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Original Article

Molecular Characterization and Sequencing of a Gene Encoding Mannose Binding Protein in an Iranian Isolate of *Acanthamoeba castellanii* as a Major Agent of *Acanthamoeba keratitis*

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Abstract

Background: *Acanthamoeba castellanii* is the important cause of amoebic keratitis in Iran. The key molecule in pathogenesis of *Acanthamoeba* keratitis is Mannose Binding Protein (MBP) led to adhesion of amoeba to corneal epithelium. Subsequent to adhesion other cytopathic effects occur. The goal of this study was to identify the molecular characterization of a gene encoding MBP in an Iranian isolate of *A.castellanii* in order to pave the way for further investigations such as new therapeutic advances or immunization.

Methods: *A.castellanii* was cultured on non nutrient agar. Extraction of DNA was performed by phenol-chloroform method. After designing a pair of primer for the gene encoding MBP, PCR analysis was performed. Finally, the PCR product has been sequenced and the result submitted to the gene data banks.

Results: An MBP gene of 1081 nucleotides was sequenced. This fragment contained three introns and encodes a protein with 194 amino acids. Homology search by Blast program showed a significant homology with the MBP gene in gene data banks (96%). Besides, the identity of amino acids with the other MBPs in gene data banks was about 86%.

Conclusion: We isolated and sequenced a gene fragment encoding MBP in an Iranian isolate of *A.castellanii*. Molecular characterization of this important gene is the first step in pursuing researches such as developing better therapeutic agents, immunization of population at risk or even developing a diagnostic tool by PCR techniques.

Key words: *Mannose Binding Protein, Acanthamoeba, Keratitis, Iran*

Introduction

Acanthamoeba are an amphizoic amoeba and it is the most ubiquitous protozoa (1-3). These free living protozoa has been isolated from various environments such as water, soil, dust, eye wash solution, sewage and even chlorinated pools (2, 3). This amoeba can cause serious human diseases such as a rare but fatal Granulomatous Amoebic Encephalitis (GAE) and amoebic keratitis (3). Keratitis due to *Acanthamoeba* is a sight-threatening disease which can lead to poor vision and finally blindness (3, 4). A brief review on epidemiological studies reflects the increased incidence of amoebic keratitis, especially since 1980. For instance the estimated rate of amoebic keratitis in 2003 was 1300 cases worldwide (4) but reports in 2007 demonstrated that there were more then 3000 patients involved with this painful corneal infection (5). In Iran, a ten year study during 1997-2007 showed that among 142 keratitis patients, 34.5% had *Acanthamoeba* infection which was a rather high rate (6). This study confirmed that amoebic keratitis continues to rise in Iran. Although the major risk factor of amoebic keratitis is wearing soft contact lenses but it is worth mentioning that corneal trauma is an important predisposing factor as well (7). Trauma may occur by wearing contact lenses, eye surgery or other noxious agent.

The first step which is very critical for amoeba

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to cause disease is its adherence to corneal epithelium. In fact, adhesion of *Acanthamoeba* to epithelial cells leads to subsequent events such as phagocytosis, apoptosis and proteolytic secretions (8). Finally, these events cause epithelial defect, stromal infiltration and cell death. Recent studies showed that *Acanthamoeba* could express Mannose Binding Protein (MBP) which is a key molecule in pathogenesis of amoebic keratitis (9). Direct evidence proved that MBP was the most virulence protein which acts like Galactose Binding protein (GBP) in *Entamoeba histolytica* (4). As it name shows, this protein bind to mannosilated glycoprotein on the corneal epithelium (10). Various studies revealed that production of these glycoproteins are up regulated by trauma and this event lead to introducing corneal trauma as a major risk factor for developing amoebic keratitis (11). Molecular investigations (12) on keratitis patients demonstrated that in Iran three species of *Acanthamoeba* were responsible for amoebic keratitis including *A.castellanii*, *A.griffinii* and *A.palestinensis.* Besides, the predominant genotype of *Acanthamoeba* was attributed to T4 in Iran.

The goal of this study was to characterize and sequence of the gene encoding MBP in *A.castellanii* isolated in Iran. Furthermore our destination is to use this gene for early diagnosis, treatment or even immunization of high risk people.

