Approaches for the vaccination and treatment of *Neospora caninum* infections in mice and ruminant models

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SUMMARY

Neospora caninum is a leading cause of abortion in cattle, and is thus an important veterinary health problem of high economic significance. Vaccination has been considered a viable strategy to prevent bovine neosporosis. Different approaches have been investigated, and to date the most promising results have been achieved with live-attenuated vaccines. Subunit vaccines have also been studied, and most of them represented components that are functionally involved in (i) the physical interaction between the parasite and its host cell during invasion or (ii) tachyzoite-to-bradyzoite stage conversion. Drugs have been considered as an option to limit the effects of vertical transmission of *N. caninum*. Promising results with a small panel of compounds in small laboratory animal models indicate the potential value of a chemotherapeutical approach for the prevention of neosporosis in ruminants. For both, vaccines and drugs, the key for success in preventing vertical transmission lies in the application of bioactive compounds that limit parasite proliferation and dissemination, without endangering the developing fetus not only during an exogenous acute infection but also during recrudescence of a chronic infection. In this review, the current status of vaccine and drug development is presented and novel strategies against neosporosis are discussed.

Key words: Apicomplexa, *Neospora*, anti-infective agents, vaccine, chemotherapy, immunotherapy.

INTRODUCTION

Apicomplexan parasites are responsible for a variety of diseases, not only in humans, but also in pets and/or farm animals. Among them are vector-borne diseases caused by *Babesia* and *Theileria* (both transmitted by ticks), *Besnoitia* (transmitted mechanically by biting insects), and orally transmitted parasitoses caused e.g. by *Cryptosporidium, Eimeria* and the cyst-forming coccidians *Sarcocystis, Neospora* and *Toxoplasma*. Diseases caused by these parasites are of great socio-economic impact worldwide (Müller and Hemphill, 2013*a*). *Neospora caninum* is phylogenetically closely related to *Toxoplasma gondii*, but several biological features distinguish these two species, including elements of their life cycle, host range, pathogenicity and ultrastructure (Buxton *et al*. 2002; Dubey, 2003; Dubey *et al*. 2007). Canids, namely dogs, wolves, dingoes and coyotes, represent definitive hosts of *N. caninum* (Buxton *et al*. 2002), while for *Toxoplasma* sexual processes are restricted to felid hosts. Both are capable of infecting and proliferating within a wide range of intermediate hosts, including cattle, sheep, goats and many more (Buxton *et al*. 2002; Dubey, 2003). Extensive proliferation can lead to cellular destruction, tissue damage and immunopathology and thus disease within these hosts. Besides infecting animals, *Toxoplasma* is also a human pathogen, most notably in immunosuppressed individuals, and when primary infection is acquired during pregnancy this can lead to abortion, hydrocephalus and other serious diseases in newborns (Saadatnia and Golkar, 2012). On contrary, *Neospora* infections in humans have never been demonstrated, but the parasite owes its importance primarily to the fact that it causes abortion in cattle, and stillbirth and/or birth of weak calves. *Toxoplasma* on the other hand is a highly important abortion-causing pathogen in sheep, but appears not so important in causing abortion in cattle (Dubey and Lindsay, 1990; Hemphill *et al*. 2006; McAllister, 2014). Natural *N. caninum* infection in sheep and *Neospora*-induced abortion problems in respective flocks have also been reported, albeit less frequently (Moreno *et al*. 2012; Gonzalez-Warleta *et al*. 2014).

Analogous to *T. gondii,* three infective stages of *N. caninum* have been identified. The proliferative and disease-causing stage is represented by tachyzoites, which can invade, and replicate within, a wide range of cell types and tissues, both *in vitro* and *in vivo* (Hemphill *et al*. 2006; Monney and Hemphill, 2014). Upon immunological and/or physiological pressure, the cyst-forming bradyzoites, are formed, which replicate much more slowly, and secrete components that lead to the formation of intracellular cysts, surrounded by a cyst wall. The third invasive stage, the sporozoites, represent the end products of a sexual process, which takes place

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in the intestine of the definitive host followed by sporulation in the environment. *Neospora caninum* also undergoes a sylvatic life cycle (Rosypal and Lindsay, 2005; Gondim, 2006), however, the importance of these parasites in wildlife as a reservoir for the transmission to domestic animals has not been definitely elucidated (Donahoe *et al*. 2015).

Within 10 countries alone, infection of pregnant cattle with *N. caninum* causes annual losses of around 1·3 billion US dollars through abortion, stillbirth, or birth of weak offspring (Reichel *et al*. 2013, 2014). In addition, *N. caninum* infection can result in birth of clinically healthy but persistently infected calves, which in turn may vertically transmit the parasite to the next generation. Besides management options aimed at reducing exposure, a number of control options to limit the economic impact of neosporosis have been proposed and modelled. Among these are (i) testing and culling of seropositive animals, (ii) discontinued breeding with offspring from seropositive cows, (iii) vaccination of susceptible and infected animals and (iv) chemotherapeutic treatment of calves from seropositive cows (Häsler *et al*. 2006*a*, *b*). In addition, embryo transfer could be another option, although only for cattle of a high genetic value. In the case of neosporosis, the ideal control options should be both highly effective and economical, thus prior to deciding on a control strategy, the economic impact has to be evaluated for each case (Larson *et al*. 2004; Häsler *et al*. 2006*a*; Reichel and Ellis, 2006). The costs of the management practices used for the control of neosporosis indicate that vaccination could be the most efficient intervention strategy (Reichel and Ellis, 2009).

In addition, modelling has shown that none of the control strategies studied to date would result in a sero-prevalence of zero due to the fact that horizontal transmission exists and control of vertical transmission will not eliminate the parasite. Thus, both vertical transmission within a herd as well as the horizontal transmission through oocyst shedding by canids, needs to be addressed (Häsler *et al*. 2008). In this context, the development of efficacious treatment options and vaccines for dogs, although not within the major focus of investigations so far, also represent interesting aspects (Monney and Hemphill, 2014).

In this paper we provide an overview on the development of vaccines and drugs for the prevention and/or treatment of *N. caninum* infections. Studies performed in laboratory models such as mice, and investigations in cattle and sheep, will be reviewed. Where appropriate, parallels to related apicomplexan parasites are highlighted.

