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Neosporosis: An Overview of Its Molecular Epidemiology and Pathogenesis

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ABSTRACT

Neospora caninum (*N. caninum*), a cyst-forming protozoan parasite, is a major cause of bovine abortions and neonatal mortality worldwide. *N. caninum* has a broad intermediate host range, and its sexual cycle occurs exclusively in canids. Another species of *Neospora*, *Neospora hughesi* (*N. hughesi*), has been identified and causes myeloencephalitis in horses. Although molecular epidemiology studies are in their infancy, the 18S ribosomal RNA (rRNA) and ITS1 regions within the small subunit ribosomal RNA (ssuRNA) and an *N. caninum* species-specific DNA probe (pNc5) have been used extensively to differentiate *Neospora* from other closely related apicomplexan parasites. While these repetitive regions have higher sensitivity and specificity than housekeeping or antigen genes, they suffer from low discriminatory power and fail to capture intra-species diversity. Similarly, although multiple minisatellite or microsatellite marker studies have shown clear geographic substructures within *Neospora*, strains are often misclassified due to a convergence in the size of different alleles at microsatellite loci, known as homoplasy. Only one strain, *N. caninum* Liverpool (Nc-Liv), has been genome sequenced and compared with its closest relative, *Toxoplasma gondii* (*T. gondii*). Hence, detailed population genomics studies based on whole-genome sequences from multiple strains worldwide are needed in order to better understand the current population genetic structure of *Neospora*, and ultimately to determine more effective vaccine candidates against bovine neosporosis. The aim of this review is to outline our current understanding of the molecular epidemiology and genomics of *Neospora* in juxtaposition with the closely related apicomplexan parasites *Hammondia hammondi* and *T. gondii*.

1. Historical perspective

Neospora caninum (*N. caninum*) is a coccidian protozoan parasite belonging to the apicomplexan phylum [1], and was often confused with the closely related apicomplexan parasite *Toxoplasma gondii* (*T. gondii*) until 1988 [2,3]. In 1984, Bjerkås et al. [4] first described cases of encephalomyelitis (brain and spinal disease) and myositis (muscular disease) in a litter of six congenitally infected Boxer pups in Norway, caused by an unidentified parasite. This parasite was formally recognized as *N. caninum* in 1988, based on a retrospective analysis of thousands of tissue sections from dead dogs and cats that succumbed to a *Toxoplasma*-like illness from 1952 to 1987 at Angell Memorial Animal Hospital in Boston, USA, which were negative for immunohistochemical reactivity with anti-*T. gondii* antibodies [2,3].

2. Life-cycle

N. caninum has a wide host range and a heteroxenous life-cycle consisting of two distinct modes of reproduction: ① asexual reproduction, which occurs in intermediate hosts; and ② sexual reproduction, which occurs exclusively in definitive canid hosts including dogs [5], coyotes [6], gray wolves [7], and dingoes [8] (Fig. 1). Although the sexual developmental stages of *T. gondii* are well defined, the developmental stages of *N. caninum*, such as schizogony and gametogony, are unknown. The only known sexual stage of *N. caninum* is the unsporulated diploid oocyst, which is environmentally resistant to freezing and drying. Unsporulated oocysts are shed in the feces of infected canids and undergo meiosis in the environment to form haploid sporozoites (Fig. 1) [3]. After ingestion of sporulated oocysts by the intermediate hosts, sporozoites are released from oocysts and convert into a rapidly growing stage known as tachyzoites, which disseminate the infection (Fig. 1). Tachyzoites invade host cells and replicate

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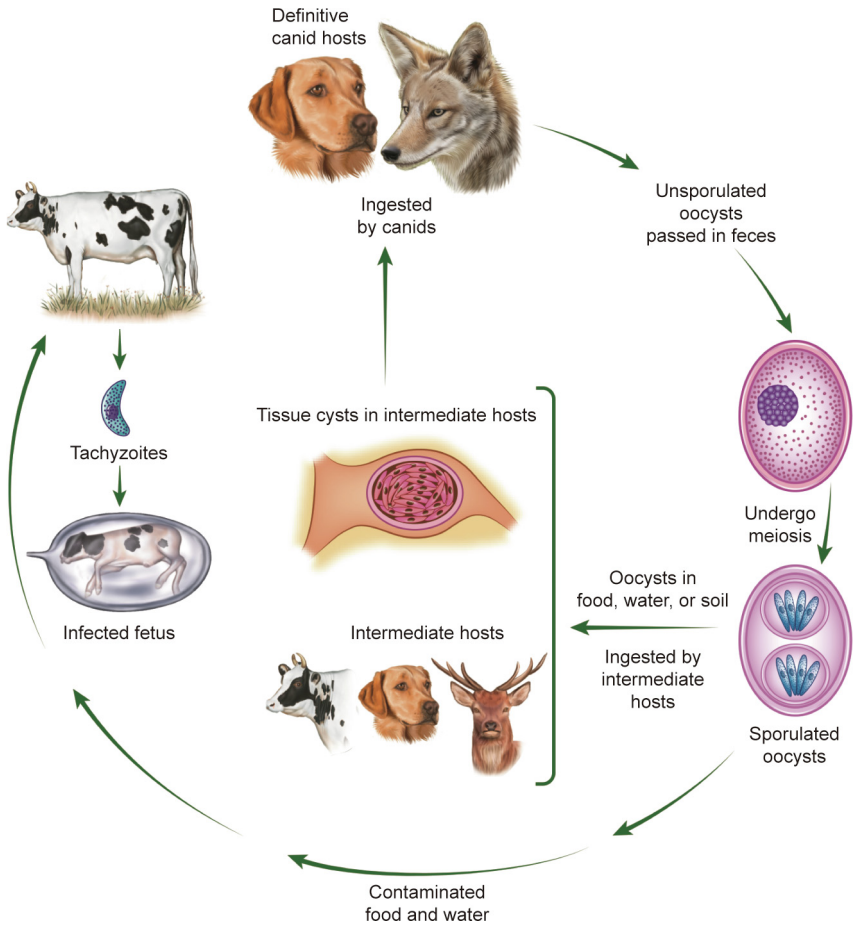

