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Research Article

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# The Potential of *Neospora caninum* Immunogens against Neosporosis

**Piraine REA[1] , Silva RAE[2*], Junior AGDS[3], Cunha RC[4] and Leite FPL[5]**

[1]*Programa de Pós Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brasil*

[2]*Laboratório de Biologia Molecular do Carrapato, Embrapa Gado de Corte (CNPGC), Campo Grande, MS, Brasil*

[3]*Programa de Pós Graduação em Veterinária, Faculdade de Medicina Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brasil*

[4]*Programa de Pós Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brasil*

[5]*Laboratório de Microbiologia, Departamento de Biotecnologia, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brasil*

[*]**Corresponding author:** Silva RAE, Zona Rural, Campo Grande, MS, Brasil, Tel: +55673368-2173; Fax: +55673368-2150; E-mail: renato.andreotti@embrapa.br

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**Abstract**

*Neospora caninum*, the parasite that causes neosporosis, is known worldwide as one of the main drivers of abortion in cattle herds, causing economic losses when raising livestock. Parasitic infection and transmission among animals is difficult to combat, and both diagnostics and controls must be applied to reduce the spread of the pathogen. For control, herd vaccinations represent an alternative, but the current lack of a safe and effective vaccine prevents this method. The parasite has a significant array of structural proteins that assist in the process of infection; surface antigens (SAGs), microneme proteins (MICs), dense granule antigens (GRAs) and rhoptria proteins (ROPs). Antigens from these proteins are currently being studied as immunogens; they are tested alone or in associations, in order to evaluate the induced immune response in animal models. In experimental vaccine studies, different approaches are used in the formulations, such as live vaccines, DNA vaccines, vaccines using biological vectors, and recombinant subunit vaccines (usually developed with the aid of reverse vaccinology). The contrasts observed (in both cytokine levels and protection rates against vertical transmission), in vaccinated and then challenged (*N. caninum*), laboratory animals show the complexity of parasite invasion mechanisms, and reveal the need for further research to isolate an effective vaccine to protect cattle against the parasite.

**Keywords:** Neosporosis; Antigens; Immunogens; Vaccines; Prospects

## Introduction

*Neospora caninum*, belonging to the phylum Apicomplexa and the agent causing neosporosis is an intracellular protozoan. It was initially identified in canine neuromuscular diseases [1], and later described by Dubey et al. [2]. It is globally recognized as a major cause of abortion in cattle [3], and accounts for global economic losses of around US $ 2 billion/year in the dairy and beef cattle industries [4].

In addition to the cost of herd animals lost, there are economic losses reported with decreased milk production [5], and low weight gains after weaning [6]. Neosporosis is widely distributed, with cases reported in Argentina, Australia, Brazil, Spain, United States, New Zealand, and other beef producing countries [4].

With more than 209 million head of cattle, Brazil has the second largest commercial herd in the world, making it one of the world's largest producers of milk and beef [7]. It is estimated that national losses related to neosporosis in the dairy industry reach up to US $ 50 million/year, while in meat production, the figure is as high as $ 100 million/year [4].

Using *Gerenpec Embrapa* Beef Cattle software, Barros et al. [8] evaluated losses resulting from neosporosis by creating scenarios for production systems with different technological levels, and comparing them with systems free of the disease. As a result, the real possibility of economic losses of up to 34% (over 10 years) in properties that have seropositive animals in their herds was highlighted.

The parasite life cycle maintains as its definitive hosts; dogs (*Canis familiaris*) [3,9], and coyotes (*Canis latrans*) [10]; and as intermediate hosts, sheep, horses, deer, other dog species, buffalo, and cattle [11].

In humans, seropositivity and the possibility of infection in individuals infected with human immunodeficiency virus (HIV), and in patients with neurologic disorders, reveal an opportunistic and pathogenic parasite with demonstrated zoonotic potential in immunocompromised individuals [12].

The sexual and asexual stages of the *N. caninum* life cycle occur in canines [9]. Being definitive hosts, dogs infect themselves by ingesting tissue cysts, and then releasing in their feces non-sporulated oocysts to the environment, which sporulate into two sporocysts.

Each sporocyst contains four sporozoites [13]. Thus, intermediate hosts ingest sporulated oocysts (stomach chemical action releases the sporozoites) which then invade the intestinal wall. At this point, tachyzoite stage conversion occurs [14], and intracellular multiplication then generates an acute parasitemia; reaching nervous, lymphatic, and vascular tissues among others [15]. Protected in the cytoplasm of infected cells in parasitophorous vacuoles (PVs), they maintain full multiplication [9].

