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Review Article: *Neospora caninum*, A Neglected Parasitic Pathogen in Field Animals

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Abstract
Neospora caninum represents one of most prevalent parasites in last three decades in many countries, worldwide. This parasite originates from canine species in particular dogs that act the definitive host to infect many domestic and wild animals that act as an intermediate host. These infections have revealed to cause different clinical symptoms among infected animals in dependence on the species of animal and the dose of ingested parasite. This leads to variable economic losses due to morbidities and mortalities, and cost of therapies and prevention. In Iraq, the main causes of abortion or even reduction in milk production or weight loss still unknown since most attempts of diagnosis based on the detectable clinical symptoms. Also, the number of carried out studies in dogs, cattle, sheep and goats remain low, and the available information are need to be supported. In addition, there is an obvious lack for governmental efforts to detect the actual prevalence of infection in herds or flocks and the role of other animals in transmission of infection through application of traditional or advanced diagnostic assays. Hence, the current study was aimed to clarify the main information concerned to *N. caninum* to list this parasite as one of the most important pathogens in field and its problematic role in field.

Keywords: Neosporosis, Domestic animals, Dogs, Diagnosis, Iraq

2.1. History

In a retrospective study, neosporosis was showed firstly in 1957 in four German shepherd dogs in Ohio (USA); however, the first description of a *Toxoplasma*-like protozoa in dogs of 2-6 months old undergo encephalomyelitis, myelitis and muscle damage but without *Toxoplasma gondii* antibody was carried in Norway by Bjerkas et al. (1984). In the UK, O'Toole and Jeffrey (1987) described a sporozoan disease in a diseased calf with negative results by immunoperoxidase for toxoplasmosis and sarcoidosis, and later identified as *Neospora caninum*. Dubey et al. (1988) isolated the parasite for first time on tissue culture and mice with proposing the name of *N. caninum* for this newly discovered parasite. The antigenicity and structure of both USA and Norway *N. caninum* isolates were compared later by Bjerkas et al. (1994).

Since 1988, *Neospora* has been implicated with abortion in cattle and isolated from aborted fetuses of cattle and dogs in many countries in Europe, Americas, Asia, Australia and Africa (Schares et al., 1998; Wouda et al., 1999; Reichel, 2000). However, several attempts that made to obtain viable *N. caninum* isolates by bioanalysis in mice or direct cell culture were failed as *in vitro* isolation of this parasite is difficult and takes lone time (more than one month) to detect parasites in early cultures (Regidor-Cerrillo *et al.*, 2008; Rezende-Gondim et al., 2017).

2.2. Classification

Kingdom: Protista
Subkingdom: Protozoa
Phylum: Apicomplexa
Class: Sporozoa
Subclass: Coccidia
Order: Eucoccidiida
Suborder: Eimeriina
Family: Sarcocystidae
Genus: *Neospora*
Species: *N. caninum* (Dubey, 2003)

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2.3. Morphology

The stages or phases of *Neospora caninum* include tachyzoites, bradyzoites (tissue cysts) and oocysts have been confirmed as very similar in structure to *T. gondii* and other related parasites (Lindsay et al., 1999a; Reid et al., 2012).

2.3.1 Tachyzoites

Depending on phase of division, the tachyzoite is oval, spherical or crescent in shape which measures 3-7 × 1-5 µm, with presence of dense granules that help the parasite to parasitize the cells of a host (Anderson, 2008). They grow endogenously (internal budding), divide rapidly within few days to form hundreds of new parasites (pseudocysts) that when reach the critical mass cause lysis in cell wall to be released and spread throughout the body and immune system (Wang et al., 2021).

2.3.2 Bradyzoite and tissue cyst

It's slightly more slender and longer than tachyzoite, 8.1 × 2 µm, and represents a slow-division phase of the parasite, which able to forming a tissue cyst having up to 100 parasites. Tissue cysts found more common in the central nervous system as well as in skeletal muscles of definitive and intermediate hosts (Khordadmehr et al., 2023).