Fig. 1. Heteroxenous life-cycle of *N. caninum*. Reproduced from Ref. [3] with permission of CRC Press, © 2017.

asexually by repeated endodyogeny within a parasitophorous vacuole (PV). After several rounds of endodyogeny, parasites egress from the host cell [9] to re-infect new host cells, subsequently generating host-specific innate and adaptive immune responses. Due to host immune responses and other environmental factors, tachyzoites convert into a slowly growing, semi-dormant stage known as bradyzoites, which are found in tissue cysts (Fig. 1). Tissue cysts can persist in a host cell for a long time and can convert into tachyzoites to generate latent infection, particularly in immunocompromised hosts [10].

3. Transmission

Despite having a wide host range and a heteroxenous life-cycle, *Neospora* infects cattle and dogs as its primary intermediate and definitive hosts, respectively [11]. Cattle can become infected either horizontally, through the ingestion of infectious oocysts that have been shed by the definitive hosts, or vertically, through transplacental or congenital transmission from an infected pregnant mother to her fetus (Fig. 1). Vertical transmission is considered the predominant and most efficient (80%) route of transmission of *Neospora* in cattle herds. Vertical transmission can occur either as exogenous transmission through oocyst-derived infection of a pregnant heifer, or through endogenous transplacental transmission as a result of reactivated latent infection during pregnancy [3]. Fetuses infected with *Neospora* can abort in any gestational age, be resorbed, mummified, autolyzed, or born with persistent infection. Transplacental transmission has also been documented in other intermediate hosts, including sheep, goats, deer, and dogs. Venereal transmission though semen and transmission through milk is considered unlikely [3]. Dogs are generally infected orally via ingestion of infected intermediate host tissue. In contrast to cats, which are the definitive host of *T. gondii* and *Hammondia hammondi* (*H. hammondi*) [12], dogs generally produce fewer oocysts of *Neospora* during sexual transmission. Given the close association of dogs and cattle in herds [1,5] and the likelihood of vertical transmission through generations of cattle [13,14], the probability of transmission is significantly higher because parasites can be maintained and propagated between the definitive and intermediate hosts for a long time within the same herd. This localized transmission cycle between cattle and dogs through asexual and sexual reproduction is probably shaping the population structure of *Neospora*.

4. *Neospora hughesi*

The identification of a new *Neospora* species, *Neospora hughesi* (*N. hughesi*), was first reported in 1998 in a horse with equine protozoal myeloencephalitis in California, USA [15]. *N. hughesi* is considered to be a separate species because it is seemingly restricted to horses [15] and shows significant antigenic and sequence divergence when compared with *N. caninum* [16–18]. For example, the surface antigen SAG1 exhibits a 6% difference in amino acid identity between NcSAG1 and NhSAG1 [16]. Thus, the NcSAG1-derived mAb 6C11 does not hybridize with *N. hughesi* SAG1 [16]. Similarly, another surface antigen SRS2 (NcSRS2 and NhSRS2) shows a 9% amino acid difference. The antigenic specificity demonstrated between these two abundantly expressed surface antigens has therefore been utilized to generate efficient molecular markers that differentiate between *N. caninum* and *N. hughesi*

[16]. Comparative analysis of the dense granule (GRA) proteins GRA6 and GRA7 between *N. hughesi* and *N. caninum* has also revealed significant differences in both amino acid sequence and structural organization [18]. In addition to these antigenic markers, sensitive molecular-detection methods have been used, based on nucleotide sequence differences within the repetitive ITS1 region that discriminate *N. hughesi* from *N. caninum* (7–9 bp) [19].

Interestingly, these genetic differences between *N. caninum* and *N. hughesi* also confer phenotypic differences during experimental rodent infection. *N. hughesi* causes predominant necrosis in myocardial tissue, whereas *N. caninum* lesions are found predominately in the liver, lungs, and brain. Furthermore, there is a striking difference in virulence between *N. caninum* and *N. hughesi* in the gerbil (*Meriones unguiculatus*) model. Gerbils are highly susceptible to oral infection with *N. caninum*, resulting in the death of most gerbils within 6–13 d post-infection [20]. Conversely, *N. hughesi* infection does not produce significant clinical signs in gerbils, with the exception of a few microscopic lesions. However, it needs to be elucidated whether the reported genetic and biological differences between these two species are due to species-specific differences or strain-specific differences, as seen for *T. gondii*. Experimental genetic crosses between *N. caninum* and *N. hughesi* may shed light on whether these two parasites should be considered to be separate species, as inter-specific crosses have been recently demonstrated to occur within the *Leishmania* field [21]. Taken together, the current consensus is that the *Neospora* population structure is composed of at least two separate *Neospora* species: *N. caninum* and *N. hughesi*, that evolved from a common ancestor and have expanded independently to adapt to their various vertebrate hosts.