To be successful in the invasion of the host cell, *N. caninum* uses organelles and sets of specialized proteins. Some of the specialized cellular invasion components are; surface antigens (SAGs), microneme proteins (MICs), dense granule antigens (GRAs), and rhoptria proteins (ROPs) [16].

Knowing the location of these antigens and their roles, and those aspects which are fundamental to the parasite-host interaction (such as

the induction of a humoral or cellular immune response, as modulated by gestational stage of the host) guarantees their being chosen as immunogens for vaccine development [17].

A commercial vaccine (comprised of inactivated tachyzoites) was recently tested for efficacy in various countries. However, in addition to the desirable result of reducing abortion rates by more than 50%, a possible link to increased embryonic death was verified, causing the vaccine to be withdrawn from sale in certain markets [15,18].

In order to provide the agricultural sector with a more effective, safe, and economical alternative for neosporosis control, recombinant vaccines (with diverse targets and formulations) are widely studied in research centers around the world [16,17,19,20].

## Analytical Discussion

### *N. caninum* invasion mechanisms

Belonging to the phylum Apicomplexa, *N. caninum* is an obligate intracellular parasite that has differing mechanisms directed toward the invasion process, the "apical complex" characteristic which gives its name to the phylum, consists of a collection of protein filaments and secretory organelles [21]. The available literature addresses certain expression patterns related to *N. caninum* virulence factors (along its life cycle stages), and differing environmental conditions. The importance of knowing such patterns in order to develop subunit vaccines, as well as the possible immune system responses induced is emphasized [22-24].

Tachyzoites move up through the extracellular matrix and seek (through receptor recognition) the best place for cell invasion. In this initial stage of the relationship between *N. caninum* and the host cell, there are only low affinity contacts with the surface membrane of the target cell, followed by more stable adhesions between them. At this stage SAG type proteins maintain this reversible contact [25]. Upon cell invasion, the tachyzoites reorient themselves perpendicularly to the cell surface membrane, where secretory organelles (micronemes and rhoptries) belonging to the apical complex release, through exocytosis, cell surface adhesive proteins that bind to glycan receptors and establish a strong and specific contact. At this time there is also an increase in parasite cytosolic $Ca^{2+}$, responsible for this, and other important events in the host-parasite relationship [25,26]. The proteins mediate protein-protein or protein-carbohydrate interactions via; lectin-like, integrin (I)-like, *thrombospondin (TSP)-like, and epidermal growth factor* (EGF)-like domains [26].

ROPs and MICs insert themselves into the plasma membrane, and enable the formation of the AMA-1 receptor, a conserved microneme protein related to "motor junction" assembly, which is responsible for the transit of *N. caninum* into the cytoplasm [21,27].

The motor junction is translocated through the parasite surface to its posterior pole while MIC proteins are released via rhomboid protease activity, ultimately forcing the engulfment of the parasite by the cell. The formation of a parasitophorous vacuole (VP), protects the parasite from the cell's defenses, the parasitophorous vacuole membrane (MVP) is constituted of the cellular membrane itself, result of the pathogen's entry into the host cell [25].

The VP is responsible for preventing the action of lysosomes, allowing *N. caninum* to multiply until reaching an intracellular critical mass, which eventually causes the rupture of the host cell. Dense granule proteins (GRA) modify the MVP and participate in the formation of an intra-vacuolar tubular-vesicle network considered to be the VP matrix, formed by lipids. The parasitophorous vacuole locates adjacently to the mitochondria and endoplasmic reticulum of the host cell [26,28].

### Surface antigens (SAGs)

The initial contact in parasite-cell interaction is mediated, in part, by two main immunodominant antigens: NcSAG1 (surface antigen-1), and NcSRS2 (SAG1-related sequence) [29]. These protein families play a fundamental role in the virulence of the parasite. The SAG1 family of proteins consists of components crucial to virulence, and is characterized by the presence of two linked disulfide domains SRS [30]. NcSRS2 is located in both the outer and inner membranes of *N. caninum* tachyzoites [29], and is also associated with dense granules and rhoptries, which relate to the connection process and entry into the host cell. From *in silico* analyses; a surface protein of 43 kDa, is predicted; containing 401 amino acids, and an Arg-Gly-Asp (RGD) site sequence which is essential for interaction with cell surface receptors [30].

NcSAG1, one of the most immunogenic and studied antigens, is expressed by tachyzoites, and undergoes a decrease in expression levels during the conversion of the parasite to bradizoite [31]. Among the heterologous antigens expressed and tested in vaccine formulations, rNcSAG1 stands out as inducing one of the greatest levels of protection [32].

