***Neospora caninum* infection in cattle: Not only an economic problem**

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Summary

Neosporosis is an infectious disease of cattle and other species with a worldwide distribution. In Poland, the seropositivity rate for *N. caninum* among dairy cattle varies between 6% and 80%, with the highest number of seropositive cows in central and north-eastern voivodeships. In the United States, in 2003 the total annual cost of *N. caninum* infections was 657 million dollars, and in Great Britain in 2014 it was nearly 14 million pounds. *N. caninum* results in severe economic losses caused by a decreased milk yield, an increased number of culled cows, a higher newborn calf mortality rate and occasional birth defects. However, the most common result of this disease is abortion, which usually occurs between the 5th and 7th month of gestation. The risk of aborting is 3 times as high for seropositive cows as it is for seronegative cows; up to 21.6% vs. 7.3%, respectively. Cattle can become infected by consuming food and water contaminated with *N. caninum* oocysts, but the principal route is transplacental transmission usually between the 70th and 210th day of gestation. The ELISA test is most often used to detect the presence of specific antibodies in blood serum. PAG-2 level in the bloodstream can be a good marker for predicting *Neospora*-induced abortions – in seropositive cows, a level of more than 4.5 ng/ml on the 120th day of gestation means a 7-fold higher risk of pregnancy loss. According to research, live attenuated vaccines are highly efficacious in preventing neosporosis. Unfortunately, the currently available methods of prevention are based on the elimination of seropositive cows from the herd and the limitation of contact with the final hosts of *N. caninum*.

Keywords: bovine neosporosis, risk factors, abortions in cattle, cattle breeding, veterinary parasitology

Neospora caninum, an obligate intracellular parasite, was first recognised in 1984 (2, 33). It is morphologically and biologically similar to another protozoan, *Toxoplasma gondii*, and therefore *Neospora caninum* invasions were often misdiagnosed as those by *T. gondii* (19). *N. caninum* is a member of the phylum Apicomplexa, class Coccidia, order Eucoccidiorida, family Sarcocystidae (19, 42). It has been isolated from many different species, such as goats, sheep, pigs, deer, llamas, rhinos, European bison and marine mammals, but most frequently from cattle and dogs (2, 8, 10, 11, 27, 34, 54). In recent years, there has been an increasing interest in neosporosis around the world, but in Poland the disease is still underestimated. Although the prevalence of *N. caninum* in cattle herds fluctuates around a few percent of the total cattle population in the country, the consequences of infection are serious (21). Clinical neosporosis results in considerable economic losses for dairy producers as

a result of bovine abortions, high newborn calf mortality, longer calving intervals, reduced milk production and excessive involuntary culling rates (2, 5, 6, 14, 42, 47). Since the beginning of the 21st century, significant neosporosis-associated pregnancy losses in dairy cattle have been noted in different European countries, including Portugal, Spain and Great Britain (5, 9, 31). In 2013, economic losses caused by *Neospora caninum* on American dairy farms were assessed at about 546 million dollars and those in beef cattle herds at 111 million dollars (44). *N. caninum* infection in 2014 cost the United Kingdom an estimated 16 million pounds (31). What is more, in 2016 *Neospora caninum* was recognised as the leading cause of infectious bovine pregnancy loss in British Columbia, Canada (53).

Epizootic situation

As in Europe and North America, *Neospora caninum* seropositive cattle have also been reported in South

America, Africa and Asia (6, 48, 56, 57). The prevalence of antibodies to *Neospora caninum* among cattle and water buffaloes in ten provinces of northern Brazil was 55% and 44%, respectively (48). The overall seroprevalence of *N. caninum* in Chinese dairy herds was 17.2% (56). The above data indicate that neosporosis is a worldwide issue in livestock production, regardless of the location or socioeconomic development of the country. It is estimated that *Neospora caninum* infection causes 25-43% of abortions in cattle herds in general (24).