EXPERIMENTAL ANIMAL MODELS TO STUDY VACCINES AND DRUGS AGAINST NEOSPOROSIS

There is a general consensus that small laboratory animals such as mice do not accurately reflect the situation that occurs during *N. caninum* infection in cattle. Physiological differences can account for discrepancies in drug efficacy, tolerance and/or toxicity and immunological differences between ruminant and mice hamper the use of small laboratory animals as models for a vaccine to be used in cattle (Monney and Hemphill, 2014). For instance, while antibodies can pass the placental tissue in mice, this is not the case in cattle. In mice, an increase of IFN-γ production is correlated to increases of IgG2a- and IgG3-synthesis, increased IL-4 production is associated with increased IgG1- and IgE-levels, and elevated TGF-β is associated with higher IgA responses. In cattle, the situation is less polarized and the classical roles of many cytokines in the laboratory mouse do not extrapolate entirely, or at all, to the situation in cattle (Estes and Brown, 2002). Compared with mice, cattle have a higher number of circulating γδ T cells. These cells can respond to antigens and pathogen associated molecular patterns (PAMPs) from different disease agents such as tuberculosis (Telfer and Baldwin, 2015), and may play a role in *Neospora* infections. In addition, *Neospora*-specific cytotoxic lymphocytes are CD4(+), while cytotoxic T cells are CD8(+) in the murine model (Staska *et al*. 2005).

Nevertheless, by far the largest numbers of experimental vaccinations and drug treatment studies have been carried out in mice (see Tables 1 and 3 for a summary of selected studies), since experiments in small animals are much more cost-effective, and despite the obvious setbacks can still provide initial information on potentially protective effects for promising vaccine and/or drug formulations. Two types of murine models are available, (i) the non-pregnant mouse model for the assessment of acute disease and/or CNS infection and (ii) the pregnant mouse model that mimics exogenous transplacental infection (Williams and Trees, 2006; Williams *et al*. 2009). For the latter, mice are made pregnant and then challenged during pregnancy, and the drug- or vaccine mediated protection of dams and offspring is assessed. Despite considerable efforts there is no laboratory animal that allows efficient monitoring of endogenous transplacental infection, namely infection of the fetus following recrudescence of *N. caninum* infection during pregnancy (Jimenez-Ruiz *et al*. 2013*a*, *b*).

Vaccination studies have been performed using live-attenuated *N. caninum* tachyzoite isolates, tachyzoite extracts, or specific polypeptides expressed in various systems. In general terms, typical vaccine trials in mice comprise different sequential steps, that include (i) verification that the test animals are not seropositive for *N. caninum* and/or already infected with a related pathogen; (ii) immunization and boosts with the vaccine formulation to be tested by different routes, and control animals receiving a placebo application; (iii)

Table 1. Overviews on selected vaccine studies on neosporosis carried out in the mouse model

Vaccine	Reference	Set-up	Results
NC-1 tachyzoites	(Kasper and Khan, 1998)	A/J, Balbc, C57BL/6; vaccinated with live NC-1 tachyzoites; challenge after various days with *T. gondii* tachyzoites	Complete protection in mice vaccinated with *N. caninum* against acute infection by *T. gondii*. Early stimulation of $CD8^+$ T-cells
SAG1, SRS2 (rE, DNA) alone or combined; crude somatic antigen.	(Cannas *et al*. 2003*a*)	C57BL/6, vaccine + RIBI (2×), challenge 28 days after the first injection (proteins). pcDNA vector with genes i.m., challenge 69 days after first injection. Euthanasia after 21 days	Protection with crude antigen. No protection with recombinant antigens as compared with adjuvant control. Protection with pcDNA in combination with recombinant antigens
MIC3 (rE)	(Cannas *et al*. 2003*b*)	C57BL/6, vaccinated with MIC3+RIBI (3× i.p.), challenge 7 days after last boost, euthanasia after 21 days	Reduced cerebral infection in MIC3 vaccinated mice as compared with adjuvant control. Th2-type humoral response associated with protection
MIC1 (rE and DNA)	(Alaeddine *et al*. 2005)	C57BL/6, recMIC1 (3× i.p.), pcDNA-MIC1 (3× i.m.) alone or in combination. Challenge with Nc Liverpool, euthanasia after 21 days	No clinical symptoms in vaccinated mice. Cerebral infection reduced in mice vaccinated with recombinant protein, enhanced in group with combined vaccination
SRS2 (native)	(Haldorson *et al*. 2005)	BALB/c, vaccinated with native protein. Mating, challenge during pregnancy	Decreased frequency of transmission in vaccinated dams, associated with Th2 type immune response
NC-Nowra tachyzoites (live, crude extracts)	(Miller *et al*. 2005)	Outbred Qs, vaccinated with tachyzoites or crude extracts before pregnancy, challenged with Nc Liverpool	Reduction of transmission to pups by live vaccine and to a lower extent by crude extracts
NC-1 tachyzoites (γ-irradiated)	(Ramamoorthy *et al*. 2006)	C57BL/6, vaccinated with irradiated tachyzoites (2×). Challenge 6 weeks after last boost (lethal; 10^7; sublethal, 10^6), euthanasia 25 days after challenge	All lethally challenged control mice died within 1 week, all vaccinated mice survived. Protection associated with mixed Th1/Th2 response
MIC1, MIC3, GRA2, GRA6, SRS2 (expressed in *Brucella abortus* vaccine strain)	(Ramamoorthy *et al*. 2007*b*)	C57BL/6, vaccinated with live *B. abortus* expressing the antigens (2×), lethal challenge (Ramamoorthy *et al*. 2007*a*) 14 days after last boost. Euthanasia after 28 days	All control mice died within 8 days. Complete protection by MIC1 and GRA6
MIC1, MIC3, GRA2, GRA6, SRS2 (in *Brucella abortus*), irradiated tachyzoites.	(Ramamoorthy *et al*. 2007*c*)	C57BL/6, vaccinated twice with live *B. abortus* expressing the antigens, mating, sublethal challenge (Ramamoorthy *et al*. 2007*a*). Euthanasia of pups after 21 days	Protection against vertical transmission by *B. abortus* expressing antigens
MIC4 (native, rE, DNA)	(Srinivasan *et al*. 2007)	C57BL/6, vaccinated 3× in 4-week-intervals, sublethal challenge, euthanasia after 21 days	Mice of all vaccinated groups showed neosporosis symptoms and had an increased mortality as compared with the control group
ROP2 (rE)	(Debache *et al*. 2008)	C57BL/6, vaccinated 3× in 2-week-intervals either with Freud's incomplete or saponin, challenge, euthanasia after 35 days	No symptoms in vaccinated mice, reduced parasite burden in brains of vaccinated mice. Th1- or Th2-type humoral response depending on the adjuvant
ROP2 + MIC1 + MIC3	(Debache *et al*. 2009)	BALB/c, single vaccines or in combination (applied 3×), mating, challenge at day 7 post mating (Lopez-Perez *et al*. 2008), euthanasia of dams and non-pregnant mice after 1 month p.p.	Reduced vertical transmission by ROP2 alone or in combination. Humoral and cytokine responses associated with a Th2 immune response
Nc expressing TgSAG1	(Zhang *et al*. 2010)	Balbc, vaccinated 2× with 10^5 NC-1 expressing TgSAG1 or GFP. Challenge with 500 *T. gondii* tachyzoites at 4 weeks after last boost	Moderate protection by Nc/GFP, good protection by Nc/TgSAG1. Immune response Th1-dominant
PDI, ROP2, MAG1 (rE)	(Debache *et al*. 2010)	C57/BL6, vaccinated with saponin as adjuvant (i.p.) or intranasally (i.n.) with cholera toxin as ajuvant (3×, 15 days intervals). Challenge 2 weeks after last boost, euthanasia after 28 days	Reduced cerebral loads with ROP1 (i. p., i. n.) and PDI (i.n. only). Protection against clinical symptoms only by i. n. PDI vaccine