Materials and Methods

Parasites A clinical isolate of *A.castellanii* from an Iranian patient who referred to Department of Medical Parasitology and Mycology, School of Public Health, Medical sciences/University of Tehran, was our sample. This parasite had been examined previously at Birkbeck College, University of London and it was proved that the species was *castellanii*. So we used these species as a reference sample (12).

Culture In order to obtain a large amount of trophozoites, gram- negative bacteria such as *Escherichia coli* were seeded on the non-nutrient agar as a food source (2). The advantage of this medium is the lack of nutrients which leads to inhibition of unwanted organism's growth. Non nutrient agar contains 1% agar in Page's Amoeba Saline (2). First, the old culture of gram-negative bacteria was applied on the surface of agar. In the next step, small piece of agar containing the cysts was added onto the fresh medium. The medium incubated at 30° C and screened daily for the presence of trophozoites. Finally, the parasites were harvested by PBS solution.

DNA Extraction Total genomic DNA of trophozoites was extracted by modified phenol-chloroform method (13). It should be mentioned that incubation of amoeba with proteinase K was about 2 h. In order to detect the DNA band, 1% agarose gel stained by Ethidium bromide were applied and the banding patterns were visualized by UV Tran illuminator.

PCR analysis A pair of primers based on a part of sequence of *A.castellanii* Mannose Binding Protein genes in Gene data banks was designed (Accession number: AY604039 and AY 604040). Primers used in this study were 5'GTC TTG ATG GTG GCC TTG TT 3'as forward and 5'CCC ACA CCT CCT TGT CCT TA 3' as reverse. These primers sequences correspond to 1000 bp of *A.castellanii* MBP gene. The PCR reaction was carried out in a separate room and sterile PCR tubes, autoclaved water and tips were used for decreasing the possibility of contamination. Each 50µl PCR mixture comprises of 0.2 mM dNTP mix, 20 ρM of each primer, 20 mM PCR buffer (with $MgCl_2$), and 1.2 Unit of Taq DNA Polymerase (Cinnagen, Tehran, Iran) and 1 µl of template DNA. The PCR cycles were as following: 94° C for 30 sec (Denaturation), 52° C for 1:30 min (Annealing) and 72° C for 2 min (Extension). After 35 cycles, program was set for 72° C for 5 min.

Detection of PCR product The PCR product were detected by using 1% agarose gel containing Ethidium bromide and the result recorded by UV transilluminator. Finally, the target PCR product was sliced and recovered by Qiagen Kit (USA).

DNA sequencing After purification, The PCR

product was submitted for sequencing (MWG-Germany).

Results

Five days after culturing, the maximum rate of trophozoites on the agar surface was obtained. Many trophozoites moved slowly in one line to the margin of plate culture. These trophozoites were collected by PBS.

DNA Extraction from trophozoites followed by gel electrophoresis showed a sharp DNA band which was visualized by UV transilluminator (Fig. 1).

Approximately 1000 base-pair PCR product was amplified from DNA samples. In order to purify PCR product and eliminate primers, excess nucleotides and non-specific bands, purification by Qiagen kit was performed and after electrophoresis only the specific band were obtained (Fig. 2).

An MBP gene of 1081 nucleotides was revealed in sequencing (Fig. 3). Sequence analysis was performed and homology search by Basis Local Alignment search tool (Blast) program in gene data banks (NCBI, NIH) showed a significant homology with the gene encoding Mannose Binding Protein in Nucleic acid level (96%) as well as its amino acid level (86%). The number of introns is estimated to be three in this fragment and 194 amino acids have been encoded by this sequence. This fragment has been submitted to the gene bank and is available for public access under accession number: EU363513

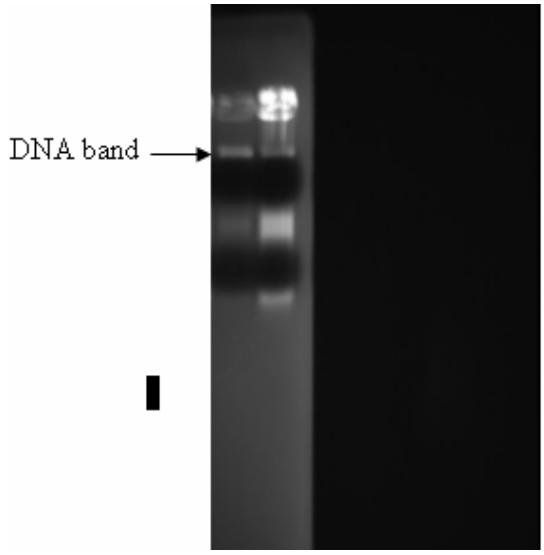

Fig. 1: 10 µl DNA of A.castellanii (agarose 1%)

