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Original Article

Cloning and Expression of Immunogenic Regions of EMA-1 Gene of *Theileria equi* From Infected Horses

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ABSTRACT

Diversity among the pathogenic strains of *Theileria equi* (*T. equi*), a major agent of equine piroplasmosis, can affect the appropriate detection of parasite and host immunization. Production of recombinant surface proteins from an infected horse in natural endemic area provides a reliable tool for immunodiagnosis of parasite. Regarding this, the present study was targeted toward the cloning, expression, and purification of the immunogenic regions of equine merozoite antigen 1 (*EMA-1* gene), as one of the most important immunodominant surface proteins in *T. equi*, from naturally infected horses in Iran. The immunogenic region of *EMA-1* gene was amplified using the blood of infected horses. *EMA-1* gene was cloned into pET26b vector. Then, recombinant plasmids (pET 26b-EMA-1) were transformed into competent *E. coli* BL21 (DE3) cells. Cloning was confirmed by polymerase chain reaction (PCR), restriction enzyme assays, and DNA sequence analysis. The recombinant protein was expressed using isopropyl β-D-1-thiogalactopyranoside as an inducer, purified using nickle-nitrilotriacetic acid column, and then confirmed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and dot blot analysis utilizing Anti-His Tag antibody. Furthermore, the immunoreactivity of recombinant protein against the serum of the infected horses was evaluated using dot blot analysis. The PCR product analysis showed a 750-bp band belonging to immunogenic regions of *EMA-1* gene. Sequence analysis revealed that cloned *EMA-1* and protein had 94% and 97% homology to *EMA-1* sequences submitted to GenBank from different countries, respectively. Restriction enzyme and sequence analyses confirmed the subcloning and correction of the orientation of inserted gene. The SDS-PAGE analysis confirmed the expression of *EMA-1* protein with a 28-kDa band. The results of the dot blot analysis revealed that the horse serum containing antibody against *T. equi* could react with the purified recombinant protein. Purified *EMA-1* protein can be used as a reliable tool for the future development of diagnostic tests or vaccines.

Keywords: *Theileria equi*, EMA-1, cloning

Le Clonage et l'Expression de Régions Immunogènes du Gène EMA-1 du Parasite de *Theileria equi* Isolé de Chevaux Infectés

Résumé: La variation parmi les souches immunogènes de Theileria equi, la principale cause de la piroplasmose chez les chevaux, peut affecter la reconnaissance du parasite et de l'immunogénicité de l'hôte. La production de protéines recombinantes à partir de parasites des chevaux infectés dans les zones endémiques fournit un outil d'identification de l'immunité parasitaire. Le but de la présente étude est le clonage, l'expression et la purification de régions immunogènes de la protéine EMA-1 (l'une des plus importantes protéines de surface immunogènes

dans Theileria equi) chez les chevaux infectés. Les régions immunogènes de la protéine EMA-1 du sang des chevaux infectés ont été reproduites. Le gène EMA-1 a été cloné dans un vecteur pET 26b. Ensuite, le plasmide recombinant (pET 26b-EMA-1) a été transféré dans le récepteur E. coli BL21. La confirmation du clonage a été réalisée par PCR, découpe de vecteurs avec des enzymes de découpe et analyse du séquençage de l'ADN. L'expression de la protéine recombinante a été induite en utilisant de l'IPTG. Une purification a été réalisée en utilisant des colonnes NI-NTA et en vérifiant l'expression en utilisant une SDS-PAGE à 10%, et le Dot blot en utilisant des anticorps anti-HisTag. La réponse immunitaire de la protéine recombinante avec le sérum du cheval infecté a été évaluée en utilisant le test de Dot Blot. L'analyse du produit de PCR a montré une bande de750 pb appartenant au gène EMA-1. L'analyse de séquence du gène et de la protéine EMA-1 avec d'autres séquences de la banque de gènes a montré une similarité de 94 et 97%, respectivement. L'analyse de séquence a confirmé la coupe avec des enzymes pour une insertion correcte du vecteur dans le vecteur. L'analyse SDS-PAGE a montré l'expression de la protéine EMA-1 avec une bande de protéine de 28 kDa. L'analyse des résultats de Dot Blot a montré que le sérum du cheval contenant des anticorps contre Theileria equi pouvait réagir avec la protéine recombinante purifiée. La protéine purifiée d'EMA-1 peut être utilisée comme un outil fiable pour la conception de tests de diagnostic et de tests de vaccins à l'avenir.