Clinical signs of neosporosis have also been recorded in Poland. One hundred and eighty-eight serum samples were collected to be tested for *Neospora caninum* antibodies. The sampled cows came from 14 small and medium-sized farms near Lublin in eastern Poland. Using the enzyme-linked immunosorbent assay (ELISA), specific IgG antibodies were detected in seven cows (3.72%). All seropositive animals originated from other farms, and the mothers of three of them had been imported from the Netherlands. One of the seven seropositive cows presented reproductive difficulties (50). According to recent population research performed in central and eastern Poland, the herd-level seroprevalence was highest in Podlaskie Voivodeship (63.3%) and Łódzkie Voivodeship (50.5%). In that study, in which 734 cows were examined, the percentage of seropositive animals in each herd varied from 6% to 80% (27).

There is a significant variation in the susceptibility of cattle to *N. caninum* and in the frequency of infections, depending on the breed and type of cattle. It has been reported that dairy cattle may be more susceptible to neosporosis than beef cattle. For instance, in Spain, antibodies have been observed in 46% of beef cattle and 63% of dairy cattle. In Argentinian herds, seroprevalence levels for *Neospora caninum* were reported as 19% in beef cattle and 43% in dairy cows (2). According to research, the Limousin breed presents the lowest seroprevalence of *Neospora caninum* infection, which is 10%. For comparison, the antibody prevalence rate fluctuates around 32% in pure Holstein-Friesians 22% in the Charolaise breed, 20% in the Belgian Blue breed, and 19% in the Piemontese breed. It has been reported that the use of beef bull semen (crossbreed pregnancies) significantly reduces the risk of abortion in seropositive dairy cows (3, 55). Neosporosis seroprevalence in aborting dams is much higher than in randomly selected population. For example, a Swiss study revealed that seropositive cows made up 44% of animals with a history of abortion and 12% of randomly chosen cows (46). In France, 17 to 45% of aborting dams presented antibodies against *Neospora caninum* (41).

Specific Immunoglobulin G (IgG) against *Neospora caninum* can also be detected in blood samples from small ruminants. According to Greek data, the seroprevalence of *N. caninum* among sheep and goats was

16.8% and 6.9%, respectively (13). In Poland, the seroprevalence of *N. caninum* infection in goats was 9.0% (10). Špilovska reported that neosporosis in sheep was much less frequent than it was in Greece: the presence of antibodies was demonstrated in 3.7% of sera (49). What is more, as stated in Argentinian sources, the percentage of seropositive goats on extensive farms was smaller (4.1%) than the percentage of seropositive cows on large-scale dairy farms (8.8%) (20).

Neospora caninum may co-exist with other infectious causes of abortions, such as *Toxoplasma gondii*, *Brucella abortus* or *Listeria monocytogenes*. According to Turkish results published in 2009, the rates of co-existence of *N. caninum* with agents mentioned above were 25%, 14% and 43%, respectively (56). In the serum of 50% of *N. caninum* seropositive sheep, the presence of antibodies against *T. gondii* was demonstrated simultaneously (49).

Sources of infection and transmission of *N. caninum* for cattle

Cattle become infected by consumption of feed or water contaminated with *Neospora caninum* sporozoites. The sporozoites proceed from the sporulation of oocysts excreted in faeces for 2 to 30 days after infection of the definitive hosts, canids (*Canidae*) (37). The sporozoites are released (by excystation) from the oocysts in the intestinal lumen of the intermediate host (cattle), after which they invade the intestine wall, where they transform into tachyzoites. These forms have a special affinity to fibroblasts, macrophages, nerve cells, endothelial cells, muscle and hepatocytes. In myocytes, tachyzoites are encapsulated to form bradyzoites (2, 19, 33). Sometimes, encapsulated forms (found in muscles and central nervous system cells) are activated, for example, as a result of stress. In a pregnant cow, a stress reaction breaks the cyst containing bradyzoites and releases the parasites into the bloodstream, which results in their spreading throughout the body. No horizontal parasite transfer has been observed so far, and there is no evidence of the invasive forms being passed on to milk (4).