5. Detection

5.1. Morphological detection

Several morphological detection methods have been developed to identify neosporosis in infected samples. Cytology by microscopic examination of cytospin smear or impression smears, followed by Diff-Quick rapid staining, Giemsa staining, and/or hematoxylin or eosin (HE) staining of lesions, is the most conventional method for the rapid detection of neosporosis in the field. However, due to morphological similarities with closely related parasites, it is difficult to differentiate *Neospora* from *Toxoplasma* and *Hammondia*, apart from the presence of thick-wall (up to 4 μm) tissue cysts, which is one of the hallmarks of *Neospora*. Transmission electron microscopy has also been used to distinguish *Neospora* from *Toxoplasma* and *Sarcocystis* based on the presence of electron-dense rhoptries (ROPs) in *Neospora*. Conversely, *Toxoplasma* ROPs are electron lucent, whereas ROPs are absent in *Sarcocystis* merozoites. Immunohistochemical staining (IHC) is another very sensitive and widely used methodology to detect neosporosis in fixed tissues [22–24]. The avidin–biotin complex indirect immunoperoxidase and peroxidase–antiperoxidase techniques are equally sensitive methods. Both monoclonal and polyclonal antibodies specific to *Neospora* have been utilized for IHC, with rabbit polyclonal antibodies exhibiting more sensitivity than monoclonal antibodies. Recently, however, a combination of NcSRS2 and NcGRA7 monoclonal antibodies [24] were shown to be more specific and as sensitive as polyclonal antibodies, obviating the potential for polyclonal antibody cross-reactivity with *T. gondii* or related coccidian parasites.

5.2. Serological detection

Since the discovery of neosporosis, several serological methods have been developed for its detection. After infection, *Neospora*-specific Immunoglobulin M (IgM) antibodies can be detected within a couple of weeks, whereas IgG antibodies take several weeks to reach detection levels, with titers peaking six months after primary infection. Several antigens have been used extensively for detection, including the surface antigens NcSRS2, NcSAG1, NcSAG4, and Ncp40; cytoskeleton protein NCPF; dense granule proteins NcGRA2, NcGRA6, and NcGRA7; serine protease NcSUB1; and microneme (MIC) proteins NcMIC6 and NcMIC10 [25]. The indirect fluorescent antibody test (IFAT) was the first antibody-based method applied for *Neospora* detection [2]. In this method, intact tachyzoites are fixed onto coverslips and incubated with test serum; they are then hybridized with a fluorescein-labeled secondary antibody directed against the immunoglobulins of the hosts. IFATs have been considered the reference test for detecting neosporosis, as this test shows very little cross-reactivity with closely related apicomplexan parasites and can be quantified by titering the sera [26,27]. A direct *Neospora* agglutination test (NAT) has also been developed by replacing the *T. gondii* antigen from the modified agglutination test (MAT) with a *Neospora*-specific antigen [28,29]. NAT has been used to test for the presence of IgM in a large number of sera from up to 16 different animal species and was found to be as sensitive as IFAT. However, the most extensively and commercially used serological detection method is the enzyme-linked immunosorbent assay (ELISA) [30], which can be automated for a large number of samples and scanned rapidly with high specificity. Various ELISA methods (i.e., indirect ELISAs (iELISAs) and cellular ELISAs (cELISAs)) have been established using mainly total tachyzoite lysate or purified native antigens for polystyrene plate sensitization [31,32]. One disadvantage of utilizing native or total antigens is the potential for cross-reactivity with other closely related coccidian parasites [33]. Thus, a wide range of *Neospora*-specific antigens are currently used for coating ELISA plates in order to establish specificity [30,34,35]. ELISA-based avidity tests using IgG, IgA, and IgE antibodies have also been established to estimate the timing of infection. Other serological techniques, such as the latex agglutination test (LAT) using NcGRA6 [36] and NcSAG1 [37], and immunoblot (IB) [38], have been developed but are not widely used.

6. Seroprevalence

The presence of *N. caninum* antibodies in the serum of cattle, beef cattle, dogs, goats, and other domestic animals has been reported throughout the world, mostly using the above-mentioned serological methods. Seroprevalence rates in cattle, dogs, goats, and sheep are depicted in Fig. 2 [3]. It is important to mention, however, that seroprevalence data from different research groups are not comparable due to the use of different serological methods, differences in cutoff values, and the lack of validation via the isolation of viable parasites from infected animals [1]. Nonetheless, viable parasites have been successfully isolated from definitive hosts in other studies particularly dogs, along with a wide range of intermediate hosts such as cattle, sheep, white-tailed deer, and water buffaloes [1,3]. Although it has been experimentally shown that *Neospora* circulate within the cattle industry exclusively between cattle and dogs, any of the above-mentioned intermediate hosts may exist as additional important reservoirs for *Neospora* transmission. For example, white-tailed deer are one of the major reservoirs of *Neospora* in the United States, and have an 88% seroprevalence rate [39]. The potential for zoonotic transmission is also unknown. It has been suggested that unlike *T. gondii*, *Neospora* and *Hammondia* are not infectious to humans. Low titers of *Neospora* antibodies have been detected in humans from Brazil, Denmark, Egypt, Korea, and the United States [40–42]. Although this observation can be explained by