Mots-clés: *Theileria equi*, Gène EMA-1, Clonage

INTRODUCTION

Theileria equi, an Apicomplexan parasite, is a tick-borne protozoan that is considered as the main cause of equine piroplasmosis (EP) in domestic and wild equines, including horses, donkeys, mules, and zebras, worldwide (Steinman et al., 2012). The animals, surviving from acute infection, are usually asymptomatic and remain as potential carriers for the infection during their lifetime with a low level of parasitemia. Transmission of *T. equi* takes place by infestation with hard-bodied ticks, mainly *Hyalomma*, *Rhipicephalus,* and *Dermacentor* species that convey protozoa in their salivary glands (Jongejan and Uilenberg, 2004). There are reports indicating that *T. equi* can also be transplacentally transmitted from carrier mare to the fetus, thereby resulting in abortion or neonatal death (Chhabra et al., 2012). Precise diagnosis requires the observation of parasites in blood smears; however, parasites are generally present in very low numbers during chronic infection that cannot be detected by the microscopic examination of blood smears. It is noteworthy that a few parasites can also be transmitted by competent tick vectors or iatrogenic means (Short et al., 2012). Asymptomatic persistently infected carriers act as the reservoirs of infection, which is a serious challenge to control the spread of *T. equi*. Therefore, the diagnosis of these subclinical infections is crucial, especially for horse racing industry, in which the movement of apparently healthy horses from enzootic districts may result in the outbreak of piroplasmosis due to *T. equi* in disease-free areas (Schwint et al., 2008). Infections can be determined by several techniques, including molecular and serological procedures. Polymerase chain reaction (PCR) is commonly utilized for the detection of many *Theileria* and *Babesia* species in particular when parasitemia is considerably low. Nonetheless, this approach cannot differentiate between the acute and chronic forms of the disease induced by the mentioned protozoa and healthy carriers as well (Salim et al., 2008). Recently, serologic procedures, such as enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test, and complement fixation test, have been used for the detection of *T. equi* (Mahmoud et al., 2016). However, these methods may result in false data due to cross-reactions with native crude antigens (Papadopoulos et al., 1996). One of the main approaches to solve these problems is to prepare the recombinant antigens for designing a sensitive and specific serologic test for the detection of etiologic agents. Many studies have focused on the evaluation of recombinant proteins to develop a suitable method for the detection of *T. equi* in the recent years. In this regard, merozoite-specific

recombinant antigens, produced by molecular techniques, are now considered as appealing alternatives for the detection of serum antibodies. Various surface proteins have been used as a target for the diagnosis of *T. equi*. Equine merozoite antigen 1 (EMA-1) is one of the most important immunodominant surface proteins in *T. equi,* belonging to major piroplasm surface protein family, which is conserved among the genus (Knowles et al., 1997). The EMA-1 is a 30-Kd protein, which plays a significant role in the recognition, attachment, and penetration of host erythrocytes. Although the surface proteins are obscure for the direct attachment of EMA on the erythrocyte membrane, a study has shown that the EMA of apicomplexean parasites interferes with the integrity of the spectrin-actin network of erythrocyte membrane (Kumar et al., 2004). This antigen can be forcefully recognized by antibodies produced in the infected animals. Therefore, it seems to be a good candidate and a reliable diagnostic molecule for the detection of antibody against the parasite. Diversity among pathogen strains of *T. equi*, a major agent of equine piroplasmosis, can affect the appropriate detection of parasite and host immunisation. The production of recombinant surface proteins from the infected horse in natural endemic area provides a reliable tool for the immunodiagnosis of parasite. With this background in mind, the present study was targeted toward cloning, expression, and purification of the immunogenic regions of *EMA-1* gene isolated from the naturally infected horse in Iran.

