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Research paper

A *Neospora caninum* vaccine using recombinant proteins fails to prevent foetal infection in pregnant cattle after experimental intravenous challenge

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ABSTRACT

The aim of the present study was to evaluate the immunogenicity and protective efficacy of rNcSAG1, rNcHSP20 and rNcGRA7 recombinant proteins formulated with immune stimulating complexes (ISCOMs) in pregnant heifers against vertical transmission of *Neospora caninum*. Twelve pregnant heifers were divided into 3 groups of 4 heifers each, receiving different formulations before mating. Immunogens were administered twice subcutaneously: group A animals were inoculated with three recombinant proteins (rNcSAG1, rNcHSP20, rNcGRA7) formulated with ISCOMs; group B animals received ISCOM-MATRIX (without antigen) and group C received sterile phosphate-buffered saline (PBS) only. The recombinant proteins were expressed in *Escherichia coli* and purified nickel resin. All groups were intravenously challenged with the NC-1 strain of *N. caninum* at Day 70 of gestation and dams slaughtered at week 17 of the experiment. Heifers from group A developed specific antibodies against rNcSAG1, rNcHSP20 and rNcGRA7 prior to the challenge. Following immunization, an statistically significant increase of antibodies against rNcSAG1 and rNcHSP20 in all animals of group A was detected compared to animals in groups B and C at weeks 5, 13 and 16 ($P < 0.001$). Levels of antibodies against rNcGRA7 were statistical higher in group A animals when compared with groups B and C at weeks 5 and 16 ($P > 0.001$). There were no differences in IFN-γ production among the experimental groups at any time point ($P > 0.05$). Transplacental transmission was determined in all foetuses of groups A, B and C by Western blot, immunohistochemistry and nested PCR. This work showed that rNcSAG1, rNcHSP20 and rNcGRA7 proteins while immunogenic in cattle failed to prevent the foetal infection in pregnant cattle challenged at Day 70 of gestation.

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1. Introduction

Neospora caninum is an intracellular apicomplexan parasite that causes abortion in cattle (Dubey and Schares, 2011). This leads to significant financial losses world-wide (Reichel et al., 2013). Transplacental transmission of the parasite from an infected dam to its foetus is the major natural route of infection (Dubey et al., 2007). Strategies for the control of this disease are based on general biosecurity procedures that aim interrupt the life cycle of the parasite and the culling of seropositive cattle, if economically viable (Reichel et al., 2014). Thus, the economic importance of neosporosis, especially in cattle, is evident and the development additional control measures, such as treatment or vaccination are urgently needed.

Protective immune responses against *N. caninum* in the host are associated with a T helper 1 immune response, mediated by cytotoxic T lymphocytes and the production of interferon-gamma (IFN-γ), interleukin-12 (IL-12), tumour necrosis factor (TNF) and immunoglobulin G_2 (IgG_2) (Staska et al., 2003; Innes, 2007). The main challenge will be to develop a vaccination strategy that will prevent *N. caninum* vertical transmission, and this vaccination strategy must result in a balanced immune response that is compatible with pregnancy. Although promising results were obtained by immunization with live parasites (Innes et al., 2001; Williams et al., 2007; Weber et al., 2013; Hecker et al., 2013), a vaccine based on a cocktail of defined antigens produced with recombinant gene technology has many advantages, such as safety, controllable composition, quality and simplified production (Reichel and Ellis, 2009).

A limited number of recombinant proteins have been investigated as vaccine candidates against bovine neosporosis. These include mostly immune-dominant proteins, such as the major surface antigens and proteins localized in secretory organelles. NcSAG1 is an immunodominant surface protein, involved in low-affinity contact between tachyzoite and host cell surface membrane (Hemphill et al., 2006). Several authors have shown that polyclonal and monoclonal antibodies directed against this surface antigen and SRS2 inhibited host cell adhesion and invasion (Hemphill et al., 1999; Nishikawa et al., 2000; Haldorson et al., 2005). It has been reported that the immunization of mice with recombinant SAG1 showed significant protection against cerebral infection with *N. caninum* (Cannas et al., 2003), but the immunogenicity of this protein has not yet been evaluated in cattle.