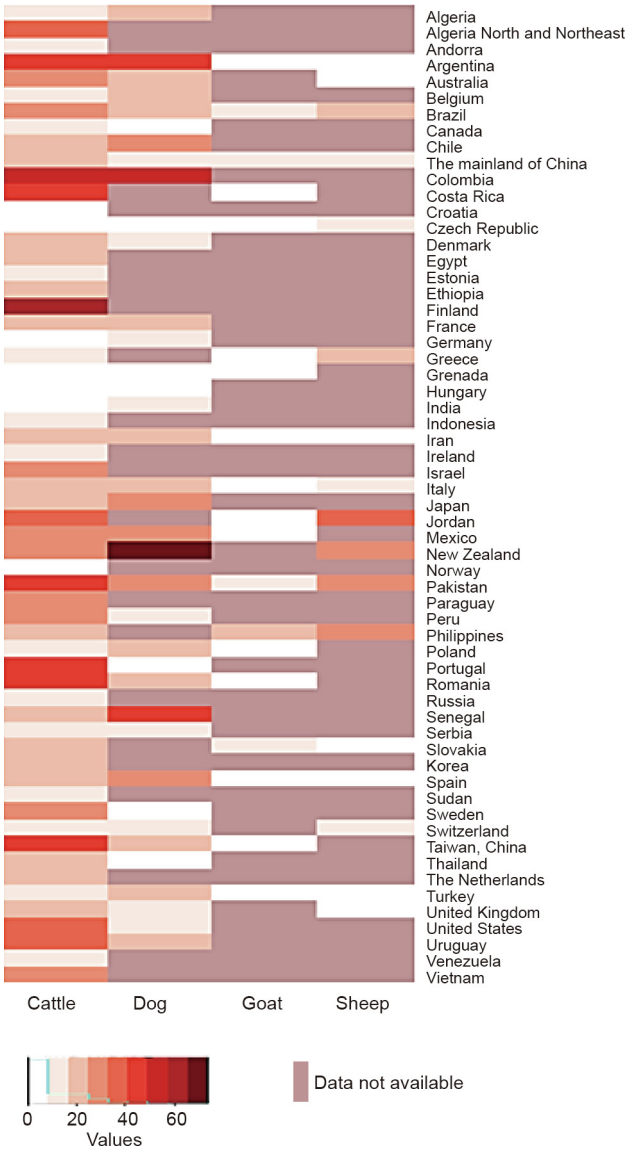

Fig. 2. Heat map of global seroprevalence of *N. caninum* in different hosts including cattle, dogs, goats, and sheep (*x*-axis). The *y*-axis represents countries/regions.

the antigenic similarity between *T. gondii* and *Neospora*, transplacental neosporosis has been described experimentally in the primate (*Macaca mulatta*) model [43,44] and *Neospora* can propagate using different human cell lines in *in vitro* culture [45]. Thus, these data provide clues regarding the possibility of zoonotic transmission. However, neither viable parasites nor parasitic DNA have yet been recovered from humans.

7. Molecular epidemiology

Successful molecular epidemiological and phylogenetic studies for the *Neospora* field require a large number of strains isolated from their extensive host and geographical range, as well as suitable genetic tools. Unlike *Toxoplasma*, *Neospora* studies lack both sufficient tools and isolates to differentiate between different *Neospora* strains and their mechanisms of host restriction. To date, characterization of *Neospora* strains has relied on a few sequenced markers that have been used to differentiate *Neospora* from closely related coccidia; however, these markers cannot distinguish between different *Neospora* strains. The most widely used molecular markers in *Neospora* are 18S rRNA and ITS1 regions within the small subunit ribosomal RNA (ssuRNA) and pNc5 (GenBank accession number, X84238) [43,44] due to their repetitive character, as single-copy genes are less sensitive for polymerase chain reaction (PCR) detection than multi-copy genes. Only a few single-nucleotide polymorphisms (SNPs) have been detected within the 18S rRNA gene between *T. gondii*, *H. hammondi*, and *Neospora*, indicating their close relationship. However, those few SNPs are adequate to differentiate *Neospora* from *Toxoplasma* and *H. hammondi* using universal primers, species-specific chemiluminescent DNA hybridization probes [46], a second round of nested PCR using *Neospora*-specific primers [47], or PCR-restricted fragment length polymorphism (PCR-RFLP) with the BsaJI enzyme [48]. Although 18S rRNA can be utilized to differentiate between closely related parasites, no sequence differences have thus far been detected between different *Neospora* isolates including NC1, *N. caninum* Liverpool (Nc-Liv), NC3, and NC-SweB1 [43,44]. Another ssuRNA marker, ITS1, is also used widely for species-specific differentiation due to its high sensitivity and specificity. An ITS1-based one-tube nested PCR for *Neospora* was later engineered in order to increase the sensitivity to detect 1–10 fg genomic DNA of *Neospora* [49]. This method is also highly sensitive and specific. However, unlike the 18S rRNA locus, a difference was detected between the strain NC-Bahia (South American origin) and different canine and bovine isolates of *Neospora,* including NC1, Nc-Liv, BPA1, NC-SweB1, and NcNZ1, which share the identical sequence at ITS1 [15,44,50,51]. While these latter markers are extremely sensitive for detecting *Neospora,* they may not be suitable for characterizing intra-specific strain heterogeneity or for phylogenetic analyses due to the high degree of genetic similarity found among circulating *Neospora* isolates. Another multi-copy gene, pNc5, has been used extensively for the detection of *Neospora* infection in a wide range of intermediate and definitive hosts. However, it is not exquisitely specific, as these primers have been reported to amplify rodent-specific DNA [52]. Recently, single-gene sequenced-based markers including 14-3-3, surface antigens (SAG1, SAG4, and SRS2), secreted dense granule proteins (GRA6 and GRA7), and several housekeeping genes such as α- and β-tubulins and heat shock protein 70 (HSP-70) were developed to genotype a few bovine and canine *Neospora* isolates from a restricted geography [16–18,53,54]. These markers appear to be highly conserved among different *Neospora* isolates, which puts into question their usefulness in differentiating among *Neospora* isolates.