Ncp29 and Ncp35, antigens, respectively similar to NcSAG1 and NcSRS2, were shown by Howe et al. [33] to be exclusively associated with the tachyzoite phase membrane of the parasite, possessing high immunogenicity and being well preserved. These characteristics make them interesting in the search for new targets, for vaccines and diagnostic tests. It is assumed that surface antigens expressed by bradyzoites such as NcBSR4, act as both protective barriers and as receptors that help in the invasion of the different cell types or as mediators of immune response evasion, enabling the spread of the parasite, as well as its survival [34]

### Microneme proteins (MICS)

Micronemes are "cigar" or spindle-shaped organelles present in large numbers in the apical complex, and along with rhoptries, are responsible for adhesion to the host cell, and localized cell membrane disruption, among other processes [35]. The protein, called "MIC" contains at least one domain that confers adhesive properties, usually related to surface receptors (carbohydrates) or other MIC(s), can possess transmembrane or cytoplasmic sites, or be associated with enzymes [21].

Among the already studied MICs we note NcMIC1, a protein secreted by the parasite as a soluble molecule that interacts with the host cell surface, by binding to specific glycosaminoglycans as part of a "molecular bridge" where different MICs participate, beginning the invasion process [36-38].

In addition to this, another protein, NcMIC2 was analyzed by Lovett et al. [39] in cell cultures infected by the parasite, the study demonstrated a gradual increase in secretion of the protein during rising cultivation temperature (25 to 37°C), as well as when agents were used that increase intracellular calcium. NcMIC2 belongs to a family of proteins that have integrin-like structure, and TSP (thrombospondin) type I sites, related to the parasite-to-cell link.

The immunodominant protein NcMIC3 is distributed over the surface of the parasite, and functions by regulating interactions with molecules of the host cell surface. Through possible physical interaction with actin/myosin, NcMIC3 provides the parasite with the machinery and driving force to actively invade the cell [40].

## Dense granule antigens (GRAs)

Dense granules are membrane bound vesicles containing large amounts of stored granular content. They vary in size, shape, and number within the apicomplex and are sometimes confused with micronemes or rhoptries in electron microscopy sections [41]. They possess antigens that are secreted into the VP during intracellular *N. caninum* tachyzoite development, and are related to nutrient uptake and waste excretion. They also stimulate humoral immunity in the host [42]. As an example of these proteins, NcGRA2 is associated with the tubular net in tachyzoite VPs, and part of the bradyzoite cell wall [43]. Equally important, NcGRA7 is highly immunogenic and related to the initial development of the intracellular parasite, but is also recognized as important during the initial invasion of the host cell [41].

The protein NcMAG1 was described by Guionaud et al. [44], principally in bradyzoites, within the dense granules, either forming the wall, or dispersed in the matrix. This protein contains highly immunogenic portions, which are related to B cell response stimulation.

## Rhoptry proteins (ROPs)

Present in greater numbers than the other organelles, the rhoptries are "club shaped", and have ducts which connect to the extreme pole of the parasite [45]. The rhoptry proteins (based on studies using *T. gondii, N. caninum* and other Apicomplexa parasites), are divided into: the *Rhoptry* neck (RONs) are conserved in the Apicomplexa phylum, and assist in the formation of the VP; the *kinases*, *phosphatases and proteases*, common to eukaryotic cells; and the *Rhoptry bulb* (ROPs), presenting homology only in closely related genres such as *Neospora*, Toxoplasma and *Sarcocystis* [45,46].

The RON complex (from interactions with components of the cell cytoskeleton) is responsible for locating strategic (stable) points to form and anchor the "motor junction" during the invasion of the host cell; and also for preventing lysosome destruction of the VP [47].

It has been suggested that the ROPs participate in VP biogenesis and in modulating the functional properties of the MVP. After secretion, they remain in the VP lumen associated with the MVP, or are released into the cytoplasm of the host cell with the intention of reaching different targets, such as the nucleus. In the ROP protein family, all precursors have signal peptides and largely depend on a “pro-region” cleavage during maturation; parasite migration and VP localization (close to the infected cell nucleus), being possibly related [45,48].

Similar to the *T. gondii* ROPs (TgROP) family, the *N. caninum* (NcROP) family is a group of rhoptry proteins with certain kinase homologies, and possesses all of the residues required to perform this function [49]. Moreover, it has sites which are exposed to the host cell cytosol and are related to the PV’s interaction with elements of the endoplasmic reticulum [50]. NcROP2 is highly conserved, immunodominant, and plays a key role during invasion and in tachyzoite maintenance; and, it has a demonstrated ability to induce host protection response [49,51]. Therefore, it presents itself as an antigen with great potential for use in the development of vaccines against *N. caninum*, and in immunodiagnostic tests.

