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Invited Review

# Epidemiology and Pathogenesis of *Neospora caninum* Infection: Special Emphasis to Neosporosis Status of Turkey

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A B S T R A C T

*Neospora caninum* is a protozoan parasite, first identified in dogs in 1988, having noteable similarities to *Toxoplasma gondii* in terms of biological and morphological features. Neosporosis, an important disease of mainly cattle and dogs, is one of the most prevalent infectious agents responsible for dairy and beef cattle abortions throughout the world. During the last two decades, abortion storms resulting from neosporosis have been increasingly reported from USA, Australia, Europe, Asia and Middle East countries including Turkey. The disease can cause milk yield loss, low fertility and abortions, particularly during the 5-6$^{th}$ months of the pregnancy. The incidence of abortion can be repeated in the subsequent pregnancies of the diseased animals and abortion rate can reach to 70% in a herd due to neosporosis. Subclinically persiste infected heifers can necessarily transmit the disease to their next progeny during their immediate pregnancy with a rate of up to 95%. Therefore, it is crucial to take cautious measures; e.g., prevention of direct dog-cattle interaction and elimination of seropositive animals from the herd, for the sake of successive herd management. There is still neither vaccine nor chemotherapeutic treatment protocol which could be effectively used in the field. In Turkey, there are several reports indicating that animals had come contact with *N. caninum* during some point in their lifespan. Seropositivity rates among some dairy cattle and dogs have been reported 4.8% to 32.7% and 10% to 28.9%, respectively. Although the role of neosporosis in epidemic and sporadic cattle abortions in some provinces has succesfully established, it is not still possible to achieve bird's eye-view of neosporosis status in Turkey. Moreover, increasing numbers of publications appear in the literature, are declaring only seroprevalence data of neosporosis in local provinces.

*Keywords: Cattle, Dog, Epidemiolog, Neospora caninum, Neosporosis, Turkey*

# *Neospora caninum* Enfeksiyonunda Epidemiyoloji ve Patogenez: Türkiye'de Neosporozise Genel Bakış

Ö Z E T

*Neospora caninum* ilk kez 1988 yılında köpeklerde tanımlanan, *Toxoplasma gondii* ile biyolojik ve yapısal olarak büyük benzerlikler gösteren bir protozoondur. Neosporozis, köpek ve sığırlarda en yaygın abort nedenleri arasında yeralır. Son yirmi yıldır, neosporozise bağlı abort salgınları Amerika, Avustralya, Avrupa, Asya ve Türkiye'nin de dahil olduğu Ortadoğu ülkelerinden artan sıklıkla bildirilmektedir. Hasta hayvanlarda, fertilite ve sütverimi kayıpları ile genelde gebeliğin 5 ve 6 ncı aylarında görülen abortlar dikkati çeker. Neosporozise bağlı abortlar, ineklerde takibeden gebeliklerde de şekillenebilir ve bir sürü içerisindeki abort oranı %70'lere kadar varabilmektedir. Bununla birlikte epidemiyolojik olarak esas problem subklinik persiste enfekte düvelerin, neosporozisi gelecek nesillere aktarmalarıdır. Anneden yavruya geçiş, %95 kadar yüksek bir oranda şekillenebilir ve başarılı bir sürü yönetimi için; köpek sığır ilişkisinin önlenmesi, damızlık adayı seropozitif hayvanların sürüden ayıklanması gereklidir. Süt sığırlarında neosporozisin kontrolüne yönelik henüz pratikte uygulanabilir aşı ya da ilaçla tedavi yöntemi bulunmamaktadır. Türkiye'de, hayvanların hayatlarının bir döneminde parazitle temas ettiklerine dair çok sayıda serolojik çalışma sonucu bulunmakta ve seroprevalans süt sığırlarında %4.8 ile %32.7, köpeklerde ise %10 ile %28.9 arasında değişmektedir. Türkiye'nin bazı coğrafi bölgeleri için, neosporozisin epidemik ve sporadik sığır abortlarındaki rolü başarıyla ortaya konulmasına karşın, mevcut durumda Türkiye'de *N. caninum* enfeksiyonlarının genel bir fotoğrafının çekilmesi mümkün görünmemektedir. Üstelik, literatürde artan bir hızla yerini alan Türkiye kaynaklı makalelerin büyük bir çoğunluğu, yalnız yerel bölgeleri temsil eden ve sınırlı sayıda hayvanın dahil edildiği serolojik analiz sonuçlarını içermektedir.

*Anahtar Kelimeler: Epidemiyoloj, Köpek, Neospora caninum, Neosporozis, Sığır, Türkiye*

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## Introduction and Brief History of Neospora caninum Infections

Neospora-like infection was firstly reported in dogs in 1984, and its definition as a new protozoon species, *Neospora caninum* was acknowledged by Dubey et al. (1988) after its clinical observation and etiologic identification in dogs. During a decade following the discovery of *Neospora caninum*, only tachyzoite and bradyzoite stages were identified in the intermediate hosts but neither its oocyst nor definitive host/s were established (Dubey et al., 1988; Barr et al., 1990; Conrad et al.,1993; Dubey, 1999a). The lack of evidence for a definitive host of *Neospora caninum*, raised questions by several scientists, whether this parasite is a really recently discovered one or was it a different genotype of *T. gondii* or *Hammondia* sp. (Mehlhorn and Heydorn, 2000). However, during the same period, it was evidenced that *N. caninum* differs from all apicomplexan protozoa in terms of ultrastructural, antigenic and molecular features (Lindsay and Dubey, 1989; Wouda et al., 1997; Dubey 1999a; Lindsay et al., 1999; Dubey 2003; Reitt et al., 2007). *Neospora caninum* also exhibits a different epidemiologic nature, causing abortions mainly in cattle and dogs in contrast to *T. gondii* (Dubey 1999a; Dubey 1999b; Atkinson et al., 2000; Dubey 2003; Gondim et al., 2004). In retrospective studies, investigating the possibility of neosporosis in clinical cases those were formerly diagnosed as *Toxoplasma*, revealed that *N. caninum* infection existences were as early as 1950s (Dubey et al., 1988; Dubey 1999a). By this way, it was shown that several cases had been misdiagnosed as *Toxoplasma* gondii, up to definition of *Neospora caninum* as a new protozoon (Dubey 1988).