Unlike the single-copy gene sequence markers, minisatellite or microsatellite markers, which are classified as a variable number of tandem repeats (VNTR) DNA, have captured extensive genetic diversity among different isolates within the species. Thus, minisatellite and microsatellite markers have been exploited extensively for the molecular differentiation of closely related *N. caninum* isolates [55,56]. A genotyping study using microsatellite markers identified genetic diversity among nine isolates of *N. caninum,* with no association between the DNA profile of each isolate and its geographic origin [55]. In another recent study, 11 *N. caninum* reference isolates collected from around the world and 96 *N. caninum* bovine clinical isolates from Spain, Argentina, Scotland, and Germany were genotyped using nine multi-locus microsatellite markers [57]. Microsatellite characterization revealed extensive genetic diversity consisting of 96 microsatellite multi-locus genotypes. Microsatellite markers do not capture the true extent of polymorphisms throughout the genome, and may misclassify strains as variants due to homoplasy [58]. Nevertheless, clear geographic substructures were identified using these markers based on *F*-statistics value (F_{ST}) and eBURST analysis, which could indicate a recent introduction of *N. caninum* isolates with the movement of cattle industries [57]. Thus, there is a need to conduct a systematic analysis that compares the relatively

non-polymorphic single-gene loci with the genetic diversity identified using microsatellite markers in order to determine the true extent of population genetic diversity. Recently, metagenomic next-generation sequencing (NGS) has evolved as a diagnostic tool to study toxoplasmic encephalitis [59]. Hence, metagenomic NGS could be developed to identify and genotype the infectious agents in a target-independent manner causally associated with neosporosis cases.

8. Genome

Although *Neospora*, *H. hammondi*, and *T. gondii* differ significantly in their host range and in other biological aspects such as virulence in the mouse model, their genomes are highly syntenic based on whole-genome sequencing (Table 1, Fig. 3(a)) [60–62]. Nc-Liv was the first *Neospora* genome sequenced using Sanger sequencing to eight-fold coverage; it clustered into a 61 Mb genome consisting of 585 supercontigs and 7121 genes (European Nucleotide Archive as project CADU00000000; www.toxodb.org). Subsequently, those supercontigs were reassembled using the *T. gondii* Me49 genome into 14 chromosomes due to their highly syntenic genomes (Fig. 3(a)) [60,61]. Although *Neospora* and *Toxoplasma* diverged from their recent common ancestor approximately 28 million years ago, according to a calculation based on the mutation rate between *Plasmodium falciparum* and *Plasmodium reichenowi* [63], only a few chromosomal rearrangements and a low net gain/loss of genetic information have been observed between these two species. One exception is the significant expansion of the surface antigen families and particularly SAG1-related sequences (SRS) in *Neospora* (227 *NcSRS* genes and 52 *NcSRS* pseudogenes) that are tandemly arrayed in multigene clusters throughout the genome (Fig. 3(b)). Of these SRSs, *Neospora* only express a subset of 25 *NcSRS* genes during the tachyzoite stage, and only one gene is expressed per multigene cluster. Recently predicted genetic structures for over one-third of the previously annotated gene models and untranslated regions (UTRs) have been corrected based on strand-specific RNA sequencing and shotgun proteomics using tachyzoites of Nc-Liv [64]. The use of RNAseq data not only significantly improved the annotation quality of the *Neospora* genome, but also led to the identification of cis-natural antisense transcripts (cis-NATs) and long intergenic non-coding RNAs (lincRNAs) [64]. Interestingly, a comparative analysis of the metabolic pathways between *T. gondii*, *H. hammondi*, and *N. caninum* found no major changes in metabolic genes, with the exception of a few differentially expressed genes and the upregulation of nitrogen metabolism genes in *N. caninum* [61].

9. Molecular genetics tools

The advent of whole-genome sequencing and transcriptional analysis has facilitated the development of molecular genetic tools to elucidate potential mechanisms of *Neospora* gene regulation and

Table 1
Comparative analysis of three closely related apicomplexan parasites.