MATERIAL AND METHODS

Parasite. Whole blood samples were taken from 20 horses with clinical signs of piroplasmosis, and then transferred to test tubes containing ethylenediamine tetraacetic acid as anticoagulant. Infection with *T. equi* was confirmed after staining the prepared methanol-fixed slides with Giemsa, examination of the clinical manifestations of equine piroplasmosis, and implementation of conventional PCR analysis. Afterwards, the blood samples were stored at -20 ^{o}C for future application.

Sequence analysis, epitope prediction, and primer design. To design the required primers, the sequences of EMA-1 gene from *T. equi* infected horses were obtained by DNA sequencing, and then aligned to the GenBank sequences using ClustalW (version 2016) software to find the conserved regions. Prediction of the antigenic regions of EMA-1 was carried out utilizing BepiPred (version 2.0), BCPred (version 2004), and SVMTrip (version 2012) software. Primers, which were designed using Primer Premier 5 software (Premier Biosoft International, USA) for cloning EMA-1 gene into pET26b could recognize a highly conserved and immunogenic 750-bp fragment of gene with restriction sites at the 5´ (BamHI) and 3´ends (HindIII) (Table 1).

Table1. Primer sequences used in the present study

Gene	Primer sequences	Restriction enzyme	Length (bp)
EMA-1	F: ATGGATCCGGAGGAGGAGAAACCCAAG	BamHI	750
	R: ATAAGCTTAATAGAGTAGAAAATGCAATG	HindIII	

DNA extraction, polymerase chain reaction amplification, and sequencing. Genomic DNA of *T. equi* was exploited from accumulated and frozen infected equine blood samples using DNA purification kit (SinaClon BioSciences, Iran) according to the manufacturer's protocol. Purified DNA was stored at -70 °C until applying as template for subsequent PCR amplifications. The PCR was carried out in a total volume of 20 µL, containing 10 µL of PCR Master Mix (Amplicon , Denmark) with 1.5 mM $MgCl_2$, 0.4 mM of each dNTP,0.2 U/ul amplicon Taq DNA polymerase, 0.25 µL of each primer (10 uM), and 3 µL (~100 ng) of template DNA. The PCR condition included denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 45 sec, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min. The amplified

PCR products were visualized by 1.5% agarose gel electrophoresis in TAE buffer stained with DNA safe stain under ultraviolet light.

Cloning of equine merozoite antigen 1 in pET26 plasmid vector. The PCR product was purified from the agarose gel using Gel DNA Recovery Kit (SinaClon BioSciences, Iran) according to the manufacturer's recommendation. The PCR product and pET-26b vector (Clontech Laboratories, Inc., USA) were double-digested with BamHI and the HindIII restriction enzymes (Fermentas, USA) at 37 °C for 2 h. To check the enzyme activity and subsequent steps, digested fragments were electrophoresed on 1% agarose gel stained by SafeStain (SinaClon, BioSciences, Iran) and purified using Gel DNA Recovery Kit based on the manufacturer's instructions. The purified linear vector and insert were subjected to ligation reaction using T4 DNA ligase (Fermentas, USA). After the deactivation of the reaction at 65 °C for 15 min, the ligation product was transformed into calcium chloride-competent *E. coli* BL21 (DE3) cells. The competent cells were transformed with 2 uL of ligation product. The transformed cells were selected on LB medium agar plates containing kanamycin (50 ug/ml). Several colonies were assayed by colony PCR using universal T7 primers. After the selection of recombinant clones, the plasmid DNA was extracted from the cells cultured overnight by using the Miniprep plasmid isolation kit (SinaClon, Biosciences, Iran) and confirmed by PCR and restriction-enzyme digestion, followed by DNA sequencing utilizing T7 primers.