2.3.3. Oocysts

It is almost circular, 11.7 x 11.3 µm in diameter, and surrounded by a thick wall measures 0.6-0.8 µm with existence of nucleus in center or in posterior part. During sexual development, unsporulated oocysts formed in intestinal part of definitive host and shed with feces to environment **(**Lindsay *et al.*, 1999a; Page, 2020).

2.4. Life cycle

Initially, *N. caninum* is thought to have a life cycle similar to *Toxoplasma gondii*, but with two important differences in their definitive hosts and their diseases (Figure 1).

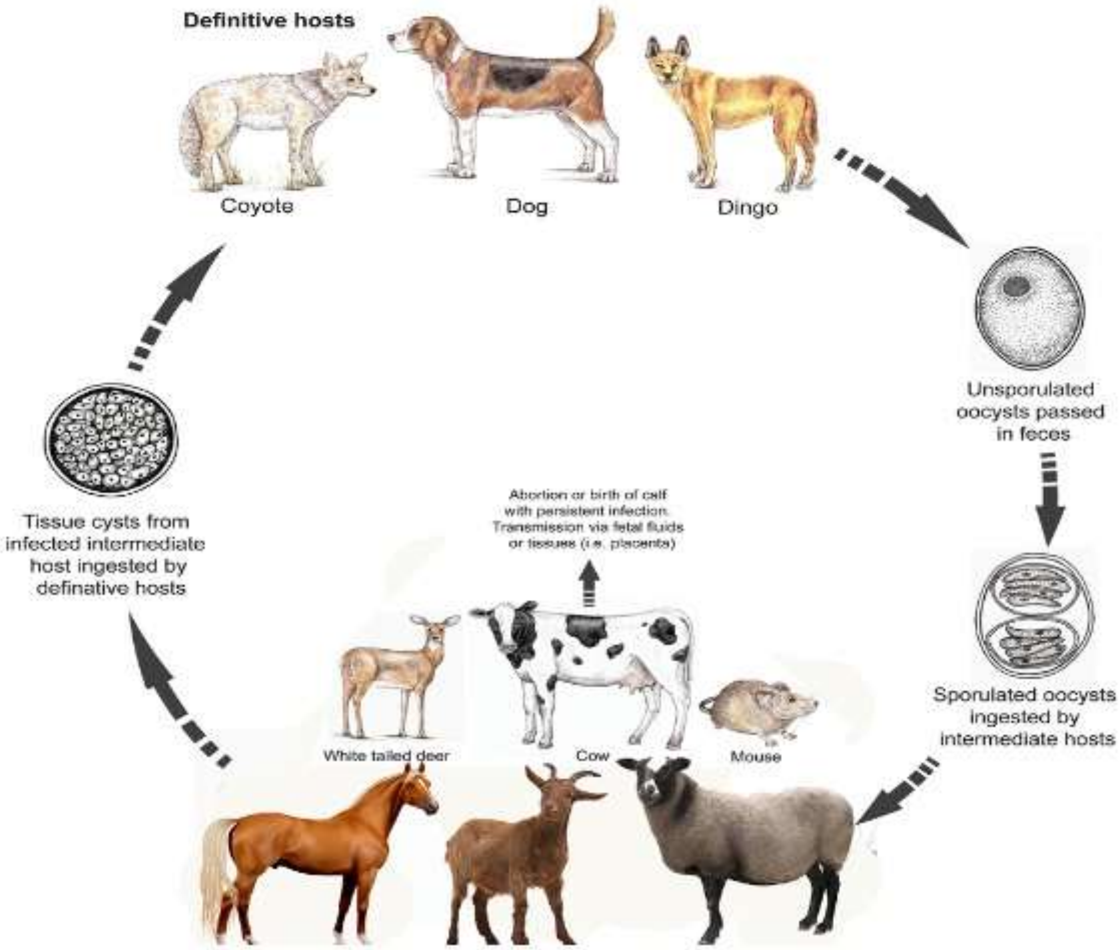

Figure (1): Life cycle of *Neospora caninum* (Dubey et al., 2007)

