Veterinary Parasitology 167 (2010) 216–226

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

In silico predicted conserved B-cell epitopes in the merozoite surface antigen-2 family of *B. bovis* are neutralization sensitive

M. Dominguez [a], I. Echaide [b], S. Torioni de Echaide [b], J. Mosqueda [c], B. Cetrá [d], C.E. Suarez [e], M. Florin-Christensen [a,f,*]

[a] *Institute of Pathobiology, Center of Agriculture and Veterinary Research, National Institute of Agriculture Technology (INTA), Castelar, Argentina*
[b] *Agricultural Experimental Station (EEA)-INTA Rafaela, Argentina*
[c] *Universidad Autónoma de Querétaro, Campus Juriquilla, Mexico*
[d] *EEA-INTA Mercedes, Argentina*
[e] *Animal Disease Research Unit-USDA, Pullman, WA, USA*
[f] *National Research Council (CONICET), Buenos Aires, Argentina*

ARTICLE INFO

Keywords:
Babesia bovis
Bovine babesiosis
MSA-2
Peptides
Subunit vaccines
B-cell epitopes

ABSTRACT

The merozoite surface antigens MSA-2 of *Babesia bovis* constitute a family of polymorphic GPI-anchored glycoproteins located at the parasite cell surface, that contain neutralization-sensitive B-cell epitopes. These are therefore putative vaccine candidates for bovine babesiosis. It was previously shown that (i) the MSA-2 antigens of the biologically cloned Mo7 strain are encoded by four tandemly organized genes: *msa-2a*$_1$, *a*$_2$, *b* and *c*, and (ii) at least one allele of each of these genes is present in the Argentine R1A strain with a moderate degree of polymorphism. The present work was aimed at defining neutralization-sensitive B-cell epitopes in the MSA-2 family, that are conserved among different *B. bovis* geographical isolates. To this end, *msa-2a*, *b* and *c* alleles from different isolates from Argentina, USA and Mexico were amplified by PCR, cloned and sequenced. Bioinformatic analysis by ClustalW alignments and B-cell epitope prediction algorithms performed on these sequences allowed the identification of several regions containing putative conserved B-cell epitopes. Four peptides representing these regions: (KDYKTMVKFCN from *msa-2a*$_1$; YYKKHIS, from *msa-2b*; and THDALKAVKQLIKT and ELLKLLIEA from *msa-2c*) were chemically synthesized, conjugated to keyhole limpet hemocyanin and used to inoculate mice to obtain immune sera. Anti-peptide antibodies recognized *B. bovis* merozoite extracts in all cases in ELISA tests. In addition, these sera reacted with the surface of merozoites of an Argentine and a Mexican *B. bovis* strains in immunofluorescence assays, and sera against two of the selected peptides inhibited invasion of erythrocytes by *in vitro* cultured merozoites. Taken together, the results show that the peptide sequences selected by bioinformatic analysis represent expressed and geographically conserved *B. bovis* B-cell epitopes that might be strong candidates for development of subunit vaccines.

1. Introduction

Infection of bovines with the tick-transmitted hemoparasite *Babesia bovis* causes important economic losses and limits cattle production in vast tropical and subtropical areas of the world. An effective control measure is vaccination of unprotected cattle with live attenuated

* Corresponding author at: Institute of Pathobiology, CICVyA, INTA-Castelar, Los Reseros y Nicolas Repetto, 1686 Hurlingham, Prov. Buenos Aires, Argentina. Tel.: +54 11 4621 1289; fax: +54 11 4621 0443.
E-mail address: mflorin@cnia.inta.gov.ar (M. Florin-Christensen).

0304-4017/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2009.09.023

strains, which allows the prevention of outbreaks in areas of enzootic instability and protects animals raised in tick-free zones when imported into endemic areas (Shkap et al., 2007). These vaccines are usually effective but have a number of disadvantages, such as the risk of co-transmission of blood-borne pathogens, the need of a cold chain, a relatively short storage life when it is not frozen, and the danger of parasite reversion of virulence. In addition, they are only safe in bovines younger than 1 year old. Some reports have pointed out sporadic failures of live vaccines (Bock et al., 1992, 1995), which could be due to the occurrence of variant parasite strains against which the vaccines do not generate protection.