Neospora caninum tachyzoites can also be transmitted vertically via the placenta in two different ways: endogenous transplacental transmission or exogenous transplacental transmission (31, 33). Endogenous transmission occurs from a dam that had acquired infection before pregnancy (and probably a dam with chronic infection as an asymptomatic carrier), and the latent parasite forms (bradyzoites) were transformed during pregnancy into invasive forms (tachyzoites) and passed to the fetus. Exogenous transplacental transmission takes place when infective oocysts are ingested during pregnancy (e.g. with contaminated food or water) and the parasite enters the placenta and then is transferred to the growing fetus. Transmission via the placenta can occur between the 70th and 210th day of gestation. As a result of vertical transmission,

parasites enter the cells of the fetus's central nervous system and cause myeloencephalitis, which may contribute to abortion or delivery of a dead or living calf with congenital malformations, such as hydrocephalus, muscle contracture, spinal cord narrowing or asymmetry. Other clinical signs, such as a decreased rate of growth, ataxia, impaired patellar reflexes and proprioception disorders, are observed in calves up to 2 months of age. Most often, however, the result of infection is an abortion, especially between the 5th and 7th month of pregnancy (2, 16, 25, 32, 43).

Neospora caninum DNA has been detected in the semen of bulls, but there is no evidence that venereal transmission plays a role in the spread of neosporosis (4).

On the other hand, according to most recent surveys, *Neospora caninum* infection may have a negative effect on bovine semen quality in terms of sperm concentration, viability and motility. Tests have shown that seropositive bulls present a significantly increased activity of GPX (glutathione peroxidase), while concentration levels of other enzymes (super oxide dismutase and malondialdehyde) are not markedly changed (7).

Risk factors

The potential risk factors associated with *Neospora caninum* infection in cattle include the presence of a definitive host (mainly dogs) on the farm, mycotoxin-induced immunosuppression, retaining and then breeding heifer calves born to seropositive dams and the coexistence of other diseases, e.g. viral diseases (40, 45).

Analysis of epidemiological data revealed that a prevalence of *N. caninum* was not related to the age of the animals, the size of the herd or the epidemiological and epidemic occurrence of abortions (12).

Effects of *Neospora caninum* infection and activation of host defence system

As already mentioned, the main sign of *Neospora caninum* infection in pregnant adult cows, besides a decreased milk yield and an increased frequency of postpartum diseases (such as metritis or placental retention), is abortion. In seropositive cows, the risk of abortion is three times as high as it is in seronegative cows, that is, 21.6% and 7.3%, respectively (6, 26, 29). The cause of premature birth, usually between the 5th and 7th month of gestation, is the death of placental cells due to the multiplication of parasites in them and an increase in the production of cytokines disturbing the maintenance of pregnancy (2, 39). Cell-mediated immunity, especially T helper cells, plays a major role in the immune response against *Neospora caninum*. The functions of T helper cells are regulated by cytokine levels. Pro-inflammatory cytokines influence Th1 cells activity, whereas Th2 cells are controlled by pro-gestation cytokines. The imbalance between Th1 cells and Th2 cells activity

(high levels of pro-inflammatory cytokines) leads to a greater risk of abortion. The most important role in the control of *Neospora caninum* infection is played by IFN-gamma secretion, which limits the multiplication of the parasite. IFN-gamma is produced, along with IL-12 and TNF-alpha, by Th1 cells. The IFN-gamma level must, however, be counter-balanced by the appropriate levels of IL-10 and IL-12. On the other hand, Th2 cells produce a series of interleukins, e.g. IL-4, IL-5, IL-6, IL-9, IL-13 and IL-25, that are responsible for the activation and maintenance of the humoral response. A new subtype of T cells has recently been described, the so-called Treg cells, which maintain the homeostasis of subsets of cells involved in the immune response as well as the production and release of anti-inflammatory cytokines IL-10 and TGF-beta1. Th17 cells, which produce signalling cytokines IL-17, IL-21 and IL-22, are also involved in the response to *Neospora caninum* infection (4).

The development of local inflammation due to infection of the placenta leads to excessive secretion of inflammatory mediators, e.g. prostaglandins, causing contractions of the uterine myometrium. The production of these factors is also an important cause of abortion in cattle (4).