Feature	*N. caninum*	*H. hammondi*	*T. gondii*
Definitive hosts	Canids	Felids	Felids
Intermediate hosts	Bovids, horses	Rodents	Mammals, birds
Human infection	Unlikely	Unlikely	Yes
Estimated size (Mb)	62	65	65
Assembly length without sequencing gaps (bp)	57 524 119	67 460 985	65 464 221
Number of chromosomes	14	14	14
Number of protein-coding genes	6 936	8 004	8 322
GC content (%)	54.8	52.5	52.2
Average length of protein-coding genes (bp)	4 892	4 868	4 778

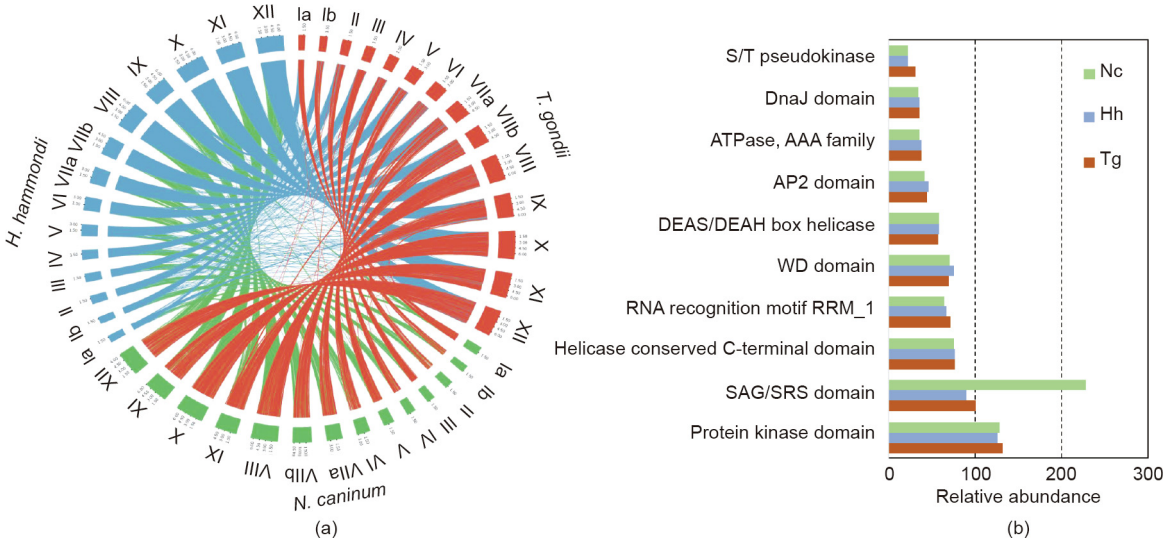

Fig. 3. Comparative analysis of closely related apicomplexan parasites *N. caninum*, *H. hammondi*, and *T. gondii*. (a) A Circos plot indicates highly syntenic genome among three closely related apicomplexan parasites. The outer circle represents the annotated chromosomes of each parasite. Nucleotide blast was performed among the reference sequences of these strains (www.toxodb.org) using NCBI blast V. 2.5 allowing a percent identity of 70% or more, an e-value less than 0.001, and a minimum segment size of 500 bp. Synteny among the reference sequences as found by the nucleotide blast were drawn using ribbon links in Circos V. 0.69. (b) The relative abundance of top 10 Pfam domains in *T. gondii* Me49 (Tg) among *N. caninum* (Nc) and *H. hammondi* (Hh) shows a significant expansion of the SAGs/SRSs domain in *N. caninum*.

pathogenesis. However, it is necessary to develop genetic tools to manipulate the genome in order to elucidate functions of the genes that have been identified via genomic studies. Due to the highly syntenic [60,61] genome with *T. gondii*, existing heterologous expression systems available for the genetic modification of *T. gondii* have been initially utilized to transfect and transform *Neospora* successfully [65]. The *Escherichia coli lacZ* gene was also stably expressed in *Neospora* using the GRA1 promoter from *T. gondii* [66], and this transgenic strain became a very important tool for screening anti-parasitic molecules. Subsequently, several genes of *T. gondii*, including SAG1, GRA2, NTPase3, and ROP2, were also transfected into *Neospora* successfully, in order to gain a better understanding of the molecular mechanism of these genes. This work ultimately led to the identification of the ROP8 gene. Thus, heterologous expression of *T. gondii* genes into the immunologically distinct *Neospora* background [67] demonstrated the usefulness of studying important *T. gondii* genes for the development of a live vaccine candidate [68]. Following the success of a stable transfection system, mutated dihydrofolate reductase-thymidylate synthase (DHFR-TS), which confers resistance to pyrimethamine [69] and the insertion of stable drug selectable markers such as chloramphenicol acetyl transferase [70] have been engineered to study genome editing in *Neospora*. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated gene 9 (CRISPR/Cas9) system from *T. gondii* has been effectively and specifically adapted for genome editing in *Neospora* [71]. Utilizing the CRISPR/Cas9 system, a green fluorescent protein (GFP) from an Nc-1 GFP expressing strain and the NcGRA7 gene from the Nc-Spain7 isolate have been successfully deleted and replaced with a pyrimethamine selectable marker [71]. Thus, the CRISRP/Cas9 system will provide efficient and reliable ways to make precise, targeted changes to the genome of *Neospora* in order to study specific gene functions [72], detect genes related to *Neospora* pathogenesis, or generate a live attenuated vaccine strain capable of providing immune protection against acute and chronic neosporosis.