Table 1. (*Cont.*)

Vaccine	Reference	Set-up	Results
Nc expressing NcSAG4	(Marugan-Hernandez *et al*. 2011)	Female Balbc, vaccinated twice with NC-1 expressing SAG4, some were mated, challenge at day 7 of gestation	Protection against vertical transmission by NC-1 wild type and NC-1 expressing SAG4, not associated with constant Th1- or Th2-type-immune response
Cyclophilin, SRS2 (rE)	(Tuo *et al*. 2011)	Female Balb/c, antigens alone or in combination with adjuvants (s.c., 2×, 2-week intervals). Control with irrelevant bacterial antigen. Challenge 3 weeks after last boost. Euthanasia 3 weeks after challenge	Humoral response against antigens. Higher protection against cerebral infection when cyclophilin was present. Lower protection with SRS2 alone
MIC1-MIC3-ROP2 (chimeric, rE)	(Monney *et al*. 2011)	Female Balb/c, immunized with combinations of antigenic domains from MIC1, MIC3 and ROP2 with saponin as adjuvant (i. p. 3×, 2-week-intervals). Challenge 2 weeks after last boost. Euthanasia 36 days post-challenge	Complete protection by one combination only (MIC3-1-R), correlated with lower parasite load in brains
Nc tachyzoites (live)	(Rojo-Montejo *et al*. 2012)	Female Balb/c, immunized with live Nc Spain H-1 tachyzoites (s. c. 2× at 3-week-intervals). For pregnant model, mating, challenge ad mid-gestation with Nc Liv	Reduction of neonatal mortality, reduction of vertical transmission, lower cerebral parasite load in non-pregnant mice
GRA7, SAG4, BSR4, SRS9 (rE)	(Jimenez-Ruiz *et al*. 2012)	Female Balb/c, pregnant, non-pregnant. Vaccinated with recombinant proteins encapsulated in poly-epsilon-caprolactone	High morbidity and mortality. No protection against vertical transmission. Low IFN-γ levels
Nc tachyzoite extract	(Mansilla *et al*. 2012)	Balb/c, vaccinated with different amounts of extract formulated with various adjuvants (s. c. 2×, 2-week-intervals). Challenge at 38 days post-vaccination with NC-1. Euthanasia 21 days post-challenge	Immune responses depend on formulation, protection not
MIC3-MIC1- ROP2 chimeric (rE)	(Monney *et al*. 2013)	Balbc, vaccine + saponin or Freund's incomplete (3×), non-pregnant or pregnant	Protection in combination with saponin in the non-pregnant model, associated with Th1/Th2 response. No protection in pregnant model. With Freund's, limited or no effects, Th1 response
SRS2-GRA7-fusion in adenovirus	(Jia *et al*. 2013)	Balbc, three times vaccinated (i. m.) with recombinant adenovirus containing the fusion or GFP as a control. No challenge	IFN-γ- and IL-4 levels elevated in adenovirus infected mice as compared with control mice. Higher levels with SRS2-GRA7-fusion than with GFP
BAG1, BSR4, MAG1, SAG4 (rE)	(Uchida *et al*. 2013)	Balbc, vaccinated with four bradyzoite antigens with PBS or bitter gourd extract as adjuvants (i. m., 2×). Challenge with Nc Liv at 3 weeks after last boost	Antigen specific IgG1 and IgG2 and IFN-γ-responses to all antigens. Protection from acute infection and lower parasite load in mice vaccinated with BAG1, MAG1 and SAG4
PDI (rE)	(Debache and Hemphill, 2013)	Balb/c, female, vaccinated with PDI and cholera toxin (subunits A and B or subunit B alone) as adjuvants, i. n. application (3×, 2-week intervals), mating, challenge at day 7 post mating	Good protection against cerebral infection by PDI with subunits A and B as compared with cholera toxin alone. No effect with subunit B alone. No protection against vertical transmission. Controls without cholera toxin are lacking
SAG1, SRS2, MIC3 (*B. mori* nucleopolyhedrovirus)	(Kato *et al*. 2014)	Mice, vaccinated with BmNPV displaying antigens or wild tpye BmNPV (i. m., 3× at 2-week-intervals). Challenge with Nc Liv after last challenge	IgG2-biased humoral response to antigens. Reduced parasite load in brains in all groups vaccinated with NPV as compared with placebo. No effect due to displayed antigens
ROP2 + ROP40 + GRA7 + NTPase	(Pastor-Fernandez *et al*. 2015)	Balb/c, female, vaccinated with recombinant antigens in QuilA adjuvant, mating, challenge	Protection by ROP2 and ROP2 + ROP40

rE, recombinantly expressed in *E. coli*; DNA, DNA vaccine; s.c., subcutaneous; i. p., intraperitoneal; i. m., intramuscular; i. n., intranasal; p. p., post-partum.