It has been demonstrated that there is a significantly lower risk of abortion in seropositive cows with clearly higher progesterone level for a given gestation period (e.g. due to the use of exogenous progesterone sources, including progesterone vaginal PRID inserts) compared to seropositive cows in which the P4 level is within the physiological norm. This phenomenon may be caused by the fact that progesterone indirectly reduces the production of pro-inflammatory factors by Th1 cells (4, 21). An increase in their production and an increase in the activity of oxidative stress markers (reactive oxygen species and butyrylcholinesterase) leading to cell damage are observed especially in seropositive cows showing clinical signs (18).

It has also been proven that *N. caninum* can negatively affect the outcome of embryo transfer in cattle. There is a higher risk of abortion and transplacental transmission in seropositive recipients. However, both seronegative and seropositive donors can be used, as their immunological status does not interfere with normal embryonic development (38).

According to British research, *N. caninum* seropositive cows, compared to seronegative ones, are significantly more prone to further abortions, stillbirths or giving birth to calves with congenital defects (43).

Research has shown that some biochemical parameters in the blood serum of seropositive cows are elevated, which may indicate a positive correlation between the occurrence of neosporosis and common metabolic disorders of cattle (1). According to the authors, the negative energy balance resulting from the multiplication of parasites in the host's tissues, and thus the increased rate of cellular metabolism, leads

to the activation of fatty reserves and, consequently, to a significantly increased concentration of betahydroxybutyrate (ketosis indicator) in plasma. Significantly increased activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) have also been observed in seropositive cows, compared to seronegative individuals, which indicates hepatocellular damage and may be associated with fatty liver syndrome (1).

PAG as an indicator of *Neospora*-induced abortions

A good indicator for predicting abortions may be the evaluation of the pregnancy glycoprotein (PAG) level in the blood serum or milk of pregnant cows (4). There are several types of PAG, but the best marker of the abortion risk is PAG-2. According to Garcia-Ispierto et al. (17), if its serum concentration in a seropositive cow is higher than 4.5 ng/ml on the 120th day of gestation, the risk of abortion is 1/7 of that for seropositive cows with a PAG-2 level lower than 4.5 ng/ml (17).

According to Almeria et al. (4), it is beneficial to crossbreed *Neospora caninum*-infected cows with different levels of pregnancy glycoproteins. The higher the level of PAG in blood, the greater the tolerance of the pregnant cow to any changes caused by the immune response to the presence of the parasite. The highest concentration of PAG-1 in serum occurs in the Limousine breed, which is consistent with epidemiological data indicating that the risk of *Neospora*-mediated abortion in this breed is the lowest (10%) (4).

Diagnosis

Diagnostic methods for neosporosis in cattle involve detection of antibodies in serum or milk samples and histopathological examination of the tissues (liver, heart, brain) of aborted fetuses or dead calves. Serological techniques for detecting antibodies against *Neospora caninum* include indirect immunofluorescence assay (IFAT), agglutination assay (NAT), immunoblotting and enzyme-linked immunosorbent assay (15, 23). ELISA, due to its simplicity and short analysis time, is most often used by field veterinarians (2, 6, 28). The test can detect antibodies in blood serum, plasma or milk (21). In order to determine the serological status of each herd (screening test), it is recommended that *N. caninum*-specific antibodies be detected in blood samples. When determining antibodies in bulk milk, it should be remembered that the test results concern only currently milked cows – they will not give information about dried cows, sick (e.g. mastitic) cows or post-partum cows which still produce colostrum.

False positive serological results can be obtained if a calf has consumed colostrum from a seropositive cow before the test. In this situation, blood testing of both the dam and the calf is recommended (43).

PCR, histopathology and immunohistochemistry are used to determine the status of *N. caninum* infection in aborted fetuses. Confirmation of *N. caninum* infection by PCR has the highest sensitivity and specificity, and

the results are not falsified by autolytic changes, as in the case of the other two methods (25). In research by Pessoa et al., *Neospora caninum* DNA was detected by PCR in up to 44.4% of intact aborted fetuses from seropositive cows. In the same study, no parasites were isolated from any of aborted fetuses of seronegative dams (39).

