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Expressão heteróloga e utilização da proteína recombinante EMA-1 de *Theileria equi* como imunobiológico

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RESUMO

NIZOLI, Leandro Quintana. **Expressão heteróloga e utilização da proteína recombinante EMA-1 de *Theileria equi* como imunobiológico***.* 2009. 67f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A Theileriose eqüina é considerada uma das principais doenças parasitárias que acometem os eqüinos, acarretando grande impacto econômico na equinocultura. A doença é causada pelo hematozoário *Theileria equi*. As perdas econômicas associadas à theileriose eqüina estão relacionadas tanto aos fatores clínicos, quanto à restrição ao trânsito internacional de animais soropositivos, já que animais portadores crônicos são passíveis de reagudizações, gerando perda de performance física e reprodutiva, e são potencialmente disseminadores da enfermidade. Nos últimos anos, os estudos sobre o diagnóstico imunológico e vacinação contra *T. equi* concentram-se na obtenção de frações antigênicas. Na membrana externa deste protozoário foram caracterizadas proteínas principais de superfície denominadas de EMAs (*equi merozoite antigen*). Dentre estas, a EMA-1 destaca-se como antígeno para diagnóstico em função de sua conservação entre diversos isolados. Seu papel também tem sido caracterizado como imunógeno por estimular forte resposta humoral com produção de anticorpos em animais infectados, podendo ser usado como ferramenta para imunodiagnóstico dessa doença. EMA-1 é também um potencial candidato como antígeno vacinal no controle da theileriose equina. Neste estudo utilizou-se o sistema eucariótico de expressão baseado na levedura metilotrófica *Pichia pastoris*, para a produção da proteína EMA-1 de *T. equi* e a avaliação quanto a sua antigenicidade e imunogenicidade. Quanto a sua antigenicidade, a proteína recombinante foi reconhecida por anticorpos de animais portadores crônicos de *T. equi*, sugerindo que epítopos nativos foram conservados na proteína recombinante. Também foi observado que a proteína recombinante foi capaz de gerar resposta imune em camundongos vacinados com esta proteína. Os dados obtidos neste estudo demonstram que a levedura *P. pastoris* é um sistema de expressão heterólogo adequado para a produção da proteína EMA-1 de *T. equi*, podendo ser utilizada como imunobiológico no desenvolvimento de testes diagnósticos e vacina recombinante.

Palavras-chave: *Theileria equi*, equinos, *Pichia pastoris*, EMA-1, proteína recombinante.

ABSTRACT

NIZOLI, Leandro Quintana. **Expressão heteróloga e utilização da proteína recombinante EMA-1 de *Theileria equi* como imunobiológico***.* 2009. 67f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Equine theileriosis is considered to be one of the most important parasitic diseases that affect horses, and has great economic impact on the equine industry. The disease is caused by the etiologic agent *Theileria equi*, which is classified as a hematozoan. The losses associated with equine theileriosis are related to clinical manifestation as well as restriction to international travel to positive horses. Chronic infected equines suffer the risk of the disease relapse which leads to losses in reproduction performance and are potentially disseminators of the disease. In the last years, studies on the immunologic diagnosis and vaccination against *T. equi* have focused to obtain distinct antigenic proteins. On the outer membrane of this protozoan, major surface proteins has been characterized and named as EMAs (*equi merozoite antigen*). Of these, EMA-1 has been used as antigen for diagnosis due to its conservation in diverse isolates. Its role as a potential immunogen has been well documented due its ability to stimulate a humoral response with production of specific antibodies in infected animals. Through this antibodies one can used as tool for immune diagnostic of this disease. EMA-1 is also a strong candidate to be use as a vaccine in the control of equine theileriosis. In this study we used the *Pichia pastoris* yeast as expression system for the production of the EMA-1 protein of *T. equi* and evaluated its antigenicity and immunogenicity. When tested for antigenicity, the recombinant protein was recognized by antibodies form chronic *T. equi* infected horses, suggesting that epitopes of the native were conserved in the recombinant protein. Also we were able to observe that this protein was immunogenic in mice. The data obtained in this study demonstrated that the yeast *P. pastoris* is an expression system of heterologous protein suitable for the production of EMA-1 from *T. equi*.

Keywords: *Theileria equi*, equines, *Pichia pastoris*, EMA-1, recombinant protein.

SUMÁRIO

1 INTRODUÇÃO GERAL

A primeira descrição do parasito foi feita por Guglielmi em 1899 na África do Sul, sendo posteriormente classificado e descrito como *Babesia equi* por Laveran em 1901. No entanto, descobertas a respeito do ciclo de vida do parasito, como multiplicação em linfócitos e ausência de transmissão transovariana nos carrapatos vetores (SCHEIN et al., 1981; SCHEIN, 1988; UETI et al., 2008), indicam que *T. equi* não é uma babésia clássica e, desde então, sua taxonomia tem sido questionada. Considerando similaridades do parasito com organismos da família *Theileriidae*, a reclassificação de *Babesia equi* como *Theileria equi* passou a ser aceita (MEHLHORN & SCHEIN, 1998). Entretanto, estudos filogenéticos, utilizando RNA ribossomal e proteínas de superfície de parasitos de ambas as famílias, indicam que *T. equi* pode representar um terceiro grupo, diferente de *Babesia* ou *Theileria* (ALLSOPP et al., 1994; KATZER et al., 1998; ZAHLER et al., 2000_a; ZAHLER et al., 2000_b; CRIADO-FORNELIO et al., $2003_{a,b}$)

A theileriose equina é uma importante doença parasitária que acomete os equinos de forma endêmica no território brasileiro, assim como, em diversos outros países (KERBER at al., 1999; PFEIFER et al., 1995). Esta doença vem sendo estudada à dezena de anos, principalmente em função do elevado número de distúrbios que pode acarretar aos animais acometidos, bem como as enormes perdas econômicas na equideocultura mundial (FRIEDHOFF et al., 1990; KNOWLES, 1996). Nos Estados Unidos, Canadá, Austrália e Japão, assim como, em alguns países da Europa e América Latina, onde o parasito não ocorre de forma endêmica, são mantidas rigorosas medidas de controle que impedem a entrada de animais soropositivos (OIE, 2008). Nestes países, apesar da doença ser considerada exótica, o risco de tornar-se endêmica é constante devido à existência dos carrapatos vetores, pertencentes aos gêneros *Amblyomma, Dermacentor* e *Rhipicephalus* (KERBER et al., 1999; GUIMARÃES et al., $1998_{a,b}$; BATTSETSEG et al., 2002; STILLER et al., 2002; UETI et al., 2008). Portanto, medidas de controle, além dos testes sorológicos, muitas vezes incluem quarentena e controle de

carrapatos (MARTIN, 1999). Estes procedimentos, além de extremamente dispendiosos, afetam negativamente o mercado internacional de eqüinos e a participação em competições eqüestres internacionais.

O parasito encontra-se mundialmente distribuído em regiões tropicais e subtropicais, sendo a prevalência da infecção diretamente relacionada com a ocorrência dos carrapatos vetores. É estimado que 90% da população mundial de eqüinos esteja exposta à *T. equi,* ainda que em alguns países a infecção não ocorra de forma endêmica (FRIEDHOFF et al., 1990). No Brasil, estudos epidemiológicos utilizando imunofluorescência indireta para detecção de anticorpos anti *T. equi* têm registrado prevalências de 49,2% em São Paulo (HEUCHERT et al., 1999), 57,9% no Rio Grande do Sul (CUNHA et al., 1996), 60,45% em Minas Gerais (RIBEIRO et al., 1999) e 72% a 100% no Rio de Janeiro (TENTER & FRIEDHOFF, 1986; PFEIFER et al., 1995).

Infecções por *T. equi* caracterizam-se pelo desenvolvimento de anemia hemolítica progressiva nos animais infectados, sendo a patogenia da enfermidade determinada principalmente pela lise de eritrócitos durante a invasão e multiplicação do parasito (KNOWLES, Jr. et al., 1994; LORDING, 2008). Quando eqüinos suscetíveis são infectados desenvolvem a fase aguda da doença, a qual cursa com febre, anemia, hemorragias petequiais de mucosas, hemoglobinúria e icterícia (ZOBBA et al., 2008). A gravidade da doença neste estágio depende da virulência da cepa, dose do inóculo e condição imunológica do animal (CUNHA et al., 1998; AMBAWAT et al., 1999). A mortalidade em infecções por *T. equi* é baixa, em geral os animais recuperam-se da fase aguda da doença, porém permanecem como portadores do parasito (UETI et al., 2005). Durante a fase crônica da infecção, sinais clínicos inespecíficos como inapetência, perda de peso e queda no desempenho físico e reprodutivo são comuns (SCHEIN, 1988). Em casos de imunossupressão a reagudização da doença é favorecida e os animais podem apresentar diferentes graus de anemia, com agravamento dos sinais clínicos (OLADOSU, 1988; OLADOSU & OLUFEMI, 1992; CUNHA et al., 1997; NOGUEIRA et al., 2005).