challenge of animals with a defined inoculum of *N. caninum* tachyzoites; (iv) monitoring of clinical signs linked to acute or chronic neosporosis. Assessments include survival of dams and offspring, neurological symptoms, parasite burdens in various organs, especially the brain, and humoral and cellular immune responses. In the case of vaccination studies in the pregnant model, mice are usually mated a few weeks following the final vaccination, and experimentally infected with *N. caninum* tachyzoites of a virulent isolate between days 7 and 9 post-mating, which produces almost 100% vertical transmission and mortality after 1 month post-partum in the offspring from non-vaccinated dams (Lopez-Perez *et al*. 2008). Experiments involving the evaluation of drug candidates follow a similar scheme, also ensuring that all animals are sero-negative, but omitting the vaccination steps. Treatments with the compounds of interest are initiated ideally 2–3 days post-infection, in order to give the parasite some time to establish before it encounters the drug treatment. Since it cannot be ruled out that a given compound affects pregnancy and offspring, in some cases, controls with uninfected dams have to be included. Ideally, in both vaccine and drug studies using the pregnant mouse model, the fate of offspring mice is followed-up over a period of 1 month post-partum (Arranz-Solís *et al*. 2015).

A major setback of many experimental studies in mice carried out to date is the fact that they cannot be accurately compared with each other, since they lack standardization. Different groups have worked with different mouse strains, different parasite isolates; have employed different culture techniques and varying routes of inoculation. With this in mind, defined conditions for the standardization and refinement of the pregnant BALB/c mouse model for *N. caninum* infection, employing the virulent Nc-Spain7 isolate have recently been suggested (Arranz-Solís *et al*. 2015). The key finding of this study were: (i) a challenge dose of 10^5 *N. caninum* tachyzoites produced identical results as so far most frequently employed dose of 2×10^6 *N. caninum* tachyzoites; and (ii) inoculation of 100 tachyzoites still resulted in 76% pup mortality at 1 month post-partum. This indicated that most experimental infections in mice carried out so far have used an unrealistically high challenge dose, rendering the identification of immuno-protective antigens or antigen-combinations, or bioactive compounds, a difficult undertaking.

Only few groups have taken on the heroic task to perform experimental vaccinations/challenge infections and even field trials in cattle, some of which are summarized in Table 2. Naturally, such trials require much larger financial resources compared with studies in small laboratory animals, but have the advantage that they are carried out in the natural and economically most important host species. Basically, two experimental vaccine strategies have been followed more recently: (i) vaccination of non-pregnant animals prior to pregnancy and with naturally attenuated low-virulence *N. caninum* isolates (e.g. NcSpain 1H and NcNowra) followed by challenge during defined time points of gestation; (ii) vaccination of already pregnant cattle with live vaccines, native antigens or recombinant antigens (see Table 2). The only drug that has been evaluated in cattle so far is toltrazuril, which was administered to newborn calves experimentally infected with *N. caninum* tachyzoites (Häsler *et al*. 2006*a*, *b*). Results on the efficacy of toltrazuril, however, were not conclusive (see Table 3).

An attractive and more cost-effective alternative to the bovine model is to perform experimental infection studies in sheep and exploit them as a small ruminant model for neosporosis. As an experimental animal model, sheep exhibit several advantages over cattle, including size, length of gestation and cost. In addition, unlike mice, sheep do not represent an artificial model, as recent evidence suggests that *N. caninum* is an important abortifacient in small ruminants (Moreno *et al*. 2012), or even the main cause of reproductive losses in some flocks (Gonzalez-Warleta *et al*. 2014). This would also account for goats (Dubey, 2003; Moore, 2005; Costa *et al*. 2014). Experimental infections in pregnant sheep (McAllister *et al*. 1996; Buxton, 1998; Weston *et al*. 2009) and pregnant pygmy goats (Lindsay *et al*. 1995) have shown that both are highly susceptible, and disease outcomes display similar characteristics as reported for cattle. As in cattle, it has been suggested that the time point of infection during gestation plays a key role in the pathogenesis of the disease (Dubey and Lindsay, 1990; Buxton *et al*. 1997, 2001; Jenkins *et al*. 2004). However, again comparison between different studies is difficult due to the lack of standardization. Most recently, the outcome of experimental infection by *N. caninum* in ewes has been investigated under standardized conditions at an early, mid-term and late period of gestation allowing evaluating the effect of the gestation period on the clinical course of disease, lesion development and parasite distribution in different organs (Arranz-Solis *et al*. 2015) This model for exogenous transplacental transmission for ruminant neosporosis can serve as a model to study the effects of vaccines and drugs.

VACCINES AGAINST NEOSPOROSIS

The costs of management practices used for control of neosporosis indicate that vaccination could be the most efficient intervention strategy (Reichel and Ellis, 2009). The only licensed *Neospora* vaccine, Bovilis Neoguard®, was composed of a tachyzoite lysate, and was available in selected countries for

Table 2. Overviews of selected vaccine studies against neosporosis in farm animals