Methods of treatment and prevention

Neosporosis is a very serious problem on dairy farms, not only because of economic losses, but also because of the lack of effective methods of preventing them. Currently, there is no vaccine or approved treatment for neospora (19, 21, 33). According to Weston et al. (52), the unique vaccine available in the market, which contains inactivated *Neospora caninum* tachyzoites, did not prevent abortion or vertical transmission of parasites and increased the risk of early embryonic death (52). In 2007, the efficacy of a live attenuated vaccine containing the Nc-Nowra strain was evaluated as a promising preventive candidate for transplacental transmissible exogenous infection. Before planned artificial insemination, cows were injected intravenously with a vaccine containing 10⁷ tachyzoites of the Nc-Nowra strain. Administration of the same amount of tachyzoites of the Nc-Liverpool strain on the 70th day of gestation resulted in a strong humoral and cellular response, as well as a significant IFN-gamma secretion. According to Weber et al. (51), the effectiveness of the above vaccine varied, depending on the type of injection (subcutaneous or intravenous), from 55% to 85%. It has also been proven that freezing the vaccine has a negative effect on immunization, which is a technical hurdle in creating a marketable live vaccine for bovine neosporosis.

In 2015, a live vaccine containing the NcIs491 strain was evaluated. The study included 520 seropositive dairy cows: 146 in the experimental group (vaccination in the middle of gestation) and 374 in the control group. The percentage of abortions was significantly lower among the immunised cows (16%) than among the others (26%). The effectiveness of the vaccine was estimated at 39% (30). So far, research results have confirmed that live attenuated vaccines are effective in preventing abortions caused by *Neospora caninum*. It is impossible, however, to use cryopreserved tachyzoites in the vaccine manufacturing process, and scientists must overcome this technical impediment for a vaccine to be successful and available (22).

A study published in April 2017 shows that the use of chemotherapeutic agents can prevent the vertical transmission of *Neospora caninum*. Researchers tested two newly discovered kinase inhibitors, BKI-1517 and BKI-1553, *in vitro* (using fibroblasts) and *in vivo* (on experimentally infected pregnant mice) (36). Within six days of application, BKI-1553 inhibited parasite endodiogenesis, while BKI-1517 prevented the formation of pre-diversion forms and showed better efficacy

in destroying infected cells. However, after 10 days of testing, the compounds lost their inhibitory power against *Neospora caninum* tachyzoites. In *in vivo* studies, BKI-1517 administered at a dose of 20 mg/kg every 24 hours significantly prevented the vertical transmission of *Neospora caninum*. The administration of the agent at a higher dose (50 mg/kg every 24 hours) resulted in decreased fertility in mice. BKI-1553 was less effective in *in vivo* studies (36).

The only currently available control tools against neosporosis in cattle herds are limiting the occurrence of risk factors and periodic (annual) monitoring of the herd's serological status. According to research, the complete isolation of canids, as final hosts, from cattle, fodder, pastures and water sources is one of the main factors preventing the disease (2). The culling of seropositive individuals with two or three occurrences of abortion is a key tool for eliminating the parasite from the herd. Moreover, because of the high risk of endogenous transmission of *Neospora caninum* in serologically positive cows (up to 63%), heifers from infected dams should also be eliminated from the herd. One cannot underestimate the importance of ensuring adequate welfare and hygiene (regular cleaning and disinfection of farm, immediate removal of aborted fetuses, removal and disposal of contaminated bedding material). As some researchers suggest, vaccination of dogs against *Neospora caninum* would be an important element in the control of this disease (2, 31).

International Embryo Transfer Association (IETS) recommends obtaining embryos from seropositive cows and transferring them to seronegative recipients. According to Moskwa's studies, *Neospora caninum* DNA is not found in these embryos. Such embryo transfer eliminates vertical transmission of neosporosis (35).

Neospora caninum, as one of the main causes of abortion, has been a major problem in cattle breeding for several decades and is of major economic significance. Despite numerous studies and observations, there are no proven control methods for the prevention or treatment of neosporosis. Vaccination has been considered a viable tool to prevent and control bovine neosporosis. Research is also underway to discover substances that may, for example, limit transplacental transmission of the parasite. Currently, only non-pharmacological methods are available, including the culling of seropositive cows, crossbreeding, maintaining proper farm hygiene and limiting direct and indirect contact between cattle and dogs.

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