Protein expression and purification. A single colony of the recombinant *E.coli* BL21 (DE3) cells, containing pET26b-EMA-1 construct, was grown in LB broth supplemented with kanamycin (50 ug/ml) overnight at 37ºC with shaking at 120 rpm. Next, 250 µl of the overnight culture was inoculated to 12 ml fresh LB broth, containing 50 ug/ml kanamycin, and placed at 37º C with shaking until the OD600 reached to 0.6. Then, the EMA-1 protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, USA), followed by incubation at 37ºC for 16 h with shaking at 90 rpm. To confirm the protein expression, two samples of recombinant bacteria were subjected to 10% SDS-PAGE before and after the addition of IPTG to the medium. The recombinant *EMA-1* protein was purified from cellular extraction under denaturing conditions using the Ni-NTA column (QiaGen, Germany). Briefly, the cells (50 ml) were harvested by centrifugation at 5000 rpm for 20 min at 4 °C. Cell pellet was suspended in 2 mL lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl) and lysed by sonication (6 cycles of 10 sec of pulses at 45% amplitude and 59 W). The suspension was centrifuged at 13,000 rpm for 10 min, and the supernatant was loaded into a Ni-NTA column with a volume of 1 mL resin preequilibrated in lysis buffer. The column was washed 8 times with 1 mL wash buffer (20 mM imidazole, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl) and 4 times with 500 μL elution buffer (200 mM imidazole, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl). All fractions were stored at 4 ºC and electrophoresed on 10% SDS-PAGE. Protein concentration was determined by Bradford method using bovine serum albumin (Sigma, USA) as a standard.

Dot blotting. Dot blot analysis was performed to confirm the expression of polyhistidine tag EMA-1 antigen and its reaction with serum horse containing antibody against *T. equi*. Briefly, nitrocellulose membranes (Millipore, USA) were pre-wetted for 5 min in TBS-T (20 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.5), and then soaked in distilled water for 2 min. The purified recombinant EMA-1 protein (10 ul~5 ug) was dotted on nitrocellulose membrane. Non-specific binding sites were then blocked using TBS-T containing 5% skim milk (Merck, Germany) for 30 min at room temperature, rinsed three times with TBS-T, and incubated for 30 min with 1:100 dilution of horse serum, containing antibody against *T.equi* or negative horse serum. The immunized serum was provided from the Department of Parasitology of the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran. The membrane was washed

three times with TBST, incubated for 30 min with rabbit anti-mouse HRP-conjugated IgG antibody (Abcam, USA) with a dilution of 1:1000. Following three washes with PBS buffer, the substrate (50 mM Tris buffer, pH: 7.8, containing 6 mg DAB, 10 uL H_2O_2) was used for the detection. For the detection of the expression of polyhistidine tag EMA-1 antigen, Anti Histag antibody (Abcam, USA) with a dilution of 1:500 was used. The PBS was utilized as a technical negative control in all experiments.

RESULTS

Characteristics of EMA-1epitopes. B-cell epitopes of EMA-1 protein were predicted using different online software. For each program, the epitopes with the highest score were selected as proper epitopes. Finally, 750-bp sequences of EMA-1 gene (nucleotide 61-812 of open reading frame) containing four conserved B-cell epitopes with the highest immunogenicity score were selected for cloning and expression (Figure 1).

