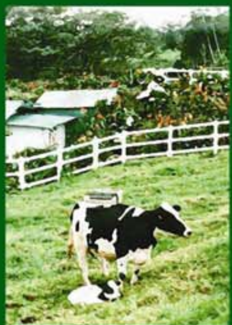

Appraisal of the Epidemiology of *Neospora caninum* Infection in Costa Rican Dairy Cattle - Juan José Romero Zúñiga - 2005

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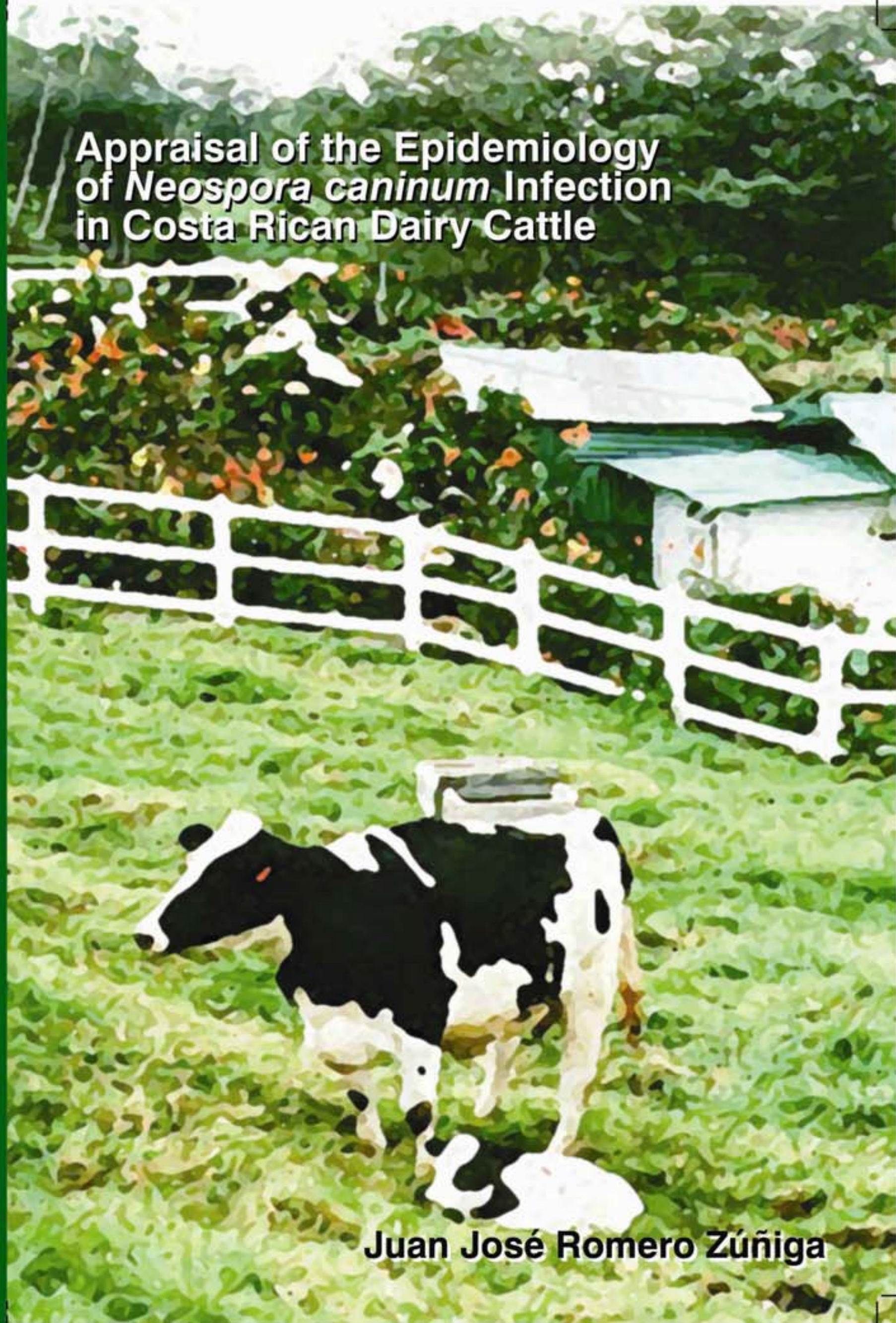

Juan José Romero Zúñiga

APPRAISAL OF THE EPIDEMIOLOGY OF *NEOSPORA CANINUM* INFECTION IN COSTA RICAN DAIRY CATTLE

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Dit onderzoek is uitgevoerd binnen de onderzoekschool WIAS (Wageningen Institute of Animal Science)

APPRAISAL OF THE EPIDEMIOLOGY OF *NEOSPORA CANINUM* INFECTION IN COSTA RICAN DAIRY CATTLE

Juan José Romero Zúñiga

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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Appraisal of the epidemiology of *Neospora caninum* infection in Costa Rican dairy cattle.

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Abstract

Romero Zúñiga, J.J. 2005. Appraisal of the epidemiology of *Neospora caninum* infection in Costa Rican dairy cattle.

In Costa Rica, milk production has increased gradually during the twentieth century, in which the activity developed from a non-technical to a technical activity. Together with the evolution of the dairy sector, the incidence of infectious and metabolic diseases increased, leading to increased economic losses. According to a VAMPP data base, the global percentage of abortion during the period between 1988 and 2003 varied between 7.5 and 12%; but at individual farms abortion rates close to 30% occurred in one or more years. Abortion is one of the most important economic disorders. Since the 90´s neosporosis (*N. caninum*) has been associated with abortion and foetal losses in cattle all over the world. In 1996, a study stated (for the first time) the presence of neosporosis in Costa Rica and *N. caninum* was diagnosed (Perez et al., 1998). The aim of this thesis is to describe the most important features of neosporosis in Costa Rican dairy cattle in order to develop strategies for the prevention and control of the infection. The main results of this thesis are: 1) no significant effects of *Neospora* serostatus were detected on (re)productive performance; 2) the association between management and environmental factors with serostatus was found to be absent 3) in the specific conditions of the dairy herds involved in this study, the serostatus of the cows should be not used as predictor of the serostatus of daughters due to the high probability of horizontal transmission, 4) the killed whole *Neospora caninum* tachyzoite preparation reduced the abortion rate in Costa Rican dairy cattle.

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GENERAL INTRODUCTION

Framework

Worldwide, there is an increased need for goods such as housing, education, food, etc., especially in developing countries as a result of population growth. The need further stems from the desire of having an increased life-quality or, at least, to get the minimum goods to survive in conditions of dignity. Related to this, a remarkable pressure was put on food of animal origin, especially meat and milk consumption has increased (Delgado *et al*., 1999). This pressure has led to increased production rates and more intensive farming practices. In some areas this has resulted in loss of biodiversity and natural habitats because the land was exploited more than it could sustain (Pérez, 1996). In Costa Rica, as result of deforestation for extraction of precious woods and the subsequent agricultural development, a destruction of almost 70% of forest areas has occurred throughout the past century. However, close to 25% of the Costa Rican territory is covered by forest, which is one of the highest around the world. A high percentage of this forest is protected,, e.g. as national parks, as part of a general Costa Rican preservation system of biodiversity and natural habitat (9° Informe del Estado de la Nación, 2003).

Productivity has been increased by optimizing breeding, herd health, herd management and animal nutrition (Vargas, 2000); but it has not dealt with the correct utilization of natural resources provided by the farm. Reproduction and herd health have been taken as major targets because milk production depends on regular calving of healthy cows. However, this way of increasing the milk production has been criticised because it depends heavily on inputs such as soy bean and maize which are the base of the concentrates. These inputs are mostly imported from temperate countries like U.S., Canada and the European Community, and therefore the long term sustainability is questionable. Besides that, these inputs represent almost 60-70% of the fixed costs of the total production costs. Sustainable animal production systems with a lower impact on environment and improved economic efficiency can be a solution. However, it is a new area in Costa Rica, which is gaining interest. An optimal level of intensification of animal production systems in developing countries must be found in making an optimum use of the land available (Nicholson *et al*., 1995; Pérez, 1996; Vargas, 2000).

Milk production in Costa Rica

Milk production has increased gradually during the twentieth century, in which the activity developed from a non-technical to a technical activity; at the same time, the human population has increased while the cattle population -beef and dairy cattle taken together- has decreased as shown in Figure 1.