## *Neospora caninum* and immunity

Abortion is the main clinical manifestation of neosporosis, both in dairy, and beef herds [3,52]. There is no specific time during pregnancy that the abortions occur, but most occur during the fifth or sixth month of pregnancy [11].

Embryo and fetus effects can vary from death, reabsorption, and mummification to clinically normal birth yet with persistent infection [3,11]. The most severe consequences arise when the pregnancy is still in its early months. This can be explained by the immaturity of the fetus immune system, being unable to effectively combat infections. Fetal immune competence begins only after 100 days of gestation, and only after 150 days is the fetus able to recognize and develop an immune response to antigens [51,53].

It has been suggested that (in defense against the parasite), cytotoxic T lymphocytes and pro-inflammatory Th1 cytokines generated by an exaggerated immune response are capable of preventing vascular nutrient distribution, and causing damage to the placenta, especially to the trophoblastic cells, leading to abortion [54,55].

One reason for the abortions is the relationship between the mother’s immune system in combating *N. caninum*, and the fetus. Being an intracellular parasite, *N. caninum* evokes an immune response involving regulatory cytokines, which is of great importance. Pro-inflammatory cytokines such as interferon-γ (IFNγ), tumor necrosis factor (TNF-α), and interleukin 12 (IL-12) may, depending on their levels, provoke a much needed immune response (to limit the proliferation *N. caninum*), but which is harmful to the fetus [56].

IFNγ is responsible for increasing the expression of class I MHC, favoring antigen presentation and increasing the chance that infected cells are recognized with a cytotoxic response. Synergistically TNF-α can act by activating macrophages and natural killer (NK) cells [22]. In this process there is also an increase in IL-12 expression, which induces an increase in the production and proliferation of IFNγ by T lymphocytes and NK cells stimulated by the parasite antigens [57].

IFNγ also has a regulatory role in IL-17 cytokine production which is important in fighting *N. caninum* infections. Peckham et al. [58] demonstrated that γδT17 lymphocytes promote death of cells in co-cultures infected with *N. caninum*, corroborating Flynn & Marshall [59], who linked increased expression of IL-17A with the immunopathology found during neosporosis.

The importance of the immune response based on this cytokine was observed in IL17R-deficient mice infected with *T. gondii*, where parasitic load increases were detected along with reductions in neutrophil numbers. In general, an exacerbated presence of IL-17 producing cells increases tissue damage, revealing its roles, in both protection, and in the pathological effects of the disease [60].

However, during pregnancy, the immune system is modulated such that there is a maternal tolerance to the fetus [61]. The maternal-fetal interface maintains cytokines such as interleukin-10 (IL-10), interleukin 4 (IL-4), and transforming growth factor beta (TGF-β) that operate from a Th2 driven immune response, and participate in decreased production of pro-inflammatory Th1 cytokines [56,62].

One of the major hormones of pregnancy, progesterone [63], (its level progressively increasing during pregnancy), inhibits secretion

(during the second half of gestation) of TNF-α, and pro-inflammatory transcription factor NF-kB (nuclear factor kappa B). Progesterone induces increased expression of its own receptor and stimulates IL-10 production in T lymphocytes, in addition to promoting the development of Th2 cells, which produce IL-5 and IL-4, thus contributing to polarization of a Th2 type response [64,65].

In mice, progesterone involvement has been observed in the modulation of differentiation, maturation, and function of dendritic cells; leading to increased production of these cells in the immature form, which thus express lower levels of T cell receptors ligands and co-stimulatory molecules [63]. This type of immunomodulation is not helpful in protecting against exogenous infection and vertical transmission, and favors parasitic invasion and infection of the fetus [61,66,67].

## Control Measures Based on Vaccination

Looking to elicit an immune response that durably protects against *N. caninum* and is capable of stimulating a properly balance between Th1/Th2, various vaccine prototypes have been and are being tested. As described by Monney et al. [68], an effective vaccine must fill certain requirements, such as: 1) prevent the proliferation of tachyzoites and their spread in the herd during pregnancy, 2) prevent/reduce the excretion of oocysts by definitive hosts, and 3) prevent the formation of tissue cysts in intermediate hosts. For over 15 years, prototypes of inactivated vaccines, live-attenuated, recombinants, and DNA, in biological vectors and with different adjuvants have been tested [15,17-19,27,56]

Adrianarivo et al. [69] conducted experiments with inactivated tachyzoites in formulations with different adjuvants, such as Havlogen®, Polygen®, Havlogen+Bay R-1005, and Montanide ISA 773, thus presenting one of the first works related to the control of the parasite using vaccinations.