Animais infectados por *T. equi* desenvolvem uma sólida imunidade que protege contra a doença clínica no caso de re-exposições ao parasito (CUNHA et al., 2006). Esta proteção tem sido atribuída à contínua estimulação do sistema imune por parasitos que persistem no organismo durante a fase crônica da enfermidade (SCHEIN, 1988). Experimentos conduzidos em diferentes modelos de infecções com

patógenos intraeritrocitários, que possuem uma patogenia semelhante à de *T. equi*, propõe que através da produção de interferon gama (IFN-γ), linfócitos T $CD4^+$ podem ativar macrófagos e estimular a produção de anticorpos por linfócitos B, mecanismos estes que atuariam na eliminação dos parasitos (IGARASHI et al., 1999; PALMER et al., 1999; HELMBY & TROYE-BLOMBERG, 2000). Macrófagos ativados não somente apresentam uma intensa capacidade fagocitária como produzem uma série de intermediários reativos de oxigênio e nitrogênio que são tóxicos para parasitos (SU & STEVENSON, 2000; SHODA et al., 2000). Anticorpos por sua vez também atuam em mecanismos citotóxicos, como opsonização e fixação de complemento, importantes no controle da multiplicação dos parasitos (AUCAN et al., 2000; CHEN et al., 2000_a; CHEN et al., 2000_b; TAYLOR et al., 2001). A participação de anticorpos na neutralização de merozoítos, impedindo diretamente a invasão de eritrócitos também tem sido demonstrada (CAVINATO et al., 2001).

Eqüinos infectados com *T. equi* desenvolvem altos títulos de anticorpos contra proteínas de superfície de merozoítos, o que sugere que os mesmos estão envolvidos no controle da multiplicação e eliminação do parasito. Assim sendo, tanto mecanismos celulares como aqueles dependentes de anticorpos parecem desempenhar papéis fundamentais no controle de hematozoários (KNOWLES et al., 1994; CUNHA et al. 2006). Em *T. equi*, a proteína *equi merozoite antigen–1* (EMA-1) é um antígeno de 34 kDa expresso na superfície do merozoíto, predominantemente reconhecido por anticorpos de eqüinos infectados por *T. equi* (KNOWLES et al., 1997). Comparações entre seqüências de aminoácidos da EMA-1, obtidas de diferentes isolados de *T. equi*, têm revelado identidades superiores a 80% (NICOLAIEWSKY et al., 2001; XUAN et al., 2001).

O desenvolvimento de vacinas representa um campo de grande interesse na pesquisa envolvendo hemoparasitos de importância veterinária. Os principais obstáculos nesta área são o desconhecimento a respeito da imunidade protetora desenvolvida pelo hospedeiro e a grande variedade de mecanismos de evasão do sistema imune utilizados pelos parasitos (JENKINS, 2001). Vários estudos têm sido realizados buscando elucidar estes aspectos e um modelo de como o hospedeiro mantém hemoparasitos sob controle e evita surtos clínicos em subseqüentes exposições ao agente tem sido proposto (BROWN & PALMER, 1999; BROWN, 2001).

Estudos desenvolvimentos no campo da biologia molecular tornaram possível expressar genes de antígenos de diferentes patógenos em sistemas heterólogos. *Pichia pastoris* é uma levedura metilotrófica, capaz de utilizar o metanol como fonte de carbono e energia que pode ser geneticamente modificada para expressar proteínas heterólogas (CEREGHINO L.G.P. & CREGG, 1999; CEREGHINO L.J. & CREGG, 2000). Durante os últimos anos, ela tem se tornado um importante sistema de produção de uma variedade de proteínas heterólogas (NIZOLI et al., 2007; DUMMER et al., 2007). A produção destas proteínas recombinantes possibilita determinar os antígenos que representam os principais alvos da resposta imune dos hospedeiros vertebrados. A partir da identificação destes antígenos, é possível testar, por imunização ativa, o potencial imunoprotetor de cada uma destas proteínas. Estes estudos tornam os antígenos protetores, ou seus epítopos mais importantes, fortes candidatos para serem utilizados como constituintes de vacinas recombinantes contra o respectivo patógeno.

Assim, os grandes desafios nas pesquisas envolvendo *T. equi* concentram-se principalmente no desenvolvimento de vacinas e no diagnóstico do parasito, especialmente na detecção de portadores assintomáticos.

2 OBJETIVOS

Objetivo Geral

Produzir a proteína EMA-1 de *Theileria equi* recombinante em *Pichia pastoris.*

Objetivos Específicos

- Clonar o gene EMA-1 em sistema *Pichia pastoris*;
- Expressar a proteína EMA-1 de *Theileria equi* em levedura *Pichia pastoris;*
- Avaliar a potencial antigênico e imunogênico da proteína EMA-1.

3 ARTIGO 1

EQUINE THEILERIOSIS: EPIDEMIOLOGICAL ASPECTS, DIAGNOSTIC AND TREATMENT

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EQUINE THEILERIOSIS: EPIDEMIOLOGICAL ASPECTS, DIAGNOSTIC AND TREATMENT

EQUINE THEILERIOSIS

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ABSTRACT

Equine theileriosis is a tick-borne protozoal disease of horses, mules, donkeys and zebras. The etiological agent is blood parasite named *Theileria equi*. Infected animals may remain carriers of these parasites for long periods and act as sources of infection for ticks, which act as vectors. The parasites are found inside the erythrocytes of the infected animals. Animals which are low-level carriers of *T. equi* parasite or ticks which may act as reservoirs pose a risk of introduction of these parasites to diseases-free areas as a result of the increased movement of horses worldwide. In this review, the biology, distribution, pathogenicity as well diagnosis and treatment of equine theileriosis are discussed.

Key words: equine theileriosis, *Theileria equi*, diagnostic, treatment

INTRODUCTION

Equine theileriosis, caused by *Theileria equi* (formerly *Babesia equi*) (1), is considered to be the most important tick-borne disease of horse in tropical and subtropical areas including Central and South America, Africa, Asia and Southern Europe (2). At present, only Australia, Canada, New Zealand, Japan, UK, and USA are considered equine piroplasmosis-free and have in place stringent regulatory important restrictions to prevent introduction of these parasites (3).

T. equi are intraerythrocytic protozoa of equids that are transmitted by ixodid ticks (4). Ticks belonging to the genera *Hyalomma*, *Dermacentor*, and *Rhipicephalus* transmit these parasites (4-7). Recent work has shown the potential for transmission of *T. equi* by *Rhipicephalus* (*Boophilus*) *microplus* (8-11).

T. equi is one of the small species of protozoa and has piroplasms in the erythrocytes that appear oval, circle, ameboid, or as double pears, and measure 2 to 3 μm in length. *Theileria spp.* has a schizogony period in the lymphoblastoid cells different from the genera *Babesia*. (12-14).

Of the estimated world population of 58 million horses, 90% are bred in endemic areas (2). In endemic countries, the control of equine piroplasmosis is important to keep international markets open to the horse industry (15). For this reason many countries prohibit the importation of horses because of the high prevalence of asymptomatic carrier animals in the region.

Sub-clinical infections have particular relevance to the horse-racing industry where the geographical movement of presumably healthy horses may aid in the spread of *T. equi* or where sub-clinical infection may negatively affect the animal's performance. It has also been shown that strenuous exercise, such as that experienced with horse-racing, can cause sub-

clinical infections to become acute (16,17). Thus there is a real need for the diagnosis of both clinical and sub-clinical infections. In this review, the biology, distribution, pathogenicity as well diagnostic and treatment of equine theileriosis are discussed.

EPIDEMIOLOGICAL ASPECTS

Thoroughbred racing industry is particularly strong affected by equine theileriosis, acute infections resulting in missed training sessions and races and hence serious loss of income to owners and trainers. Income is also lost to owners through abortion in stud mares which are *T. equi* carriers (2,18).

Authors have demonstrated occurrences varying from 49.2% to 100% in the southern and south-eastern states of Brazil. They cited different epidemiologic conditions but with high tick infections (19-24).

The effects of the variation of prevalence has been observed with different categories and breeding systems in different regions of Brazil (21,25). Previous studies have shown that the prevalence of equine theileriosis in Brazil is serious (26-28). Transmission of theileriosis is usually influenced by the dynamics of vector populations, and these are directly influenced by climatic conditions (29).