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McAllister et al. (1998) discovered that dogs are the definitive host of parasite; however, next studies discovered that parasite could be carried by other canide species such as coyote (Gondim et al., 2004), and fox (Jakubek et al., 2001). Life cycle of *N. caninum* involved 3 infectious phases; tachyzoites, bradyzoites, and oocysts (Dubey et al., 2007). Dogs, coyote, and fox when shed unsporulated oocyst by feces into environment, they sporulate during days to become infectious for intermediate hosts such as cows, sheep, goats, deer, cows, and horses during feeding or drinking of contaminated materials (Donahoe et al., 2015). In intestine, sporozoites were released from oocyst, invade, develop within the host cells and multiply by binary fission to form tachzoites that invade additional cells in the body such as macrophages, fibroblasts, vascular endothelial, muscle, liver and kidney, and nerve as well as influencing on immune response. Tissue cysts can persist in infected animals for months or even years without causing obvious clinical symptoms, and can cross placenta to infect the fetus or result in abortion (Marugan-Hernandez, 2017). Dogs could gain infection when ingest the contaminated tissues of intermediate host which contain bradyzoites or tachyzoites (Goodswen et al., 2013). When a tissue cyst is detected by the host, bradyzoite is released, infect the nerve cells and skeletal muscle fibers and undergo schizogony (asexual production). When the schizonts matured, many merozoites were released into the body (Cole et al., 1995; Al-Qassab et al., 2010).

2.5. Transmission

Although, horizontal method was reported for transmission of tachyzoites, tissue cysts or bradyzoites of *N. caninum* by eating and drinking of water, vertical transmission of parasite from mother to fetuses by placenta was also detected (Bartels et al., 2007; Bartley et al., 2013; Shaapan, 2016). Transmission of infection directly from cow to cow was not confirmed though a number of cows were ingested the remnants of placenta of other aborted cows (Davison et al., 2001). Davison et al. (1999) assessed the relative importance of horizontal and vertical infections in an affected herds revealing that 95.2% of animals were infected vertically while 4.8% of animals were infected horizontally. This study showed that transplacental transplantation is the most important form of parasite transmission. In another Dutch study, the findings showed that 80% of serologically positive cows were resulted in congenitally infected calves (Schares et al., 1998). Jensen et al. (1999) discovered that calves fed colostrums from infected cattle were not affected concluding that this method of infection is not important.

Postnatal infection rates are differed depending on region, country and applied test. Buxton et al. (2002) summarized that type of parasite, relationship between host and parasite, and genetic susceptibility of a host are the factors that influence the effects in prevalence of *Neospora* infection. Dijkstra et al. (2001) showed that the ingestion of infected placenta for dogs had resulted in infecting of these dogs; however, ingestion of colostrums of infected pitches was not produced in the positive animals.

2.6. Impact on public health

The risk of *Neospora caninum* infection in humans is unknown; however, positive sera were identified in immunocompetent and immunocompromised patients (Lobato et al., 2006; Robayo-Sánchez et al., 2017). However, many studies have been demonstrated that *N. caninum* infection is not zoonotic parasite (McCann et al., 2008; Reid et al., 2012; Calero-Bernal et al., 2019).

2.7. Epidemiology

2.7.1. Neosporosis in dogs

In experimentally (McAllister et al., 1998; Lindsay et al., 1999b) and naturally (Basso et al., 2001; McGarry et al., 2003; Schares et al., 2005) studies, dog was identified as the definitive host of *N. caninum*. Dijkstra et al. (2002) reported that the presence of new dogs in a cattle herd was increased the risk of epidemic abortion-related to neosporosis suggesting the role of dogs in transmission of infection between cattle and its role as a definitive host. However, only a few reports have confirmed the natural role of dogs in excretion of oocysts (Asmare et al., 2014; Robbe et al., 2016).