Development of subunit vaccines could prove useful for the control of bovine babesiosis. Experimental vaccines based on single recombinant antigens have been successfully developed for some apicomplexan hemoparasites of veterinary importance. Such is the case for *Theileria parva*, where vaccination of cattle with a recombinant form of the sporozoite surface antigen p67 significantly reduced the development of East Coast Fever after a natural tick challenge in Kenya (Musoke et al., 2005). Likewise, immunization of gerbils with a recombinant form of Bd37, a glycosyl-phosphatidylinositol (GPI)-anchored protein of *B. divergens*, induced protection against challenge with different virulent strains of this parasite (Hadj-Kaddour et al., 2007). However, attempts to elicit protective immune responses against *B. bovis* using single recombinant proteins derived from known strong immunogenic proteins such as MSA-1 and RAP-1 proved to be so far unsuccessful (Hines et al., 1995; Norimine et al., 2003). A recombinant vaccine based on the *B. bovis* antigens 11C5 and 12D3 was recently tried with encouraging results (Hope et al., 2005), although full protection was not achieved. Thus, these results suggest that a protective *B. bovis* subunit vaccine may require the inclusion of several antigens or alternatively, a combination of effective B- and T-cell epitopes derived from multiple antigens.

Promising candidates for *B. bovis* subunit vaccines include members of the merozoite surface antigen (MSA)-2 family. This family was originally identified in transcripts from the virulent Kv Australia strain and termed *Babesia* recombining genes (BabR) (Cowman et al., 1984). The gene family was afterwards identified and their products characterized in North American *B. bovis* strains. Interestingly, members of this family contain a proline-rich hypervariable region and present a considerable degree of sequence variation among strains, although they do not seem to change rapidly during the course of infection, and thus the family was renamed as variable merozoite surface antigen (VMSA), including the MSA-1 and MSA-2 genes (Reduker et al., 1989; Hines et al., 1989, 1992; Berens et al., 2005; LeRoith et al., 2006). They are a group of immunodominant, GPI-anchored glycoproteins, homogeneously distributed over the surface membrane of both merozoites and sporozoites (Reduker et al., 1989; Hines et al., 1989; Mosqueda et al., 2002). Importantly, VMSA proteins were shown to contain neutralization-sensitive B-cell epitopes and are thought to participate in erythrocyte invasion (Hines et al., 1992; Suarez et al., 2000; Florin-Christensen et al., 2002; Mosqueda et al., 2002; Wilkowsky et al., 2003). Definitive characterization of the genomic *msa-2* locus in the Mexican Mo7 strain (Florin-Christensen et al., 2002) showed a tandem organization of four related genes, which were named: *msa-2a*$_1$, *msa-2a*$_2$, *msa-2b* and *msa-2c*. A similar structure was later found in the virulent Texas T2Bo strain (Berens et al., 2005; Brayton et al., 2007). At least one allele of each *msa-2* gene was identified in the Argentine R1A strain, and comparison of R1A and Mo7 sequences showed a moderate to high degree of conservation (Florin-Christensen et al., 2002). However, characterization of the *msa-2* locus in 12 Australian strains demonstrated a completely different structure and, with the exemption of *msa-2c,* a high degree of polymorphism among strains (Berens et al., 2005).

A variable B-cell epitope recognized by the monoclonal antibody 23/70.174 was identified and mapped in a repetitive region of MSA-2 in the Texas and Mexico strains of *B. bovis* MSA-2a (Goff et al., 1988; Palmer et al., 1991; Jasmer et al., 1992). However, the moderate degree of sequence conservation later found between R1A and Mo7 MSA-2 predicted proteins allowed us to hypothesize that this family might contain other B-cell epitopes that, in contrast to the epitope recognized by mAb 23/70.174, are conserved among American geographic isolates. This work has been aimed at identifying and characterizing such B-cell epitopes, with the long-term goal of developing a subunit vaccine that would be effective against otherwise antigenically different strains of *B. bovis*.

2. Materials and methods

2.1. Strains and isolates

The following *B. bovis* strains and isolates were used in this study: Mo7, RAD, Pullman, Veracruz and Tabasco, from Mexico; R1A, S2P, M1A, M2P, and M3P from Argentina and T2Bo from USA. Mo7, a biologically cloned Mexican strain and the isolates T2Bo (Texas, USA), R1A (vaccine strain initially isolated from a clinical case in Santa Fe, Argentina in 1990 and attenuated across passages in splenectomized bovines, Anziani et al., 1993), and S2P (pathogenic isolate from Salta, Argentina) were propagated in cultured bovine erythrocytes. The M2P and M3P isolates derive from clinical cases in the province of Corrientes, Argentina, and were amplified in splenectomized calves. M1A was isolated from a clinical case in Salta and attenuated after several passages in splenectomized calves in Mercedes, Argentina. It is used for vaccination in the NE of this country. The Mexican strains RAD (vaccine strain), Veracruz and Tabasco derive from clinical cases and were propagated *in vitro* or amplified in splenectomized bovines. The Pullman isolate was obtained from infected ticks in Mexico, 1975, and was amplified in splenectomized bovines as well.

2.2. PCR, cloning and sequencing

Genomic DNA was purified by standard phenol/chloroform extraction. In the case of *in vitro* cultured parasites, *B. bovis* merozoite-enriched suspensions were first obtained by differential centrifugation (4000 × *g*, 20 min, and 10,000 × *g*,