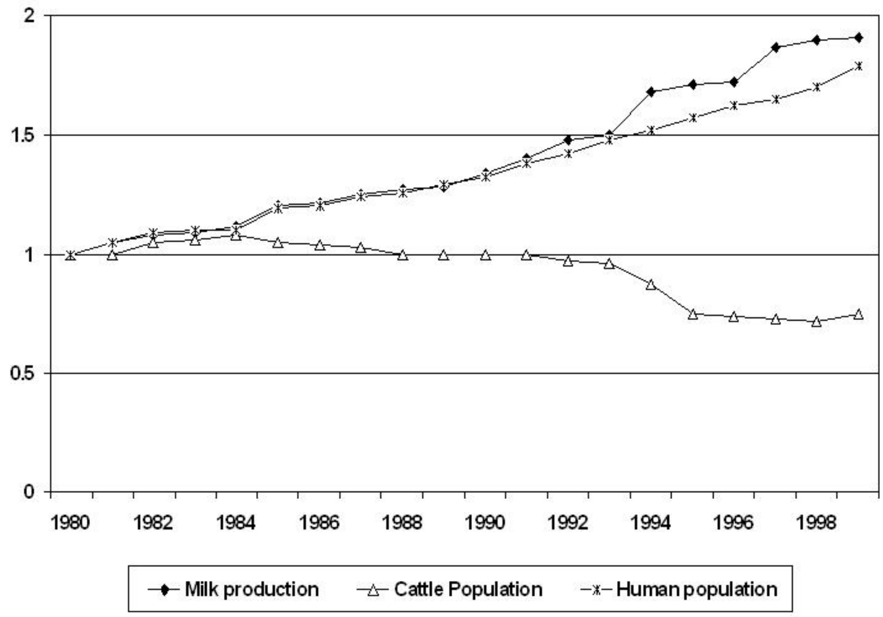

Figure 1. Trends in milk production in relation to human and cattle population (Production for the base-year 1980 is set to 1 and corresponds to 318,000 TM milk, 2,18 million cattle and 2,84 million inhabitants). Modified from Vargas, 2000; based on FAO stats.

Originally, native breeds were used for milk production, which were the result of the natural selection from the cows brought to Costa Rica by Spaniards during colonisation from the 15th century onward. Recently, these breeds were replaced with specialised dairy breeds like Holstein and Jersey, by importation of live animals or sperm for artificial insemination. This process was driven by an increasing market demand to a higher quality and a broader variety of milk products. Therefore, dairy cooperatives and private companies encouraged dairy producers to improve their milk production in both quantity and quality. Also the dairy farms in the lowlands became more and more specialised almost like dairy farms in the highlands. Now, most of the farms have a milking parlor, electric fences, improved pastures and breeds specialised in milk production. Also, the Brown Swiss as purebred or in crosses with Holstein or Jersey is used in dual purpose systems, especially in the lowlands.

Three milk production systems can be defined in Costa Rica: 1) Specialised dairy farms in the highlands, 2) Specialised dairy farms in low lands, and 3) Dual purpose farms. The first two are highly technical with productivity levels very close to those found in countries with a temperate climate. Also, this system is the highest in costs per Kg of milk (Vargas, 2002). The second and third system produce almost 60% of the total milk production in Costa Rica. These systems have lower costs per Kg of milk than specialised farms in the highlands due to a lower use of concentrates. Besides, the dual purpose system is characterised for use pastures of low quality and the use of cows not highly specialised like in the highlands (crossbreds or purebreds adapted to the lowlands). A brief characterization of the Costa Rican dairy areas is briefly presented in Table 1, taken and adapted from Vargas (2000).

As part of the increased specialisation, information systems have been adopted as management tool for the farmers (Pérez *et al*., 1989). In this way, the CRIPAS project (Regional Centre in Informatics for Sustainable Animal Production) of the Universidad Nacional de Costa Rica, adopted the VAMPP system (Veterinary Automated Management and Production control Programme, [Noordhuizen and Buurman, 1984]) in collaboration with the Faculty of Veterinary Medicine of the University of Utrecht (The Netherlands). Nowadays, VAMPP is used by almost 600 of the 1500 most specialised dairy farms in Costa Rica (Figure 1). Due to this relationship between CRIPAS and the farmers, the Universidad Nacional is able to perform in-depth studies (Pérez, 1996; Herrero, 1997; Vargas, 2000, Solano, 2000).

Bovine abortion in Costa Rican dairy farms

Together with the evolution of the dairy sector, the incidence of infectious and metabolic diseases increased, leading to increased economic losses. According to the data base, the global percentage of abortion during the period between 1988 and 2003 varied between 7.5 and 12%; but at individual farms abortion rates close to 30% occurred in one or more years. Therefore, abortion is one of the most important economic disorders. Historically, brucellosis was the most important infectious disease causing abortion until the beginning of the eighties.

A very intensive vaccination campaign initiated by the Ministry of Agriculture and Livestock reduced the national prevalence at animal level below 2% (Ministry of Agriculture and Livestock, personal communication). Then, other causes of abortion were relatively more important such as bovine rhinotracheitis, bovine virus diarrhoea and leptospirosis. However, a high percentage of abortions could not be related to any infectious abortifacient, which is in agreement with the literature (Anderson, 1990; Yaeger and Holler, 1997).

Table 1

Main characteristics of milk production systems in Costa Rica.

Characteristic	Highlands	Lowlands	Dual-purpose
% of income from milk	> 95	70-85	45-55
Location (m.a.s.l.)	1,200-3,000	0-1,200	0-900
Temperature (°C)	10-20	20-30	20-35
Breeds	Holstein Jersey	Holstein Jersey Holstein x Zebu Brown Swiss Brown Swiss x Zebu	Holstein x Zebu Brown Swiss Brown Swiss x Zebu
Milk yield per lactation (305-day milk production [*in Kg*])	4,500-7,500	2,500-5,000	1,200-2,300
Average herd size (range)	115 (46-372)	83 (48-778)	67 (39-132)
Concentrate (Kg/cow/day)	3-10	< 5	0-2
Age at first calving (months)	25-30	27-35	30-35
Lactation length (days)	328	< 300	210
Calving index (%)[1]	85.3	…	63.3
Most common pastures[2]	Kikuyu, Ryegrass	Star grass, Brachiaria, Ratana	Jaragua, Natural, Imperial, Brachiaria
Cost/kg milk ($U.S.)	< 0.23-0.25	0.20-0.22	0.18-0.20
Profitability / kg milk (%)	< 22	25-30	35-50
Kg milk/ farm/ year	40,000-60,000	>20,000	33,000-125,000
VAMPP users (%)	30.6	64.0	5.4

[1] Calving index: Number of calvings/number of cows available

[2] Ratana: *Ischaemun ciliare*; Brachiaria: *Brachiaria ruziziensis*/*brizantha*, *Brachiaria decumbens*, kikuyu: *Pennisetum clandestinum*; Star grass: *Cynodon nlemfuensis*; Natural: *Paspalum notatum*; Imperial: *Axonopus scoparius*.

In 1996, a study stated (for the first time) the presence of neosporosis in Costa Rica and *Neospora caninum* was diagnosed (Perez *et al*., 1998). Since then, studies carried out in Costa Rica have documented a high prevalence of neosporosis leading to a risk of abortion, which exceeds that of other infectious diseases (Perez *et al*., 1998, Romero *et al*., 2000). Cows that were seropositive to neosporosis had a 12 times increased risk for abortion compared with seronegatives, while the seropositivity towards other diseases (brucellosis, leptospirosis, IBR and BVD) did not have a relation with abortion (Pérez *et al*., 1998). Based on these findings research was initiated to get more and reliable information on neosporosis and abortion (in general) in Costa Rican dairy cattle. Results of this research are presented in this dissertation.

Outline of this thesis

The aim of this thesis is to describe the most important features of neosporosis in Costa Rican dairy cattle in order to develop strategies for the prevention and control of the infection. In order to achieve this goal the following objectives were defined:

1. to make an extensive literature review about neosporosis, including reports from Costa Rica;
2. to establish the serostatus towards neosporosis, at both farm and cow level;
3. to quantify the effect of neosporosis on the (re) productive performance of the cows,
4. to identify factors associated with neosporosis;
5. to assess the vertical and horizontal transmission rates,
6. to determine the effect of a commercial vaccine on the crude abortion rate,

This thesis is structured in chapters that will deal with the previous objectives. **Chapter 1** is an extensive literature review that served as a starting point of this investigation. **Chapter 2** is related to objectives 2 and 3. A serum bank of 2495 samples (originating from 94 dairy farms located in the most significant dairy areas in Costa Rica), was assayed to determine the serostatus of the cows. Besides, the effect of the serostatus on the (re)production performance of the cows was assessed. **Chapter 3** deals with factors associated with neosporosis (objective 4). The relationship between seropositivity and some intrinsic and extrinsic factors of the cows were studied by means of a cross-sectional study in 25 specialised dairy farms of a specific area in Costa Rica. In **Chapter 4** vertical and horizontal transmission of neosporosis is quantified using a cross-sectional study. **Chapter 5** describes a standard clinical trial to assess the efficacy of a commercial vaccine. A total of 438 matched pairs (vaccinated – control matched by herd, parity number and days of gestation) belonging to 25 dairy herds of different areas were used in this study. Finally, a general discussion with the general consideration about the epidemiology of neosporosis in Costa Rica is presented.