Between 1990 to 2000, neosporosis became one of the most important abortifacient agents in cattle (Conrad et., 1993; Jardine and Wells, 1995; Wouda et al., 1997; Dubey 1999a; Gonzales et al., 1999; Atkinson et al., 2000; Reichel, 2000). *Neospora caninum* infections caused high incidence of abortions and increased economical losses in dairy cattle in some states of USA, Brasil and England, and disease took more interest than other infectious agents responsible for abortions, e.g. brucellosis and BVD, in those countries (Dubey 1999a; Dubey 1999b; Dubey 2003). Mc Allister et al., (1998) found that the dogs are definitive host of *N.caninum* and thus, one of the important pieces of the puzzle, oocyst stage, was accomplished. Afterwards, the importance of cattle-dog contact and oocyst shedding by dogs in the epidemic abortions of dairy cattle (exogenous transmission) were clarified (Dubey 2003; Gondim et al., 2004).

At the beginning of the second millennium; neosporosis has been reported from different geographical sites of the world with an increasing rate, and it is re-established that neosporosis is a disease of mainly cattle and dogs (Reichel 2000; Coskun et al., 2000; Dubey 2003). Pregnant cattle show abortion generally during the second trimester of the pregnancy and it was shown that abortion could be repeated in their later pregnancies (Dubey et al., 2007). Otherwise, neosporosis can be successfully transmitted to the fetuses during pregnancy and infected but non-clinically sick newborn calves may bear. This latter is epidemiologically more important than abortion, and, vertical transmission seems to be the major route of the parasite's survival through the next progenies in cattle (endogenous transplacental transmission) (Dubey et al., 2007; Dubey and Schares, 2011). When neospora-infected-born female calves reach to their puberty ages, they can also give a healthy, but neospora infected birth and *Neospora caninum*-infection prevalence increases by time in that herd, which is not implemented on successful herd management (Dubey et al., 2007).

At this moment, following issues still need to be properly understood; reactivation mechanism of the parasite during gestation, pathogenesis of abortion, placental and maternal immunologic reactions and development of efficacious vaccine and/or chemotherapeutic protocols (Dubey et al., 2006; Dubey and Schares, 2011).

## Etiology and Epidemiology

*Neospora caninum* can invade and cause clinical symptoms in its intermediate hosts such as cattle, sheep and goats (Dubey et al., 2007; Dubey and Schares, 2011). There are two developmental forms (tachyzoite and bradyzoite) of *Neospora caninum* in its intermediate hosts. Tachyzoites in crescent shape and 2x6μm in size, have a centrally localized nucleus (Figure 1a). Tachyzoites, -the rapidly proliferating form- can invade the brain, spinal cord, heart, skeletal muscle, liver, adren, lungs, spleen and lymph nodes (Figure 1b) (Barr et al., 1990; Conrad et al., 1993; Dubey et al., 1988; Dubey 2007). Bradyzoites are 1.5x6.5μm in size and they are the slowly growing developmental form, and they contain abundant amounts of amylopectin granules. Tissue cysts may reach up to 107μm in size, depending on how many bradyzoites they contain, and the cyst wall can be as thick as 2-4μm (Figure 1c). *Neospora caninum* tissue cysts have been detected only in brain, spinal cord and skeletal muscles (Dubey 2003; Dubey 2007; Kul et al., 2009). While it is thought that vertical transmission takes place after somehow reactivation of the tissue cysts, tissue cysts in adult animals couldn't be demonstrated (Wouda et al., 1997; Dubey 2011). Thus, probably a fewer tissue cysts are responsible from vertical transmission in persistently infected pregnant animals. The oocysts of *Neospora caninum* are shed by definitive hosts, dogs (*Canis familiaris*), coyotes (*Canis latrans*) and gray wolves (*Canis lupus*) and they sporulate within 24 hours (Mc Allister 1998; Dubey and Schares 2011). Systemic neosporosis may also develop in dogs and red foxes. A sporulated oocyst, which is round to oval in shape and 10-11μm in size, contain 2 sporocysts and four sporozoites in each (Figure 1d) (Lindsay et al., 1999; Dubey 2003). *Neospora caninum* oocysts show great resemblance to the oocysts of *Hammondia hammondi* in cat and *H. heydorni* in dog faeces. Copro-PCR technique is helpfull in the identification of these oocysts in faeces, as they cannot be differentiated morphologically (Dubey and Schares 2006).
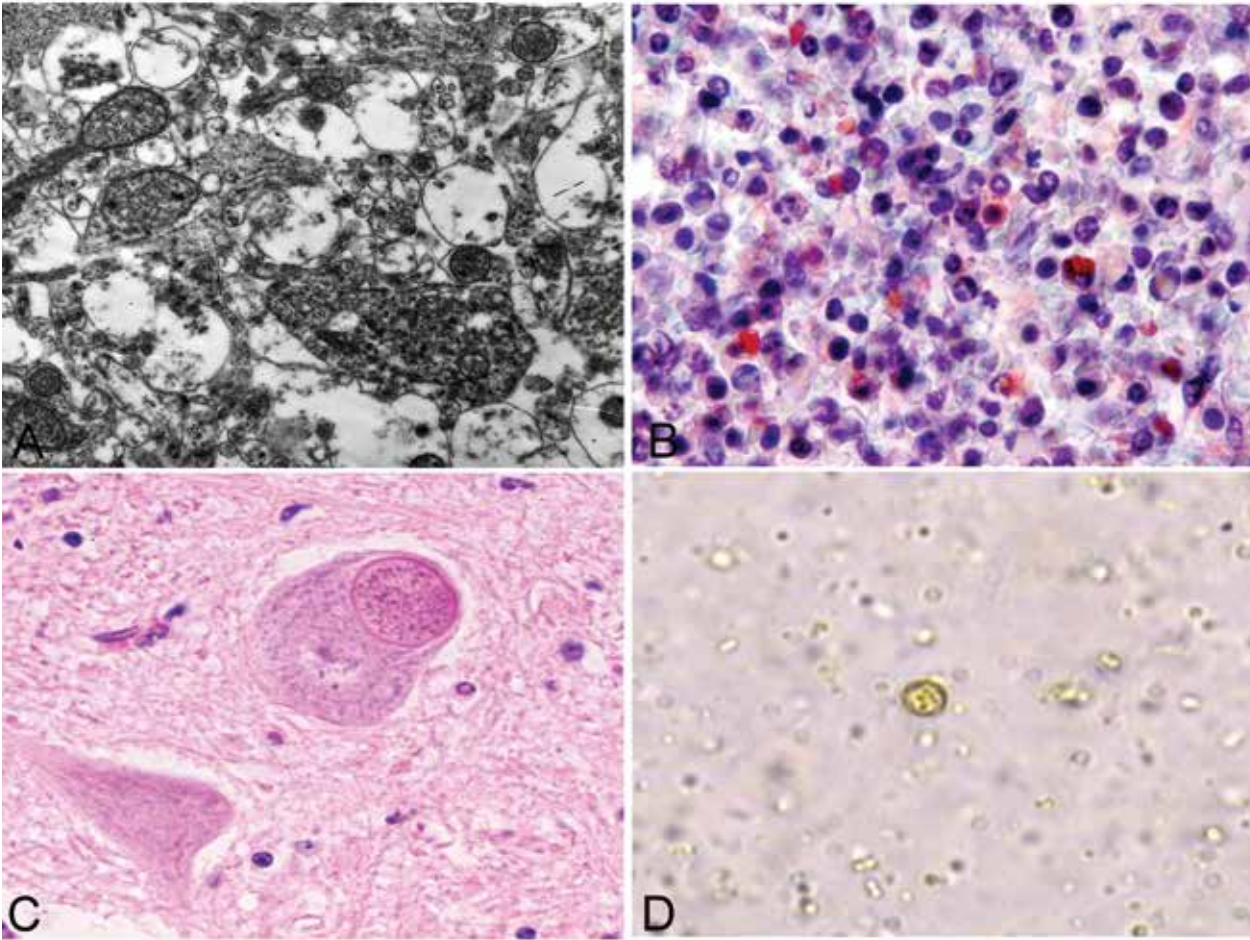
**Figure 1.** Developmental stages of Neospora caninum. a) Ultrastructural appearance of a tachyzoite in the brain of a calf. Carl Zeiss EM9000, X 7 000. b) Phagocytized Neospora caninum tachyzoites in the cytoplasm of sinusoidal macrophages. Mesenteric lymph node, calf. N. caninum primary antibody, HRP peroxidase, AEC chromogen and Mayer's hematoxylin counterstaining. X 320. c) Tissue cyst localized in a neuron cytoplasm, X 160. d) Sporulated oocyst X 500.