Animals sensitive to the disease die within 24 to 48 hours after the development of first clinical signs (30). In chronic cases, the disease continues for months and these animals deteriorate to a worse condition within 3 to 4 years. The protozoa may not be seen microscopically during the subclinical phase of the disease; therefore, serologic tests need to be done to diagnose the disease (31-33).

Clinical signs of infection vary from asymptomatic to acute, fever, jaundice, anaemia, icterus, and even death may occur (30). Intra-uterine infections with *T. equi* may result in

abortion and neonatal death (30,34). Horses that recover from acute infection may act as reservoirs for transmission to ticks which subsequently infect other equids. Various authors postulate that *T. equi* persists for life-long infection in its equine host (2,18,35,36).

In *T. equi* infection, clinical parasitaemia may exceed 20% but 1-5% parasitaemia is more commonly observed in field conditions (37). In latent carrier equids, it is very difficult to demonstrate the parasite in stained blood smears because the parasitaemia is extremely low. Equids reared in endemic areas usually get infected at early age and become immune tolerant throughout their life span. Nevertheless, outbreak of *T. equi* infection may occur in these equids consequent to unfavorable health conditions (16).

Foals born of pre immune mares are naive at birth and acquire passive immunity through colostrum (38). Maternal antibodies, against *T. equi*, decline steadily to extinction by about four months from birth (38); however in endemic areas it is expected that primary infection takes place before they decrease. It has been demonstrated that under these conditions foals acquire the infection shortly after birth with the majority showing patent parasitemia before 42 days of age (39). Regarding *T. equi*, transplacental transmission can occurs and takes place during the first four months of gestation and appears to be a rule rather than an exception, as recently demonstrated by a DNA probe (40). Recently was demonstrated the presence of DNA of *T. equi* parasite in neonate foal's blood born from a latently infected mare, using specific DNA probe (41). They opined that parasite transmission occurred during pregnancy despite the fact that latently infected mare was having normal placenta as the foaling and foals were normal. Passage of infected erythrocytes or extra cellular parasitic form across the placental barrier is the probable mode of transmission of *T. equi* and this may occur as a result of damage to placental vessel in the event of abortion which could have lead to mixing of maternal and foetal blood (42).

Kumar et al. (43) indicated that new-born foals were born naive and passively transferred immunity is transitory which wanes after a period of time rendering the foals susceptible to natural *T. equi* infection, after 63-77 days after foaling. This will help in reducing the losses due to disease condition in new-born foals by following suitable management practices at farm after waning of antibody titers.

Positive titers for *T. equi* from the first day of life on can be explained by maternal antibodies, especially in those cases where the foals became negative thereafter. This occurred between the first and fourth month. Maternal antibodies for *T. equi* have been demonstrated before until about 4 months of age (in the CF test) in foals of field-infected mares (38).

Immunocompetent young horses infected with *T. equi* preferentially produce antibodies to erythrocyte-stage antigens of 30 and 34 kDa during resolution of acute infection (15,44). These antigens (equi merozoite antigens-1 and -2) are erythrocyte-stage which possess a surface epitope that is conserved worldwide and induce specific high antibody levels (15, 45, 46). Knowles Jr.(1991) had shown that a protein epitope shared by EMA-1 and EMA-2 was immunodominant for antibody induction and shared by isolates worldwide (46).

To survive and replicate in the erythrocyte, members of the Apicomplexa phylum and the intra-erythrocytic parasite *Plasmodium falciparum* export proteins that interacts with and dramatically modify the properties of the host erythrocyte. As part of this process, *P. falciparum* appears to establish a system within the erythrocyte cytosol that allows the correct trafficking of parasite proteins to their final cellular destinations (47).

The pathways and components of these complex trafficking processes are fundamental to the survival of *P. falciparum in vivo*, and are major determinants of this parasite's unique pathogenicity (47). Recently has been showed that the parasite *T. equi* EMA-2 in the infected erythrocytic cytoplasm can be exported to the membrane and this can affect the parasite's erythrocytic binding behavior (48).

DIAGNOSTIC TESTS

Several direct and indirect detection methods, including blood smears (49), in-vitro cultures (50,51), DNA probes (13) and serology (52), have been used to diagnose *T. equi* infection.

Microscopic detection from blood smears has been used for the most standard diagnosis of equine theileriosis (53); however, it has many limitations. The technique is relatively laborious when large numbers of blood smear samples need to be simultaneously quantified. While the identification of parasites in blood smears constitutes the definitive diagnosis of equine infection, it bears certain limitations, particularly during apparent or chronic infection due to low parasitemias (54).

Among the molecular tools, PCR is the most commonly used, including other PCR-derived tools, such as the multiplex and nested PCR (13,55). However, these molecular tools require thermo cyclers and several other expensive operations, which make them unsuitable for routine diagnosis, especially in resource-poor countries (56).

A primary PCR has been shown to detect *T. equi* infection in horses however, in all cases the parasitemia level was sufficiently high for the organism to be detected by microscopic examination (30). More recently, a nested PCR for *T. equi* based on the merozoite antigen *ema*-1 gene sequence has been reported to give increased sensitivity (13).

A variety of serological tests such as indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and complement fixation test (CFT) have been used to detect specific antibodies. Both the complement fixation test and the immunofluorescent antibody test have been used for many years for diagnosis (57).

These methods have a low sensitivity for detecting latent infections. However, it has been reported that because of its low sensitivity and specificity, the complement fixation test fails to discriminate accurately between negative and carrier animals (19).

Compared with ELISA, however, IFAT is time consuming and requires large amounts of parasites. Moreover, the estimation of the intensity of fluorescence is subjective and requires the participation of experts, which has hindered the standardization and comparability of the results (58).

Recently, the competitive ELISA (cELISA) using recombinant antigens was developed as a more specific method than CFT, IFAT and other iELISA for the serodiagnosis of piroplasmosis (32,47,59-64). Thus, several serological assays such as ELISAs are often more sensitive and specific have been developed to advance diagnosis of equine piroplasmosis.

A competitive inhibition ELISA, employing a monoclonal antibody and a recombinant *T. equi* merozoite protein (EMA-1), was shown to be specie-specific for anti-*T. equi* antibodies, as horses infected with *B. caballi* tested negative (46). It has been shown that an ELISA using recombinant EMA-1 expressed in insect cells by baculovirus (65) and *Pichia pastoris* (66) can be a useful diagnostic reagent for detection of *T. equi* infection in horses, being more sensitive than CFT and IFAT.

TREATMENT

Treatment of piroplasmosis varies depending on the location of the horse and the goal of treatment. In endemic regions, suppressing clinical signs without eliminating the organism from the body is desirable because premonition depends on the continued presence of the parasite at low levels (67).

The use of the dipropionate of imidocarb is recommended in solution 10%, 2.4.mg.kg^{1} four times, with break of 72 hours. However, the protocol of one dose of 1.2 mg.kg^{1} is efficient to *T. equi*, not presenting difference of the treated animals with 2.4 mg.kg^{1}, with the advantages of reduce costs and diminish risks of effects to the horses (17).

Babesiacidal/theilericidal drugs used in the treatment of equine theileriosis are limited and are either ineffective in completely eliminating the parasites and/or cause severe side effects (68,69). Thus, a continuous search for alternative and effective chemotherapeutic drugs is necessary.

The most reliable method to control equine piroplamosis remains preventing entry of infected equine and ensuring that animals entering from endemic countries are thoroughly checked and found to be free of ticks.

CONCLUSIONS

As a result, equine theileriosis is a very important equine disease. Serologic examination is better than microscopic examination to determine the prevalence of the disease. To combat the disease, the animal owners should be informed about the importance of the disease and the danger of tick infestation. Considering the high prevalence of *T. equi* infection in Brazil further studies are required to define the population of the infection. Control strategies for horses used for recreational riding should based on reducing their exposure to ticks by preventing them grazing areas with high tick infestation, preventing contact with high tick infestation, preventing contact with wild horses, spraying horses with acaricides and treating positive horses. In conclusion, the hardest challenges in the researches involving *T. equi* concentrate mainly in the development of treatment protocols and in the diagnosis of the parasite, especially in the detection of chronic cases of equine theileriosis.