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Chapter 1

Bovine Neosporosis: A review

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1.1. Introduction

Neosporosis is a parasitic disease caused by *Neospora caninum*, a protozoan that until 1988 was misdiagnosed as *Toxoplasma gondii* because of close structural similarities (Dubey, 1992; Dubey and Lindsay, 1993). This disease has been diagnosed in a wide range of hosts such as cats (Dubey *et al*., 1990d), sheep (Dubey and Lindsay, 1990; Dubey *et al*., 1990a), dogs (Dubey *et al*., 1990b; Cochrane and Dubey, 1993), goats (Dubey *et al*., 1996a), horses (Dubey and Portfield, 1990; Daft *et al*., 1997) and cattle (Anderson *et al*., 1995; Boulton *et al*., 1995; Wouda *et al*., 1997; Paré *et al*., 1998; Bergeron *et al*., 2000; Reichel, 2000; Atkinson *et al*., 2000); causing various clinical signs. Also, it has been diagnosed by means of serology in water buffaloes (Dubey *et al.*, 1998; Huong *et al.*, 1998), camels (Hilali *et al.*, 1998), foxes and other wild canids (Barber *et al.*, 1997), and in non-human primates by means of experimental studies (Barr *et al.*, 1994a; Ho *et al.*, 1997a). No antibodies to *N. caninum* have been detected in humans (Petersen *et al.*, 1999; Graham *et al*., 1999). However, Tranas *et al.* (1999) found some evidence of seropositivity when screening blood of donors for antibodies by indirect fluorescent antibody (IFA) tests and immunoblotting. Sixty-nine out of 1,029 (6.7%) had titers of 1:100 by IFA testing. Although the antibody titers in healthy donors were low, these data provide some evidence of human exposure to *N. caninum*.

Without doubt, most studies have been done in cattle, and more specifically in dairy cattle, probably due to the economic impact that neosporosis has to the cattle sector.

In some countries *N. caninum* is the main cause of bovine abortion or foetal losses, of infectious origin. Thilsted and Dubey described this effect for the first time in 1989. Besides, it can cause paralysis and death in dogs, and neonatal mortality and abortion in goats, sheep and horses as well (Dubey, 1992; Lindsay *et al.*, 1996b; Daft *et al.*, 1997, Dubey, 2003).

The life cycle was finally elucidated by McAllister *et al.* in 1998. They found that the dog is the definitive host, and observed for the first time the oocysts by means of an experimental study. Later this finding was confirmed by others (Lindsay *et al*., 1999; Basso *et al*., 2001). Both ways of transmission vertical (transplacental) and horizontal (postnatal), have been documented, but the vertical one is responsible for the majority of animals that test positive (Paré *et al*., 1996; Thurmond *et al*., 1997a; Schares *et al*., 1998; Davison *et al*., 1999; Dubey, 1999; Dijkstra *et al*., 2001; McAllister and Latham, 2002).

In Costa Rica *N. caninum* was discovered in 1996 causing abortion in a goat (Dubey *et al.,* 1996a), and in the same year it was related, for the first time, with abortion in dairy cows by means of an epidemiological study (Perez *et al.,* 1998). Other studies have proven the wide spread of the disease in the most important dairy areas of the country with within-herd seroprevalences varying between 10 and 88% (Romero *et al*., 2002, Chapter 3).

The aim of this review is to summarise the most relevant knowledge with regard to *Neospora caninum* and, specifically, regarding to bovine neosporosis. First a general description of the agent will be given and secondly a description of the disease.

1.2. History

The first report of neosporosis was under the name of *Toxoplasma*-like protozoan by Bjerkas *et al.* (1984). They reported encephalomyelitis and myositis in dogs between 2 and 6 months of age that showed neurological disorders; besides, they found similar organisms as *Toxoplasma gondii* in lesions in the central nervous system (CNS) and muscles, but the dogs had no antibodies against *T. gondii* (Bjerkas *et al.,* 1984). In 1988, a similar parasite was found in 10 dogs in the USA, and the parasite was named *N. caninum* (Dubey *et al.,* 1988a). In retrospective studies *N. caninum* was found in dogs in the USA that died in 1957 and 1958 (Dubey *et al.,* 1990c). In 1992 Bjerkas and Dubey compared the structure and antigenicity of the parasites in fixed tissues from dogs of Norway and USA, and concluded that the parasite in the Norwegian dogs, was *N. caninum* or closely related to it.

Since 1988 *Neospora*-related abortions and other symptoms in cattle have been observed in Europe, America, Asia, Australia and Africa (Obendorf *et al.,* 1994; Trees *et al*., 1994; Boulton *et al.*, 1995; Jardine and Wells, 1995; Venturini *et al.,* 1995; Yamane *et al.,* 1996; Perez *et al.,* 1998).

1.3. Description of the agent

1.3.1. Structure

Morphological studies by electron microscopy on *N. caninum* have shown that this organism has a subcellular structure typical of parasites classified in the family Sarcocystidae, subclass Coccidiasina of the phylum Apicomplexa (Ellis *et al.,* 1994). This parasite is structurally very close to *T. gondii* (Barr *et al.,* 1997) and specimens of the genus

Hammondia (Hill *et al*., 2001; Dubey *et al*., 2002). The known infectious stages of *N. caninum* are tachyzoites, tissue cysts and oocysts.

Tachyzoites are ovoid, lunate or round, approximately 2-3 x 5-7 μm (Figure 1). Depending on the stage of division *N. caninum* is located in brain, myocardium, lungs and placenta of the host, but primarily in the CNS (Jardine and Wells, 1995; Dubey, 2003). In the host, tachyzoites are located within the cell cytoplasm with or without a parasitophorus vacuole, and have organelles typically found in *T. gondii* tachyzoites (Dubey, 1992). Tachyzoites have three layered plasmalema, 22 subpellicular microtubules, two apical rings, a conoid, a polar ring, one to three mitochondria, up to 150 micronemes, eight to twelve rhoptries anterior to the nucleus and four to six rhoptries posterior to the nucleus, a Golgi complex, rough and smooth endoplasmic reticulum, a nucleus and a nucleolus (Bjerkas and Prestus, 1989; Speer and Dubey, 1989; Dubey and Portfield, 1990; Barr *et al.*, 1991; Conrad *et al.*, 1993; Barr *et al.*, 1997; Sonda *et al*., 2000).

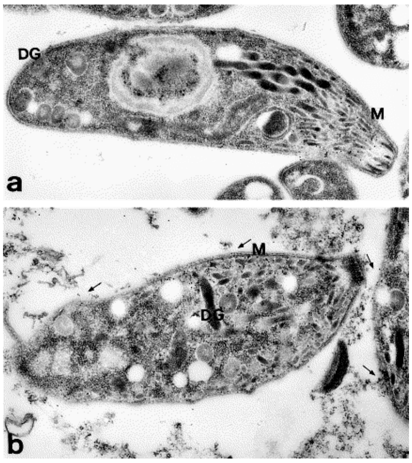

Figure 1. Immunogold labelling EM of LR-White embedded *N. caninum* tachyzoites. Extracellular (a) and intracellular (b) tachyzoites were labelled with affinity-purified anti-recNc-p38 antibodies and secondary goat anti-mouse conjugated to 10 nm gold particles. Note the micronemes at the apical end of the cells (M) and some dense granules (DG). *Source: Sonda S, Fuchs N, Gottstein B, Hemphill A. 2000. Molecular characterization of a novel microneme antigen in Neospora caninum. Mol. Biochem. Parasitol 108, 39-51.*

Tissue cysts are often round to oval, up to 107 μm long and are found in several species affecting mainly the neural tissues, like brain and spinal cord (Dubey, 1992; Wouda *et al.*, 1997; Daft *et al.*, 1997). The cyst wall is smooth and up to 4 μm tick, depending on how long infection has existed. In most tissue cysts, the cyst wall is 1-2 μm thick. Tissue cysts contain branched tubule-like structures (Bjerkas and Prestus, 1989). There is no secondary cyst wall, and septa are absent (Dubey, 1992). The cyst contains stages of the parasite called bradyzoites, that are slender structures (6-8 x 1.1.8 μm) and contains the same organelles as are found in tachyzoites except that there are fewer rhoptries and more PAS positive granules in the bradyzoites. Furthermore, tubular vesicular structures are present in between bradyzoites, which may contain micropores (Bjerkas and Prestus, 1989; Bjerkas and Dubey, 1992).