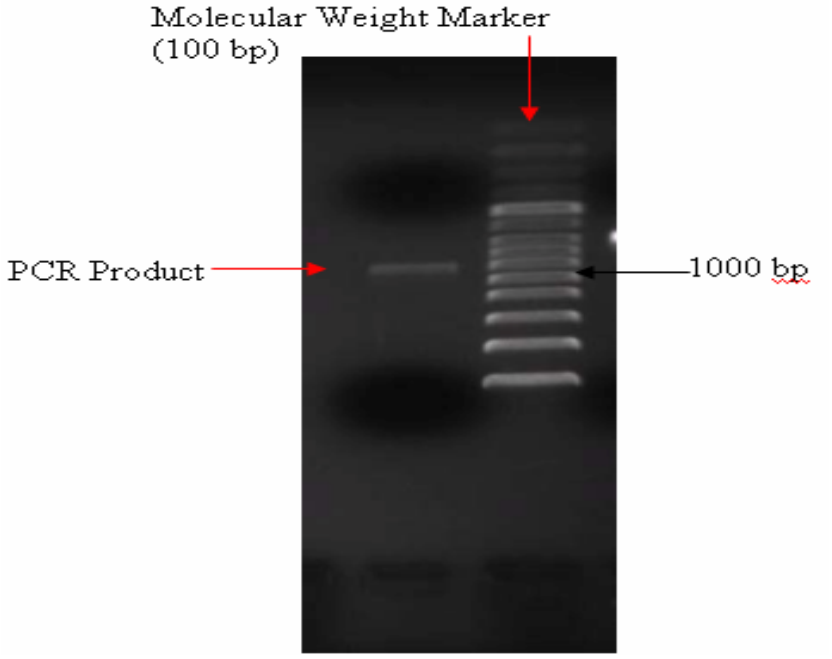

Fig. 2: 5µl PCR- product of the A .castellanii MBP (agarose 1%)

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F   E   A   V   G   S   D   G   T   C   N   L
ttc gaa gcc gtc ggc tcc gac ggc acc tgc aac ttg
gtacatacccagccccgtttcatctcctcctccggccctctcttcctgtcgattttgttctcaagaaaggcttcccttgatcgtccctctttttttg
agaggagaactcagttcaccttcgtaggtgaaccaaaactaataaaaatctgttctcattctctctctctctctctctacag

 F   G   P   I   K   Q   P   G   L   D   C   S   A   T   S   C   F   I   T
ttc ggt ccc atc aag cag ccc ggc ctt gat tgc tcg gct aca agc tgc ttc atc acg
gttccccccccaagctaccactacactcggacgttcagctgatctcccctcgccccgtttttcgttccttccttcctttgtacgaaacagtcag

 S   G   T   F   P   F   P   L   P   Q   A   E   T   Y   D   S   F   Y   S   W
agc ggc acc ttc ccc ttt ccg ctg ccc cag gcc gag acc tac gat agc ttc tac agc tgg

 I   L   G   L   I   G   T
att ctg ggc ctc atc ggc ac
gtacggcctccacccactcctcatcctcgacaccacacgcctcgccactcgcgggtttttaatgtccgacgcgttcgttgtggccgtggttacgca
tatattaatttgtttaataatgccaccaatcagcggagaccgcatcactcttaggttacctttttttatatctatgcaacgaccaataccaacact
cgacacaacaacaacaacaatgtaccaacag

    T   H   G   A   T   V   N   A   Q   Y   V   D   Y   T   K   A   D   P   N
  g acc cac gga gcc acg gtg aac gcg caa tat gtc gac tac acc aaa gcc gac ccc aac

 I   Y   F   T   A   G   Q   A   N   C   M   V   N   S   T   F   V   Y   D   V
atc tac ttc acg gcg ggt cag gcc aac tgc atg gtc aac agc acc ttc gtc tac gac gtg

 A   F   Y   R   T   S   M   G   Y   F   T   F   S   R   D   S   M   P   T   S
gcc ttc tac cgt acc agc atg ggc tac ttc acc ttc agc cgt gac tcc atg ccc acc tcc