Neosporosis is a disease of worldwide occurrence which has been reported in many regions in Europe, United States, Canada, Australia, Japan and South Africa. Barber et al. (1997) reported that 11% of 300 Belgian dogs have positive serum prevalence; while in United Kingdom, Trees et al. (1993) recorded 17% of 163 dogs were positive for indirect fluorescent antibody (IFAT). In another study, 0.5% of 398 dogs were found to have a positive reaction to enzyme-bound immunoadsorption assay (ELISA) (Björkman et al., 1994). In Denmark and USA respectively, studies have reported positivity in 15.3% sera of 98 dogs (Rasmussen and Jensen, 1996) and 2% sera of 229 dogs (Lindsay et al., 1990). Dubey et al. (1988) reported

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that clinical signs of *Neospora* could occur in dogs of any age between 2 and 20 weeks, while confirmed events occur in dogs between 2 and 15 years. In Switzerland, a total of 3289 fecal specimens were examined by floatation and molecular assay which revealed on 25 and 1 positive samples for *N. caninum* (Sager et al., 2006). In Portugal, microscopic and histological, immunological and molecular methods were succeed in detection of neosporosis in feces of naturally infected dog 8 years old (Basso et al., 2001). In Iran, Razmi (2009) reported that the microscopic prevalence of neosporosis in 174 fecal samples of farm dogs was 2.2%. In UK (McGarry et al., 2003), New Zealand (McInnes et al., 2006) and Germany (Schares et al., 2005), molecular studies have confirmed the presence of *N. caninum* DNA in feces of dogs. In Iraq, only 1.67% of 180 fecal samples were positive by molecular assay (Mallah et al., 2012).

2.7.2. Neosporosis in cattle

Neospora caninum infections have been reported among many parts of the world. Also, many studies conducted on large numbers of dairy cattle demonstrated that 12-42% of aborted fetuses were positives to *N. caninum*. However, the number of positive sera could variable from country to country, region to region as well as between the serological tests and the level of CUTOFF (Fort et al., 2015). The prevalence rates of abortion and stillbirth have showed to increase significantly in serologically positive cows (Takashima et al., 2013; Al-Gharban et al., 2017; Ansari-Lari et al., 2017). Reichel et al. (2020) mentioned that little is known about the prevalence of *N. caninum* in beef cattle; however, the first case of abortion in beef cows was reported in Andorra (Armengol et al., 2007). Subsequently, the authors showed that the positive beef cows may remain positive serologically for 1 year after abortion. In both beef and dairy cows, the risk of seropositivity can be increased with age due to horizontal transmission of infection as a result of feeding of shed oocysts from the infected dogs (Eiras et al., 2011; Altaee et al., 2022).

Nonetheless, management practices involving selective replacement can decrease the risk of seropositivity even in absence of treatment or vaccine for precention and regardless the age of exposed cows (Lagomarsino et al., 2019). Moore et al. (2002) discovered that prevalence rates of neosporosis in beef and dairy cattle were 18.9% and 43.1%, respectively, and that the most extensive breeding used in both dairy and beef cattle can increase the rates of transmission of infection between animals.

An important feature of bovine neosporosis is the ability of parasites to maintain in cattle as a chronic disease and passing vertically to fetus during gestation. In some cattle, most infected cattle may give an apparently healthy birth but congenitally infected with the ability to passing the disease to the next generation during pregnancy and maintaining the infection in a herd (Goodswen et al., 2013; Reichel et al., 2014). Vertical and congenital transmission appeared as the most method of *N. caninum* maintenance as supported by evidence of the familial distribution of positive cattle serologically throughout the successive generations (Pare et al., 1996). In dairy herds, both sporadic cases and outbreaks of abortion-related to *N. caninum* were identified in different regions, worldwide. However, the conducted studies have been assessed the prevalence and factors contributed to disease, and estimated that the prevalence rates of *Neospora* were ranged 16.8-70% (Waldner et al., 1998).