**Şekil 1.** Neospora caninum gelişim aşamaları. a) Bir buzağının beyin dokusunda saptanan takizoite ait elektron mikroskobik görünüm, Carl Zeiss EM9000, X 7000. b) Sinüzoidal makrofaj sitoplazmalarında fagosite edilmiş Neospora caninum takizoitleri, mezenteriyal lenf düğümü, buzağı. Neospora caninum primer antikoru, HRP peroksidaz, AEC kromojen ve Mayer's hematoksilen karşıt boyama X320. c) Nöron sitoplazmasında doku kisti, X160. d) Sporlanmış ookist X 500.

Horizontal transmission of susceptible intermediate hosts occurs by ingestion of oocysts shed in dog faeces (Wouda et al., 1997; Dubey and Schares, 2006). Cow to cow horizontal transmission does not seem very likely, except for calves fed tachyzoites containing milk in experimental conditions (Dubey 1999b; Ortega-Mora et al., 2007). Although it has not been completely shown, it is speculated that a limited number of infective oocysts shed within a short period of time after gametogony stage in dog intestines. There is still no scientific evidence of schizogeny and gametogony stages that are probably eventuating in intestinal epithelia of dogs (Dubey et al., 2007). In bioassay studies; the dogs orally fed tissue cysts obtained from experimentally infected mice and gerbil, did not shed oocysts (Dubey et al., 2007; Ortega-Mora et al., 2007). Nevertheless, after ingestion of tissues of an aborted fetus originated from a cow with neosporosis, *N. caninum* oocysts were successfully isolated from excrement samples for 2 days. Ingestion of infected placental tissues by dogs also results the same (Dubey et al., 2007; Paradies et al., 2007).

When intermediate hosts ingest food, contaminated with dog faeces containing *Neospora caninum* oocysts, oocyst and sporocyst walls rupture and infective sporozoites first invades intestinal mucosa and it is thought that they reach to the blodstream and regional lymph nodes (Innes et al., 2007). If a pregnant cow acquires *N. caninum* infection with horizontal transmission (oocysts shed by dogs), neosporosis can result in abortion or vertical transmission via passing through placental barriers (Exogenous transplacental transmission) (Gondim et al., 2004; Dubey et al., 2006; Dubey et al., 2007). If incidences of abortions with a rate of 10 to 12.5% among risk group pregnant cows take place within 6 to 8 weeks, this is considered as "epidemic abortion" which is closely related with point source infection. The role of horizontal transmission during epidemic abortions have been supported by the detection of seropositive newborn calf while its mother is seronegative and higher positive correlation between age and seropositivity in a herd (Dubey et al., 2006; Dubey and Schares, 2011). Higher neosporosis prevalences have been reported in dairy cattle ranches that have *N. caninum* infected dogs (Dubey 1999b; Aguiar et al., 2006). Besides, the dogs are raised in urban areas have lower seroprevalances

in contrast to those are having direct contact with dairy cattle in rural areas (Ferroglio et al., 2007). In an additional epidemiologic study focused on neosporosis status of dairy farms holding domestic dogs, neosporosis in family type ranches has higher seroprevalences than integrated ranches both for cattle and dogs (Unpublished observation).

