one

ofwaspositive.

Conclusion By doing PCR technique, Nocardia bacteria is seen in samples of people who suffer MS more than the healthy people.

Keywords: Multiple Sclerosis, Nocardiabacteria, PCR

INTRODUCTION:

s are the important part of debilitating neurological disease among young adults. Multiple sclerosis (MS) is a disease of the central nervous system myelin sheath surrounding nerve fibers in the central nervous system is damaged and disrupted the neurological flow.Multiple sclerosis can affect the white matter of the brain, spinal cord and optic nerve II (David 1999).According to studies, MS is an autoimmune disease that the cause is not completely clear and factors such as genetic, geographic region, race infectious agents are known .Multiple sclerosis based on lesions and disease progression is divided in to four categories Manufacturer- relapse remitting, secondary progressive, and primary progressive and progressive – relapsing (Bala 2006).

Nocardia is a group of positive aerobic bacteria with a diameter of 0.5 to 1.2 micrometers, half Acidophilic, positive catalase and non-mobile. Nocardiosis is a dangerous infectious disease that is usually caused by entering bacteria into the respiratory or damaged skin.Lungtype is the most common. This type of Myelin disease infection can be seen often asbrain abscess and meningitis, but fortunately not highly prevalent.

Nocardia brain abscess do not usually have obvious clinical symptoms and often involves the brain and upper membrane can occur as single or multiple. Different species of Nocardia are common causes of brain abscess, but there are many reports of the incidence of brain abscess by Nocardia Ciriacy Jorjica. Another reports of other species of Nocardia in the incidence of brain abscess have been publishedincluding the role of Farsinica Nocardia, Nocardia Favieh, Nocardiaasteroids (Eshraqi, 2013).

Today by the growth of science and technology, it is possible to diagnose the cause of disease within a day or some hours by PCR. Recent investigations indicate that DNA tract of Nocardia bacteria, the case of this study by PCR molecular method is a sensitive, specialized, and secure method in the primary level of disease (Shahhosseini, 2005)

In 2009 attoicnarDiego F on serum and cerebrospinal fluid of MS patients tested by Elisa method.He divided them into 3 groups, 54 patients with MS who 34 of them were active, 10 patients with non-infectious neurological disease and 15 were healthy. He looked for herpes viridae in these samples by ELISA and PCR method.The test results were only a sample of people with MS in the active phase were positive for Epstein-Barr virus (EBV) DNA in the CSF of this person (DiegoF, 2009).

In the research of Levin and his colleagues in 2003, the correlation between the Epstein-Barr virus and MS was studied.In this study, serum levels of antibodies against EBNA-1 and VCA stored in the sample showed that the serum levels of antibodies increases before the onset of the disease.

In the study of Khaki and colleagues in 2009 the correlation between the herpes virus type 6 and multiple sclerosis patients was studied. 33 patients with MS who were diagnosed at an early stage and no specific treatment program was not carried out on them(Khaki, 2009).

In the study of Atefi and colleagues in 2013 that the diagnosis of herpes simplex virus in patients with multiple sclerosis through amplifying the same temperature was accomplished by (LAMP) ring (Atefi, 2013).

In the study of Ebringer 26 patients with MS sufferers in the UK were tested, the levelof antibodies against 5 strains of Sino bucket was measured. In this study, 20 ELISA and 25 patients Cerebrovascular accidents attended.The study showed that in patients with IgM, IgG, IgA antibody classes are significantly higher than the other two groups of MS patients. Among the 5 strains studied,Acinetobacter, Acinetobacter strains of Acinetobacterlwoffi 11171 and calacoaceti highest titer antibody, respectively, so that the surface antibodies was synthesized (Ebringer2001).

The aim of this study is accurate and fast diagnosis Nocardia bacteria in patients with multiple sclerosis (MS).

METHODOLOGY

Supplying standard strain

Nocardia brasiliensis strain PTCC.1422 was prepared in funguses and bacteria center of Iran in lyophilized (vial).

Culture of Bacterial

This strain opened under the biological hood Class II 0.5 cc environment Brain Heart infusiom broth was added till Lyophilized powder to be resolved, then we add the value of this solution to the BHi-Agar and BHi-BRoth for 48 hours, we incubated at 37 ° C until colonies

appeared. Several isolated Nocardia strains were compared.