Vaccine	Reference	Set-up	Results
NcSRS2 expressed in CHV	(Nishikawa *et al*. 2000)	Dogs immunized with inactivated CHV expressing NcSRS2. No challenge	IgG production against NcSRS2
N. caninum tachyzoites (killed)	(O'Handley *et al*. 2003)	Seronegative ewes, vaccinated twice with tachyzoites plus adjuvant. Challenge during pregnancy	Humoral response against *N. caninum* in vaccinated ewes higher than in control ewes. Lower *N. caninum* DNA levels in lambs from vaccinated ewes
Natural infection by *N. caninum*	(Williams *et al*. 2003)	Naturally infected and naïve cows, challenged at week 10 of gestation	Natural infection protected against abortion induced by challenge, but not against vertical transmission
N. caninum tachyzoites (killed)	(Jenkins *et al*. 2004)	Ewes, vaccinated twice with tachzoites plus adjuvant. Challenge 30 days after last boost.	Protection against abortion, but not against vertical transmission
N. caninum tachyzoites (killed)	(Romero *et al*. 2004)	Field trial with dairy cattle. No challenge.	Reduction of abortion from 20% in the placebo group to 11% in the vaccinated group
N. caninum tachyzoites (killed)	(Weston *et al*. 2012)	Clinical trial with a killed tachyzoite vaccine (Bovilis Neoguard™) on 5 dairy farms (s.c. 2×, 4-week-intervals)	Vaccination increased the risk of vertical transmission. In one of five herds, vaccination reduced abortion
N. caninum tachyzoite extract	(Mansilla *et al*. 2013)	Cattle, vaccinated twice with aequous tachyzoite extract at various concentrations with soybean based adjuvant. No challenge	Increased IgG1 and IFN-γ levels in vaccinated animals as compared with controls. Stimulation of CD4(+)-T-cells
GRA7 (rE)	(Nishimura *et al*. 2013)	Cattle, GRA7 (50–200 μg) entrapped in oligomannose microsomes (s. c. application twice). Challenge with NC-1 27 days after last boost. Euthanasia at 85–87 days post infection	IgG and IFN-γ levels increased as compared with controls. Lower parasite load in brains in cattle immunized with 50 μg formulation
N. caninum tachyzoites (live)	(Rojo-Montejo *et al*. 2013)	Seronegative heifers, immunized with non-virulent Nc-spain H1 (2×), challenge with Nc-1 post-mating	Strong IgG and IFN-γ reponses post-immunization. No foetal loss in immunized non-challenged heifers. In challenged heifers, 50% protection against fetal loss
N. caninum tachyzoites (live or frozen)	(Weber *et al*. 2013)	Cattle, 96 seronegative animals, immunized with Nc-Nowra (s. c. or i. v.), mating. Pregnant heifers were challenged with Nc-S197	Protection against abortion by vaccination, best with live tachyzoites applied by i. v. administration
SAG1 + HSP20 + GRA7	(Hecker *et al*. 2014)	Pregnant heifers, immunized twice with recombinant proteins formulated with ISCOMs (s. c.). Challenge with NC-1 at day 70 of gestation	Immune responses against antigens. No IFN-γ response. No protection against vertical transmission
N. caninum tachyzoites (live)	(Mazuz *et al*. 2015)	Field trial: 520 pregnant, seropositive heifers; 146 vaccinated with live vaccine (NcIs491), 374 non-vaccinated controls	Lower incidence of abortion in vaccinated as compared with control cows. No protection against vertical transmission

CHV, canine herpes vector; s. c., subcutaneous; i. v., intravenuous; ISCOMs, immune stimulating complexes.

several years (Barling *et al*. 2003). However, this vaccine exhibited only moderate efficacy in field trials (Romero *et al*. 2004), and one study suggested that vaccination itself could increase the risk of early embryonic death (Weston *et al*. 2012). As the vaccine has been taken off the market, farmers have been left without an alternative (Reichel *et al*. 2015). Therefore, there is a need for the development of effective vaccines to prevent *N. caninum* infection (Monney and Hemphill, 2014).

More recently, an *in silico* approach has been proposed for the development of a vaccine against neosporosis (Goodswen *et al*. 2014). This approach is based on exploiting the current genomic and transcriptomic information on the *N. caninum* Nc-Liv isolate, and using bioinformatics tools to assess the suitability of expressed proteins as vaccine candidates by identifying those antigens containing T and B cell epitopes by reverse vaccinology. This approach could be helpful in providing a priority list of potential vaccine candidates. However, besides assessing the physicochemical properties of proteins based on their predicted peptide sequence, this approach does not take into account other important factors that are crucial for vaccine development against *N. caninum* infection, such as the extensive immunomodulation that takes place during pregnancy, routes of vaccine delivery, infection route,

genetic background of parasites and hosts, immunization dose, challenge dose and timing, as well as the effects of the adjuvant and the non-protein components of an antigen preparation such as lipids and carbohydrates (Monney and Hemphill, 2014). Nevertheless, with the improvement of novel bioinformatics tools *in silico* vaccinology could become an interesting approach, but will not replace the tedious work in the wet lab in the near future.

Both, live vaccines and subunit vaccines have been studied in experimental settings. So far, the largest numbers of experimental vaccinations has been carried out in mice (see Table 1), while few studies have been performed in cattle and sheep, a selection of which is summarized in Table 2. While live vaccines have clearly shown superior efficacy in experimental trials compared with any subunit vaccine formulation, the pharmaceutical industry has been reluctant to introduce these live vaccines into the market, and have clearly favoured an approach that includes subunit vaccines (Reichel *et al*. 2015). For the closely related *T. gondii,* considerable efforts have been put into the development of vaccines to reduce oocyst shedding in cats and tissue cyst formation in mammals. However, the only licensed vaccine is a live-attenuated strain (Toxovax®), which is maintained in cell culture and licensed for veterinary use in sheep only (Zhang *et al*. 2013). Also for other livestock apicomplexan diseases, including theileriosis, babesiosis and coccidiosis, live vaccines are used as effective tools for the prevention of infection and serious disease (McAllister, 2014). In the case of *Theileria parva,* the live vaccine is composed of a cocktail of three strains, but it has been shown that there is not always cross-protection against other strains (Morrison *et al*. 2015). For besnoitiosis, a live vaccine has been produced and made available in Israel, but its efficacy and safety have not been reported. A live vaccine for neosporosis prevention ideally would use attenuated parasites, but concerns such as high production costs, short shelf life of the product, maintenance of a cool-chain prior to application of the vaccine and the risk of reversion to virulence have prevented the introduction of several live-*Neospora* vaccine candidates into the market. In addition, as live vaccines might result in chronic infection of the host, there is a risk that the life cycle could ultimately be completed again, if tissues from vaccinated animals were fed to canid definitive hosts (Reichel *et al*. 2015). Toxovax® comprises a *T. gondii* tachyzoite strain that has lost the ability to form tissue cysts in the vaccinated host however, the molecular basis for this failure to persist long-term in the host has not been defined (Monney and Hemphill, 2014).

For the development of subunit vaccines, many researchers have exploited the fact that *N. caninum* has developed distinct adaptations to its intracellular lifestyle. Survival depends on successful invasion of host cells, and on the ability to differentiate into the slowly proliferating and cyst-forming bradyzoite stage under physiological stress conditions (Hemphill *et al*. 2013*a*). Knowledge of the molecular basis of these processes is essential for understanding the pathogenic mechanisms underlying infection and for designing strategies to combat these diseases. Thus, the concept of using subunit vaccines has largely relied on identifying defined parasite fractions or proteins that play essential roles in host cell invasion and/or tachyzoite-to-bradyzoite stage differentiation, and targeting these components by generating humoral and/or cellular immunity against them.