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4 ARTIGO 2

CLONING AND EXPRESSION OF *MEROZOITE ANTIGEN* 1 OF *Theileria equi* GENE IN THE METHYLOTROPHIC YEAST *Pichia pastoris*

Artigo científico formatado de acordo com as normas da revista *Protein Expression and Purification*

CLONING AND EXPRESSION OF *MEROZOITE ANTIGEN* 1 OF *Theileria equi* GENE IN THE METHYLOTROPHIC YEAST *Pichia pastoris*

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ABSTRACT

The equine piroplasmosis caused by *Theileria equi* is a tick-borne protozoal infection of horses, causing damage to animal health and economic losses. In *T. equi*, two merozoite surface proteins, *equi merozoite antigen* EMA-1 and EMA-2, have been identified as the most immunodominant antigens, suggesting that they might be used as immunobiological tools. This study focused on expression and purification of truncated recombinant EMA-1 of *T. equi* in *P. pastoris*. The DNA encoding EMA-1 was cloned by PCR from strain Pelotas of *T. equi*. After PCR and construction of expression vector, EMA-1 was expressed in *P. pastoris* yeast X-33 and secreted into the culture medium. The secreted EMA-1 was purified using ammonium sulfate at 80%. The recombinant protein was imunogenic and antigenic. This study provides a new alternative for expression and utilization of recombinant protein EMA-1.

Key Words: *Theileria equi*; Recombinant vaccine; *Pichia pastoris*; EMA-1

Running headline: Truncated EMA-1 expressed in yeast *Pichia pastoris*

INTRODUCTION

The equine piroplasmosis caused by *Theileria equi* is a tick-borne protozoal infection of horses, it causes great damage to animal health and important economical losses. The disease is a haemoparasitosis caused by *Theileria equi* and *Babesia caballi*, is a tick-borne hemoprotozoan disease affecting horses worldwide [1]. Through infecting and destroying erythrocytes, it can compromise the equine function, leading to loss of vitality and decrease in the performance of infected animals. Equine piroplasmosis caused by *T. equi* is more pathogenic and widespread in horses than that by *B. caballi* [2], it causes a persistent infection for which drug therapy or vaccination are not available [1,3]. The *T. equi* infection is characterized by fever, anemia, icterus, and hepato and splenomegaly [4].

Merozoite surface proteins are important in the pathogenesis of hemoprotozoan diseases because of their role in parasite recognition, attachment to and penetration of host erythrocytes [5,6]. In *T. equi*, two kinds of merozoite surface proteins, *equi merozoite antigen* EMA-1 and EMA-2, have been identified as the most immunodominant antigens [7,8,9]. EMA-1 is geographically conserved among all *T. equi* isolates [8,10] and shares significantly high identity in amino acid sequence with the counterpart proteins of many *Theileria* parasites [7,11]. Additionally, EMA-1 has glycosyl-phosphatidylinositol (GPI) anchor-specific motifs in their sequence, suggesting that these proteins might be expressed on the outer surface of merozoite with a GPI anchor [11]. The *T. equi* merozoite express surface protein of molecular mass 34 kDa, which are strongly recognized by antibodies produced in infected animals [11,12]. Further, EMA-1/2 are members of the major piroplasm surface protein (MPSP) family that is conserved among the genus *Theileria*, but the biological role of MPSP has not yet been clarified. Therefore, functional studies on EMA family proteins may also provide

insight into the biological significance of MPSP expression in the intra-erythrocytic development of *Theileria* parasites.

The methylotrophic yeast *Pichia pastoris* is one of the dominant expression systems in molecular biology due to its stable and high-level expression of heterologous proteins [13,14]. Over 400 proteins from prokaryotes, eukaryotes, and viruses have now been successfully expressed in this yeast [15]. The *P. pastoris* expression system offers many advantages, including its ease of usage relative to other eukaryotic expression systems, the possibility of high-level expression of foreign proteins. The yeast is also able to introduce eukaryotic post-translational modifications such as glycosylation and proteolytic processing [16].

In this study, we report the successful cloning of the truncated DNA sequence of *T. equi* gene. Its expression in *P. pastoris* and characterization of the recombinant protein were also investigated. This is the first report of the cloning and expression of EMA-1 of *T. equi* in *P. pastoris* system.

MATERIALS AND METHODS

Strains, plasmids and yeast culture media

Escherichia coli TOP10 cells were used as a host for plasmid propagation. For yeast transformation, the *P. pastoris* transfer plasmid pPICZαB containing the 5' alcohol oxidase 1 (AOX1) promoter and the 3' AOX1 transcription termination sequences was used. pPICZαB also contains the dominant selectable marker antibiotic zeocin, which is bifunctional in both *Pichia* and *E. coli*. *Pichia pastoris* host strain X-33 was used for protein expression experiments. These products were purchased from Invitrogen (Carlsbad, CA).

Pichia pastoris cells were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% D-glucose) or BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen

base, $4x10^{-5}$% biotin, 1% glycerol and 100 mM potassium phosphate [pH 6.0]). YPDS plates (YPD medium plus 1 M sorbitol and 2% agar (w/v)) containing 100 μg/ml zeocin (Invitrogen, San Diego, CA) were used for selection of *Pichia* transformants. BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4x10^{-5}$% biotin, 0.5% methanol, and 100 mM potassium phosphate [pH 6.0]) was used for protein induction.

DNA extraction and PCR amplification

The EMA-1 gene (GenBank accession no **AF261824**) without the native signal peptide sequence was amplified by PCR using DNA obtained as previously described [17]. Amplification reaction was performed in a thermocycler (Mastercycle Eppendorf). The PCR was subjected to amplification in a 25 μl mixture containing ~20 ng of genomic DNA, 0.2 μl Taq DNA polymerase (5U/μL), 2.5 μl 10x PCR buffer, 1.0 μL 50 mM $MgCl_2$, 0.5 μl 10 mM dNTP, 1.0 μl 10 pmol/μL primer 1 and primer 2 (Table 1) and ddH2O, under the following conditions: 94 °C for 5 min (1 cycle); 94 °C for 60 sec, 54 °C for 60 sec, 72 °C for 1 min (40 cycles); and 72 °C for 7 min (1 cycle). All primers used in this study were synthesized in MWG-Biotech AG (USA).

Construction of expression plasmid pPICZalpbaB-EMA1

The amplified PCR products after purification and cleavage with *EcoR*I and *Kpn*I were cloned into expression vector pPICZαB digested by the same restriction enzymes and the resultant plasmid was transformed to TOP10F competent cells. The transformants were cultured in LB plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) with zeocin (25 μg/ml), for screening the positive clones. The recombinant plasmids with EMA-1 truncated gene were identified with ultra-rapid microprep plasmid extraction [18], proper insert orientation was tested by restriction endonuclease cleavage, and confirmed by

sequencing with the DYEnamic ET terminators sequencing kit (GE Healthcare, Giles, United Kingdom) following the manufacturer's protocol. Sequence determination was performed in a MegaBACE 500 automatic sequencer (GE Healthcare). Sequencing reactions were performed using primers 5'AOX1 and 3'AOX1 vector-specific and primers used for PCR amplification (Table 1). Sequence analyses were assembled using Vector NTI 10, AlignX and ContigExpress (Invitrogen). Homologies analyses were performed with the NCBI database and BLAST [19].

Transformation and screening of* P. pastoris *expression strains

The plasmid pPICZαB-EMA1 was linearized by digestion with restriction enzyme *Pme*I for integration into the chromosomal DNA of *Pichia pastoris* X-33. Transformation of the linearized plasmid was carried out as described in the Invitrogen Pichia Expression kit manual. Approximately 250 colonies were obtained on the YPDS plates containing 100 µg/ml Zeocin after growing for 3 days.

For confirm integration of the EMA-1 gene into the yeast genome, the colonies were subsequently screened by colony blotting. The colony blotting assay was performed as previously described [20], with some modifications. Briefly, a hundred Zeocin recombinant colonies were plated onto BMMY agar medium (1% yeast extract, 2 % peptone, 1.34% yeast nitrogen base, $4x10^{-5}$% biotin, 0,5% methanol, 100 mM potassium phosphate [pH 6.0] and 1% agar) and incubated at 28°C for 3 days. Every 24 h, 1% of total medium volume of absolute methanol was added on the top of plates. Pre-cut nitrocellulose membrane (*Hybond* C, Amersham Biosciences) was then left standing for 3 h at 28°C on the surface colonies. Membrane was then washed with PBS (pH 7.4) plus 0.05% Tween-20 (PBS-T) prior to remove adhering colonies fragments. Membrane was blocked for 1 hour at room temperature with gentle agitation in PBS-T buffer containing 5% of non-fat milk. After, the membrane

was incubated for 1 hour with monoclonal antibody (MAb) anti-His peroxidase conjugate. The reaction was developed with diaminobenzidine (Sigma).