A study by Jardine (1996), with tissue cysts and bradyzoites of *N. caninum*, originating from dogs and cattle, indicated that there are no ultrastructural morphological criteria differentiating *N. caninum* in dogs and cattle.

In 1998, by means of an experimental study carried out by McAllister *et al.*, the sexual stage of this coccidian parasite, the oocyst, was detected. Unsporulated and non-infective oocysts, of 10 to 12 μm in diameter, were observed in fresh faeces. Oocysts become sporulated and infective within 3 days outside the host. Sporulated oocysts have 2 sporocysts, each containing 4 sporozoites (Fig. 2).

There is a description of a new *Neospora* species (*N. hughesi* n. sp.), isolated from the central nervous system of an adult equine from California. The ultrastructural characteristics are very similar with *N. caninum*; however, the isolates of *N. hughesi* showed phenotypic differences in immunoreactive proteins (Marsh *et al.,* 1998).

1.3.2. Life cycle and transmission

Initially, it was suggested that the parasite had a life cycle like *T. gondii,* with a carnivore as definitive host (Dubey, 1992). McAllister *et al.* (1998) discovered that the dogs are the definitive host of *N. caninum.* With this finding, the life cycle was almost defined; even so, the ways of horizontal transmission remained unclear. Dubey (1999) suggests a life cycle as is shown in Figure 2. The vertical transmission route has been established almost simultaneously with the discovery of the parasite. *N. caninum* can be transmitted transplacentally in a very efficient way in dogs, cats, cattle, sheep, goats, horses and mice (Dubey *et al.*, 1990c; Barr *et al.*, 1997). Horizontal transmission has not been observed in studied species, but some studies support this way of transmission (Paré *et al.*, 1997; Schares *et al.,* 1998; Waldner *et al.,* 1998; Dubey, 1999; Bergeron *et al*, 2000; Romero and Frankena, 2003). The postnatal infection rates are variable depending on country, region within the country, the test used and the cut-off values used (Dubey, 2003). Natural infections have been diagnosed in dogs, cattle, goats, horses, sheep, deer (Barr *et al.*, 1997), buffaloes (Huong *et al.*, 1998; Dubey *et al.*, 1998) and camels (Hilali *et al.*, 1998).

Experimental infection with *N. caninum* has been achieved by subcutaneous, intraperitoneal, intramuscular and oral routes (Dubey and Lindsay, 1990; Lindsay *et al.*, 1995; Buxton *et al.*, 1997). Recent investigations conducted by Uggla *et al.* (1998) suggest that the oral infection via olostrums might be a possible route of transmission. In their experiment two neonatal calves infected with *N. caninum* tachyzoites by feeding bottle showed DNA residues in their brain, although no pathological lesions were seen; parasites were not detected by immunohistochemistry, and it was not possible to re-isolate *N. caninum* of culture brain cells. These studies support the possibility of horizontal transmission, by means of the ingestion of oocysts in grass, water or some contaminated feed.

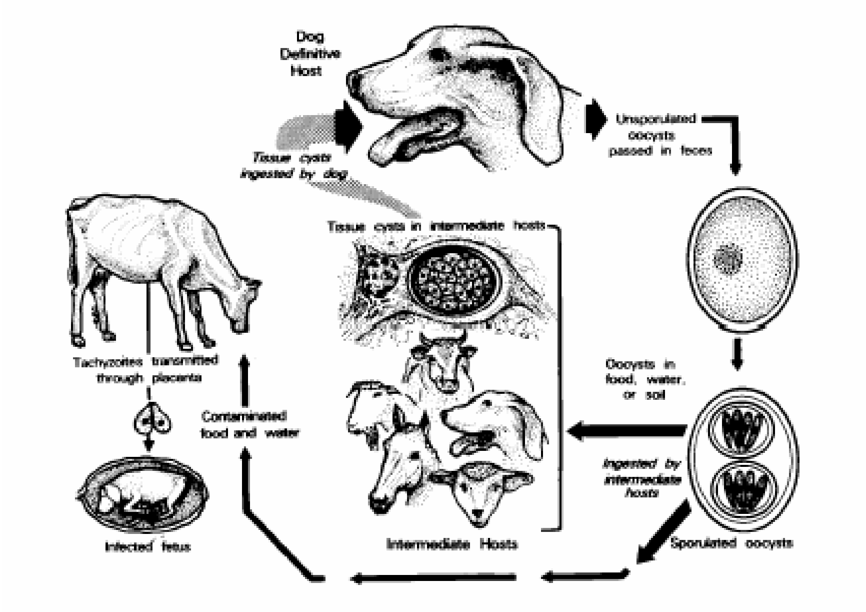

Figure. 2. Assumed life cycle of *Neospora caninum.* Source: Dubey, J.P. 1999. Neosporosis in cattle: biology and economic impact. J. Am. Vet. Med. Assoc. 214:1160-1163.

The possibility of venereal transmission or by embryo transfer has been tested but no clear evidence of transmission was found. Ortega-Mora *et al*. (2003) found DNA in fresh non-extended and frozen extended semen by real-time PCR. The mean load of parasites in positive fresh semen was low (varying between 1 and 2.8 parasites/ml of semen). They observed *N. caninum* DNA most frequently in the cell fraction, but no specific DNA was present in the seminal fluid. These authors suggest that tachyzoites were associated to any type of cell. Based on this study, further studies are needed to determine whether semen from *N. caninum*-infected bulls can infect inseminated heifers or cows.

On the other hand, Baillargeon *et al*. (2001) conducted a study in which seronegative and seropositive cows received embryos from seropositive or seronegative donors. None of the 70 foetuses or calves born from seronegative recipients became seropositive, whereas 6 out of 8 born from seropositive recipients were seropositive to *N. caninum*.

How a dog acquires the parasite is not completely clear; however, it seems to be by ingestion of contaminated material: aborted foetuses or placentas. The second one is very likely because the parasite has been found in naturally-infected placentas (Bergeron *et al*., 2000), and dogs that were fed placentas of naturally infected cows shed *N. caninum* oocysts (Dijkstra *et al*., 2001). Also, Basso *et al*. (2001) identified *N. caninum* oocysts by bioassay and polymerase chain reaction in faeces of naturally infected dogs.

1.3.3. Host-parasite relationship

Clinical signs

In cattle the main clinical sign is abortion between 3 and 9 months of gestational age, with a mean age of 5.6 months (Dubey and Lindsay, 1993). Also, *Neospora* may cause foetal death, retention or resorption early in gestation and, in some instances, only skeleton or mummified foetuses are aborted or retained until full gestation. Mummification appears to be an important clinical finding in outbreaks of *N. caninum* associated abortions in cattle (Nietfeld *et al*., 1992; McAllister *et al*., 1996; Campero *et al*., 2003) but calves may also be born alive with clinical signs or be born clinically normal but chronically infected. Seropositive cows are more likely to abort than seronegative cows (McAllister *et al*., 1996; Perez *et al*., 1998; Jensen *et al*., 1999; Dubey, 2003). Abortion due to neosporosis may be endemic or epidemic (Wouda *et al*., 1999). To these authors, abortion was considered as epidemic when more than 10% of cows at risk aborted within a period of 6-8 weeks. Repeated abortion in cows due to neosporosis is infrequent (Barr *et al*., 1993; Anderson *et* *al*., 1995). Additionally, Thurmond and Hietala (1997a) reported a decreased *N. caninum* induced abortion risk in subsequent pregnancies, especially in heifers. They attributed this fact to the selective culling of cows that aborted.

In dogs, and probably in other hosts, the main sign of neosporosis is severe neuromuscular disease (Dubey, 1992). Dogs infected in a natural way exhibit ascending paralysis, rigid hind limb hyperextension, which is a characteristic pattern of neosporosis. The hind limbs are more severely affected than front legs (Dubey and Lindsay, 1993; Barber and Trees, 1996). The limb cannot be flexed even with the patient under anaesthesia (Barr *et al.*, 1997). Other dysfunctions include difficulty in swallowing, paralysis of the jaw, muscle flaccidity, muscle atrophy, head tremors, forelimb ataxia, and even heart failure due to myocarditis (Dubey and Lindsay, 1993; Barber and Trees, 1996).