Small heat shock proteins (sHSPs) are recognized for their participation in protein trafficking, signal transduction and cytoskeleton dynamics being their principal function to assist in the folding of newly formed proteins during synthesis. De Miguel et al. (2005) identified at least five sHSPs in *Toxoplasma gondii*. One of them, HSP20, was reported to be localized on the outer leaflet of the inner membrane complex (IMC) and at the conoid of *T. gondii* (De Miguel et al., 2005, 2008). HSP20 was also observed in this subcellular location in *N. caninum*. Vonlaufen et al. (2008) and Cóceres et al. (2012) suggested that HSP20 could have a similar role in different apicomplexan parasites because this protein has a highly conserved structure. Montero et al. (2008) reported that antibodies against *Babesia divergens* HSP20 blocked that parasites growth. In addition, Cóceres et al. (2012) showed that rabbit anti-*T. gondii* HSP20 antibodies reduced gliding motility and invasion of not only *T. gondii* but also *N. caninum*. Furthermore, *B. bovis* HSP20 was found to be recognized by $CD4^+$ T lymphocytes from cattle that have recovered from infection and thus, HSP20 was proposed as a candidate vaccine antigen for inclusion in a vaccine for babesiosis (Norimine et al., 2004); we consider that the protective immunity role of NcHSP20 should be analyzed.

The dense granules are globular organelles containing molecules that are secreted shortly after the invasion of host cells (Hemphill et al., 1999). In addition, a number of dense granule proteins such as NcGRA1, NcGRA2 and NcGRA7 are secreted during *in vitro* stage conversion and are incorporated into the cyst wall (Vonlaufen et al., 2004). The dense granule protein NcGRA7 has shown be an immunodominant antigen which is highly immunogenic and associated with active replication of the parasite (Jenkins et al., 1997; Huang et al., 2007). It has shown promising results with regard to protection against *N. caninum* challenge in mice (Liddell et al., 2003; Jenkins et al., 2004) and cattle (Nishimura et al., 2013). Nishimura et al. (2013) demonstrated that M3-NcGRA7 could induce protective immune response in a model using male calves seronegative to *N. caninum*. As a pregnant cattle model would be more suitable for evaluating the vaccine efficacy against *N. caninum*, in the present study we have tried to evaluate the efficacy of rNcGRA7 against vertical transmission in pregnant cattle challenged with *N. caninum*.

Numerous adjuvants have been evaluated in the formulation of inactivated vaccines against *N. caninum* (Andrianarivo et al., 1999; Moore et al., 2005; Williams et al., 2007). Immune stimulating complexes (ISCOMs) are 40 nm nanoparticles used as delivery system for vaccine antigens. The ISCOMs are made up of saponin, cholesterol, lipids and antigen (Morein et al., 2004). Immune stimulating complexes (ISCOMs) have been successfully used in the development of vaccines for ruminants (Morein et al., 2004). Promising results have been obtained in terms of immunogenicity and IFN-γ levels when using inactivated *Neospora* immunogens in calves (Moore et al., 2011). Moreover, partial protection against tissue cyst formation was provided by using crude rhoptries antigens from *T. gondii* formulated in ISCOMs in pigs (García et al., 2005).

Based on the mentioned previous studies, the aim of this study was to test whether a cocktail of NcSAG1, NcHSP20 and NcGRA7 recombinant proteins formulated with ISCOMs are able to stimulate an immune response that is protective against vertical transmission in pregnant cattle experimentally challenged.

2. Materials and methods

2.1. Recombinant protein production and vaccine preparation

N. caninum isolate used in the current study was strain NC-1 (Dubey et al., 1988). The cloning and purification of the recombinant protein NcSAG1 (rNcSAG1) was already described by Wilkowsky et al. (2011). Briefly, rNcSAG1