Expression of rEMA-1 on shaker and bioreactor cultures

The selected positive clones were used to inoculate 5 ml BMGY medium and incubated overnight at 30° C with shaking at 250 rpm. A 0.3 ml aliquot of the culture was used to determine cell density and viability. The remainder was harvested by centrifugation at 5,000 x *g* for 5 min. To induce protein expression, the cells were resuspended in BMMY. A total of 10 ml cell suspension in BMMY was incubated at 30 °C with shaking at 250 rpm for 7 days. The cultures were supplemented with 100% methanol to a final concentration of 1%, and this step was repeated every 24h. After methanol induction for various periods of time, aliquots were withdrawn for cell viability assay and expression analysis. Recombinant EMA-1 was detected by Dot-Blotting procedure using MAb Anti-6xHIS HRP conjugated. One transformant with high expression level in the culture supernatant was used for large-scale expression. The transformed strain with the highest activity screened in the shake flask expression was cultivated in bioreactor (*Biostat B,* B. Braun Biotech). For large-scale expression, recombinant X-33 was pre-inoculated in YPD medium for 24h at 28°C in agitation of 250 rpm and then cells were placed in reactor containing BMGY medium. Temperature was controlled at 30ºC and agitation was set at 300 rpm and aeration rate of 1 vvm. After complete consumption of glycerol in the medium, 1.0% of methanol was added every 24 h to induce expression during 4 days. Recombinant EMA-1 expression was detected by Dot-Blotting as mentioned before. After that, cells were harvested in 5,000 x *g* at 4ºC for 10 min and supernatant stored at 4°C until purification.

Precipitation of protein

For precipitation, the culture (1 L) was first centrifuged at 5,000 x *g* for 30 min at 4°C, and the resultant supernatant containing the secreted EMA-1 was pooled and mixed with ammonium sulfate to a saturation of 80% and incubation at 4°C for 16 hours. The mixture was centrifuged at 12,000 x *g* for 30 min, and the resultant pellet was resuspended in PBS (pH 7.0), followed by overnight dialysis against the same buffer at 4° C using a membrane of 30 kDa cut-off. After dialysis, the samples were centrifuged to remove any insoluble materials; the resultant supernatant was pooled and stored at -20°C. The protein quantitation was determined using the BCA^{TM} Protein Assay Kit (Pierce Chemical Company) with bovine serum albumin (BSA) as a standard.

SDS-PAGE and Dot-Blotting

Purified proteins were boiled for 10 min in SDS-PAGE loading buffer and separated on 10% separating gel and then submitted to electrophoresis in Bio-Rad Mini-PROTEAN Tetra Electrophoresis System. Gel was stained with Coomassie Brilliant Blue R250. For Dot-Blotting analysis was performed as described elsewhere [21], with some modifications. Supernatant of yeast X-33/pPICZαB was used as negative control. Proteins adsorption was carried out by spotting 5 µl in nitrocellulose membrane pieces (2.0 x 1.0 cm). After blocking and washed, membranes were probed also as described and detected with DAB solution. Reactions were stopped with distilled water washes. Membrane was blocked with 5% non-fat dry milk in PBS-T pH 7.4 for 1h and after several washes with PBS-T, antigenic proteins spots were detected by incubating membrane with the following sera: MAb Anti-6xHIS HRP conjugated (SIGMA); anti-EMA-1 monoclonal antibody (*Babesia equi* Antibody. Test Kit, cELISA, VMRD. Inc, Pullman**)**; sera of horse naturally infected with *T. equi* or sera of negative horse, also for 1 h and then washed with PBS-T. Membranes were incubated for 1 h

with anti-mouse (1:2.000) and anti-horse (1:10.000) immunoglobulins HRP conjugated. After that, membranes were washed again and then placed in DAB solution (0,6 mg Diaminobenzidine, 0,03% nickel sulfate, 50 mM Tris-HCl [pH 8.0], and hydrogen peroxide [30 vol]) until colored reaction began to appear, and then stopped with distilled water washes.

RESULTS AND DISCUSSION

Construction of expression vector and P. pastoris *transformation*

The *P. pastoris* gene expression system is an attractive method with which to produce a variety of intercellular and extracellular proteins [13]. Since, we previously isolated a DNA encoding this protein, we decided to clone the DNA sequence encoding EMA-1 without its native signal peptide in the pPICZαB plasmid, in frame with the yeast a-factor signal sequence, for secretion of protein containing a 6His-Tag at its C-terminus. PCR amplification of the EMA-1 gene yielded a 755 bp DNA (Fig. 2A) fragment with the expected sequence. The amplified EMA-1 gene was inserted into the vector pPICZαB. The positive insert in the recombinant plasmids was screened by restriction endonuclease cleavage with *Xho*I and three expected fragments were showed (Fig. 2B). Then, constructed expression vector pPICZαB/EMA-1 (Fig. 1) was used for *E. coli* strain TOP10F transformation, which result in several colonies Zeocin resistant. About 50 colonies were picked for an ultra-rapid procedure screening [18] which shown 11 recombinants. One of these was replicated and then sequenced. Sequencing analysis showed that the EMA-1 gene from *T. equi* was sequenced. The lengths of the sequenced regions varied from nucleotide 121 to 735. A consistent alignment provided by 615 nucleotides in EMA-1 gene revealed high degrees of identity with others sequences EMA-1. The sequence was submitted at GenBank (accession no **FJ628171**) (data not show).

After the recombinant vector was constructed and verified experimentally to be correct, it was transformed into *P. pastoris* strain X-33 competent cells by electroporation, and approximately 250 colonies were obtained on the YPDS plates after growing for 3 days. These colonies were picked up and spotted on 100 μg/ml Zeocin YPD plates.

Expression of EMA-1 on shaker and bioreactor cultures

The screening was performed by colony blotting assay which cells growing on BMMY plates are induced with methanol and the secreted proteins can be detected with MAb Anti-6xHIS HRP conjugated (Fig. 3). No transformed X-33 was used with a negative control and recombinant B subunit of heat labile enterotoxin from *E. coli* (LTB) with HIS-tag was used as positive control added on nitrocellulose membrane after induction. From one hundred colonies chosen for Colony blotting assay, three shown positive recombinant protein expression in different levels and one of these positive colony with apparently higher expression level was chosen for shaker flasks and bioreactor growth.

Recombinant *P. pastoris* that expressed EMA-1 of *T. equi* on higher level chose by Colony blotting assay was used for small scale expression on shaker in order to verify the best expression time after induction. Secreted protein was detected by Dot-Blotting assay and best results were obtained with 96 h of induction (Fig. 4). As negative control, no transformed *P. pastoris* X-33 was also growth in BMGY and induced with 1% of methanol on BMMY medium. In bioreactor, fed-batch process continued for a period of 4 days was performed. After 24 h of glycerol growth, when this was exhausted, cells were induced with 1% methanol. Protein secretion was detected with Dot-blotting, as described above.

Precipitation and characterization of protein rEMA-1

In the precipitation of recombinant EMA-1 expressed in *P. pastoris* X-33 cells, ammonium sulfate precipitation was carried out to concentrate the protein, while the best EMA-1 precipitation yield was reached at 80% ammonium sulfate saturation level. SDS-PAGE analysis the culture supernatant of recombinant strain revealed that the EMA-1 secreted into supernatant compared to no-transformant and indicated a major protein band at a molecular mass of ~45 kD, which is consistent with the molecular mass of EMA-1 (Fig. 5). The final yield of the purified protein was quantified by BCA Proteins Assay and resulted in a yield of ~389 mg of rEMA-1 per liter of cell culture supernatant.

Dot blot is a technique for detecting and identifying proteins, similar to the *Western blot* technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane [21]. Antigens may be applied directly to nitrocellulose membrane as a discrete spot (dot) to give a simple and reliable assay [22]. Purified EMA-1 was utilized to evaluate antigenic responses. Dot blot analysis showed a positive reaction of the supernatant of recombinant strain using anti-histidine monoclonal antibody, monoclonal antibody anti-EMA-1, and serum of equine positive carrier of *T. equi*, and did not show reactivity with serum of negative animal. No reactivity was observed with control negative protein. The immunogenicity of rEMA-1 protein was demonstrated by IFAT using sera from recombinant protein immunized mice using aluminum hydroxide as adjuvant. All animals vaccinated with rEMA-1 developed a high specific antibody response (data no showed) [23].

The *P. pastoris* expression system has been used for the production of a wide variety of proteins [16]. However, to the best of our knowledge this is the first report on the cloning and expression of EMA-1 protein in *P. pastoris.*

CONCLUSION

In conclusion, the production and purification of rEMA-1 in the methylotrophic yeast *P. pastoris* was effective, permitting a high-yield production of this protein. Thus, in this work we were able to clone in a secretory expression plasmid and purified EMA-1 protein in *P. pastoris.* Further studies will focus in apply these recombinant antigen for use in immunodiagnosis assays, and possible as a candidate as vaccine antigen for theleiriosis.