*Neospora caninum* can pass to fetuses through damaged placenta in pregnant cattle, sheep, goats, cats, dogs, non-human primats and mice (Osawa et al., 1998; Dubey 1999b; Gonzales et al.,1999; Armangol et al., 2006; Bartels et al., 2006; Dubey and Schares, 2006). Neosporosis is basically a disease of pregnant uterus. Thus, endogenous transplacental transmission is the major route of infection. *Neospora caninum* mostly become reactivated during the 5th and 6th months of the pregnancy and causes abortion or congenitally infected born (Dubey 1999a; Dubey et al., 2006; Reitt et al., 2007; Kul et al., 2012). Congenitally infected newborn female calves that reached to their puberty ages can also have miscarriages or give birth to subclinically ill calf (Dubey 1999a; Dubey 1999b; Dubey 2003; Kul et al., 2009; Kul et al., 2012). Transferring of neosporosis through succeeding generations via neospora infected but clinically healthy female calves can cause incrementally higher prevalence in a breeder dairy cattle ranch than it is foreseen because of overlooked and/or neglected diagnostic procedures (Dubey and Schares, 2006; Dubey et al.,2007; Innes et al., 2007; Reitt et al., 2007). In a study conducted in California, 240 *N. caninum* seropositive pregnant dairy cows were followed up during their pregnancy period with monthly interval. Vertical transmission rate in these cows was detected 88%, by evaluation of their pre-colostral sera (Pare et al, 1997). In another study conducted in a European country, vertical transmission and infected birth rate of *N. caninum* is found to be 92% but abortion risk was 3.13 in dairy cattle ranches, which, previously experienced abortion (Wouda et al 1998).

In congenital infections, mummification, abortion, stillbirth and clinical- subclinical births may occur depending on the stage of the pregnancy (Innes et al., 2007). While early embryonic death, resorbtion or mummification of the fetus may take place in the first trimester of pregnancy, abortion and stillbirth are more frequently observed in the second trimester. During these periods, the immune system of fetuses is either not developed or unsufficiently developed. Thus, the outcome of the infection is more severe in the tissues and organs of the fetus than a fully developed one (Dubey et al., 2006). On the other hand, developed immun sysytem of the fetus can deal with the infectious agent in the third trimester, and more likely subclinically infected live births may occur (Barr et al., 1990; Atkinson et al., 2000; Dubey et al., 2006; Dubey et al., 2007; Innes et al., 2007; Kul et al., 2012). Gondim et al., (2004) reported that 5 out of 19 calves were born with neosporosis from 19 beef cattle inoculated with *N. caninum* oocysts in their 70-176th days of pregnancy. In that study, it was shown that 3 out of 5 congenitally infected born calves were obtained from 4 cows experimentally infected in 162-

176 days and vertical transmission of neosporosis to the next generations occurs more frequently in the last stage of pregnancy (Gondim et al., 2004).

Clinical neosporosis has been reported in calves and dogs (Jardine and Wells 1995; Gonzales et al.,1999; Reichel, 2000; Kul et al., 2009; Kul et al., 2010). It is rarely seen and only 1 to 5% of all pregnancies in cattle with neosporosis result in clinical signs in newborn animals. In affected calves, difficulty in standing up and walking, incoordination, tremors, hyperextension or flexion in fore and hind-limbs are characteristic features (Dubey 1999a; Dubey 1999b; Dubey 2003; Kul et al., 2009; Kul et al., 2010). In slightly affected cases, arcur in carpal phalanges, exophtalmus and weak patellar reflex can be observed. In these animals, pupillar and anal reflexes are normal (Kul et al., 2010).

In human, while specific protein structures and antibodies are demonstrated, there is no information regarding systemic neosporosis associated with abortion or clinical neosporosis (Aydın et al., 2003; Dubey et al., 2007).

**Diagnosis**

In serologic diagnosis of neosporosis, commercial c-ELISA and IFAT are frequently used in practice (Björkman et al., 1994; Williams et al., 1997; Coskun et al., 2000; Akca et al., 2005; Aguiar et al., 2006; Bartels et al., 2006; Ferroglio et al., 2007; Yildiz et al., 2009). Detection of *Neospora caninum* specific antibodies in an animal serum shows only exposure to the parasite and it is not in the meaning of ongoing neosporosis. Otherwise, seropositive results obtained from either pre-colostral sera of newborn calves or peritoneal, pericardial and cerebrospinal fluids taken from aborted fetuses are the indicatives of transplacental neosporosis (Williams et al., 1997; Osawa et al., 1998; Dubey 2003; Dubey and Schares 2006).

Definitive diagnosis of neosporosis can be achieved by any combination of at least two analyses e.g. serology, PCR, pathology and immunohistochemistry (Lindsay and Dubey, 1989; Dubey 1999b; Dubey, 2003; Dubey and Schares 2006; Reitt et al., 2007). Blood serum, blood and/or body fluids (e.g. cerebrospinal, pericardial and ascites fluids) brain, hearth, liver and placenta are the most suitable samples to be sent to laboratory for diagnostic purposes (Dubey and Schares 2006; Dubey and Schares 2011). Immunohistochemistry (IHC), allows labelling and demonstration of *N. caninum* specific proteins in lesioned tissue sections, is the golden standard in diagnosis of neosporosis (Lindsay and Dubey, 1989; Wouda et al., 1997; Dubey 1999a, Dubey 1999b). Sometimes, organs of aborted fetuses may be destructed by autolysis in a short time, and tissues can be liquified. Even if they aren't suitable for histopathologic examinations, IHC analyses can be conveniently used after their fixation in 10% buffered formalin, routine pathologic follow up and sectioning (Lindsay and Dubey, 1989; Dubey 1999a). Polyclonal anti-*Neospora caninum* antibodies, which are produced in several laboratory animal species, using immunization techniques, are available in different