The unique vaccine available in the market was comprised of tachyzoite lysate and presented results of close to a 50% decrease in the number of abortions; under study in Costa Rica [70]. However, in subsequent studies, this vaccine increased the chances of embryonic death, and did not prevent placental or fetal infection in vaccinated cows [15,18,56]

Based on these and other studies, the vaccine was removed from the market in certain countries [15,27]. The problems (related to a vaccine containing the inactivated pathogen) can be explained by the variety of antigens present in *N. caninum*, and also that some antigens are responsible for modulating a protective immune response, while others have the opposite effect, favoring the invasion by the parasite and causing damage the host [16].

This was evidenced when using experimental NcMIC4 native protein vaccines (purified from parasite), and recombinant was unable to induce protection and was found responsible for the increased susceptibility of mice to *N. caninum* infection [71].

After immunizing heifers with inactivated tachyzoites, without obtaining a protective immune response, it was suggested that epitopes remained absent or were modified due to changes during the inactivation process, and thus a variety of epitopes not as relevant to the development of a protective immunity had been presented to the immune system [69].

## Live Vaccines

In the search for vaccines against neosporosis, live vaccines (less virulent or genetically modified strains of the parasite) are also studied. As highlighted by Hecker et al. [17], the protection provided by this type of vaccines make them important tools in combating the disease, however, there are limitations because of the possibility of causing chronic infections with subsequent vertical transmission.

The difficulties and risk in cultivating the microorganisms, the possible difference between *in vitro* and *in vivo* patterns of expression, and the differences in protein expression profiles for different stages of the pathogen's life cycle are some of the difficulties and limitations faced by laboratories developing effective vaccines [72]. The use of live attenuated vaccines brings great concern with regard to the strains used and with the methods of attenuation; since there may be a risk of *N. caninum* transmission to the fetus, resulting in congenitally infected animal births [73]. These vaccines also tend to be relatively unstable and have short shelf life [74].

Two isolates, Nc-Nowra [75] and Nc-Spain 1H [76], have been identified as less virulent and when tested in mice, there were no clinical signs or deaths resulting from the disease [17,76].
A naturally attenuated strain Nc-Spain 1H was analyzed by Rojo-Montejo et al. [77] as an experimental vaccine for the prevention of congenital transmission of the Nc-Liv strain in female BALB/c mice. While the non-vaccinated group of animals did show a rate of 84% for post-natal mortality in their litters, the group immunized with two doses of Nc-Spain 1H (at a concentration of $5 \times 10^5$ living tachyzoites) showed a rate of 2.4%. When analyzed by polymerase chain reaction (PCR) the presence of parasite DNA in the brain of immunized animals was not detected. Regarding vertical transmission, the group of non-immunized animals showed a rate of 89.1%, whereas the group of animals that received doses of the attenuated strain, showed a rate of only 2.3% [77].

Other approaches such as isolation of temperature sensitive mutants, irradiation of tachyzoites by gamma ray, attenuation by successive passages in cell culture and genetic manipulation of the parasite, result in vaccine candidates that are under control [78,79].

*N. caninum*, strain NC-1 was attenuated by γ radiation and inoculated in two doses at a concentration of $1 \times 10^6$ tachyzoites for immunization of C57BL6 mice. These were subsequently challenged with tachyzoites at a concentration of $2 \times 10^7$. Serology of the vaccinated animals detected antibody response of isotypes IgG1 and IgG2a, suggesting a balance between Th1/Th2. Splenocyte cultures detected significantly increased levels of IFN-γ and IL-10, while IL-12 and IL-4 were not detected. Followed for 25 days after the challenge, no mice from the vaccinated group had died or presented signs of neosporosis, while these had already occurred at 7 days post challenge for the control group [79].

Using genetic engineering Marugán-Hernandez et al. [80] presented an interesting approach in which tachyzoites were obtained that constitutively express NcSAG4, an important and specific bradyzoite antigen. Thus, they had obtained a strain with low persistence (in the brains of rats), which was capable of inducing immune response against NcSAG4 prior to the encysted stage of the parasite [80]. However, the disadvantages of live attenuated vaccines, such as high costs, the difficulty to produce tachyzoites, and the persistence of chronic infection in vaccinated animals still exist.

## Vaccines using Biological Vectors

Biological vectors such as Herpesvirus and Vacciniavirus, carriers of recombinant protein sequences have aroused the interest of researchers for their efficacy against protozoan infections [17].