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FIGURES

Tabela 1. PCR primers used in this study.

Primer	DNA sequence (5′ to 3′)	restriction enzyme
Primer 1	CGGAATTCACAAAATGGAGGAGGAGAAACCCAAG	*Eco*RI
Primer 2	GGGGTACCAATAGAGTAGAAAATGCAATG	*Kpn*I
5’ AOX1	GACTGGTTCCAATTGACAAGC	
3’AOX1	GGATGTCAGAATGCCATTTGC	

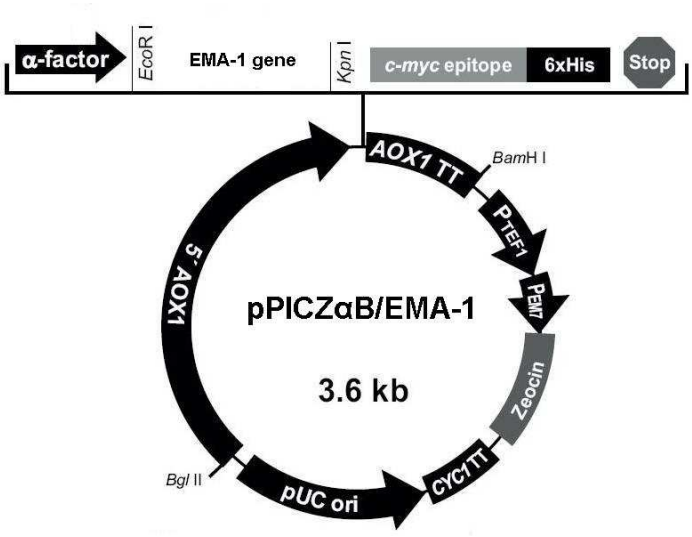

Figure 1. Map of the pPICZαB/EMA-1 expression vector.

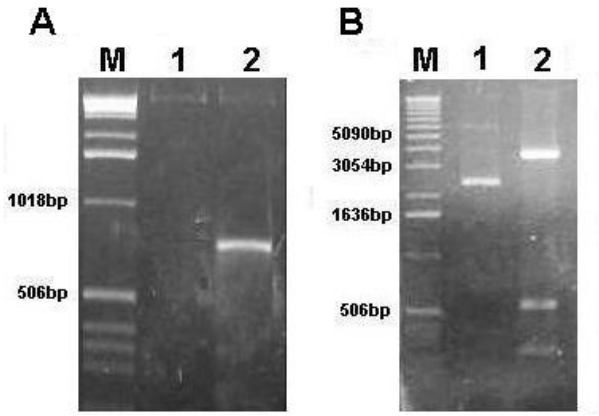

Figure 2. Analysis of amplified EMA-1 gene and recombinant of pPICZαB/EMA-1. (A) PCR amplification of EMA-1 gene. Lane-M: DNA marker, (1) control (2) EMA-1 gene. (B) Restriction analysis of recombinants pPICZαB/EMA-1. Lane-M: DNA marker, (1) pPICZαB (2) restriction recombinants.

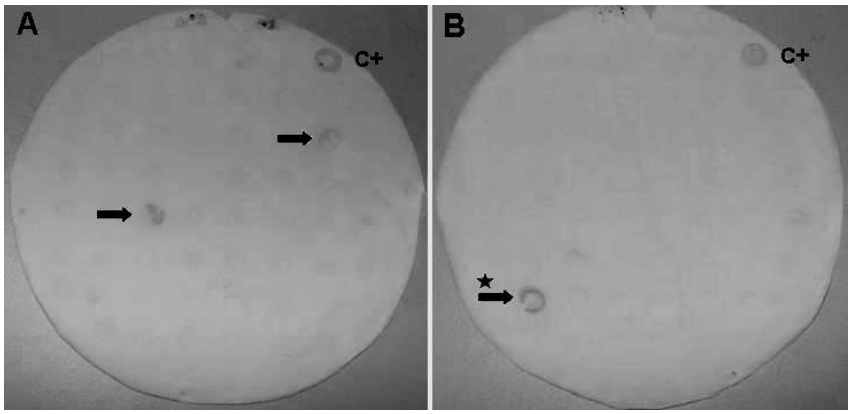

Figure 3. Colony blotting analysis of transformed *P. pastoris* strain X-33 with MAb Anti-6xHIS HRP conjugated. Membranes A and B: Arrows indicate positive colonies expressing EMA-1; selected recombinant colony for scale up expression test are indicated with an asterisk; C+ positive control.

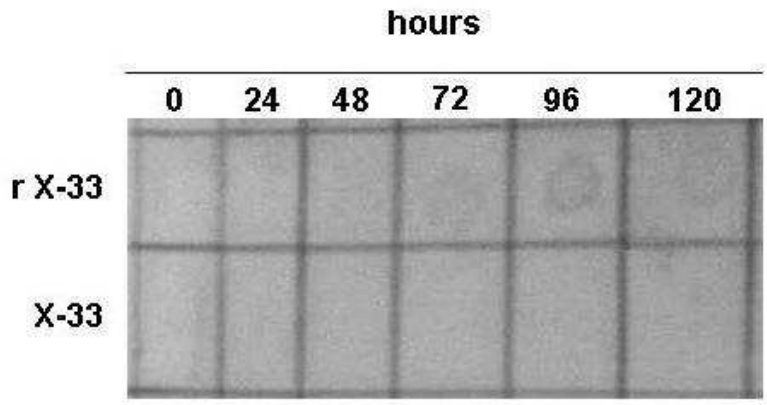

Figure 4. Time-course of rEMA-1 secretion in shake-flask cultures of *Pichia pastoris* positive clone. Dot Blotting of supernatant collected after each 24 h of induction with 1% methanol.

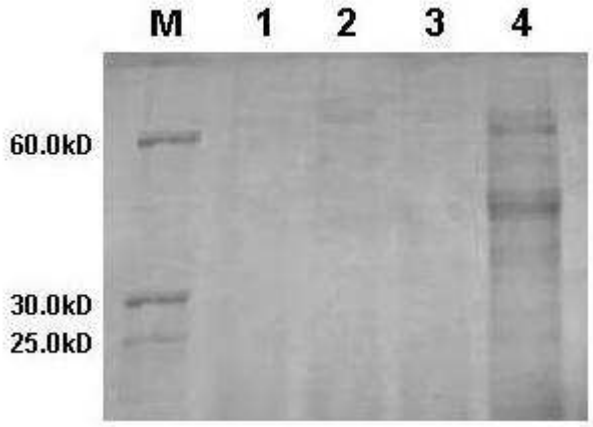

Figura 5. The 10% SDS-PAGE. Lane-M: marker; Lane 1-2: supernatant of non transformed yeast cells before and after methanol induction used as negative control; Lane 3-4: supernatant of transformed yeast cells before and after methanol induction.

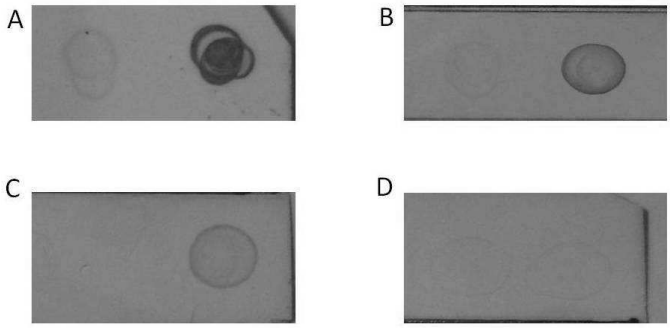

Figure 6. rEMA-1 *Dot blot*. Reactivity against different antibodies (A) anti-histidine monoclonal antibody; (B) anti-EMA-1 monoclonal antibody; (C) sera from positive equine; (D) sera from negative equine. In left of membranes was spotted a negative control and right the rEMA-1 protein precipitated with ammonium sulfate.

5 ARTIGO 3

IMMUNOGENICITY AND ANTIGENICITY OF THE RECOMBINANT EMA-1 PROTEIN OF *Theileria equi* EXPRESSED IN THE YEAST *Pichia pastoris*

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Imunogenicidade e antigenicidade da proteína recombinante EMA-1 de *Theileria equi* expressa em *Pichia pastoris*

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ABSTRACT

The equine piroplasmosis caused by *Theileria equi* is one of the most important parasitic diseases of the equine, causing damage to animal health and economic losses. In *T. equi*, two merozoite surface proteins, *equi merozoite antigen* EMA-1 and EMA-2, have been identified as the most immunodominant antigens. Thus, suggesting that these antigens might be used as immunobiological tools. The EMA-1 of *Theileria equi* was cloned and expressed in the yeast *Pichia pastoris*. The transformed yeast was grown at high cell density expressing up to 389 $mg.L^{-1}$ of recombinant protein. The protein was concentrate, purified and detected in *Dot blot*. The recombinant product was antigenically similar to the native protein as determined using monoclonal antibodies, and polyclonal antibodies obtained from naturally infected equine with *T. equi*. The immunogenicity of rEMA-1 protein was demonstrated by IFAT using sera from recombinant protein immunized mice using aluminum hydroxide as adjuvant. All animals vaccinated with rEMA-1 developed a high specific antibody response. This results suggest that rEMA-1expressed in *P. pastoris* might be used as an antigen for immune diagnostic as well as vaccine antigen.