DNA extraction

To extracting DNA of Nocardia bacteria in this study, DNA extraction was used by DNG Sinaclon kit.

Samples:

50samples from patients with MS and 50 healthy control samples were collected in 2013 from the hospital ShahidSadouqi in Yazd and serum was isolated. DNA samples were extracted by Kit DNP (Sinaclon- Iran).

Primer Design:

Primer was selected fromAlfaresiarticle (2006).

The primer sequence is as follows:

Nocardia primer:

NG1 (5-ACCGACCACAAGGGGG-3)

NG2 (5-GGTTGTAAACCTCTTTCGA-3)

These primers encodea product with 595 bp size product and the target gene of primers in Nocardia bacteriais 16SrRNA.

PCR test

10X PCR Buffer(2.5 µl)-dNTp(0.5 µl) primerForward(0.5 µl)

MgCl2(0.75µl)- Reverse primer(0.5µl)-TaqDNA Pol(0.3µl)

DNA template(5 □l)= 25□l

Investigating limit of detection (LOD) and features of PCR Test

To determine the limit of detection of optimized PCR test, the DNA of sample (positive control) Serial dilution up to 10^{-7} was provided and on each of these dilutions PCR test put.

The initial number of Nocardia DNA, in samples of formula Genome Copy Number and genome size brasiliensisNocardiawas calculated.

To determine the specificity of the used primers in this study, on DNA of Nocardia with DNA of other organisms: human, mouse, Saccharomyces Crozier, cytomegalovirus, adenovirus, hepatitis, PCR test were performed.

Cloning of PCR products

After purification of PCR product,by kit T / ACloning company Fermantas (Cat: K1214) and vector pTZ57R of this kit, the practice of cloning the 595 bp PCR product was performed.

RESULTS

Using DNA extracted from standard strains and by negative control, PCR test was accomplished and fragment 595 bp was amplified (Fig. 1).

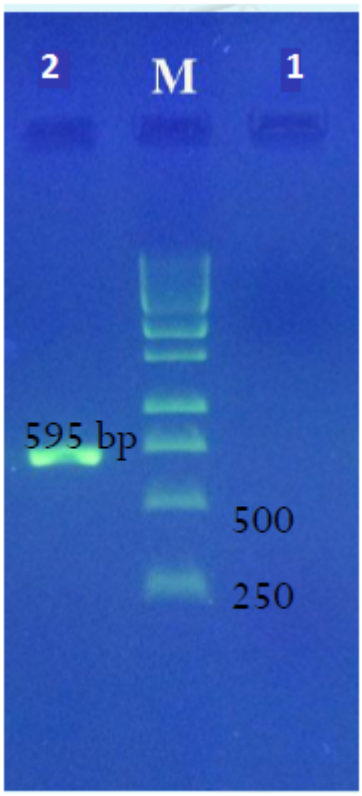

Fig 1: The Optimized PCR test in Nocardia (SPP)
1. Negative control; M. Fermantas size marker DNA lader 1Kb; 2. DNA standard strain of Nocardia

The limit of detection of PCR test was determined up to 10 bacteria.