Nishikawa et al. [81] constructed vectors (Vacciniavirus) expressing NcSRS2 and NcSAG1 used for immunization of BALB/c mice, that presented high levels of IgG1 antibodies before and during the challenge with *N. caninum* (concentration of $4 \times 10^4$ living tachyzoites). They were sacrificed at 5, 8, and 10 days post-infection for PCR detection of parasite DNA from the total DNA extracted from brains and livers. No DNA was detected in the brains and livers of *N. caninum* vaccinated animals with the vector expressing NcSRS2, different from that observed in those vaccinated with the vector expressing NcSAG1. In liver analyses, neither vector used was capable of promoting a significant difference from the group control [81,82].

Attenuated *Brucella abortus* (RB51 strain) was also used as a vector expressing five immunodominant antigens of *N. caninum*: NcMIC1, NcMIC3, NcGRA2, NcGRA6 and NcSRS2. In this study, with vaccination and subsequent challenge of C57BL/6 mice, the tested proteins (individually or in combination) showed satisfactory results, such as the 90% level protection with RB51-SRS2 and 100% for RB51-GRA6 [32]. Also, the ability to induce a predominantly cellular immune response was revealed while combining both *N. caninum* and *B. abortus* antigens, (aiming to control two important abortive bovine diseases) an interesting approach [32]. However, using biological vectors, particularly viruses, requires high levels of biosafety, vectors with reduced or eliminated pathogenicity are essential to avoid damage to the immunized organisms [83].

## DNA Vaccines

Vaccines based on plasmids containing gene sequences from pathogen antigens have joined the list of candidates toward the development of neosporosis control. Monney et al. [68] set up associations (chimeras) of partial gene sequences from three different proteins, NcMIC1, NcMIC3, and NcROP2 using regions predicted to be more immunogenic and built four different sequences for expression: NcMIC3E-NcMIC1E, NcROP2E-NcMIC3E-NcMIC1E, NcMIC3E-NcROP2E -NcMIC1E, NcMIC3E-NcMIC1E-NcROP2E. Even containing the same parts of these antigens, the association in the form of NcMIC3E-NcMIC1E-NcROP2E showed an immune response able to protect 100% of animals after challenge with *N. caninum*; this was not achieved by the other chimeras, which induced no protection above 70%. Both constructs stimulated a response based on IL-4 with low IFN-γ levels (Th2) during vaccination, and subsequently, when challenged with the parasite, IL-4 levels were lower than those for IFN-γ, similar to that found in the control group [68].

Cannas et al. [84] immunizing mice with DNA vaccines containing NcSRS2 or NcSAG1, followed by a booster with recombinant SRS2 (rSRS2) and SAG1 (rSAG1) obtained respective protection levels of 60% and 75% for mice challenged with *N. caninum.* Another study also developed a DNA vaccine containing the gene sequence NcSRS2 for immunization of BALB/c mice (with subsequent splenocyte cultivation) [85]. In this study, the researchers reported a low specific response to the protein in ELISA, but observed an increase in the concentrations of nitric oxide, IL-2, and IFN-γ in cell cultures stimulated with recombinant NcSRS2.

Liddell et al. [86] constructed two plasmids for expression of different proteins: NcGRA7 belonging to the dense granules of tachyzoites, and NcsHSP (a small heat shock protein), both little studied, but having highly regulated expression during parasite development. *In vitro* tests using human fibroblast cells transformed with pCMVi-NcGRA7 and pCMVi-NcsHSP plasmids confirmed the expression of these proteins, and vaccinations carried out with the recombinant proteins in BALB/c pregnant female mice conferred partial protection against congenital transmission of the parasite. The animals vaccinated with plasmids were subsequently challenged with *N. caninum* and when compared with the controls, they yielded litters with about 50% fewer pups (PCR) positive for the parasite.

## Recombinant Subunit Vaccines

The expression of peptides/proteins in recombinant expression systems such as plants, viruses, fungi, bacteria, insect and animal cells for use as subunit vaccines has aroused the interest of researchers [87].

The use of isolated peptides (containing important immune response epitopes) in vaccines can stimulate protection (with a response according to each tested peptide), yet without the antagonistic effects or immune reactions often caused by other structures of the pathogen. When combined (fused peptide fractions), they can lead to better responses in certain diseases, or orient the immunization towards more than one type of pathogen strain or serotype [68,88,89]. Immunization of experimental animals has become a major tool for protection analysis and for seroconversions (starting from such fractions) [15,17].

Three different recombinant proteins of *N. caninum* have been expressed in *E. coli*; rNcPDI, rNcROP2 and rNcMAG1. These proteins were inoculated by intranasal and intraperitoneal routes in C57B1/6 mice, seeking to evaluate which of these would be the best to stimulate a protective response against the challenge. Analysis of the data suggests that immunization pathways play an important role in the induction of specific immunity; 28 days after infection, rNcPDI intraperitoneal administration was rated at 20% protection, while intranasal administration conferred 90% protection. Conversely, rNcMAG1 revealed 10% protection in the same period using intranasal inoculation and 50% when applied intraperitoneally. Regardless of the route of administration, the rNcROP2 protein conferred protection levels above 60%, even at 28 days from the challenge [90].