Some vaccines based on native antigens of apicomplexan parasites are currently commercially available. For instance, CoxAbic™ is composed of affinity-purified gametocyte antigens from *Eimeria maxima* and confers protection to hens and their offspring against coccidiosis by transmission of specific antibodies via egg yolk (Sharman *et al*. 2010). Another marketed vaccine, Nobivac Piro™, is composed of soluble antigens from two *Babesia* species and confers protection against babesiosis in dogs (Schetters *et al*. 2009). However, these vaccines are derived from parasite cultures, and to date there has been no vaccine candidate composed of bacterially expressed subunit antigens showing convincing protection against apicomplexan parasites.

There are a few common characteristics in all *Neospora* vaccines studies carried out so far: a primary characteristic is that only live vaccines have conferred convincing protection against abortion and/or vertical transmission upon experimental or natural challenge in cattle. Vaccination of animals prior to pregnancy with naturally attenuated low-virulence *N. caninum* isolates (e.g. NcSpain1H and NcNowra) and then challenged during defined time points of gestation demonstrated substantial protection against abortion and fetal loss (Williams *et al*. 2007; Rojo-Montejo *et al*. 2013; Weber *et al*. 2013). Vaccination of pregnant cattle with tachyzoites of a live-attenuated vaccine strain (Hecker *et al*. 2013), iscom preparations of native *N. caninum* extract (Hecker *et al*. 2013), or recombinant antigens (SAG1, Hsp20, GRA7) incorporated into iscoms (Hecker *et al*. 2014) demonstrated partial protection against transplacental fetal infection with the live vaccine, but not with the native or recombinant subunit vaccines. In addition, a live vaccine isolate (NcIs491) was assessed in a field trial comprised of 520 pregnant and *N. caninum* seropositive cows, of which 146 were vaccinated at mid-gestation and 374 served as controls (Mazuz *et al*. 2015). While this field trial showed that the live vaccine reduced the abortion to 16% compared with 25% abortion losses in unvaccinated cows, these losses upon endogenous transplacental transmission are unusually high, much higher than in typical endemic situations. Interestingly, this vaccine reduced abortion, but not

the rate of vertical transmission. Similar efficacy of live vaccines was demonstrated in the mouse model (Miller *et al*. 2005; Rojo-Montejo *et al*. 2012). A second important point is that subunit vaccines, either tachyzoite-derived or expressed as recombinant antigens exhibited promising protective efficacy in non-pregnant models (mainly mouse models), but in pregnant mice, the same or virtually identical formulations have been found to be largely non-protective (reviewed in Monney and Hemphill, 2014; see also Table 1). Thus, in these cases pregnancy has led to the loss of subunit vaccine-induced protective immunity. Protection against infection in non-pregnant cattle was achieved using oligomannose-coated liposome entrapped NcGra7, but there is no further information whether this protection would be retained during pregnancy (Nishimura *et al*. 2013). In addition, in both pregnant and non-pregnant mouse models, combinations of antigens as polyvalent vaccine exhibited a higher efficacy compared with- single antigens applied as monovalent vaccines, indicating that only a combination of recombinant antigens will induce protective immunological responses (Debache *et al*. 2009; Pastor-Fernandez *et al*. 2015). However, in some cases, vaccination rendered mice are more susceptible to infection, demonstrating that some antigenic components of the parasite could exhibit immune-modulating properties (Srinivasan *et al*. 2007).

Another difficulty in interpreting the results from studies employing recombinant vaccines comes from the fact that many of these antigens are expressed in *Escherichia coli* and affinity purified from crude extracts. Therefore, the presence of immunomodulating agents derived from bacterial contaminants such as lipopolysaccharides (LPS) cannot be ruled out, and controls with irrelevant proteins expressed in the same system or with LPS -depleted protein fractions (Basto *et al*. 2015) should be included. Surprisingly, only a minority of *Neospora* vaccine studies performed to date have addressed this point. LPS and other bacterial contaminants will greatly influence the way innate immune pathways are activated, and to what extent polarization of the cellular immune response is elicited later during infection (Basto *et al*. 2012; Basto and Leitão, 2014). This fact could be exploited, by actually generating LPS-free subunit vaccine candidate formulations, and then mixing them with PAMPs in a controlled way, or even physically link these antigens with PAMPs as described by Basto *et al*. (2012, 2015).

DRUGS AGAINST NEOSPOROSIS

In general, chemotherapeutic treatment of *Neospora*-seropositive animals has not been regarded as an economically viable option, since no effective and safe drugs are available, and depending on the compounds used, milk or meat from drug-treated animals would remain unacceptable for consumption for some time (Dubey *et al*. 2007). Nevertheless, experimental studies have revealed potentially interesting effects of several compounds *in vitro* and in laboratory animal models *in vivo* (Müller and Hemphill, 2011) (see Table 3). Many of these compounds or compound classes were shown previously to be active against other intracellular protozoan parasites, including *Trypanosoma cruzi,* the causative agent of Chagas Disease, and *Leishmania* species responsible for cutaneous and visceral leishmaniasis, and some exhibited broad-spectrum anti-parasitic activity against various protozoan and helminth species. However, other approaches identified compounds that inhibited targets that were conserved almost exclusively within the group of apicomplexan parasites, and were shown to be active against different *N. caninum* isolates, as well as other apicomplexans including *Plasmodium, Toxoplasma, Cryptosporidium* and others. The most interesting drugs, however, are most notably derived from screenings carried out in the framework of *Plasmodium* research. Their application against other apicomplexans would thus be a good example of drug repurposing (Andrews *et al*. 2014; Sateriale *et al*. 2014).