 V   G   S   L   T   L   K   P   V   F   S   D   A   T   A   D   C   S   G   T
Gtc ggc agc ctc acc ttg aag ccc gtg ttc agc gat gcg acg gca gac tgc agc ggt acg

 S   S   Q   T   L   A   G   T   S   C   L   A   P   G   S   N   I   S   F   G
tcc agc cag acg ctg gcc ggc acc tct tgc ctc gcg ccc ggc tcg aac atc tcc ttc ggc

 P   F   S   S   T   Q   A   V   G   F   Y   L   K   H   D   S   L C   S   G
ccc ttc agc tcc acc cag gcc gtc ggc ttc tac ctc aag cac gac tcc ctc tgc agc ggc

 T   T   T   F   Y   S   V   D   A   L   T   K   V   T   S   R   W
acc acc acc ttc tac tca gtc gac gcc ctc acc aag gtc acc tcc cgc tgg
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Fig. 3: 1081 bp nucleotide sequence of DNA and deduced amino acid sequence of MBP gene of *A. castellani*

Discussion

In this study, we have isolated and sequenced a fragment of gene encoding MBP in an Iranian isolate of *A.castellanii*. This fragment encodes a protein with 194 amino acids. Alignment analysis of amino acids of this fragment by protein BLAST search showed a low identity with other proteins. In contrast, the above fragment revealed a significant homology (86%) with the MBP of *A.castellanii* presented in the gene data banks.

This result is in agreement with homology analysis of MBP of *A.castellanii* (9) in 2004 which revealed that this protein had 42% homology with 220-kDa silk protein, 44% with hypotetical protein from microbulbifer degradans and 32% with extracellular E- selectin precursor. We can conclude that MBP has a little homology with any other proteins.

Cloning of MBP gene (9) in 2004 revealed that this gene composed of about 3620 bp with 5 introns and 6 exons and encodes a protein with 833 amino acids. This team proved that MBP was a transmembrane protein. MBP protein acts as a receptor for the mannose residue of mannosilated glycoprotein of cornea. Other finding of these researchers revealed that MBP in *A.castellanii* itself contained mannose (8). According to our study amino acid analysis of MBP showed a high amount of serine (12.58%) and threonine (12.37%) and the low amount of tryptophan (1.03%), histidine (1.03%), methionine (1.54%) and arginine (1.54%). In addition, the joint of exons was in 1 to 36، 216 to 272، 368 to 447 and 673 to 1081.

To date, it has been shown that amoeba adherence to corneal epithelium is the first step for developing this chronic disease and MBP is the key molecule (2, 4, 9, 14). Indeed, subsequent to adhesion, cell death as a result of phagocytosis, apoptosis and proteolytic secretions occur (2). In addition, binding of *Acanthamoaba* to cell protects them from being washed out by tear film. Garate et al. (8) in 2005 introduced MBP as a cell surface protein of *Acanthamoeba* trophozoite. Recent article by Khan stated that the binding of *Acanthamoeba* can be inhibited by the presence of exogenous α- mannose, this is similar to free galactose which inhibits the adherence of *Entamoeba histolytica* by Galactose binding protein (4). Results of this finding are somewhat controversial. Cao et al. (15) demonstrated that amoeba can loss its cytopathic effect following the inhibition of binding by exogenous mannose, in contrast, Leher et al. (16) showed that mannose lead to exacerbation of cytopathic effects of amoeba.

Studies by Khan (4) in 2003 revealed that some of the virulent genotypes such as T_2, T_4 (*A. castellanii*) and T_{11} exhibited higher binding ability to the corneal epithelium. Following the above mentioned studies, we decided to work on *A.castellanii* MBP as a virulent isolate since the lack of this protein in some non virulent genotypes is possible. In support of this, another study carried out (8) in 2006, suggested that the pathogenicity potential of *Acanthamoba* was directly dependent on the level of MBP which is expressed on the surface of the amoeba.

Besides, Garate et al. (14) suggested that antibodies to MBP in tear film lead to protection against amoebic keratitis and oral immunization with recombinant MBP could ameliorate AK in animal models. Finally MBP can provide protection against the disease which is very important for further studies.

In conclusion, identification and sequencing of this important gene is the first step to pursue future research such as developing better therapeutic agents, immunization of population at risk or even developing a rapid diagnostic tool by PCR techniques. The sequence analysis of the gene fragment characterized in this study is still under investigation.

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The authors declare that they have no Conflict of Interests.

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