Experimental vaccines were developed using recombinant proteins rNcROP2, rNcROP40, rNcGRA7, and rNcNTPase, and combinations of these expressed in *E. coli* to evaluate humoral immune response and the protection afforded against the vertical transmission process in female BALB/c mice. The animals were vaccinated and challenged with the antigens and with the Nc-Liverpool strain prior to the gestation period, thus permitting parasite congenital transmission evaluation. The rNcROP2 protein represented an increase of 6.3% in offspring survival rate, while the chimera rNcROP2/NcROP40 showed a 16.2% increase, these results contrast to the other vaccinated groups rNcROP40, rNcGRA7, rNcNTPase, and rNcGRA7/NcNTPase that at 30 days postnatal, showed a 100% mortality rate for the litter. All of the mice groups showed an immune response based on IgG1, and from splenocytes of the vaccinated animal groups, the group inoculated with rNcROP2 had a significantly higher production of IFN-γ when compared to the others [91].

From phage libraries, two host cell binding proteins, NcGRA7 and the first description of NcP78, a 78 kDa protein were isolated and

expressed, and separately inoculated in BALB/c mice in 3 doses at a 2 week interval. After the first immunization, IgG1 levels were significantly increased in both groups vaccinated with NcGRA7 NcP78, while IgG2a levels in the groups increased significantly after the 3rd immunization. Further, using sera from the immunized animals, the inhibitory effect was evaluated in Vero cell invasion by *N. caninum*, demonstrating that sera of the NcGRA7 immunized group were more effective (*in vitro* inhibition) [92].

The same expression system (*E. coli*) was used to obtain four recombinant bradizoite proteins: rNcBAG1, rNcBSR4, rNcMAG1 and rNcSAG4. Mice were vaccinated with two doses in separate groups, with formulations containing the test protein plus oil-in-water adjuvant; after five weeks they were challenged with *N. caninum* tachyzoites. The groups vaccinated with rBAG1, rMAG1 and rNcSAG4 showed few or mild clinical signs of disease, whereas those vaccinated with NcBSR4 developed a severe form of the disease [93].

## Reverse Vaccinology

An *in silico* approach for finding immunogenic fractions of subunits can be more interesting than searching in traditional attenuated or inactivated vaccines. In this type of analysis, it is possible to predict which proteins among the various present in the parasite are able to stimulate a desired immune system activity; from T lymphocyte epitopes (which can bind to the major histocompatibility complex (MHC), or from surface or secreted proteins containing lymphocyte B epitopes [22].

The sequencing of the genome and transcriptome of *N. caninum* Liverpool strain tachyzoites helped in the study of new peptides targeted for synthesis or recombinant expression beginning with a comparison to sequences of *T. gondii* in search of similarities and differences [23]. Reverse pan-genome vaccinology analyzes different genomes of the same species of pathogen, seeking genetic variability patterns that can be taken into consideration during the construction of a vaccine. The pan-genome can be defined as a global gene repertoire belonging to the species, and divided into pieces, such as "core-genome" which concentrates invariant and conserved genes, "genome dispensable" for genes that are present in some but not all strains, and "strain-specific," genes those present in a single isolated strain [94].

The availability of microorganism gene sequences in databases enabled the use of "Reverse Vaccinology" which, when using *in silico* analysis may predict target proteins that contain important epitopes for inducing a protective immune response by the host, demonstrating an alternative to using attenuated or inactivated vaccines [95].

The potential of the “omes” (genome, transcriptome, proteome, and metabolome), is both recognized and used as a tool to understand pathogens, and in the search for vaccines and drug targets. The information available on *N. caninum* reveals that the strains are extremely similar, yet there are significant differences in parasite behavior, especially as related to virulence [14].

However, as of yet, there are no reports on the use of peptides designed and synthesized by reverse vaccinology with the aim of stimulating an immune response against *N. caninum* in cows.

## Conclusion

The main problem of neosporosis in cattle is its persistence by means of vertical transmission in asymptomatic animals, this, especially in the absence of a definitive host. Moreover, the economic impact of neosporosis, is mainly due to its causing abortions as a result of this same asymptomatic transmission [54], because there are no further expenses (such as those with control programs), when disposing infected females from the herd, even when including their treatment.