<u>Lesions</u>

Neospora caninum is an intracellular parasite in their forms of tachyzoites and tissue cysts, then it can cause cellular death by active multiplication of tachyzoites. The lesions are a result of an inflammatory reaction against the parasite. *Neospora caninum* is capable of producing grossly visible lesions in a few days and destroys a variety of neural cells including those of cranial and spinal nerves (Dubey, 1992). In aborted foetuses of cattle, multifocal non-suppurative encephalitis, myositis, myocarditis, periportal hepatitis with or without focal hepatocellular necrosis were repeatedly observed microscopically (Jardine and Wells, 1995; Danatt *et al.,* 1995; Wouda *et al.*, 1997). *N. caninum* tachyzoites have been identified immunohistochemically in brains, hearts and livers. Tissue cysts were observed in brain, spinal cord and muscles. Lesions consisting of a central focus of necrosis surrounded by inflammatory cells (glial or mononuclear) are indicative of *Neospora* infection in cattle. Only a small number of tachyzoites are present in these lesions, and they are difficult to identify without the aid of immunohistochemistry (Dubey and Lindsay, 1993).

<u>Immune response in the host</u>

Humoral response

All studied species develop *N. caninum* IgG antibodies after 2 weeks of inoculation, indicated by higher titers 3 weeks after inoculation (Dubey *et al.,* 1996b); therefore, serologic tests are useful for detection of *N. caninum* infection in all species. Other studies evaluating the humoral immune responses in cows revealed the production of both IgG1 and IgG2, in some cases with predominance of one of them, or with a mixed response in some other cases (Andrianarivo *et al*., 2001; De Marez *et al*., 1999).

Specifically in cows, a detectable immune response develops within 3 weeks with Immune Fluorescence Assay (IFA) titers of 1,600 at day 21 post infection and optical density ratios (OD) in ELISA greater than 0.750. At 3 months post infection, antibodies are at the highest levels. In samples of cows that aborted because of natural infection with *N. caninum*, antibody titers ranged from 1:1,600 to 1:25,000 or more in the IFA test (Dubey *et al.,* 1996b). There was a good correlation between the antibody titer in bovine foetal fluids and the presence of lesions in the late stages of gestation (after 6 months). This correlation reflects the ability of foetuses to develop an antibody response for antigens in the later half of pregnancy (Barr *et al.,* 1997).

Reports of cows repeatedly aborting *N. caninum*-infected foetuses suggest that maternal *N. caninum* antibodies *per se* do not prevent foetal infection (Barr *et al.,* 1993; Anderson *et al.,* 1995). However, Paré *et al.* (1997) and Piergili-Fioreti *et al*. (2000) indicated that seropositive cows with high antibody levels at third trimester of gestation were less likely to abort than cows with low antibody levels at those times. Furthermore, these studies revealed that if the cow becomes infected during gestation, the foetus might not necessarily become infected. These results indicate that acquisition of infection during pregnancy is not necessary for congenital infection or abortion to occur, and suggest that maternal immune response influences congenital infection and abortion. Moreover, the foetal immune response may be responsible for the decrease in abortion risk during the third trimester of gestation, when the foetus becomes immuno-competent (Osburn, 1986; Piergili-Fioreti *et al*. 2000).

Cell-mediated immune response

As *N. caninum* is an intracellular parasite, the host defence most likely includes cell mediated immunity. Specific cell-mediated immune responses involving proliferation of cells and production of interferon (IFN) have been observed in both natural and experimental infections with either tachyzoites or oocysts (De Marez *et al*., 1999; Lunden *et al*., 1998; Andrianarivo *et al*., 2000, Andrianarivo *et al*., 2001). Some studies observed that *N. caninum* was able to induce significant amounts of IL-12 and IFN gamma, most evident shortly after infection. Also, these observations suggest that *N. caninum* induces a T-cell immune response that is at least partially mediated by IL-12 and IFN gamma (Khan *et al.* 1997; Almeria *et al*., 2003). Furthermore, infected dams showed a rise in lymphocyte subpopulations compared to uninfected pregnant animals (Innes *et al*., 2001). Increased levels of T lymphocytes were also observed in infected foetuses (Almeria *et al.*, 2003).

1.4. Diagnosis

In the first years after de discovery of *Neospora caninum*, the diagnosis was made by means of clinical signs and histopathology, describing lesions found in CNS, muscles and other organs of the foetuses. This kind of diagnosis had the problem that *N. caninum* was misdiagnosed as *T. gondii* due to the close structural similarities (Dubey, 1992; Dubey and Lindsay, 1993). Because this similarity, it was necessary to develop specific tests to differentiate between *N. caninum* and *T. gondii*. Thus, immunohistochemistry, serology, and molecular biology have been important in supplying specific tests to diagnose exactly, rapidly and sensitively those animals with neosporosis.

1.4.1. Immunohistochemistry

Bjerkas and Presthus (1989), in the first report of *Toxoplasma*-like infection in dogs, used an immunohistochemical technique. However, the first immunohistochemical test specific to detect *Neospora* was an avidin-biotin-peroxidase complex immunoperoxidase staining method, developed to detect *N. caninum* in formalin-fixed paraffin-embedded tissue sections (Lindsay and Dubey, 1989). This test was able to distinguish *N. caninum* from other parasites. In the subsequent years, new immunohistochemical tests were developed to make a more specific diagnosis of *N. caninum* (Dannatt *et al.*, 1995; Barr *et al.*, 1994b; Barber *et al.*, 1995; Daft *et al.*, 1997; Wouda *et al.*, 1997). This test has been used to state the presence of the parasite in tissues from foetuses suspected to originate from *Neospora*-induced abortion. Besides, immunohistochemistry has been used as gold standard test to validate serological tests (Baszler *et al*., 2001).

1.4.2. Serology

Neospora induces antibody production in the host. Hence, a serological test may be useful to detect seropositive animals. However, a positive result to antibodies only indicates exposure to the parasite and infection with high probability. Histological examinations of tissues from aborted foetuses might be done to corroborate a definitive diagnosis. Even so, sometimes it is not possible to identify the agent in the tissues assayed; but some lesions might be very indicative of infection to a qualified pathologist.

The main serological tests used in studies for *Neospora* diagnosis have been the indirect immunofluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) and immunoblot; however, the IFAT and ELISA are the most frequently used tests because they are relatively cheap.

The ELISA´s have higher sensitivity and specificity for serodiagnosis of *Neospora* infection in cattle compared to IFAT. These characteristics of the ELISA´s range between 88-100% and 94-96% respectively, compared to IFAT (Bjorkman *et al.,* 1994, 1997; Williams *et al.,* 1997). In most studies carried out during the last years, the ELISA´s have been the main test used because of its sensitivity and specificity (Paré *et al.,* 1995; Baszler *et al.,* 1996; Thurmond and Hietala, 1996; Thurmond *et al.,* 1997; Jenkins *et al.,* 1997; Osawa *et al.,* 1998; Romero *et al*., 2002). Furthermore, the ELISA has been used recently for *Neospora* diagnosis in milk, showing an agreement of 95% between serum and milk ELISA (Bjorkman *et al.,* 1997).

A very important characteristic of IFAT is its ability to detect IgM and IgG antibodies produced by the foetus in response to *Neospora* infection (Barr *et al.,* 1997). Some other tests have been validated and standardised using the IFAT as gold standard test (Paré *et al*., 1995; Osawa *et al*., 1998; Romero *et al*., 2002).

Later than IFAT and ELISA, the Immunoblot tests for *Neospora* were developed, being a useful diagnostic tool to differentiate between *Neospora* and *Toxoplasma* species. This test has been used to confirm the findings of ELISA and IFAT tests (Bjorkman *et al.,* 1994; Paré *et al.,* 1995; Baszler *et al.,* 1996; Hemphill and Gottsein, 1996; Beckers *et al.,* 1997; Stenlund *et al.,* 1997; Schares *et al*., 1999).