ACGGGTTGCGGGGCAGGATGGACGAAGTGGCAGGAGCAGGATGACAACGGGTTTTGGTGAGGCTTCATGCGAGGT

CTCGCGATGCCGTCCTCACGCATGAGGCCGGAGAATGAGAGCGATTTCCAGGTGTCTTCCTTCTGAGTCGGGTTGT

GTTTGGCTGTGAAGGACAGGGTTGGGTATCGCGGTGGAGCTGGGTTGCTGTGCTCGCTGGGACTTCAGGAGCGGCA

TCGGAGGACATCGCTCACTGACTGGAGGCACGCTGAACACCGTATGTCGTAAATCGGAGTTGCTTCTATGTGGCAT

TCCTCTTGCGAAGCATTGTTGGTGGAGCAGCGCAGCGGTGTTGGCTGGAGGCGGCAGCAGCGAGGGAGGTGTGGT

GGAGGTATAGGAGAGAGAATTTCGTGGAAGGGCGGAATGGAAGGAATTGAGTGGAGGCAGGGGGGA`GGGTGTGC` `GTCCAATCCTGTAAC`GT`GTTGCTCTGCTGACGTGTCGTTG`TTGGGCGCAGCCTGCGGCAGCAAGGCTCCTTTTTGTT

TGTGACTATAGTGTGTGAACGGGTGAACCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGTGGTTA

GTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGAGGACAGTGTGTCAATGATACTTAT`CGAGAGTTCAGTGT` `TCTGTGTTGAG`GCAACACCGGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCAAGCGGACGAGGGAAGGG

GC`AGAAGACGTAGGTTGACTGGCGAG`TGCCCCCGAGGAGACGAGAATGAGTTTCGTTCGCATTGGGAGGCACAAG

AAAGCATCCAGGTATGGGAGCCAAATAAGGGAACCGTGGTATAGACTGGATGCCCGTCGAGGTAGGTGGTCAATG

TCGACACTGCTGGCGAACGGAGAGCGCCTGCGGGGAGGCGACACGTCTCCGGTAGAGTTTTTGGAATCGTGGGCC

GAGTGTAACGAGGTCGATGTGAACGGGCAGGCGGAAGAGCGGCCTGGTAGATGGGTTTGCAGAGCGTTGTGCACT

TGCGTGTTTAGGTGACAGATGTGAGTTTTTTTATCATTGCTGTGGAGACGCTGTCCAGCGTTAGCATACGCCGCACA

AAAGGAATATCATGAAGGAATCGGATTCGTGGTGGCAGTGATGAATGGGTAGGGAGTAGAGGTGAGTGACCTG

**Figure 2.** Demonstration of amplified DNA sequences which are specific to Nc-5 gene of Neospora caninum. First PCR step; Purple label is Np21+ forward primer, Yellow label is complementary of Np6+ reverse primer. Second internal PCR; green label is Np9 forward primer and red label is complementary of Np10 reverse primer.
**Şekil 2.** Neospora caninum Nc-5 geni üzerinde yükseltgenen spesifik bölgenin gösterimi. İlk aşama; pembe renk Np21+ forward primer, sarı renk komplementer Np6+ reverse primer. İkinci internal PCR; yeşil renk Np9 forward primer ve kırmızı renk komplementer Np10 reverse primer.

research laboratories (Lindsay and Dubey 1989; Wouda et al., 1997; Dubey 2003). There are some prerequisites, e.g. parasite's itself or its immunogenic antigens that should be completed before immunization procedures. Thus, these sources, even anti-*Neospora* specific primary serum, have been kindly shared between related laboratories with international collaborations. Using these tachyzoite specific antibodies, neosporosis have specifically been diagnosed in the lesioned tissues of aborted fetuses, placenta and died animals following clinical neosporosis (Wouda et al., 1997; Wouda et al., 1998; Kul et al., 2010; Kul et al., 2012). Positive and negative tissue controls should be used in each round of immunohistochemical tests, especially where the *Neospora* specific IHC will be firstly carried out. Unless standardization, working dilution setting of primary antibody and positive control sections, IHC tests will be inconclusive and requires additional confirmatory tests e.g., PCR etc.

Specificity and sensitivity of PCR usage on diagnosis of neosporosis is closely depending on sampling technique, degree of autolysis and PCR procedures (Dubey and Schares 2006; Reitt et al., 2007). Sampling from non-lesioned areas or necrotic lesions may cause false negative results. However, PCR positive results can't be used on the evaluation of viability, infectivity or quantity of the parasites (Reitt et al., 2007; Dubey and Schares

2011; Kul et al., 2012). Nested PCR techniques are open to cross contamination and negative control lanes are very valuable. Cross contaminations take place mostly during the manipulation of micro-centrifuge tubes and transfer of amplified DNA after first PCR reaction (Ortega-Mora et al., 2007). Neospora specific DNA can also be amplified from formalin fixed and paraffin embedded tissues (FFPE). Methyl bridges form permanent bounds between DNA molecules after formalin fixation, so the first step should be DNA retrieval methods, such as heating up at 95$^o$C for 20 minutes. However, every minute of heating over 60$^o$C may also cause DNA break and it is explaining that why a positive DNA sample of FFPE hardly amplified in PCR (unpublished data). In a study, histopathologically Neospora consistent lesions were labelled and paraffin samples were forwarded to PCR analyses. Interestingly, PCR positivity was higher in paraffin sections than in fresh tissues (Reitt et al., 2007).

For final amplification of 224 bp *Neospora caninum* Nc-5 gene specific area, nested-PCR technique constituted of consecutive two steps has been used **(Figure 2)**. *Neospora caninum* Nc-5 gene specific nested PCR primers in the first PCR reaction are; Np21+ (forward primer 5'-GGG TGT GCG TCC AAT CCT GTA AC-3') and Np6+ (Reverse primer 5'-CTC GCC AGT CAA CCT ACG TCT TCT-3') (Muller et al.,1996; Liddell et al.,1999). In the second reaction of nested PCR; internal primers are Np9 (5'-GTT GCT CTG

CTG ACG TGT CGT TG-3`) and Np10 (5'-CTCA ACA CAG AAC ACT GAA CTC TCG-3').

## Pathogenesis and Pathologic Findings

If an adult animal is firstly infected by *N. caninum*, infective stages such as sporozoites and tachyzoites invade and pass mucosal barriers and then they reach to blood stream after replication in mesenteric lymph nodes and spleen (Wouda et al., 1997; Wouda et al., 1998; Dubey 1998a; Innes et al, 2007; Dubey and Schares, 2011). Following this phase fluctuating parasitemia takes place, where host immune system mostly limits the replication of the parasite and tissue cysts of *N. caninum* form in brain and spinal cord. Chronic subclinical infection may likely continue until the end of host's life in concordance with the immun system (Wouda et al., 1998; Dubey 2003; Innes et al., 2007; Kul et al., 2012).