The strategies to identify anti-parasitic agents are discussed elsewhere (Müller and Hemphill, 2013*a*, *b*). Briefly, drug candidates are identified and initially characterized by *in vitro* tests, during which suitable host cells (e.g. fibroblasts) are infected with *N. caninum* tachyzoites in the presence of the test compounds or of a solvent control. After a given time period (i. e. when the controls show a high level of infection) the experiment is stopped and the tachyzoites are quantified by a suitable method. Quantitative real time polymerase chain reaction will work with all *N. caninum* isolates, but is laborious, time consuming and costly, if many samples need to be processed. For higher throughput screening, transgenic parasites expressing an easily detectable marker are more practical. For instance, a *N. caninum* NC-1 strain expressing *E. coli* beta-galactosidase under the control of a GRA1 promotor (Howe and Sibley, 1997) has been used for such initial drug screenings. At first, these *in vitro* studies will provide inhibition constants (e.g. IC_{50}) and data concerning host cell toxicity. Furthermore, it can be determined whether a compound is parasitocidal or only parasitostatic, whether it affects intracellular parasites or only parasites prior to infection, and to which extent resistance formation can occur. Combined with morphological studies using scanning and transmission electron microscopy, such *in vitro* studies have already provided a detailed picture on how a given compound affects the parasite. For recent examples on *Neospora* see Table 3. Moreover, a detailed study dealing with all these aspects has been performed with *T. gondii* strains and pentamidine derivatives (Kropf *et al*. 2012).

Table 3. Overviews of selected *in vitro* and *in vivo* studies with chemotherapeutics against neosporosis

Compound	Class	Reference	Type	Result
Compound screen	43 various classes	(Lindsay *et al*. 1994)	*in vitro*	Few selected compounds with *in vitro* activities inhibit proliferation of tachyzoites
Toltrazuril, ponazuril	Triazinone	(Gottstein *et al*. 2001)	*in vivo*	Protection against cerebral infection by daily application (20 mg kg^{-1}) in drinking water
Ponazuril		(Kritzner *et al*. 2002)	*in vivo* (calves)	Protection against symptoms, lower parasite burden in organs
Toltrazuril		(Ammann *et al*. 2004)	*in vivo*	Protection only in immunocompetent mice
Toltrazuril		(Gottstein *et al*. 2005)	*in vivo*	Reduction of placental transmission
Toltrazuril		(Haerdi *et al*. 2006)	*in vivo* (calves)	Treatment of congenitally infected calves does not affect seropositivity
Toltrazuril		(Strohbusch *et al*. 2008)	*in vitro*	Treatment with 30 mg L^{-1} during 14 days is parasitocidal
Toltrazuril		(Strohbusch *et al*. 2009)	*in vivo*	Reduction of placental transmission (3 treatment with 30 mg kg^{-1} each)
Nitro- and bromo-thiazolides	Thiazolides	(Esposito *et al*. 2005)	*in vitro*	Inhibition of proliferation is independent of nitro group
Nitro- and bromo-thiazolides		(Esposito *et al*. 2007)	*in vitro*	Induction of egress of tachyzoites from infected cells
Nitazoxanide		(Debache *et al*. 2011)	*in vivo*	150 mg kg^{-1} day^{-1} during 6 days p. o. has no effect, i. p. kills the mice after 2 days
DB750	Dicationic arylimidamide	(Debache *et al*. 2011)	*in vivo*	2 mg kg^{-1} day^{-1}, during 6 days i. p. is well supported, better survival, reduction of cerebral parasite burden
DB745		(Schorer *et al*. 2012)	*in vitro in vivo*	IC_{50} 80 nM. Reduction of cerebral burden in mice after 14 daily applications of 2 mg kg^{-1} day^{-1}
Mefloquine	Trifluoromethylhinolin	(Müller and Hemphill, 2011)	*in vitro*	IC_{50} 0·5 μM, EC_{50} for HFF 3 μM.
Miltefosine	Alkylphosphocholine	(Debache and Hemphill, 2012)	*in vitro in vivo*	IC_{50} 5·2 μM. Treatment with 25 μM for 20 h parasitocidal. Reduction of symptoms and of cerebral burden
Artemisone	Sesquiterpene lactone	(Mazuz *et al*. 2012)	*in vitro in vivo* (gerbil)	Inhibition of infection by 15 mg L^{-1}. Partial clearance of pre-infected cells by 50 mg L^{-1}. Reduction of symptoms and of cerebral burden
		(Müller *et al*. 2015*b*)	*in vitro*	IC_{50} around 3 nM. Parasitocidal only by long-term treatment with 5 μM
Ruthenium	Heavy metal	(Barna *et al*. 2013)	*in vitro*	IC_{50} around 10 nm. Parasitocidal activity only by long-term-treatment with 100 nM
Bumped kinase inhibitors	Substituted pyrazolopyrimidines	(Ojo *et al*. 2014)	*in vitro in vivo*	Good structure-activity correlation. IC_{50} s around 100 nM. Inhibition of infection. Reduction of symptoms and cerebral parasite burden in non-pregnant mice
		(Winzer *et al*. 2015)	*in vivo*	Protection against vertical transmission
Buparvaquone	Naphtoquinone	(Müller *et al*. 2015*a*)	*in vitro in vivo*	IC_{50} of approximately 5 nM, IC_{100} 100 nM (short-term). Long term adaptation to 100 nM. Parasitocidal activity after 6 days treatment with 1 µM. Prevention of acute neosporosis

If not indicated otherwise, *in vivo* studies were performed with mice. p. o., per os (oral application); i. p., intraperitoneal; HFF, human foreskin fibroblasts.

Several studies have been performed with toltrazuril, a triazinone derivative effective against various coccidians including *Eimeria* (Steinfelder *et al*. 2005), and commercialized under the proprietary name Baycox™. The mode of action of toltrazuril and of its main metabolite toltrazuril sulfone (ponazuril) does not consist only in the inhibition of dihydroorotate dehydrogenase and thereby pyrimidine biosynthesis, but also in the inhibition of the respiratory chain of the parasite (Harder and Haberkorn, 1989). Whereas the effects against coccidian infections are well documented in poultry (Mathis *et al*. 2003) as well as in cattle (Mundt *et al*. 2005), it remains unclear whether toltrazuril is a suitable drug against neosporosis because the efficacy results in cattle do not support this conclusion (Haerdi *et al*. 2006; see Table 3). Thiazolides including nitazoxanide, the prototype compound of this class (Hemphill *et al*. 2013*b*), have good effects against *N. caninum in vitro*, but fail *in vivo* when applied orally or are even toxic when applied intraperitoneally (see Table 3). This is most likely due to induction of host cell apoptosis (Müller *et al*. 2008). The most promising drug candidates against neosporosis come from compounds initially developed against *Plasmodium* (artemisinin-derivatives) and *Leishmania* (dicationic pentamidine derivatives (Soeiro *et al*. 2013) and spiroindolones, a novel class of antimalarials (Rottmann *et al*. 2010) inhibiting a Na^+-efflux pump in *Plasmodium* (Spillman *et al*. 2013). In addition, there is a link between anti-cancer chemotherapeutics and anti-parasitic activities, since many anti-cancer drugs, which target mechanisms that lead to increased cellular proliferation, also affect the proliferative stages of parasites (Klinkert and Heussler, 2006). For instance, exoerythorcytic and erythrocytic development of *Plasmodium* parasites is blocked by the proteasome inhibitor MLN-273 (Lindenthal *et al*. 2005). Moreover, artemisinin and derivatives, which are active against *N. caninum* and *T. gondii*, also impact the proliferation and viability of many cancer cells (Das, 2015). Another example is given by organometallic ruthenium compounds originally developed for the treatment of cancer, and also active against *N. caninum* and *T. gondii* tachyzoites *in vitro* in the low nanomolar range (Barna *et al*. 2013). Ruthenium drugs were originally thought to bind to DNA, but more recent investigations showed that they also interact strongly with proteins (Ravera *et al*. 2004; Scolaro *et al*. 2007) and potential targets in cancer cells were postulated, including cathepsin B, P-glycoprotein, and glutathione *S*-transferase P1 (Casini *et al*. 2008).