10. Host-pathogen interaction

Over the course of evolution, hosts have developed complex immune defenses to combat pathogens, while pathogens have evolved strategies to evade host surveillance and achieve successful infection. *Neospora* infects a wide range of intermediate hosts. Thus, entering host cells and circumventing host immunity is paramount to its survival and persistent infection. In order to enter host cells, *Neospora* parasites first develop a low-affinity contact between the tachyzoite and host cell surface, followed by adhesion to the host cell. Host cell surface proteoglycans particularly chondroitin sulfate glycosaminoglycans (GAGs) for tachyzoites and terminal sialic acid residues for bradyzoites act as adhesion receptors to promote *Neospora* infection [73,74]. *Neospora* surface antigens, including NcSAG1 and NcSRS2, are the parasite factors that facilitate the initial contact between the host and parasite. Interestingly, whole-genome sequencing has identified a significant expansion of surface antigens in *Neospora* (223 SRS genes, Fig. 3(b)) in comparison with closely related *T. gondii* (135 SRS genes) [61]. This striking difference in the expansion of SRS genes and their differential impact on the modulation of initial host immunity and the regulation of parasite virulence remains unknown. After initial contact, using active gliding motility and constitutively expressed proteins present in their secretory organelles namely, MICs ROPs, and GRAs, the parasite enters the host cell through a moving junction to form PVs [75–77]. Unlike the mechanism that occurs in *T. gondii*, an inhibitor of aspartyl protease in *Neospora* known as pepstatin plays an important role in assembling and trafficking the MIC and ROP proteins into host cells, and has a significant impact on parasite invasion [77,78]. Immunofluorescence studies with rabbit anti-N54 identified another *Neospora* protease, NcSUB1 (formerly known as NC-p65), which localizes in the MIC organelles of the parasites and is involved in the invasion of host cells [79]. Formation of a moving junction leads to the injection of ROP proteins into the PV. Forward genetic-based studies, quantitative trait locus (QTL) analyses, and reverse genetic-based gene knock-out studies in *T. gondii* have established that ROP18/ROP5 complexes are the major virulence effectors during the infection of susceptible laboratory mice. ROP5 proteins bind to immunity-related GTPases (IRGs) to block their oligomerization and activation, following the ROP18 mediated phosphorylation of IRGs [80–87]. It was recently shown that ROP18/ROP5 complexes also include another ROP kinase, ROP17, which has a high affinity toward oligomerized IRGs [88]. Interestingly, a genome-wide comparison of ROP18 sequences indicated that ROP18 is pseudogenized in *Neospora* and contains upstream regions (UPS) that are present in nonvirulent type III strains, but not in virulent type I and intermediately virulent type II *T. gondii* strains [61,89]. However, transgenic expression of ROP18 from the *T. gondii* type I virulent RH strain in *Neospora* (Nc1) makes them significantly more virulent in mice [90]. Although ROP18 is pseudogenized in *Neospora*, it has been well documented that interferon (IFN)-γ is the major cytokine mediator for resistance against *Neospora* [91,92]. Interestingly, IFN-γ activated mesenchymal stromal cells not only inhibited the growth of *Neospora*, but also showed the involvement of IRGs (irga6, irgb6, and irgd) and guanylate-binding proteins (GBPs; mGBP1 and mGBP2) in the anti-*Neospora* effect [93]. Thus, taken together, these findings suggest that pseudogenized ROP18 and the involvement of IRGs in parasite clearance are responsible for the nonvirulent phenotype of *Neospora* in the murine model. In addition to IFN-γ, *Neospora* is a potent activator of the toll-like receptor (TLR3) dependent type I (α/β) IFN [94]. Forward genetic studies in *T. gondii* also identified a non-ROP2 ROP protein, ROP16, which traffics to the host cell nucleus and phosphorylates STAT3/STAT6 in a strain-specific manner, leading to significant activation of innate immune signaling, and decreasing the virulence in mice by attenuating IL-12 signals [61,95,96]. In contrast to *T. gondii*, *Neospora* ROP16 only phosphorylates STAT3 and not STAT6, resulting in the induction of host cell apoptosis [97]. ROP proteome analyses have identified other ROP components in *Neospora*, including NcROP1, NcROP5, and NcROP30, which have a high homology to those described in *T. gondii*.

Similarly, GRAs are secreted at high levels during invasion and are expressed constitutively in the PV to modulate host signaling pathways. Although several GRAs have been identified as the critical modulators for the maturation of the PV in *T. gondii* [98], very few of these namely, GRA15 [99], GRA16 [100], and GRA24 [101] are associated with the host cell nucleus. GRA15 has been shown to activate the NF-κB pathway and control the induction of IL-12 secretion after *T. gondii* infection [99]. Conversely, GRA16 is involved in cell-cycle progression and the p53 tumor suppressor pathway [100], whereas GRA24 modulates host p38α MAP kinase [101]. In contrast to the case of *T. gondii*, GRAs are not well characterized in *Neospora*, with the exception of NcGRA6 and NcGRA7 (originally named NcDG1 [102] and NcDG2 [103]). NcGRA7 is secreted into the PV and is known to localize within the PV matrix (PVM) [76]. Other GRAs including NcGRA1, NcGRA2, NcMAG1, and NcNTPase have been characterized and are expressed within tachyzoite dense GRAs, and localize within the PV matrix [76,104]. Although GRA15 activates the NF-κB pathway in *T. gondii* [99], this gene is pseudogenized in *Neospora*.