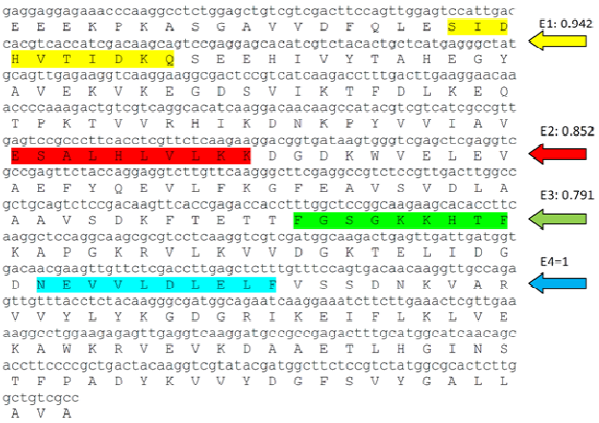

Figure 1. B cell epitopes (E1-E4) predicted in EMA-1 protein of Teileria equi using BepiPred, BCPred, and SVMTrip software (numbers show the immunogenecity score of each region analyzed by SVMTrip software. The sequence was obtained from infected horses from Khuzestan province, Iran, sequenced and submitted to GenBank under Gene ID No: 2030335.)

Polymerase chain reaction amplification, cloning, and nucleotide sequence analysis. After transforming the recombinant pET-26b plasmid containing the gene (EMA-1) to *E. coli*, direct colony PCR was applied for the accuracy of transformation. As shown in Figure 2, a 1100-bp band was observed on 1% electrophoresis gel corresponding to 750-bp EMA-1 gene and 350-bp flanking regions of plasmid. This confirms the accuracy of recombinant plasmid transformation in the bacteria.

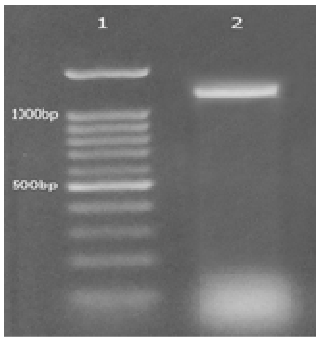

Figure 2. Polymerase chain reaction (PCR) product analysis of pET26b vector containing EMA-1 gene on 1% electrophoresis gel; lane 1) 100 bp DNA Ladder, lane 2) 1100 bp PCR product on pET26b-EMA-1 recombinant vector

As illustrated in Figure 3, the EMA-1 gene is broken in both ends by restriction enzymes, and the 750-bp EMA-1 gene was separated from the vector, which suggests the existence of EMA-1 gene in expressive pET26b vector. The sequencing of recombinant plasmids was also performed with universal primers to confirm the accuracy of EMA-1 sequence after amplification and cloning. The obtained sequence was submitted to GenBank (ID No: 2030335) and analyzed, using nBLAST online tool.

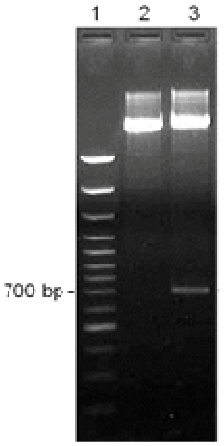

Figure 3. Analysis of the enzyme digestion of recombinant pET26b-EMA-1 vector; lane 1) 100 bp DNA Ladder, lane 2) recombinant plasmid before digestion, lane 3) 750-bp *EMA-1* gene separated from recombinant pET26b-EMA-1 digested using BamHI and HindIII enzymes

Based on the findings, cloned EMA-1 gene sequence had 94% homology to EMA-1 sequences submitted to GenBank from different sources (Accession No: XM_004829445.1 and CP001669.1) (Figure 1).

Expression of EMA-1 recombinant protein. As indicated in Figure 4A, EMA-1 protein band was placed at around 28 kDa, whereas no band was detected in the control samples. Furthermore, a 28-kDa protein band was well-separated from the NI-NTA column (Figure 4A). Purified EMA-1 protein could react with Anti Histag antibody (Figure 4B). These findings confirmed the expression of the cloned EMA-1 gene.