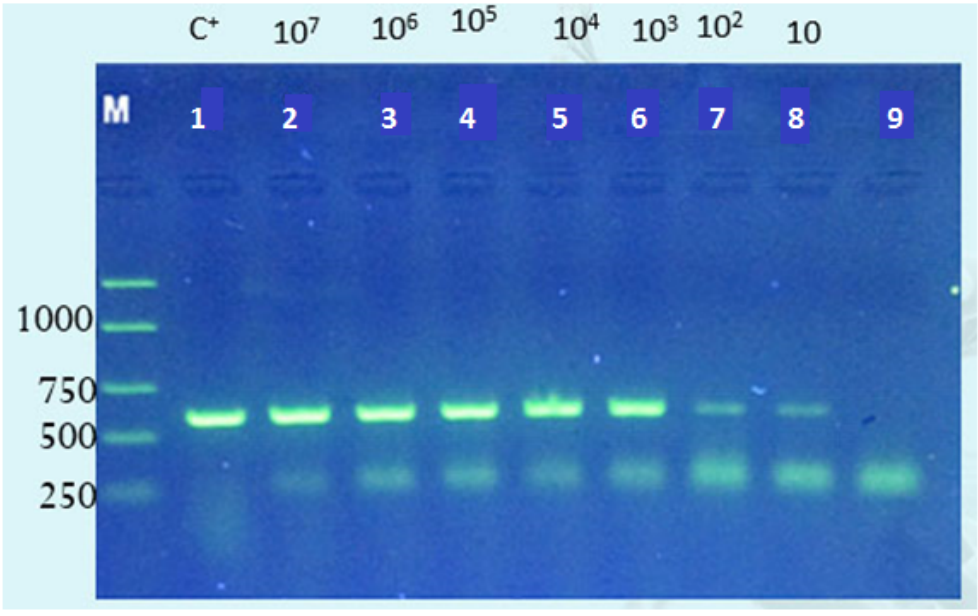

Figure 2. The limit of detection of optimized PCR test in bacteria of Nocardia (SPP):
M: size marker 1Kb DNAladerFrmntas
1. Positive control
2. DNA of ten million bacteria
3.DNA of one million bacteria
4.DNA of hundred thousand bacteria
5.DNA of ten thousand bacteria
6.DNA of thousands of bacteria
7. DNA of hundred bacteria
8. DNA of ten bacteria
9. Negative control

To ensure this issue that the intended premiers are identified by DNA of

Nocardia, the test with the DNA, Adenovirus, cytomegalovirus, and hepatitis B, DNA of Saccharomyces cerevisiae, human DNA and rat DNA and Haemophilusinfluenzae.

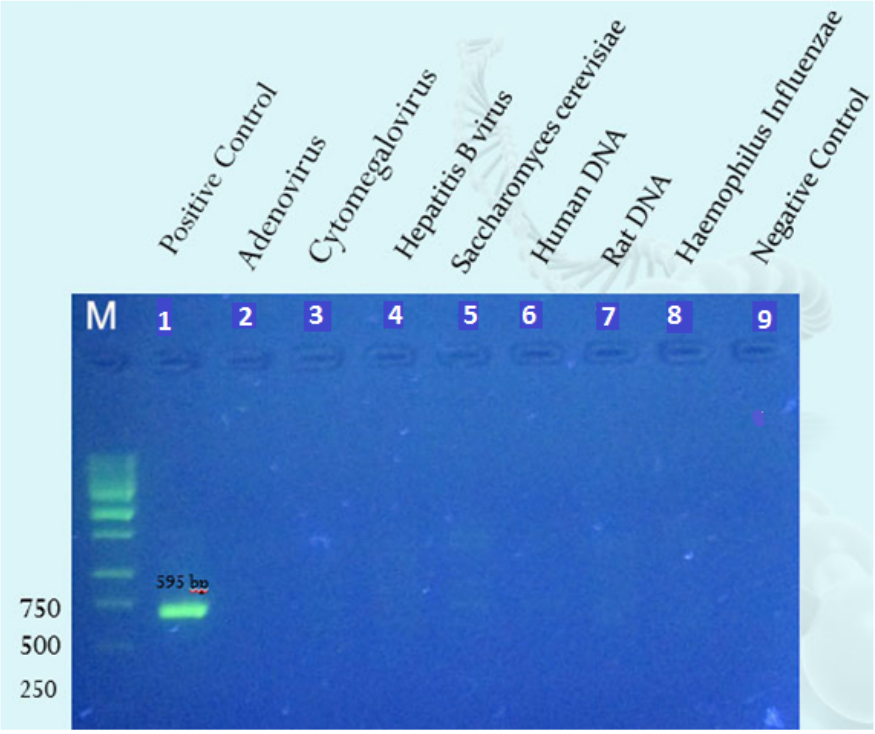

Figure 3. Specificity of optimized PCR testin Nocardia (SPP)

M. Fermantas company size marker 1Kb DNAlader

1. Sample of positive control of Nocardia (SPP)

2. Rat DNA

3. Saito Mgalo virus DNA

4. Adenovirus DNA

5. Hepatitis B virus DNA

6. Saccharomyces cerevisiae DNA

7. Cocos Staphylococcus staphylococcus DNA

8. Negative control

As it can be seen in Figure 3,only the product of 595 bp with the DNA of Nocardia are resulted and were not amplified by other factors.

Optimized PCR test of Nocardia (spp) for all 50 patients and 50 controls were performed.