Several studies have been found a significant correlation between the prevalence rate of neosporosis and the number of dogs in dairy herds with occurrence of abortion throughout all seasons of a year (Vural et al., 2006; Öcal et al., 2014; Venturoso et al., 2021). In Netherland, Wouda et al. (1999) reported that abortion is more incidences in summer; while in USA, Rinaldi et al. (2005) mentioned that the peak of abortion can occur at autumn and winter. Pessoa et al. (2016) found that serologically positive cows have a greater risk of abortion in subsequent pregnancy. In Venezuela, Lista-Alves et al. (2006) reported 11.3% seropositive infections totally 459 examined samples; whereas in China, Yu et al. (2007) detected that the prevalence of neosporosis was 20.2% in aborted cows compares to 16.6% in non-aborted cows but without significant relationship to age. In Hungary, Hornok et al. (2007) examined a total of 1063 cows by an indirect fluorescent antibody test (IFAT) with revealing the parasite is more prevalent in dairies (3.4%) than beef cattle (1.9%); while in Croatia, Beck et al. (2010) found that 5.8% of examined dairy cattle were seropositive. In Iraq, Nema-Alhindawe (2010) found that 19.56% of 92 tested cows were seropositives; whereas, Al-Gharban et al. (2017) indicated that 27.22% and 12.36% of 327 examined cattle were positive for serological (ELISA) and molecular (PCR) methods, respectively.

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2.8. Pathogenesis

This associates with the balance between the ability of tachyzoite to invade and replicate the cells and the ability of a host to prevent the spreading of parasites (Marugan-Hernandez, 2017). The cell penetration process has two different steps including adhesion and entry into the cell, which might take up to 5 minutes (Lei et al., 2005). In the first step of contact, release of microneme-content proteins act as a more specific ligation for direct attachment to the host cell and invasion. However, availability of many receptors on cell surface of a host is necessary for invasion of *Neospora* into the cytoplasm of a cell (Silva and Machado, 2016). To initiate the invasion of host cells, tachyzoite migrates directly to the host cell membrane and flows into the cytoplasm to be surrounded by parasitophorous vacuole (PV). Then, *Neospora* attracts the microtubular cytoskeleton in addition to other cell parts as mitochondria, lysosomes and Golgi apparatus to their PV, and remove cholesterol from the organelles to saving it in lipid bodies and regenerating of sphingolipids from Golgi vesicles (Nolan et al., 2015; Mayoral al., 2020). Also, the parasite attracts mitochondria to PV and uses it to production of energy.

During the prepatent period (5-8 days after ingestion of tissue cyst), cysts can be detected in fecal samples, but little is known about the presence of cysts in the body organs or in affected structures (de Barros et al., 2018). The parasite can cause a necrosis that can be identified within a few days, and resulting in a cell death due to proliferation of tachyzoites with inducing of neuromuscular infections in canine and other domestic animals as a result of damages caused to a large numbers of neurons and disturbances in conductibility of nerve impulses (Kuruca et al., 2013).

2.9. Pathology

Neospora caninum infections and brain damage are most common in the submeningeal and periventricular regions of the brain (Platt, 2016). Several experiments have been performed in birds to indicate the pathogenesis of *N. caninum* and to understand the clinical signs of these pathological changes. In these studies, the finings revealed a presence of inflammatory cell infiltration in the brain such as histiocytes, lymphocytes and plasma cells in brain which caused over-accumulation of tachyzoites (Leepin et al., 2008; Du et al., 2015; Gharekhani et al., 2020). In other experiment, the findings of inoculation of embryonated chicken eggs with tachyzoites were revealed in embryonic death in the first week, infiltration of polymorphonuclear cells in liver, hear and brain with existence congestion, hemorrhage and necrotic lesions (Mansourian et al., 2009). de Barros et al. (2018) observed an inflammation of foot joints, infiltration of macrophages in the synovium, thickening of chorioallantoic membrane after hatching. In dogs, Kul et al. (2015) reported non-purulent encephalitis, myocarditis, fibrinohemorrhagic enteritis, necrosis and multifactorial pulmonary consolidation in young puppies infected experimentally with neosporosis.