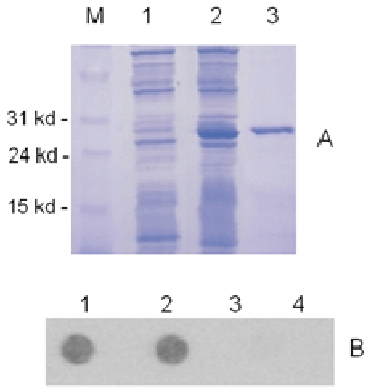

Figure 4. Characterization of EMA-1 protein after expression in *E. coli* and purification using NI-NTA column; A) sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis showing the expression of EMA-1 protein with a MW about 30 Kd (M: Protein ladder), 1A) crude extract of *E. coli* with pET26b, 2A) crude extract of *E. coli* containing pET26b-EMA-1 vector, 3A) purified 30 kD EMA-1 protein from the cellular extract of *E. coli* using the Ni-NTA column, B) positive reaction of purified polyhistidine tag EMA-1 antigen using dot blot analysis, 1B) cell lysate of *E. coli* containing pET26/EMA-1 recombinant vector, 2B) purified EMA-1 antigen after purification using NI-NTA column, 3B) cell lysate of *E. coli* containing pET26 vector, 4B) PBS as negative control.

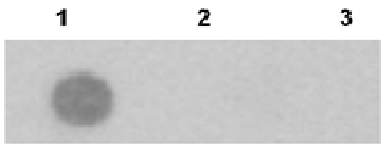

Figure 5. Result of dot blot analysis (purified recombinant EMA-1 protein react with the serum of horses infected with *T. equi* [1], while it showed no reaction with the serum of the healthy horses [2] and negative control [PBS] [3]).

Immunoreactivity of recombinant EMA-1 protein. The results of the Dot blot analysis revealed that the horse serum, containing antibody against *T.equi*, could detect the purified recombinant protein and react with it. However, in the negative horse serum and control samples, no reactivity was detected (Figure 5).

DISCUSSION

In this study, EMA-1gene of *T. equi* obtained from the horse of endemic areas of Khuzestan province, South of Iran, was sequenced, characterized, and produced as a recombinant form. The researchers determined the nucleotide sequence of the EMA-1gene from *T. equi* Khuzestan strain and compared it with other published sequences available in GenBank. The results indicated that even in the strains already isolated worldwide, there were sequence differences reflected in the derived amino acid sequence. In addition, the immunogenic regions of EMA-1 protein based on the Iranian strain was produced as polyhistidine tagged antigen and tested for the detection of antibody to *T. equi*. To the best of our knowledge, this is the first analysis reporting the immunogenic regions of EMA-1 and production of recombinant EMA-1 from *T. equi* Iranian isolates. Equine piroplasmosis is considered as an endemic disease in Asia, which has been transmitted all over the tropical and subtropical parts of the world (Friedhoff et al., 1990). Accurate diagnosis of *T. equi* plays a key role in the control, prevention, treatment, and presumably eradication of equine piroplasmosis. Traditional diagnostic methods have some limitations that negatively affect the results. Therefore, researchers have recently paid more attention to utilize the recombinant antigens in order to increase the sensitivity and specificity of methods to detect the different stages of the disease. Equine merozoite antigens, including EMA-1, EMA-2, and EMA-3, are immunodominant proteins, which play pivotal roles in pathogenicity and the survival of equine piroplasms. These proteins are targeted by the host immune responses during the host-parasite interaction, indicating their importance to be applied as subunit vaccines and diagnostic reagents