In a pregnant animal; cellular immunity is supressed in favor of materno-fetal connections and the maintenance of the pregnancy. In this case, while T helper 1 cytokines (IL-2, IL-12, TNF-alpha and IFN-gamma) are downregulated, T helper 2 cytokines (mainly IL-4 and IL-10) are upregulated (Innes et al., 2007; Ortega-Mora et al., 2007; Dubey and Schares 2011). In *in-vitro* studies, it has been shown that elevated levels of IFN-gamma are effective on tachyzoite to bradyzoite stage conversion of the protozoon, and vice versa. Several studies in mice and cattle have demonstrated that immunity to *N.caninum* infection involves predominantly Th1-type immune response. High levels of Th1 response during protozoal infections compromises the viability of the fetus, whereas Th2 response facilitates the maintenance of the pregnancy (Wouda et al., 1998; Innes et al., 2007; Ortega-Mora et al., 2007).

Tissue cysts of the parasite reactivate with an unknown mechanism and tachyzoites invade placenta. At this stage, depending on the severity of placental damage, parasite load and the developmental status of the immunity of the fetus, either abortion, or, subclinically infected but healthy calf birth is seen (Wouda et al., 1998; Atkinson et al., 2000; Dubey and Schares 2011). If the parasite reactivates during the last trimester of the pregnancy, a limited and slight infection develops in fetus by the effects of competent fetal immun system and increased fetal age. In this case, pregnancy results with transplacentally infected but healthy calf birth (Wouda et al., 1998; Gondim et al., 2004).

Abortion mechanisms in neosporosis are quite complicated. First of all, it is thought that cytokine exaggregations originated from placental lesions may cause de-attachment on chorionic villi connections and then fetal hypoxia (Dubey et al., 2006). Second abortion mechanism is activation of luteolysis by placenta-derived prostaglandins arising from lesioned placenta. In this instance, fetal ACTH is mainly responsible for the abortion. The third reason for abortion in neosporosis is, severe fetal infection, and death of the fetus caused by transplacentally acquired tachyzoites (Wouda et al., 1998; Gonzales et al., 1999; Dubey 2003; Dubey et al.,

2006; Ortega-Mora et al., 2007).

The most characteristic pathological finding in aborted fetuses is encephalomyelitis (Dubey et al., 1988; Barr et al., 1990; Dubey 1999a; Dubey 1999b; Kul et al., 2006). Multifocal gliosis, necrosis of the gray matter, demyelinization and perivascular mononuclear cell infiltration are noticed in the brain, spinal cord and cerebellum (Lindsay and Dubey, 1989; Wouda et al.,1997; Wouda et al., 1998; Dubey, 2003). In some instances, brain lesions can be restricted with mild gliosis, astrocytosis and neuronal degenerations, where the tachyzoites are present. Multifocal leucomalacia suggests placental hypoxia and no tachyzoites are found in these necrotic areas (Dubey et al., 2006; Ortega-Mora et al., 2007). *Neospora caninum* tissue cysts are localized in neuron cytoplasms and neuropil of the gray matter. Tissue cysts are quite rare features of fetal pathology and they arbitrarily encountered during a detailed histopathological examination. In heart, non-purulent interstitial myocarditis is associated with degeneration and necrosis of myocardium. There are numerous free and intracellular tachyzoites around necrotic lesions. Skeletal muscle lesions are similar to those of myocardium and characterized by necrotic myositis (Lindsay and Dubey 1989; Dubey 1999a; Dubey 2003; Ortega-Mora et al., 2007; Kul et al., 2009; Kul et al., 2010; Kul et al., 2012).

In liver, periportal mononuclear cell infiltrations and hepatocyte necrosis occur and periportal hepatitis in aborted fetuses is a more frequent feature of exogenous horizontal transmission (Barr et al.,1990; Wouda et al., 1997; Wouda et al., 1998). In a large scale and well-organized study, which is focused on fetal pathology; encephalitis and myocarditis were seen in all of 82 fetuses. In the same study, adrenalitis (80%), myositis (72%), nephritis (66%), hepatitis (62%) and placentitis (53%) have been reported as other most frequently detected lesions, respectively (Bar et al., 1990). However, in another study conducted in Holland, it was reported that encephalomyelitis, myocarditis and hepatitis associated with *Neospora caninum* tachyzoites in 91% of all histopathologically examined fetuses (n=80) (Wouda et al., 1997).

Placental pathology can also be helpful in diagnosis and explanation of vertical transmission-pathogenesis, but tachyzoites are rarely encountered in the placenta. Placental lesions comprise mononuclear cells in materno-fetal interface, replicated tachyzoites in villi chorialis, and villous necrosis. Increased level of placental Th1 cytokines is closely associated with invasion degree of *Neospora caninum* tachyzoites (Dubey et al., 2006; Dubey and Schares 2011; Kul et al., 2012).

## Neosporosis in Turkey

In Turkey, there are several reports indicating that animals had come into contact with *N. caninum* during some point in their lifespan (Table 1). Seropositivity rate among dairy cattle herds has been reported between 4.8% and 32.7% (Akca et al., 2005; Aktas et