Multiple differential and strain-specific secretory effectors have been identified that impact *T. gondii* host interactions and virulence in the murine model. Although strain-specific differences in *Neospora* have not been well studied, several instances of differential immune outcomes have been documented. For example, the reference strain Nc-Liv was shown to induce severe clinical symptoms of neosporosis in mice, including inflammatory infiltrates and highly necrotic lesions, compared with the avirulent Nc-SweB1 [105] isolate. Another nonvirulent isolate, Nc-Nowra, caused mild to moderate nonsuppurative encephalitis in laboratory mice, whereas Nc-Liv induced severe nonsuppurative encephalitis [106]. Mouse virulence analysis has also demonstrated that NC1 is more virulent than NC3 [107]. Based on these observations, it has been postulated that similar to *T. gondii*, differences in virulence are due to strain-specific differences in *Neospora* parasite factors. To support this postulation, comparative difference gel electrophoresis (DIGE) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) techniques were conducted and showed that the proteome expression profiles differ considerably between virulent and attenuated strains; furthermore, these analyses identified unregulated expression of NTPase in virulent *Neospora* strains [108]. A recent *Neospora* immunome study revealed strain-specific differences between virulent (Nc-Liv, Nc-Spain1H) and attenuated (Nc-Spain7) strains, and showed consistently higher expression of four proteins in the virulent strains: a serine-threonine phosphatase 2C, superoxide dismutase, GAP45, and NcGRA1 [109]. As has been done for *T. gondii*, forward genetic analysis including the development of genetic crosses followed by QTL analysis or genome-wide association tests will potentially identify additional secretory determinants responsible for strain-specific differences in *Neospora* pathogenesis.

11. Vaccination

Bovine neosporosis is a huge concern worldwide due to its economic impact. According to a systematic review, economic loss by cattle neosporosis worldwide is estimated at 1.298×10^9 USD·a^{-1}, and ranges as high as 2.380×10^9 USD·a^{-1} [110]. Two-thirds of these losses were incurred by the dairy industry (8.429×10^8 USD·a^{-1}), because the median-specific abortion risk due to *Neospora* is higher in dairy cattle (14.3%) than in beef cattle (9.1%). Moreover, two-thirds of the global losses were incurred by North America (65.7%), followed by South America (18.5%) and Australasia (10.6%), whereas losses in three European counties (the Netherlands, Spain, and the United Kingdom) were only estimated to be 5.3%. Thus, Brazil, Mexico, and the United States are the primary target markets for vaccination against neosporosis [110]. Although it has been postulated that the global market for a vaccine against cattle neosporosis is very large, there is as yet no treatment or vaccine to prevent the transmission of abortion-inducing neosporosis. The only licensed *N. caninum* vaccine was Bovilis Neoguard (Intervet International B.V., the Netherlands), which is composed of inactivated *N. caninum* tachyzoites (3×10^6 mL^{-1} at harvest), 10% Havlogen adjuvant, 5% stabilizers, and 5% phosphate-buffered saline. Unfortunately, several follow-up studies in Costa Rica and New Zealand demonstrated either low efficacy (20%) or increased transplacental transmission with increased risk of early embryonic death, ultimately leading to the withdrawal of this vaccine from the market [72,111]. Recently, a soluble fraction of tachyzoite-extract vaccine with soya lecithin/β-glucan adjuvant (sNcAg/AVEC) showed immunogenicity and induction of high IFN-γ responses in pregnant cattle [112]. Nevertheless, live tachyzoite vaccines consisting of naturally attenuated or less virulent isolates such as Nc-Nowra [113], Nc-Spain1H [114], and the Argentinian isolate Nc-6 [115] provide a significant increase in *N. caninum* antibody responses and have led to significantly reduced abortion rates. However, there are some inherent disadvantages to using live vaccines, such as bulk preservation of live parasites and reversion risk of pathogenicity after immunization [116]; thus, inactivated or subunit vaccines are the more attractive options. Unfortunately, recombinant NcGRA7 (50–200 μg) entrapped in oligo-mannose microsome (M3-NcGRA7) [117], as well as bacterially expressed and purified recombinant proteins including rNcSAG1, rNcHSP20, and rNcGRA7 [118], show very little promise, as they have failed to prevent infection in pregnant heifers. Therefore, the identification of a novel, more efficient vaccine approach such as live attenuated strains (including Ca^{2+}-dependent protein kinase 2 deficient tachyzoites) of *Neospora* is critical to ensure long-lasting protection against neosporosis [119].

12. Conclusion

The apicomplexan parasite *N. caninum* is the leading cause of bovine abortion and stillbirth, or neuromuscular disorder in dogs. Bovine neosporosis has a great economic impact in both the meat and dairy industries, and no efficacious control methods exist. Thus, there is an urgent need for new drugs, vaccines, and/or tools to combat neosporosis. To identify new methods to mitigate neosporosis, it is necessary to determine its population genetic structure in order to anticipate how the pathogen is evolving; it is also necessary to better understand host-pathogen interactions. Currently available genetic markers (both RFLP and sequence based) are not adequate to distinguish among *N. caninum* isolates. The recent use of microsatellite markers has led to the identification of substructures within the *Neospora* population; however, fixation (F_{ST}) statistics and Bayesian clustering algorithms determined using microsatellite markers were confounded by a homoplasy that infers a more complex population structure than likely exists. Hence, whole-genome sequencing of multiple strains from a wide host range throughout the world should be performed. This will expand the realm of genetic markers available and generate better models to determine the extent to which transmission is occurring via asexual or sexual propagation, in order to devise better-informed strategies to reduce transmission risk and improve immune protection against acute and chronic infection of *Neospora*.

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Compliance with ethics guidelines

Asis Khan, Jahangheer S. Shaik, Patricia Sikorski, Jitender P. Dubey, and Michael E. Grigg declare that they have no conflict of interest or financial conflicts to disclose.

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