(Knowles et al., 1997). A highly conserved surface protein, namely EMA-1, expressed by the merozoites of *T. equi,* has been utilized as a diagnostic antigen (Xuan et al., 2001a; M. et al., 2014). This protein is not expressed in all merozoite developmental stages. There are differences among the EMA-1 sequences (about 10%) and antigenicity of *T. equi* strains isolated from various countries (Heuchert et al., 1999; Nicolaiewsky et al., 2001). As a result, the production of recombinant antigen from the infected horses is necessary for the development of more specific and sensitive immunoassay detection method. In the present study, the researchers expressed the *EMA-1* gene of *T. equi* exploited from the blood of horses living in one of the most important epizootic areas of *T. equi* infection in Asia using a recombinant pET26b plasmid. *E. coli* is widely utilized for recombinant protein production systems. The main advantages of *E. coli* expression system include cost-effectiveness, simplicity, high amount of purified expressed protein, simple plasmid construction, and easy cultivation (Huang et al., 2003). Furthermore, the powerful promoter of pET26b vector allows the system to over-express the cloned gene**.** Several diagnostic tests have been developed to detect *T. equi* using recombinant *EMA-1* in *E. coli* and other expression systems. Baldani et al. (2011) sub-cloned and expressed *EMA-1* gene from *T. equi* into pET28a plasmid and E*. coli*, respectively. They accomplished an ELISA test using the recombinant protein to detect *T. equi* in horses from Brazil and demosntrated that the test was highly sensitive and specific to differentiate between *T. equi* infection and the other infections. They finally reported that the recombinant *EMA-1* expressed in *E. coli* can be a powerful diagnostic tool for the detection of antibodies against *T. equi*. In another study, Xuan et al. (2001b) showed the high titer of antibody against *T. equi* in mice inoculated with recombinant *EMA-1*. In some studies, serodiagnostic methods with the recombinant antigen were conducted for the diagnosis of *T. equi* infection in horses. Accordingly, the use of recombinant *EMA-1* was reported to increase the sensitivity and specificity of the method. In a study carried out by Nizoli et al. (2009), *EMA-1* was successfully cloned and expressed in *Pichia pastoris* using pPICZαB vector. They also reported a high immune response in mice vaccinated by recombinant *EMA-1,* suggesting that recombinant *EMA-1* might be a promising antigen candidate for the development of future immunodiagnosis and vaccine studies. To the best of our knowledge, the present study is the first attempt regarding the development of a recombinant pET26b-expressed immunogenic region of *EMA-1* in Iran. In our study, the reaction of recombinant *EMA-1* with *T. equi*-infected horse sera was confirmed by dot blot analysis, indicating the ability of the protein to react with specific anti-*T. equi* antibody. This finding is in line with the data presented by Huang et al. (2003) and Kumar et al. (2004). Dot blot technique is a common immunological method used in research and analytical/diagnostic laboratories as well. It has been successfully applied for the diagnosis of many infectious and parasitic diseases, such as toxoplasmosis and visceral leishmaniasis, to present a faster response (Kamikawa and Vicentini, 2015). Although this research was carefully prepared, it had one limitation. The research was conducted for testing the reactivity of EMA-1 antigen against the serum obtained from the horses infected with *T. equi*. However, this is not enough for the development of a reliable immunologic test, such as ELISA, in future. The present study only involved the investigation of EMA-1 antigen reactivity against *T. equi* antibody for the determination of the purity and functionality of purified EMA-1 harvested from the Iranian isolate. The reactivity of purified EMA-1 should be determined against other related parasites, such as *Babesia caballi,* before the clinical application of purified EMA-1 for the diagnosis of *T.equi* infection in horses in the future. Finally, it was concluded that the recombinant *EMA-1* can be proposed as a reliable tool to be used in diagnostic tests and vaccine development for *T. equi* in the future. Additionally, pET26b was shown as a

suitable vector to express *EMA-1*. Further studies are recommended to use *EMA-1* in order to develop a more sensitive and specific diagnostic method, new vaccines, and new drug targets. In conclusion, the production of EMA-1 protein could be a preliminary step for further research in designing a sophisticated diagnostic kit or an effective vaccine against equine theileriosis in Iran.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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