Keywords: Theileria equi; recombinant vaccine; *Pichia pastoris*; EMA-1.

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RESUMO

A piroplasmose eqüina causada por *Theileria equi* é uma das mais importantes doenças parasitárias de eqüídeos, causando danos a saúde animal e perdas econômicas. Em *T. equi*, duas proteínas de superfície de merozoítos, *equi merozoite antigen* EMA-1 e EMA-2, têm sido identificados como antígenos imunodominantes. Sugerindo que estes antígenos possam ser usados como produtos imunobiológicos. O gene EMA-1 de *T. equi* foi clonado e expressado na levedura *Pichia pastoris*. As leveduras transformadas foram cultivadas a alta densidades celulares expressando 389 $mg.L^{-1}$ de proteína recombinante. A proteína foi concentrada, purificada e detectada em *Dot blot.* O produto recombinante foi antigenicamente similar a proteína nativa quando determinado usando anticorpo monoclonal e anticorpos policlonais obtidos de eqüinos naturalmente infectados com *T. equi*. A imunogenicidade da proteína rEMA-1 foi demonstrada por RIFI usando soro de camundongos imunizados com proteína recombinante usando hidróxido de alumínio como adjuvante. Todos animais vacinados com rEMA-1 desenvolveram uma alta resposta específica de anticorpos. Esses resultados sugerem que rEMA-1 expressa em *P. pastoris* possa ser usado como antígeno para diagnóstico imunológico bem como antígeno para vacinas.

Palavras chaves: *Theileria equi*, vacina recombinante, *Pichia pastoris*, EMA-1

INTRODUCTION

The equine piroplasmosis is one of the most important parasitic diseases of the equines, it causes great damage to animal health. The disease is a haemoparasitosis caused by *Theileria equi* and *Babesia caballi*, is a tick-borne hemoprotozoan disease affecting horses worldwide (SCHEIN, 1988). Through infecting and destroying red blood cells, it can compromise the equine function, leading to loss of vitality and decrease in the performance of infected animals. Equine piroplasmosis caused by *T. equi* is more pathogenic and widespread in horses than that by *B. caballi* (DE WAAL, 1992), causes a persistent infection for which drug therapy or vaccination is not available (SCHEIN, 1988; FRIEDHOFF, 1990). The *T. equi* infection is characterized by fever, anemia, icterus, and hepato and splenomegaly (OIE, 1989).

Merozoite surface proteins are important in the pathogenesis of hemoprotozoan diseases because of their role in parasite recognition of, attachment to, and penetration of host

erythrocytes (JACK & WARD, 1981; KUMAR et al., 2004). In *T. equi*, two kinds of merozoite surface proteins, *equi merozoite antigen* EMA-1 and EMA-2, have been identified as the most immunodominant antigens (KAPPMEYER et al., 1993; KNOWLES et al., 1991; KNOWLES et al., 1992). EMA-1 is geographically conserved among all *T. equi* isolates (KNOWLES et al., 1991; CUNHA et al., 2002) and shares significantly high homologies in amino acid sequence with the counterpart proteins of many *Theileria* parasites (KAPPMEYER et al., 1993; KNOWLES et al., 1997). Additionally, EMA-1 has glycosyl-phosphatidylinositol (GPI) anchor-specific motifs in their sequence, suggesting that these proteins might be expressed on the outer surface of merozoite with a GPI anchor (KNOWLES et al., 1997). The *T. equi* merozoite express surface protein of molecular mass 34 kDa, which are strongly recognized by antibodies produced in infected animals (KNOWLES et al., 1997; CUNHA et al., 2006). Further, EMA-1/2 are members of the major piroplasm surface protein (MPSP) family that is conserved among the genus *Theileria*, but the biological role of MPSP has not yet been clarified. Therefore, functional studies on EMA-1/2 family proteins may also provide insight into the biological significance of MPSP expression in the intra-erythrocytic development of *Theileria* parasites.

The methylotrophic yeast *Pichia pastoris*, which is one of the dominant expression systems in molecular biology due to its stable and high-level expression of heterologous proteins (CREGG et al., 1993, ROMANOS et al., 1998). Over 400 proteins from prokaryotes, eukaryotes, and viruses have now been successfully expressed in this yeast (CEREGHINO et al., 2002). The *P. pastoris* expression system offers many advantages, including its ease of usage relative to other eukaryotic expression systems, the possibility of high-level expression of foreign proteins. The yeast is also able to introduce eukaryotic post-translational modifications such as glycosylation and proteolytic processing (CEREGHINO & CREGG, 2000).

The present study aims to express truncated EMA-1 gene by *Pichia pastoris* system and subsequently determine the immunologic and antigenic properties of recombinant EMA-1 protein.

MATERIALS AND METHODS

Transformation of *P. pastoris* and expression in shaken flasks

The transformation of *P. pastoris* and cultivation in shaken flasks were performed according to the EasySelect™ Pichia Expression Kit (Invitrogen, Catalog No. K1740-01). A

DNA encoding the EMA-1 from *T. equi* was obtained as previously described Nicolaiewsky et al. (2001). The EMA-1 gene (GenBank accession number AF261824) was amplified by PCR with the following primers: forward (5′-CGGAATTCACAAAATGGAGGAGGAGAA ACCCAAG-3′) and reverse (5′-GGGGTACCAATAGAGTAGAAAATGCAATG-3′), containing a *Eco*RI (forward) and an *Xba*I site (reverse), respectively. After purification and digestion, the amplified DNA fragment was cloned into the vector. The *Escherichia coli* TOP10F were used as a host for plasmid propagation. The *P. pastoris* wild type strain X-33 and pPICZαB vector were used as a host for expression of EMA-1 in *P. pastoris*. The *P. pastoris* transformants were selected in YPD containing 100 mg/mL zeocin.

Precipitation of protein

Proteins in the media were precipitated by addition of solid ammonium sulfate to 80% and incubation at 4°C for 16 hours. Precipitated protein was pelleted by centrifugation at 12.000 *g* for 15 min and the pellet was resuspended in buffer PBS. The solution was desalted by overnight dialysis against buffer PBS at 4°C. Protein quantitation was determined using the BCA™ Protein Assay Kit (Pierce Chemical Company) with bovine serum albumin as a standard.

Dot blot

Dot blot assays were performed using 7,5 µg of recombinant protein. As a negative control, 20 µL of a membrane preparation from a *P. pastoris* clone transformed with the empty expression vector. The proteins were spotted onto a nitrocellulose membrane (*Hybond* C, Amersham Biosciences). The nitrocellulose membranes were blocked for 1 hour at room temperature in PBST buffer containing 5% of non-fat milk. One membrane was incubated with monoclonal antibody anti-His peroxidase conjugate and other membrane with monoclonal antibody anti-EMA-1. Two membranes were incubated with serum of equines negative and positive for infection of *T. equi*, respectively. Membranes were incubated with secondary antibodies for 1 hour with goat anti-horse or anti-mouse IgG peroxidase conjugate. The reaction was developed with diaminobenzidine (Sigma).

Immunization of mice

The recombinant purified protein was prepared for immunization of mice (*Mus muscullus*), female BALB/c mice (six weeks old) were randomly divided into three groups

(four mice per group) and subcutaneously immunized twice at 10 days intervals. One group (GI) was injected with EMA-1 protein (50 µg) without adjuvant. The other group (GII) was injected with 50 µg of EMA-1 protein formulated with the adjuvant aluminum hydroxide. The final group (GIII) was used as a negative control and injected with 100 µl of sterile PBS. Serum samples were collected from the retro-orbital plexus immediately before immunization and about 10 days after each of the immunizations and used in serological tests. The experiment was approved by the UFPel Committee of Ethics in Animal Experimentation.