1.4.3. Polymerase chain reaction (PCR) test.

Since 1993 the polymerase chain reaction (PCR) has been used to identify *Neospora* DNA in tissues or sera from cattle, especially to distinguish this parasite from related species, like *Toxoplasma* (Brindley *et al*., 1993). A study was made to identify the *N. caninum* phylogeny, which was based on DNA sequence analysis of products derived by asymmetric PCR to determine the nucleotide sequence. The results confirmed the placing of *N. caninum* in the family of Sarcocystidae as a sister group of *T. gondii* in the phylum

Apicomplexa (Ellis *et al.*, 1994). After this study, Guo and Johnson (1995) confirmed these findings, but additionally, they showed that *N. caninum* has a high level of genetic divergence with regard to *T. gondii* and other Sarcocystis species. Other studies confirm the usefulness of PCR as an important tool in specific and exact diagnosis of *N. caninum*, besides immunohistochemistry and serologic tests (Holmdahl and Mattson, 1996; Lally *et al.,* 1996; Muller *et al.,* 1996; Yamage *et al.,* 1996; Ho *et al.,* 1996; Ho *et al.,* 1997a,b,; Sreekumar *et al*., 2003). Today, this technique is more and more used in epidemiological studies, even in antemortem samples (Schatzberg *et al*., 2003). These authors developed a multiplex polymerase chain reaction (PCR) assay for the detection of *T. gondii* and *N. caninum* DNA in canine and feline biological samples of animals with serological evidence of neosporosis or toxoplasmosis.

1.5. Treatment

Several studies have been carried out to find the best treatment for *Neospora* infection or its effects on the species affected. However, there is currently no commercial chemotherapy available. A well known problem is that drug treatment for neosporosis in cattle leads to milk withdrawal when used in lactating dairy cows (Barr *et al.,* 1997). However, this problem is not very important in other species, in which the duration of chemotherapeutic treatment has less impact. Another problem in all species is the resistance of tissue cysts and bradyzoites to the drug; there is no 100% guarantee of effectiveness in clearance of these stages of the parasite in the animal (Barr *et al.,* 1997).

The sulphonamides is the main group of drugs used for *Neospora* treatment -solely or in combination with other drugs- because their historical utility in the treatment of toxoplasmosis. Lindsay and Dubey (1989) tested the effectiveness of the drugs: Lasalocid sodium (0.05 μg/ml), monensin sodium (0.05 μg/ml), piritrexim (0.01 μg/ml), pirimethamine (0.05 μg/ml), and trimethoprim (5.0 μg/ml). These drugs were effective in preventing development of intracellular *N. caninum* tachyzoites in bovine monocyte cultured cells. Treatments that combine sulphonamides with trimethoprim are able to diminish clinical signs and prevent death, but are not able to restore the health of infected animals completely (Lindsay and Dubey, 1990; Hay *et al.,* 1990; Mathew *et al.,* 1991). In an extensive study, in which 43 chemotherapeutic agents were tested against *N. caninum* tachyzoites in cultured cells, the results indicated that some drugs have coccidiocidal activity while others act coccidiostatic (Lindsay *et al.,* 1994). Besides, a study carried out by Lindsay *et al.* (1996a), demonstrated the efficacy of a treatment that combines 7 sulfonamides and 5 dihydrofolate reductase/thymidylate synthase inhibitors against tachyzoites of *Neospora caninum* in cultured cells. The better results were obtained with suboptimal concentrations of DHFR/TS inhibitors and sulfonamides.

Further, the activity of decoquinate, an coccidiocidal drug, was tested, showing that it acted quickly to kill intracellular stages at coccidiocidal concentrations (Lindsay *et al.*, 1997). Gottstein *et al.* (2001) tested the toltrazuril and ponazuril for prevention cerebral lesion formation using mouse models. DNA detection in a PCR was reduced by >90%. The efficiency of ponazuril was also examined in experimentally infected calves by Kritzner *et al*. (2002). They reported that after ponazuril treatment parasites were not detectable in the brain and other organs, while 50% of non-treated calves became PCR-positive in brain and muscles. Besides, anti-*Neospora* antibody concentrations developed after infection were significantly lower, when compared to non-treated animals. With these findings, the researchers are encouraged to develop efficacious chemotherapy against neosporosis in cattle.

1.6. Prevention and control

Although the life cycle of *N. caninum* is now known, it is difficult to make specific recommendations about preventive measures because the possibility of other species than canids could be definitive hosts (Barling *et al*., 2000a). Nevertheless, farmers should be encouraged to protect feed and water from faecal contamination by canine faeces (Barr *et al.*, 1997; Trees *et al.*, 1998). The importance of horizontal transmission in the total amount of new infections has not been fatefully established. However, the horizontal infection rate can be higher than reported initially, especially in some parts of he world (Romero and Frankena, 2003, Chapter 4), though vertical transmission is the most important way of transmission (> 90%), and its control requires specific management control strategies (Trees *et al.*, 1998). As part of those strategies, culling of seropositive cows has been considered as a unique control strategy to prevent birth of animals that are congenitally infected, but there is no evidence that this control measure has economic benefit. Therefore it is necessary to assess the economic impact of neosporosis and to assess the cost of culling of seropositive cows. Thurmond and Hietala (1995) recommended two general ways to prevent *N. caninum* transmission; a) control of congenital transmission, and, b) control of postnatal transmission. These recommendations were taken into account by French *et al.*

(1999) in mathematical models in which they evaluate the effectiveness of some control measures. They assessed that selective culling (seropositives) and reduction of the possibility of vertical and horizontal transmission (control of dogs into the farm) were effective to control the infection at long term. Reduction of congenital transmission can be achieved by removing all infected cows. Furthermore, they recommend using only seronegative heifers for replacement. The second way involves the removal of all tissue sources that are possibly infected with infective stages of *Neospora*; e.g. placentas, foetuses, dead calves, etc., which could serve as a possible source of infection for the definitive host.

Ortega-Mora *et al*. (2003) detected *N. caninum* DNA by a real-time PCR in non-extended fresh semen samples and frozen extended semen straws of five seropositive bulls. The parasite mean load in positive fresh semen samples varied between 1 and 2.8 parasites/ml of semen. No *N. caninum* DNA was amplified in any of three similar uninfected bulls (controls). Embryo transfer (ET) has been recommended to avoid vertical transmission of the parasite -when the embryo was received by a seronegative cow-. Several studies have been carried out to evaluate the efficacy of embryo transfer to prevent congenital infection, all of them showing no evidence of congenital infection of the calves (Baillargeon *et al*., 2001; Landmann *et al*., 2002; Campero *et al*., 2003).

A more recent effort to prevent *Neospora*-induced abortion is vaccination. The knowledge to develop an effective vaccine and vaccination strategy against it is increasing (Hemphill and Gottstein, 2000; Innes *et al*., 2001). Experimental studies in cattle, under laboratory and field conditions, have shown the effectiveness of several vaccinia preparations, based on killed tachyzoites with adjuvants, to elicit a response at both cellular and humoral level (Andrianarivo *et al*., 2000; Choromanski and Block, 2000). There is only one vaccine commercially available against *Neospora*-infection; but the effects of vaccination on the probability of bovine abortion and/or its effect on prevention of infection in susceptible animals have not yet been well documented. Nevertheless, one paper reported a decreasing number of abortions in a Minnesota dairy herd after a year of vaccination using this vaccine (Choromanski *et al*., 2001). Heuer *et al.* (2003) reported an efficacy (prevented fraction) to prevent abortion such as 0.7%, 39.0%, 54.2%, 31.4%, and –5.2%, in 5 seasonally calving, commercial dairy farms in New Zeeland. Besides, in a field trial carried out in 25 Costa Rican dairy farms by Romero *et al.* (2004, Chapter 5) vaccination was associated with a 46.2% decrease of the abortion rate. However, the efficacy of vaccination to prevent *Neospora*-infection -or its consequences- in cattle is not well demonstrated until now.

1.7. Epidemiological features

Neosporosis has been related with epizootic and sporadic abortion in dairy herds worldwide. Since the discovery of neosporosis, some studies have been conducted to assess the prevalence and to identify factors related to the disease. Prevalences have been estimated in ranges between 16.8% and 70% (Paré *et al.,* 1996; Paré *et al.,* 1997; Thurmond *et al.,* 1997; Waldner *et al.,* 1998). No specific studies have been carried out to assess incidence rates because of the low odds of horizontal transmission. However, Paré *et al.* (1997) reported a rate of seroconversion of 8.5/100 cows/year; besides, Romero and Frankena (2003, Chapter 4) reported a probability of apparent horizontal infection of 22%; however, this probability increased when the within herd seroprevalence increased.