2.10. Clinical symptoms

In general, clinical symptoms are similar to those commonly found in toxoplasmosis, but neurological and muscular disorders are more common in forelimbs (Dubey, 2003). Symptoms such as connective tissue disease, peripheral movement, and hindlimb paralysis have been found in newborn older than 3 weeks (Furuta et al., 2007). In young puppies aged 3-9 weeks, forelimb atrophy and persistent muscle stiffness are the most important clinical signs that distinguish neosporosis from other degenerative diseases. In some cases, hind limbs might be affected more than the forelimb with formation of arthrogryposis and articular deformation and paralysis that leads to muscles and rigidity of affected joints (Dubey and Schares, 2011). Some neonatal dogs may show the signs of joint deformity, cervical weakness, loss of appetite, and megaesophagus (Dubey et al., 2005). Dogs older than 6 months are more likely to develop the disease by reversing the latent infection (Nishimura et al., 2015). In adult dogs, neosporosis is asymptomatic, but may develop polymyositis and / or medullary encephalomyelitis with clinical signs associated with many lesions of the central nervous system especially in the brain (Lindsay and Dubey, 2000; Kul et al., 2015). As a result of over dose exposure to tachyzoites, dogs can develop the signs of myocarditis, dermatitis, pneumonia, ascites, peritonitis and multiple proliferationin addition to ataxia, nausea, vomiting, stuffy nose, headache, limb paralysis, neck and hyperesthesia (Detmer et al., 2016; Lye et al., 2020); while in chronic phase, most animals remain asymptomatic (Kul et al., 2015).

2.11. Immunity

Many studies have been hypothesized to the role of CD8 and T lymphocytes in protecting the host against infection by Apicomplexan (Jordan and Hunter, 2010). Fereig et al. (2015) reported that IFN-γ

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production which corporate with the humoral immune response is the major defense mechanism achieved by CD8 + T cells during *Neospora* disease. However, many factors including cytokines such as IL-2 and IL-12 can influence the development of the CD8 + T cell response and contribute to T cell proliferation, survival, and function (Li et al., 2021).

2.12 Diagnosis

Several methods were described for identification of *N. caninium*; but serologic tests, polymerase chain reaction (PCR), or histopathology are the most preferable tools. Although, parasitic isolation is usually the most accurate tool, it is very difficult to carry out due to difficulties in recovering a large number of live *Neospora* as well as isolation of parasites in cell culture can take several weeks (Dubey et al., 2007; Donahoe et al., 2015).

2.12.1. Serological examination

In cattle, serologic tests are usually performed using the Enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), *Neospora*-agglutination test (NAT), and immunobloting assay (IBA). The ELISA test can be used with milk or serum with identification that this technique is one of the best diagnostic assays in examination of cattle in herds (Wapenaar et al., 2007; Tamponi et al., 2015). Many studies demonstrated that ELISA is highly sensitive, specific and more accurate and reliable than other serological assays in particular IFAT (Staubli et al., 2006; Casartelli-Alves et al., 2014; Mansilla et al., 2020).

2.12.2. Histopathology

Histopathology plays a role in diagnosis and detection the distribution of lesions and parasite with confirmation of clinical symptoms with providing an important support for visual infections (Van Maanen et al., 2004; Donahoe et al., 2015). Several researchers have been reported that histopathological examination is more invasive than serologic examination, and the preparation of tissue samples were not always available until the animal is deceased (Guy et al., 2001; Duarte et al., 2020). However, serological testing can be made beside solely or beside the histopathology (Donahoe et al., 2015).