Immunofluorescence assay

Antibody titers of the serum samples against the recombinant protein were measured with IFAT. The slides were prepared with infected horses erythrocytes in which are visible as compact inclusion by IFAT staining. IFAT was performed according to the Cunha (1993). Then polled serum from the different groups were added incubated, and applied FITC-conjugated goat anti-mouse IgG (Invitrogen), per well diluted at 1:400 in PBS buffer. The visualization slides were in epifluorescent microscopy (Olympus, BX-FLA).

RESULTS AND DISCUSSION

Expression and purification of rEMA-1 in *P. pastoris*

The *P. pastoris* gene expression system is an attractive method with which to produce a variety of intercellular and extracellular proteins (CREGG et al., 1993). Since, we previously isolated a DNA encoding this protein, we decided to clone the DNA sequence encoding EMA-1 without its native signal peptide in the *P. pastoris* pPICZαB plasmid, in frame with the yeast a-factor signal sequence, for secretion of protein containing a 6His-Tag at its C-terminus. The pPICZαB-EMA-1 plasmid was then transformed and targeted to the *P. pastoris* genome by means of homologous recombination. The presence of the EMA-1 coding sequence in the genomic DNA isolated from *Pichia* transformants was confirmed by PCR. In the *P. pastoris* expression system, recombinant protein expression is strictly controlled by the AOX1 promoter. Expression was induced by addition of methanol to a final methanol concentration of 0.5%. A protein that was not present before methanol induction was detected by SDS-PAGE (data not shown).

One of the main advantages in producing heterologous proteins as secreted products in *P. pastoris* is the easy isolation of the recombinant product from the medium in which it is produced. Indeed, the initial purity of recombinant molecule in culture medium is high

because the level of endogenous secreted proteins is very low. Thus, after removal of the yeast cells by centrifugation, proteins in the culture supernatant were precipitated with 80% ammonium sulfate fractionation. The final yield of the purified protein was 389 mg of rEMA-1 per liter of cell culture supernatant.

Detection of reactivity to recombinant EMA-1

Dot blot is a technique for detecting and identifying proteins, similar to the *Western blot* technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane. Antigens may be applied directly to nitrocellulose membrane as a discrete spot (dot) to give a simple and reliable assay.

In order to evaluate the reactivity of recombinant EMA-1 with sera from *T. equi* positive and negative equine, we tested by *Dot blot*. Positive samples were considered all of those that developed a reaction color. Negative controls samples did not develop any color.

In *Dot blots*, we are able to observe that the rEMA-1 protein reacted with the serum from naturally infected horse by *T. equi*, but did not with the serum of the negative horse.

The figure 1 show the reactivity of the rEMA-1 protein using anti-histidine monoclonal antibody, monoclonal antibody anti-EMA-1, and serum of equine positive carrier of *T. equi*, and did not show reactivity with serum of negative animal. No reactivity was observed with control negative protein.

Immunogenicity of the rEMA-1 protein in mice

Mice immunized with the recombinant protein showed high antibody response, were able to react with native EMA-1 of *T. equi* as observed by IFAT. Sera from the negative control group did not react with native EMA-1, showing the specificity of the response (Figure 2). These results indicate that immune response was generated in all the vaccinated groups, showing that recombinant protein has capability to generated antibodies that react with the native protein. We also observed that the antibody titers have significant difference between the groups with without adjuvant. This worth noting that rEMA-1 expressed in *P. pastoris* maintain the same three-dimensional structure as the native protein since it was recognized by antibodies generated by naturally infected horses.

The *P. pastoris* expression system has been used for the production of a wide variety of proteins (CEREGHINO & CREGG, 2000). However, to the best of our knowledge this is the first report on the cloning and expression of EMA-1 protein in *P. pastoris.*

In conclusion, the production and purification of rEMA-1 in the methylotrophic yeast *P. pastoris* was effective, permitting a high-yield production of this protein. Thus, rEMA-1expressed in *P. pastoris* might be a strong candidate to be used as an antigen for immune diagnostic as well as vaccine antigen.

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FIGURES

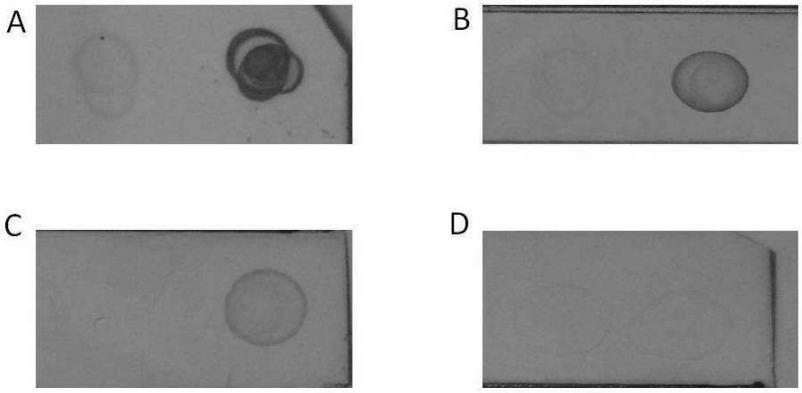

Figure 1. rEMA-1 *Dot blot*. Reactivity against different antibodies (A) anti-histidine monoclonal antibody; (B) anti-EMA-1 monoclonal antibody; (C) sera from positive equine; (D) sera from negative equine. In left of membranes was spotted a negative control and right the rEMA-1 protein.

Figure 2. IFAT of sera from immunized mice with rEMA-1. (A) Pool of sera from immunized mice twice in the presence of adjuvant; (B) Mice immunized with sterile PBS; (C) IFAT positive control (equine sera with FITC anti-equine).

6 CONSIDERAÇÕES FINAIS

Durante a interação parasito-hospedeiro, as proteínas de superfície do parasito são as principais responsáveis pela resposta imune do hospedeiro, sendo que, os antígenos de superfície são promissores para uso como vacinas de subunidade e reagentes para diagnóstico.

As tentativas para controlar a epidemia da doença causada pelo protozoário *T. equi* tem sido focalizadas em estudos biológicos, bioquímicos, estruturais do parasito e no desenvolvimento de novas drogas. As proteínas de superfície do merozoito desempenham um papel chave no estabelecimento da infecção, a interação da EMA-1 com as células do hospedeiro é crucial para a sua infectividade (KUMAR, et al., 2004). Sabendo-se que *P. pastoris* é um sistema de expressão de proteína de grande eficiência e reproduz as melhores condições de expressão de proteína de eucariotos superiores, neste estudo, este organismo foi selecionado como o ideal para a produção de EMA-1 com a finalidade de utilizá-la como antígeno vacinal recombinante.

A escolha do sistema utilizado para a expressão da EMA-1 recombinante, assim como, a definição dos objetivos foi extremamente importante. O entendimento de que EMA-1 seria utilizada não só para a produção de vacinas, mas também para a padronização de um teste imunológico para diagnóstico e para teste de protocolos terapêuticos, nos remeteu à necessidade de uma molécula extremamente semelhante à nativa.

No entanto, a capacidade de tradução de uma seqüência de DNA seria igual em qualquer vetor de expressão. Porém, as diferenças se baseiam principalmente na capacidade de produzir glicosilação da proteína, e na quantidade de proteína que o vetor é capaz de produzir. A glicosilação em proteínas pode influenciar na bioatividade, farmacocinética, biodistribuição e imunogenicidade da molécula. Células procariontes não conseguem adicionar carboidratos (glicosilar); leveduras bem como outros sistemas eucarióticos são capazes de glicosilações mais simples normalmente sem a capacidade de sialização e com baixa capacidade de

glicosilação ligada ao O. A escolha do modelo de expressão da EMA-1 foi baseada nos dados citados acima.

Várias proteínas já foram expressas em *P. pastoris*, entretanto, até o momento não há relato de expressão de EMA-1 de *T. equi* nesta levedura para ser utilizado como um antígeno recombinante. Desta forma, este trabalho é pioneiro quanto à utilização de *P. pastoris* como vetor de expressão de proteína EMA-1 de *T. equi* na tentativa de ser utilizada como antígeno em produtos imunobiológicos.

Com os resultados deste estudo desenvolvemos ferramentas que poderá ser utilizadas para melhor entender a patogenicidade deste importante parasito bem como sua utilização em estudos de vacinas e de diagnóstico.

7 CONCLUSÕES GERAIS

- A levedura *P. pastoris* é capaz de expressar a proteína recombinante EMA-1 de *Theileria equi*;

- A proteína EMA-1 expressa por *P. pastoris* mostrou ser antigênica e imunogênica;

- A proteína EMA-1 é um promissor alvo á ser utilizado como imunobiológico no desenvolvimento de testes diagnósticos e na formulação de vacinas recombinantes.

- Primeira citação da clonagem e expressão do gene EMA-1 de *T. equi* em sistema de expressão heterólogo *P. pastoris*;

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