**Table 1.** Neosporosis seroprevalences in Turkey.
**Tablo 1.** Türkiye'de genel neosporozis seroprevalansı.

| Specie | No. Exam | Test | City | % Positivity | Reference |
|---|---|---|---|---|---|
| **Dog** | 100 | IFAT | Bursa | 10% | Coskun et al., 2000 |
| | 1 | IFAT | Bursa | Clinical neosporosis | Batmaz et al., 2004 |
| | 121 | IFAT | Kırıkkale | 28.9% | Yıldız et al., 2009 |
| **Cattle** | 3290* | c-ELISA | Ankara<br>Çankırı<br>Eskişehir<br>Kayseri<br>Kırıkkale<br>Kırşehir<br>Nevşehir<br>Yozgat | 10%<br>6.9%<br>5.4%<br>10.8%<br>32.7%<br>19.5%<br>5.1%<br>20.3% | Bıyıkoglu et al., 2001 |
| | 92 | c-ELISA | Sakarya | 9.2% | Öncel and Bıyıkoglu, 2003 |
| | 513* | c-ELISA | Elazığ<br>Malatya<br>Bingöl<br>Muş | 15%<br>4%<br>4.7%<br>4.8% | Aktas et al., 2005 |
| | Local 228<br>Imported 73 | ELISA | Kars | 0%<br>8.2% | Akça et al., 2005 |
| | 305 | c-ELISA | Şanlıurfa | 23% | Sevgili et al., 2005 |
| | 186* | c-ELISA | Kayseri | 7% | İça et al., 2006 |
| | Cattle 40*<br>Heifer 25<br>Calves 6 | c-ELISA | Kırıkkale | 60%<br>40%<br>33.3% | Kul et al., 2009 |
| | 557* | c-ELISA | Kırıkkale | 10.77% | Yıldız et al., 2009 |
| | 134 * | c-ELISA | Ankara | 35% | Pişkin and Ütük, 2009 |
| | 450 | c-ELISA | Van | 4.8% | Alan et al., 2011 |
| **Cattle-Dog** | Cattle 1100<br>Dog 210 | CI-ELISA<br>IFAT | Kars | 7.2%<br>25.2% | Mor and Akça, 2012 |
| | Cattle 427<br>Dog 18 | c-ELISA<br>IFAT | Kırıkkale<br>Aksaray<br>Burdur<br>Elazığ | 37.7%<br>55.5% | **Unpublished Observation |
| **Goat** | 128 | c-ELISA | Erzurum<br>Kırşehir | 10.4% | Ütük et al., 2011 |
| | 180 | c-ELISA | Şanlıurfa | 5% | Sevgili et al., 2003a |
| | 181 | c-ELISA | Niğde | 25.9% | Cayvaz, 2011 |
| **Horse** | 90 | c-ELISA | Şanlıurfa | 8.8% | Sevgili et al., 2003b |
| **Human** | 190 | IFAT | Bursa | 0% | Aydın et al., 2003 |

*These test results contain aborted cow sera. ** Project results of the author's research group.*

al., 2005; Ica et al., 2006; Kul et al., 2009; Yildiz et al., 2009). Though there is not enough epidemiologic data representing overall situation of neoporosis in Turkey, the role of neosporosis in epidemic and sporadic cattle abortions in some provinces has successfully been established. However, it is not still possible to achieve bird's eye-view of neosporosis status in Turkey, despite there are increasing numbers of publications appear in the literature. But, a few of them have been indeed containing comprehensive data and helpfull for understanding of neosporosis epidemiology and impact on animal health in Turkey (Akca et al., 2005; Aktas et al.,

2005; Ica et al., 2006; Kul et al., 2009; Yildiz et al., 2009; Kul et al., 2010; Mor and Akca 2012).

According to serologic examination results of aborted dairy cows in Kars Province; it was reported that 6 out of 73 (8.2%) imported Simmental cows and none of indigenous cows sera samples were positive (Akca et al., 2005). The results of this study are very important because they concluded that neosporosis is not prevalent in local animals and imported breeder cattle have a greater risk on neosporosis transmission. In another study carried out in Eastern Anatolia, 7.01% of examined dairy cattle (n=571) were found seropositive

**Figure 3.** Clinical neosporosis in a Holstein calf exhibiting neurological signs. Notice that decubitis wounds on left hind-limb because of paraplegia.
**Şekil 3.** Sinirsel klinik belirtiler gösteren bir Holstein buzağıda klinik neosporozis. Parapleji nedeniyle sol arka bacakta dekübit yaraları dikkati çekiyor.

and *N. caninum* antibody was detected only in one out of 32 aborted cow (Aktas et al., 2005). They found that older dairy cows had higher seroprevalence than young stock up to 1 year old. But, the difference was not statistically important. Nonetheless, Simsek et al. (2008) reported that neosporosis seropositivity was 13.48% in repeat breeder dairy cows (12 out of 89) and 3.19% in healthy pregnant dairy cows. Thus, they demonstrated that neosporosis can important cause of repeat breeder in dairy cows in Turkey. Ica et al. (2006) declared 7% (13 out of 186) seropositivity in dairy cows and 3 of those seropositive cows were aborted. Yildiz et al. (2009) have reported co-association between *N. caninum* and *T. gondii, Brucella abortus, Listeria moncytogenes* seropositivities in aborted (n=234) and pregnant (n=323) dairy cattle. Serologic co-associations of these agents are *N. caninum/T.gondii* 24.77% (138/557), *N. caninum/B. abortus* 13.82% (77/557) and *N. caninum/L. monocytogenes* 42.85% (162/378), respectively.

In Turkey, the first report declaring epidemic abortions due to *N. caninum* infections in dairy cattle and clinical neosporosis in a calf was published in 2009 (Kul et al., 2009). The authors of this study suggested that vertical transmission of neosporosis through succeeding generations would be much higher than expected in Turkey. Because they followed up a closed dairy cattle ranch having over 15% abortion history for 3 years and high seropositivity rates in infertile cattle (60%), female heifers (40%) and calves (33.3%) were revelaed. They also described clinical neosporosis in a 20-day-old Simmental calf showing incoordination, tremors and hyperextension. Neosporosis was etiologically identified in that calf with ultrastructural demonstration of *N.caninum* tachyzoites and immunopositive reactions in the necrotic and degenerative neurons and in cardiomyocytes (Kul et al., 2009). In another study, *N. caninum* seropositive nine calves exhibiting neurological symptoms, difficulty in suckling, incoordination, exophtalmus, phalangeal contracture, and fore and hind limb paralyses, were detected in different 4 dairy cattle ranches during 5 years of follow up (Figure 3) (*N.caninum* seropositivity was between 23% to 40%, in total 2000 dairy cattle) (Kul et al., 2010). It is reported that five out of nine calves died, and in pathologic examinations *Neospora caninum* infection was confirmed by histopathology and immunopositive reactions detected mainly in the necrotic and degenerative neurons, cardiomyocytes, spleen, thymus and epithelial lining of the oral mucosa. Clinical neosporosis is a quite rare manifestation in calves (1 to 5%). As the author's knowledge, if clinical neosporosis diagnosed in a calf, tip of the iceberg, it will be an indicative of high seroporevalance in that ranch and intensity of neosporosis at the herd level.