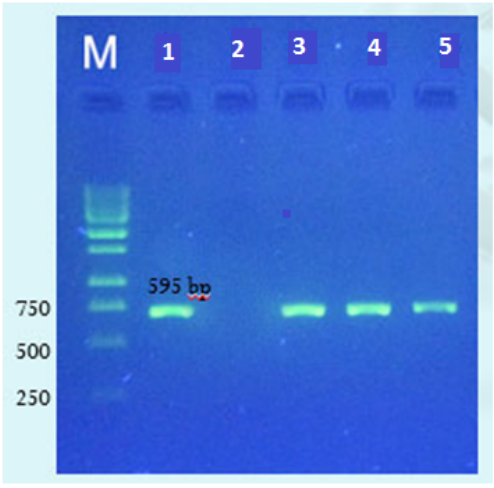

Figure 4- PCR test for diagnosis of Nokardia (SPP) in serum samples of those who suffered MS
M- Fermantas company size marker 1Kb DNAlader; 1. Positive control wit;h DNA Nokardia (SPP); 2. Negative control 3. 3-5 positive samples ; Among 50 patients in PCR test, 3 samples were positive.
Among 50 healthy people, one sample was positive.

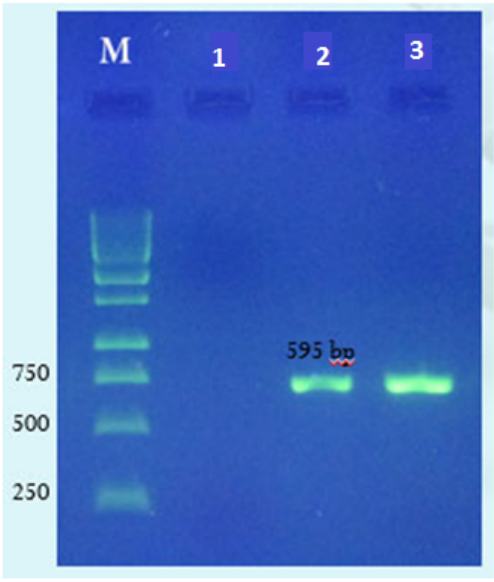

Figure 5- PCR test for diagnosis of Nokardia (SPP) in serum samples of control group
M- Fermantas company size marker 1Kb DNAlader; 1. Negative control; 2. Positive control; 3. Control group

Argument

MS is a neurodegenerative, inflammatory and demyelinating central nervous system disease withunknown etiology.

The peak incidence is between the ages of 20-40 years of age, but it may also occur in children and people over 60 years.

Women are almost twice more likely than males (Stuve & Oksenberg, 2010). The most plausible theory about the cause of MS is an autoimmune mechanism. In this way, an environmental factor such as viral and bacterial infections stimulate the immune system and to form antibodies against myelin nerve tissue causes the

neurological symptoms (Finlayson ML2006).

The techniques of PCRhas lofty and important position in various aspects of genetic engineering, diagnostic microbiology, sequencing and the evolution of lives studies that

DNA polymerases resistant to heat in PCR technique is one of the important developments in accessibility of this method in clinical laboratories (Persing DH, 2003).

Today, with the development of science and technology by PCR, the cause of disease can be diagnosed in less than one day or even a few hours and in the new version of it within 20-15 minutes. Recent investigations indicate that DNA tract of Nocardia bacteria, the case of this study by PCR molecular method is a sensitive, specialized, and secure method in the primary level of disease. This test not only for the detection of bacteria, but can be utilized to track and evaluate the results of treatment with anti-bacterial agent (Shahhosseini, 2005).

In 2006, Alfaresi and colleagues using real -time PCR with Cyber Green and analysis curve flux could isolate and detect the DNA of Nocardia from biopsies and abscess pus and stated that the sensitivity and specificity of themethodis 90 and 100 percent.

Real-time PCR test new primer, in comparison with the culture method is more suitable, the sensitivity, specificity and predictive values for the diagnosis of the disease.

By this method,diagnosis and treatment can be faster, and other complications be prevented.

In 2012, Hong and colleagues reported first nova Nocardia in brain abscess of a patient with immune deficiencies.This patient had a brain operation six month ago, and then he suffered brain abscess, and it was diagnosed by PCR method sequence 16S rRNA, nova

In 2013, Eshraqi and colleagues used molecular methods to identify the species of Nocardia classic and used 180 lavage fluid samples.Of these, 103 patients (57.22%) were male and 77 patients (42.78%) were women. The samples were cultured and grown colonies were purified and determined.