2.12.3. Polymer chain reaction (PCR)

PCR analysis is a highly sensitive, specific and accurate diagnostic method, which having the capability for testing different samples such as tissues, blood and body fluids (Barry et al., 2019), as well as detection of live and dead parasite with identification the presence of *N. caninum* DNA (Sharifzadeh et al., 2012). Wang et al. (2018) reported that the *Nc5* gene and the *Internal Transcribed Spacer* (ITS) gene are the most commonly used to detect *N. caninum* in different samples in particular brain due to notorious nature of the parasite to nervous tissues. Al-Shaeli et al. (2020) reported the ability of *N. caninum* to accumulate in different organs and tissues, so that; the performing of PCR on brain tissue alone reduces the chance of diagnosis of infection.

2.13. Sequencing

Information concerned to genetic variation of *N. caninum* is increased, and many studies have revealed that there are significant genetic differences between isolates. It has many purposes in the genome used to study differences between isolates (Rico-San Román et al., 2020).

2.14. Treatment

Worldwide, many studies have attempted to find the best therapy for neosporosis. Lefkaditis et al. (2020) discovered that treatment of bovine neosporosis need to withdrawal period in particular in dairy cows; however, they pointed out that this problem was not serious and chemotherapy has less impact. Another problem is that there was no drug guarantee 100% of affectivity against tissue cysts and bradyzoites due to drug resistance (Imhof et al., 2022). However, several studies demonstrated that sulfanilamide has a historical interest in treatment of toxoplasmosis and, in the basis, might be effective alone in treatment of *N. caninum* or in combination with other drugs such as trimetoprim to reduce the clinical symptoms and prevent death, but this is not entirely possible (Prescott, 2013; Baneth and Solano-Gallego, 2020). Other suggested therapies include lasalocid sodium, monensin sodium, trimetoprim, pirimethamine and piritrexim which have been shown to be effective in preventing the development of tachyzoites in bovine neosporosis (Pereira et al., 2017; 2018). In mouse model, Qian et al. (2015) suggested the using of toltrazuril and ponazuril to prevent the development of brain damage.

2.15. Control

Despite the life cycle of *N. caninum* is now known, it is difficult to make specific precautionary or recommendations because other non-canine species may act as a definitive host (Sokol-Borrelli et al., 2020). However, farmers should be encouraged to protect their food and water from dog contamination (Masatani et al., 2018). Other studies mentioned that the vertical transmission is the most important mode of transport, and prevention requires several control strategies such as culling of serologically positive cows and destroying the milk of serologically positive cows (Häsler et al., 2006; Guido et al., 2016). However, most of these control methods is economically feasible due to the cost of such methods, and the two common ways to prevent *N. caninum* infections include control of congenital and post-natal transmission of infection (Demir et al., 2020).

2.16. Vaccination

Experimental studies have shown the effectiveness of several vaccines based on killed tachyzoite that generate humoral and cellular responses (Weston et al., 2014). In USA, one approved vaccine has revealed an efficacy inimmunization of cattle against *N. caninum* through eliciting a strong immune response in addition to its safety for animals through reducing the abortion rates (Marugan-Hernandez, 2017). According to one study, vaccination with live tachyzoites before pregnancy provided 100% of protection; whereas, vaccination with tachyzoit lysate did not provide this protection (Williams et al., 2007). Experimental inoculation of live tachyzoites to cattle can develop an active immunity against vertical transmission of infection but with different challenges at mid-gestation (Mazuz et al., 2021).

Conclusion

Neospora caninum represent relatively a neglected parasitic pathogen in Iraq, in which, several morbidities and mortalities were diagnosed mainly based on clinical signs. Recent studies in last decade using of serological and molecular assays have demonstrated the prevalence of parasite among farm animals as well as dogs. However, the role of other canines as definitive hosts requires further investigations. In addition, there are many challenges for preventing the vertical transmission of parasites after subsequent parasite infection during pregnancy. Therefore, moreover studies are of great importance to establish new methods for treatment, control and prevention.

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