Seroepidemiological studies have assessed the increased risk for abortion in seropositive cows (Thurmond and Hietala, 1997a; Perez *et al.*, 1998; Waldner *et al.*, 1998; Wouda *et al.*, 1998) which also have higher risks for stillbirth (Waldner *et al*., 1998), a seropositive offspring (Paré *et al.,* 1997), culling for reproductive reason (Thurmond and Hietala, 1996; Waldner *et al.*, 1998) compared to seronegative ones. It is expected that infected cows have higher odds of subsequent abortions.

There are no conclusive data regarding economic losses due to *Neospora* infection, but it has been estimated to be millions of US dollars, due to direct costs and values of the lost foetuses but also because of the indirect costs (Barr *et al.*, 1997; Chi *et al*., 2002, Dubey, 2003). Other studies have documented the effect on (re)production parameters (Thurmond and Hietala, 1997b; Barling *et al.*, 2000b, Hernandez *et al.*, 2001). Trees *et al*. (1999) summarise that neosporosis might have impact on the global performance of the herd due to: 1) increased risk of abortion, 2) stillbirths and neonatal mortality, 3) early foetal death and resorption leading to return to service, increased time to conception or infertility, 4) increased culling, 5) reduced milk production, and 6) reduced value of breeding stock.

Romero *et al*. (Chapter 2, Theriogenology *in press*) did not find significant effects of *Neospora* serostatus on productive and reproductive performance, specifically on 305-day milk production, calving interval, and the number of services per conception.

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The most difficult thing in the world is to know how to do a thing and to watch somebody else doing it wrong, without comment.

T. H. White

Chapter 2

Effect of neosporosis on productive and reproductive performance of dairy cattle in Costa Rica

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Theriogenology (In press)

Abstract

A cross-sectional study was carried out to assess the effect of neosporosis on productive and reproductive parameters. 2,743 cows from 94 farms located in the most important dairy areas in Costa Rica were used in the study. The size of the herds varied from 32 to 379 females (mean= 110, median=125). An indirect ELISA was used to determine the serostatus of the animals towards *N. caninum*. The effect of neosporosis on milk production was analysed by a mixed linear model. Besides, the effect on calving interval (days) and calving-conception interval (days) was analysed by survival analysis. The risk of abortion in relation to the *N. caninum* serostatus was assessed by logistic regression with herd as random effect. Out of 2,743 animals, 1,185 (43.3%) were seropositive to neosporosis. Eighty-nine out of 94 (94.7%) farms were classified as *Neospora*-seropositive. It was estimated that a cow produces 84.7 litres milk more when it is seronegative to *Neospora* (p = 0.6). The serostatus did not show a significant effect on the length of the calving interval in the Cox proportional hazard survival analysis (Hazard ratio= 1.2, 95% CI: 0.9, 1.4). The logistic regression model showed a weak positive association between serostatus and abortion (OR 1.7, 95% CI: 0.8, 3.9), but did not show a strong association between serostatus and the number of services per conception (OR= 0.95, 95% CI: 0.7, 1.3). In this study no significant effects of *Neospora* serostatus were detected on productive and reproductive performance.

Keywords: *Neosporosis, Cattle, Milk production, Reproductive performance*

2.1. Introduction

Neosporosis is a parasitic disease caused by *Neospora caninum*, a protozoan that until 1988 was misdiagnosed as *Toxoplasma gondii* because of close structural similarities (Dubey, 1992; Dubey and Lindsay, 1993). It has world wide been recognised as a problem in the dairy livestock sector.

Neospora caninum primarily causes brain and heart damage in bovine foetuses, resulting in abortion between three and eight months of gestation (Dubey, 1999). It can cause abortion storms with incidences as high as 10%, but it is more commonly found as an endemic cause of abortion. The organism has been around for a long period but has been diagnosed with increased frequency over the past 10 years. The definitive host of the parasite is the dog (McAllister *et al*., 1998). Two ways of transmission are possible in cows: 1) vertical transmission, through placenta from dam to calf, and 2) horizontal transmission, by ingestion of sporulated oocysts shed by infected dogs (Dubey, 1999; Anderson *et al*., 2000; Bergeron *et al*., 2000). In some countries *N. caninum* is considered to be the main cause of abortion by infectious agents in cattle (Anderson *et al*., 1995; Wouda, 1998).

Not many studies have related neosporosis to economic losses, but studies in California and Canada estimated important losses by direct and indirect costs (Barr *et al*., 1997; Chi *et al*., 2002). Besides, Trees *et al*. (1999) discussed the possible economic consequence of neosporosis due to an effect on milk production and reproduction of the cows. Specifically, several studies have determined a detrimental effect of neosporosis on milk production (Thurmond and Hietala, 1997; Hernandez *et al*., 2001; Hobson *et al*., 2002) and reproduction (Waldner *et al*., 1998; Moore *et al*., 2003) and on premature culling (Thurmond and Hietala, 1996; Waldner *et al*., 1998).

In Costa Rica *N. caninum* was discovered in 1996 causing abortion in a goat (Dube, 1996) and, in the same year, it was related with abortion in dairy cattle (Perez *et al*., 1998). Furthermore, other studies found a 100% seroprevalence at the herd level, with within-herd seroprevalences varying between 25 and 70% (Romero *et al*., 2002).

Because *N. caninum* causes abortion, it is likely that it will affect the reproductive parameters as well as the productive parameters. More quantitative knowledge about the effect of this infection might convince farmers to take more efficient measures to reduce or to prevent transmission.

The main objective of this study is to quantify the effect of *N. caninum* seropositivity on the productive and reproductive parameters of Costa Rican dairy cattle. The hypothesis is that seropositivity to *N. caninum* does have a negative effect on the productive and reproductive parameters.

2.2. Material and methods

2.2.1. Study design

In a cross-sectional study to detect the presence of leptospirosis at the herd level, about 4,000 blood samples were collected from 145 farms between September 1998 and July 1999. The sampling design consisted of a random sampling stratified by age. The age categories were 0.5-2, 2-4, 4-6, and >6 yr old. The number of samples needed per herd was calculated using the software FreeCalc 1.02, which uses the hypergeometric exact model described by Cameron and Baldock (Cameron and Baldock, 1998). Parameters used in this calculation fitted satisfactorily to the detection of N. caninum at herd level because the number of samples based on the expected prevalence for leptospirosis and neosporosis (25% and 37% resp.), sensitivity (90% and 87.8% resp.) and specificity (95% and 96.4% resp.) of the tests were quite similar. The number of samples per herd varied from 28 to 38.

Blood samples of 5-10 ml were taken from the caudal vein using tubes without anticoagulant (Vacutainer®, Becton Dickinson VACUTAINER Systems). Samples were transported at 4-6ºC to the laboratory. Serum was separated by centrifugation (4000 rpm x 8 min.); afterwards, 2 ml aliquots were stored at -20°C until analysed.

Due to the specific inclusion criterion that farms should use VAMPP - Veterinary Automated Management and Production control Program (Noordhuizen and Buurman, 1984) - as management information system and loss of samples, 2,743 samples from 94 farms remained for use in our study, which is about 69% of the original amount. The size of these 94 herds varied from 32 to 379 females with a mean of 110 and a median of 125. The 94 farms were located in three dairy areas in Costa Rica in the central zone of Costa Rica. Although this area is relatively small, several ecozones have been identified: moist-low mountain forest, moist-pre mountain forest, very moist-low mountain forest, very moist-pre mountain forest, very moist-tropical forest and rainy-low mountain forest (Holdridge, 1989).

Information at animal level such as breed, parity and milk production was extracted from the VAMPP database. Of cows that were present with more than one lactation in the database, the first lactation after the sampling moment was selected. Also, the ecozone and abortion rate for the period at study of the farms were derived from this database. The abortion rate was defined as all foetal losses after a positive diagnosis for pregnancy divided by all pregnancies confirmed in a period. In all cases, pregnancy diagnosis was done by rectal palpation by a veterinarian three times after insemination at 35 d with confirmations at 120 d and 210 d.

The parameter used as indicator for milk production was the 305-d milk production. VAMPP calculates this figure based on the following rules: 1) if the cow produced more than 305 d, it cuts the production at 305 d and makes an interpolation of the last milk recording before and the first after 305 d; 2) if the cows does not have milk recording records after 305 d, but it has records between 200 and 304 d, it cuts the production and the last milk weight and then makes and extrapolation, 3) for cows that do not have milk recording records of production over 200 d, the 305-d production is not calculated. Due to this, 95% of the records showed a time span between sampling and 305 d production calculation of 245 to 568 d.