NG1 and NG2, as well as primers to amplify a 598 bp fragment specific 16SrRNA Nocardia were used.After culturing samples and purified five strains were obtained net (2.78%). Based on specific biochemical tests, five specimens belonging to the Nocardiaasteroides complex. After DNA extraction and PCR assay on the samples collected, 19 samples (10.56%) were

diagnosed by a positive test which in turn can be rapid and accurate diagnosis of Nocardia species for the treatment of severe infections and the prevention of brain abscess is necessary.This study showed that PCR compared with culture and biochemical tests to identify Nocardia higher sensitivity and accuracy requirements.Depending on the speed, accuracy, sensitivity and specificity of molecular assays is better in the future of this technique with other phenotypic methods for the diagnosis of Nocardia in laboratories, medical centers and research we use.

In the study of Levin and his colleagues in 2003 the correlation between the Epstein-Barr virus and MS was studied.In this study, serum levels of antibodies against EBNA-1 and VCA stored in the sample showed that the serum levels of antibodies before the onset of the disease increases, as well as the concentration of IL-12, IFN-gamma and IL-4 secreted by peripheral blood mononuclear cells in patients with MS showed that the concentration of cytokines was significantly higher than the control group.So it seems that the Epstein-Barr virus plays an important role in the pathogenesis of MS.

In the study of Khaki and colleagues in 2009 the correlation between herpes virus type 6 and 31 MS patients was studied. Multiple sclerosis patients who were diagnosed at an early stage and no specific treatment program was not carried out on them.

60 healthy individuals with indicators such as age, sex and location matched with healthy controls were analyzed in terms of G and M antibodies against human herpes virus 6 by ELISA and immunofluorescence.

The result was as follow: 74.2% of case group and 34.2% of control group in terms of G and M antibodies against human herpes virus 6 were positive and this difference was statistically significant ($p = 0.001$). The risk of multiple sclerosis in positive cases the presence of antibodies against HHV -6 M is 5 times more than the patients without these antibodies.The mean antibody titer against the virus G statistically significant difference was observed between the two groups ($p = 0.019$). Finally, they concluded that acute infection of human herpes virus 6 can be a factor for MS.

In 2005, Itano and his new technique Loop-mediated Isothermal Amplification (LAMP) to identify Nocardiaseriolae that nocardiosis in fish, were used.Loop-Mediated Isothermal Amplification this new technique (LAMP) is one of the isothermal gene amplification reaction

temperature from start to finish in one place.Despite this technique simply has very high sensitivity and precision, and as the need for a device such as a thermocycler is over, the reaction is carried out with minimal cost.

In this technique, a special target of 4 primers as internal primers (FIP, BIP) and external (F3, B3) and a DNA polymerase with strand displacement activity (Bst large fragment DNA polymerase) is used to cause the formation of shoot the ring.

The high sensitivity of this method as a way to identify Nocardia stated.

In 2004, Loeffler and his colleagues both culture and PCR in mice infected brain samples were compared to Nocardia asteroids,in this study, they stated that the rapid and highly sensitive PCR method to identify Nakardia that tend to brain tissue, and also stated that the cultivation methods, forms of L-form nocardia is detected by PCR but this type of Nocardia is detected.And also based on standard culture tests and PCR tests that culture experiments 72-48 hours, but by PCR after 35 cycles of more than one million copies of the desired DNA is created.Given that the main objective of this study was to compare two techniques and PCR in the identification of Nocardia asteroids, as a result, samples collected with the technique and the results showed that the sensitivity and specificity of PCR was 100% and 98%.

Bogdanos and colleagues in 2005 studied the association between autoimmune hepatitis b and against components of myelin membranes of neurons, which can be seen in multiple sclerosis.The amino acid in the structure of the peptide hepatitis b virus surface antigen myelin membrane proteins were evaluated in 147 adult patients that obtained similarities were significant in this field.

The author concludes that the made antibody against HBsAg can be as an agent against myelin membrane, and the cause of multiple sclerosis disease.

CONCLUSION:

PCR is a fast and accurate method to identify Nocardia.Also, the PCR method showed that contamination was higher in patients with MS compared to controls, as well as the results were analyzed by statistical software Grap pad prism that it indicates lack of correlation between the disease and the bacterium of Nocardia.

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