CALCIUM DEPENDENT KINASE 1 (CDPK1) AS A PROMISING DRUG TARGET

Amongst novel compounds, inhibitors of CDPKI, deserve particular interest. CDPK1 is essential for microneme secretion, host cell invasion, and egress of *T. gondii* (Lourido *et al*. 2010). A particular class of inhibitors, the bumped kinase inhibitors (BKIs), has bulky C3 aryl moieties entering a hydrophobic pocket in the ATP binding site. BKIs selectively inhibit CDPK1 from apicomplexans in a good structure-activity-relationship (Keyloun *et al*. 2014; Zhang *et al*. 2014) but do not inhibit mammalian kinases because they have larger amino acid residues adjacent to the hydrophobic pocket, thereby blocking the entry of the bulky C3 aryl group. Some BKIs, especially BKI-1294 (Ojo *et al*. 2014), have a good efficacy against *N. caninum in vitro* and *in vivo* (see Table 3). *In vitro* studies, however, indicate that the BKI 1294 is not directly parasiticidal. Only upon long-term *in vitro* treatment of infected human foreskin fibroblasts monolayers, a complete clearance of viable tachyzoites can be observed (Ojo *et al*. 2014). For different *Neospora* isolates, but also for *T. gondii* strain RH and ME49, clearance of intracellular parasites is preceded by the formation of large, multinucleated complexes with deregulated gene expression as evidenced by the expression of bradyzoite as well as tachyzoite antigens (Winzer *et al*. 2015). The upregulation of bradyzoite antigen expression, as exemplified by the heat shock protein hsp20 (or BAG1) has also been reported upon treatment of *N. caninum* infected monolayers with artemisone and derivatives (Müller *et al*. 2015*b*). In the pregnant mouse model, BKI-1294 was the only compound tested so far that achieved a good protection against vertical transmission of *N. caninum* (Winzer *et al*. 2015).

A COMBINED IMMUNO-CHEMICAL STRATEGY AGAINST NEOSPOROSIS?

During the last decade, numerous *in vitro* and *in vivo* trials have yielded several promising vaccine and drug candidates that could be potentially used for the prevention or treatment of neosporosis. None of them achieved complete protection against transplacental transmission of *N. caninum*, the goal that should ultimately be achieved in cattle. In addition, it is very unlikely that a vaccine can be developed that protects against endogenous transplacental transmission from a chronically infected dam to its offspring (Williams and Trees, 2006). Nevertheless, the promising results of both approaches suggest that they could be combined for immuno-chemotherapy.

One possible strategy could include application of a live-attenuated vaccine in combination with a compound that has exhibited high efficacy in previous *in vitro* and *in vivo* studies. Such an approach has been applied for a long time against East coast fever, in which cattle are vaccinated with live sporozoites together with a drug that affects parasite viability such as tetracycline, or alternatively buparvaquone, the only, but highly effective, drug against *Theileria*

available (Irvin and Morrison, 1989; Mutugi *et al*. 1988; Brown, 1990). BKIs (Ojo *et al*. 2014; Winzer *et al*. 2015) would constitute a suitable class of chemotherapeutics for co-application. They exhibit little or no side-effects and prevent the infection of cells by tachyzoites. The fact that they do not directly kill the parasites, or take an extended period of time until they act parasiticidally, renders them suitable for a combined immunization plus treatment protocol. As mentioned above, intracellular parasites treated with these compounds express a variety of tachyzoite and bradyzoite antigens. By turn-over of the infected cell, these antigens may be presented to the immune system thereby eliciting stable immune responses against tachyzoite as well as bradyzoite stages.

Another approach could include the simultaneous application of suitable chemotherapeutics and polypeptides such as recombinant antigens acting as classical vaccines or immunostimulants. Recombinant proteins produced in *E. coli* or in any other organism may contain impurities and are very expensive, especially when produced in high purity at a large scale. On the other hand, the chemosynthesis of peptides has become increasingly cheaper. Highly antigenic peptides could thus be produced by chemosynthesis, coupled to a high molecular weight carrier to render them immunogenic and/or to a ligand recognized by a toll-like receptor (Casal *et al*. 1995; Xin *et al*. 2012).

Taken together, the results achieved in the last years suggest that the ultimate aim of a one-shot-therapy against neosporosis in cattle will be difficult to achieve, but still is a realistic goal. Similar to *Toxoplasma, Neospora* has developed important means of adaptation and protection by differentiating into bradyzoites and forming tissue cysts. These can only be tackled by parasite-specific compounds that cross the blood brain barrier, and they could inactivate reactivated parasites early during recrudescence. A major problem, however, is that we do not know the exact timing of recrudescence during pregnancy. More insight is also needed into the immune mechanisms that are required to combat infection, but also how these can be maintained during pregnancy, without affecting the viability of the fetus. Potentially, a combined immune-chemotherapeutical approach could provide a solution. More *in vitro* as well as *in vivo* research is required, and ideally researchers join forces to apply appropriate and standardized models, that allow accurate comparisons of results achieved in different laboratories. In addition, it is important to ensure that any standardized model is truly appropriate otherwise standardized testing methods are not predictive of protection in the field.

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