Seeking to control infection and transmission of this disease in cattle herds, various experimental vaccines are being developed with formulations containing; living parasite [76,96], biological vectors [97,98], DNA vaccines [68,85], and recombinant subunit vaccines, (usually proteins related to the invasion process) [92,93].

Assays based on immunization in animals models (usually mice) focus on the ability of these vaccines to induce a protective response which prevents infection by the parasite, (and especially its vertical transmission), through stimulating cytokine production, critical for both the cell mediated immune response (e.g., IFN-γ and TNF-α) and humoral immune response (e.g., IL-4 and IL-10).

In the literature, different levels of protection induced by vaccination are observed. Some vaccines provide up to 100% protection where in all animals of a challenged group remain without any clinical signs of the disease [68]. Others are unable to stimulate the immune system to fight effectively against congenital tachyzoite transmission and infection [69].

However, for control of neosporosis, among other relevant factors involved in the process of vaccine efficiency analysis, it is important to note that the varied studies usually differ in the following: per dose protein concentrations, number of immunizations, inoculation method, vaccine adjuvant, animal model, challenge strain, and concentrations of tachyzoites. These variables present difficulties when comparing levels of protection conferred by the vaccines tested; this type of study should be done judiciously, while considering the aforementioned variables [22]. Moreover, even if a vaccine has been tested in murine models and presented effective protection against neosporosis, it should still be tested and validated in bovine animal models. There remains a gulf between the vaccine prototypes being surveyed today and the availability of a product able to control bovine neosporosis.

The search for experimental vaccine candidates using conventional vaccinology, (based on the culture of pathogens and further purification of their protein structures), is still an active field research [88]. However, with the availability of annotated genomic sequences, transcriptomic, and proteomic databases as made possible through bioinformatics, the prediction of antigens with more immunogenic potential, known as reverse vaccinology has become possible [94].

*In silico* analyses were performed by Monney et al. [68] to predict peptides capable of inducing a protective immune response; and, after definition of the amino acid sequences, the authors developed chimeras for murine immunization models. Similar to this group, our research group has studied experimental vaccines, in addition to immunodiagnostic tests using immunodominant regions and chimera proteins such as NcROP2 and NcSRS2 [99-101], associated with immune response-modulating proteins, such as LTB (the B subunit of *E. coli* heat-labile enterotoxin), and OprI (outer membrane lipoprotein of *Pseudomonas aeruginosa*) has already been described .

LTB is a valid alternative for the carrier molecule function, since: it is composed of five identical polypeptides (with a molecular weight of 11.6 kDa each), is able to modulate the immune response, and is

characterized as a potent mucosal adjuvant [102]. OprI is a TLR2 ligand, with humoral and cellular immune response modulation characteristics, having potential use as a mucosal adjuvant [103-106].

In the search to reduce economic impacts, it is common to refer to herd abortion decreases as a positive response to the vaccine. However, one must consider that abortion prevents the spread of *N. caninum*, and that the decrease in the abortion rate does not necessarily exclude vertical transmission, and that the vaccine may have influenced the infection of the fetus, perhaps by inhibiting the action of a molecule responsible for infection pathogenicity, reducing its potential.

We believe that a solution can be obtained through activation of the immune system mucous IgA secretory cells, inhibiting intestinal invasion by sporozoites by applying vaccinations via the mucous membranes. However, even if you obtain a vaccine to protect cattle against infection with sporozoites, the infected animals will still have to be separated from reproduction and go to slaughter. Only then would a reduction in the spread of the parasite be achieved.

Simple alternatives such as using different adjuvants could also be helpful. By using Montanide, which is a water/oil microemulsion comprised of stabilized squalene with surfactant [107], a high antibody secretion, high T cell proliferation, and a balanced profile of Th1/Th2 cytokines stimulating an effective immune response against the spread of *N. caninum* would be possible. This adjuvant is also known to increase the recruitment, activity, and migration of antigen presenting cells to the lymph nodes, as well as for interacting with cell membranes, promoting the capture of antigens [108].

Thus, an effective vaccine should not only decrease miscarriages, but inhibit infection and reduce the spread of *N. caninum*. The disease could then be controlled and neosporosis abortion cases reduced without impact on breeding cows.

More research is needed to increase the range of today's existing antigen options. Further, it remains necessary to test promising vaccines in cattle. However, before testing, a secure system must be determined, with challenge standardization, in order to compare studies, and reach as accurate a comparison as possible.

Still, we believe that the existing gap in the market for reliable and high efficiency vaccines will soon be supplemented by the development of biotechnological products that upon reaching the market will then be applied in cattle farming as an alternative to fight neosporosis.

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## Conflict of Interest

The authors declare that there is no conflict of interest with regard to this work.

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