Indeed, high rate of co-association between *N. caninum* seropositivity (35%) and abortion-stillbirth in dairy cattle was declared by Piskin and Utuk (2009). However, it also means, in fact 35% of all examined aborted cows might be contacted with the parasite somehow in a point of their life. In addition, if it was given, demographic data of regarding cows such as; age, breed and previous abortion history would increase the credibility of abortion and stillbirth descriptions.

In a serologic study that was carried out in stray dogs in Bursa Province, 10% (15 out of 150) *N.caninum* seropositivity was detected (Coşkun et al., 2000). Later on, the first clinical dog neosporosis in Turkey was reported in 2004 with neurological symptoms such as hyperextension of hind limbs and paraplegia in a 9-week-old Doberman Pincher puppy (Batmaz, 2004). As stated by the authors, this puppy unresponded to sulfadiazine and trimethoprim treatment and died. Then, it was forwarded to necropsy but no characteristic histopathologic findings observed. Thus, diagnosis was based on *N.caninum* specific antibodies at 1/800 IFAT titer and clinical findings. The other clinical and fatal dog neosporosis was observed in a mixed breed Kangal puppy, which was died shortly after its birth from a *N. caninum* seropositive mother. Neosporosis was diagnosed in this Kangal puppy by observation of severe multifocal necroses and gliosis in the brain, necrotic bronchopneumonia and *N. caninum* antigen immunopositive reactions in tissue sections (Figure 4) (Unpublished observation). A seroepidemiologic study showed that neosporosis prevalence in dogs were 28.9%, and 54.2% of which was also carrying *T. gondii* antibodies (Yildiz et al., 2009). In this study, it is speculated that there is no difference between dogs living in urban and rural areas, and high seropositivity rate of *T. gondii* co-association with neosporosis seropositivity might be originated from environmental contamination by domestic cats.

In a large scale study, blood samples collected from dogs (n=210) and dairy cattle (n=1100) located in 12 villages of Kars Province, were tested and neosporosis seroprevalence was detected as 7.2% and 25.2% in cattle and dogs, respectively (Mor and Akca, 2012). They also detected *N. caninum*-like oocysts in 10 out of 125 dog faeces. Concluding remark of this study; dog-cattle contact may have a greater risk in transmission of

neosporosis in Kars Province as naive and seronegative cattle proportion are still in majority. In another research results showed that cows and dogs present in integrated and modern dairy cattle ranches had much lower neosporosis prevalences than rural-family type ranches in Turkey (Unpublished data).

In goats, neosporosis was detected 5% (9 out of 180) in Şanlıurfa. In this study, difference between seronegative and seropositive goats was not statistically important (Sevgili et al., 2003a). In another study, serologically tested purebreed Arabian horses (n=90) showed 8.8% positivity with an increased rate in older horses (Sevgili et al., 2003b).
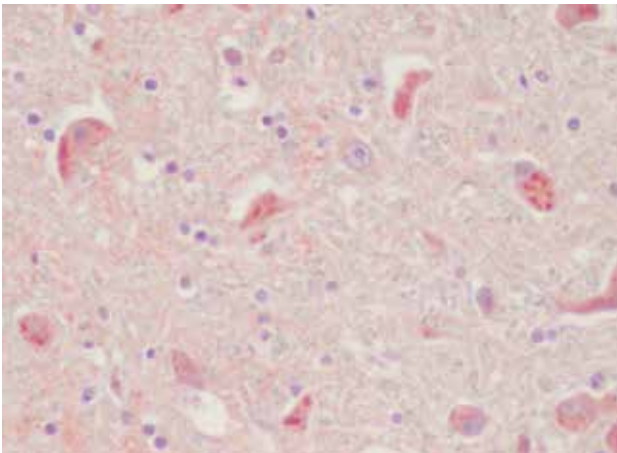


**Figure 4.** *Neospora caninum* antigen immunopositive reactions in degenerated and necrotic neuron cytoplasms. Brain of Kangal puppy with clinical neosporosis, X 200.

**Şekil 4.** Dejenere ve nekrotik nöron sitoplazmalarında *Neospora caninum* antijen immunopozitif reaksiyonlar. Klinik neosporozisli Kangal yavru kopek, X200.

## Control and Treatment Strategies of Neosporosis

Currently, no effective vaccine is available to protect newborn calves from neosporosis (Ortega-Mora et al., 2007; Dubey and Schares 2011). It has been shown that pregnant cows with a high antibody titer may have abortion or give birth to infected calves. Even a strong humoral response is not adequate enough to challenge a neosporosis infection alone. An alive attenuated vaccine is quite effective in naïve animals for protection from a novel *N. caninum* infection but the efficacy in subclinically infected cattle is still questionable (Innes et al., 2007; Dubey and Schares, 2011).

It is known through in vitro studies that toltrazuril and ponazuril have parasitostatic and parasitocidal effects on *N.caninum* tachyzoites, causing cytosolic vacuolization and damage to mitochondria and endoplasmic reticulum in tachyzoites as well as inhibiting parasite division. In *in vivo* studies, toltrazuril has been shown to prevent vertical transmission in pregnant mice so that reduces abortion rate and infected birth (Kritzner et al., 2002; Kul et al., 2012). Similarly, it reduces the spread and severity of infection in experimentally infected calves. Sulphonamide derivates are confirmed to be effective to kill *N. caninum* tachyzoites in cell cultures.

Additionally, Cuteri et al. (2005) showed that, toltrazuril and sulphonamide administration to seropositive cattle reduced the abortion rate of neosporosis in the following year.

In neosporosis, repetead abortions can normally be seen in pregnant cows. So, it is very important to the kill *N. caninum* tissue cysts in seropositive heifers before they don't became pregnant for a succesfull herd management. So, the most effective therapeutic protocols against neosporosis are still investigated for routine usage in veterinary practice.

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