2.2.2. Determination of the serostatus of the cows

An indirect ELISA was used to assess the serostatus of the animals towards *N. caninum*. The sera were defrosted in a refrigerator at 4°C the day before testing. The ELISA was performed as described elsewhere (Paré *et al*., 1995). The sensitivity and specificity of the ELISA were estimated as 87.8 % (CI 95%: 81.3, 94.3) and 96.4% (CI 95%: 93.3, 99.5), respectively (Romero *et al*., 2002).

The software FreeCalc 1.02 (Cameron and Baldock, 1998) was used to calculate the cut-off for positive reactors to define a farm as seropositive. Using the sensitivity and specificity of the ELISA described above and an estimated prevalence of 37.0% (Romero *et al*., 2002), a farm was defined as seropositive when 3 or more reactors were found.

2.2.3. Data analysis

Descriptive statistics for animal and environmental characteristics

Descriptive statistics about the general characteristics of the cows and the serological status stratified by ecozone, breed and parity, were performed using SAS -Release 8.0- (SAS/STAT® release 8.0). In this analysis, all 2,743 samples from the 94 farms were taken into account. The record keeping in VAMPP (Noordhuizen and Buurman, 1984) was not carried out with the same rigour and periodicity in all farms; so the number of cows may vary for each parameter.

Statistical analysis

From the database used in the descriptive statistics, after a process of editing and reduction, 850 and 406 cows remained available for the analysis of the effect of *Neospora* serostatus on productive and reproductive parameters respectively. Major reason for dropping cows was missing information to calculate the 305-d milk production. Besides, heifers or young animals without reproductive data, and subsequently without productive information were excluded.

The relation between milk production and neosporosis status was analysed by a mixed linear model using Proc MIXED from SAS/STAT® release 8.0, while adjusting for parity number, breed of the cow and the ecological zone in which the farms are located. An analysis of residuals was done to evaluate the model fit, then the Kolmogorov-Smirnov statistic was used to assess the goodness of fit for normal distribution.

The statistical model was:

$$Y_{ijklmn} = \mu_0 + neo_i + par_j + breed_k + eco_l + a_m + e_{ijklmn}$$

Where:

Y_{ijklmn} = individual milk production (305-d milk production)

μ_0 = general mean

neo_i = fixed effect of i^{th} *Neospora* status (i = 0 - 1; 0 = negative, 1 = positive)

par_j = fixed effect of j^{th} parity class (j = 1 - 4; 1 = parity 1, 2 = parity 2&3, 3 = parity 4&5, 4 = parity 6 or greater)

$breed_k$ = fixed effect of k^{th} breed (k = 0 – 3; 0 = Holstein, 1 = Jersey, 2 = Holstein/Jersey, 3 = Others)

eco_l = fixed effect of l^{th} ecozone (j = 1 - 9)

a_m = random effect of m^{th} herd (m = 1-94) nested within ecozone

e_{ijklmn} = random residual effect

To avoid effects of herds with very few animals in the dataset, the minimum number of animals in a herd was set at 5.

The effect of the *N. caninum* serostatus, adjusted for breed, parity and ecozone, on calving interval (days) and calving-conception interval (days) was analysed by survival analysis using Proc LIFETEST from SAS -Release 8.0-. For this analysis the exit time to define censoring for the calving interval was 550 d which equals the mean (402 d) + 2 times the standard deviation (SD). In the same way, the exit-time for the calving-conception interval was 250 d (2 SD above the mean of 132 d). Besides, Kaplan-Meier curves were made to visualise whether the curves for both interval variables differed between seropositive and seronegatives animals; the Wilcoxon test was used for hypothesis testing.

The relation between abortion (yes/no) and the number of services per conception (SPC, 2 categories: 1 or more than 1) and the *N. caninum* serostatus was assessed by logistic regression with herd as random effect and breed, parity and ecozone as potential confounders. The logistic procedure consisted of 2 steps: 1) univariable analysis 2) multivariable analysis. In the second step, all variables with P <0.25 (likelihood ratio test) in the univariable analysis were included. A backward model building strategy was followed (Hosmer and Lemeshow, 1989). After the exclusion of each variable from the multivariable model, presence of confounding was evaluated by comparison of the estimated coefficients in the new model with the estimated coefficients and likelihood ratio of the previous model. Confounding was deemed present if at least one coefficient had changed more than 0.1 (if the coefficient had a value between –0.4 and 0.4) or if at least one coefficient changed more than 25% (if the coefficient had a value < –0.4 or > 0.4). Finally, biologically plausible interaction terms were added to the model and statistically evaluated using the likelihood ratio test. The significance for each explanatory variable in the final model was determined by the likelihood ratio test and the confidence interval for the odds ratio (OR). Logistic regression was performed in EGRET® 2.0 (1999).

2.3. Results

2.3.1. Descriptive statistics for animal and environmental characteristics

Out of 2,743 animals, 1,185 (43.3%) were seropositive towards neosporosis. Eighty-nine out of 94 (94.7%) farms had at least three seropositive animals. Most samples were taken in the very moist pre mountain forest (1,070). The highest percentage of seropositive cows (60.7%) was found in the tropical dry forest, while the lowest were found in the rainy low mountain forest and the moist low mountain forest being 29.6% and 22.8%, respectively.

Holstein is the most frequently used breed. The percentage of seropositive cows was higher in Jersey (49.1%) and Jersey/Holstein crosses (50.3%) compared to pure Holsteins (43.2%) ($p < 0.05$)

The average 305-d milk production was 5,562 kg. (range 2,408-11,844). Information at cow level on calving interval and age at first conception is presented in Table 1; besides, information on culling and abortion rate, and serostatus at farm level is presented in the same Table.

Table 1.

Description (number, mean, minimum and maximum values) of the most important productive and reproductive parameters in the farms sampled.

Parameter	No. of cows (herds)	Mean	Min-Max
Milk production. (305-d milk production)	850 (51)	5,562.5	2,408.0 - 11,844.0
Calving interval (days)	605 (81)	401.8	291.0 - 690.0
Age at first conception (months)	701 (81)	20.0	9.0 - 36.0
Culling rate (%)**	- (94)	24.4	13.2 - 31.8
Abortion rate (%)**	- (81)	12.9	4.1 - 42.9
Neospora serostatus (%)	2,743 (94)	38.7	0.0 - 100.0

2.3.2. Statistical analysis

2.3.2.1. Effect on milk production

Based on records of 850 cows from 51 farms, the serostatus of the cows did not have a significant effect on the 305-d milk production when adjusted for ecozone, parity and breed. It was estimated that a cow produced 84.7 litres milk more when it was seronegative to *Neospora* (Table 2, $p = 0.62$). Parity and breed, showed an expected relation with milk production (production increases with age and Holsteins produce more than Jerseys or crossbreds).

2.3.2.2. Effect on reproductive parameters

To perform these analyses 406 cows from 39 farms were available. The Kaplan-Meier curves of the calving interval (Fig. 1) differ significantly (Wilcoxon test, $p<0.05$) between seropositive and seronegative animals. However, after adjustment in the Cox proportional hazard analysis, serostatus did not show a significant effect (Table 3, HR = 1.2, 95% CI: 0.9, 1.4). Other variables in the model were breed ($p < 0.01$), ecozone ($p< 0.01$) and parity ($p = 0.90$). Although parity did not have a significant effect, it had to remain in the model as it was a confounder for the estimate of serostatus.

Table 2.

Effect estimates on 305-d milk production of explanatory variables, their overall significance (F and p(F)) and the significance of differences between levels (p (t)), based on a mixed model.

Variable	Level of variable	Freq. (%)	F value*	*p(F)*	*Estimate*	*p* (t)	*Diff. LSM* ⴲ
Intercept					4,810.3	0.00	
Serostatus			0.25	0.62			
	Negative	57.7			84.7	0.62	a
	Positive	42.3			0.0	.	a
Parity			23.06	0.00			
	1	35.1			-1,386.5	0.00	b
	2-3	33.8			-312.5	0.20	a
	4-5	21.4			64.3	0.80	a
	≥ 6	9.7			0.0	.	a
Breed			6.10	0.00			
	Holstein	57.7			605.4	0.06	a
	Jersey	26.6			-465.2	0.18	b
	Holstein*Jersey	6.7			203.2	0.61	a,b
	Others	8.9			0.0	.	a,b
Ecozone			4.99	0.00			
	Moist-low mount. forest	7.0			1,352.0	0.10	a
	Moist-pre mount. forest	31.3			2,386.6	0.00	a,b,c
	Very moist-low mount. forest	8.0			1,818.2	0.07	a,b,c
	Very moist-pre mount. forest	22.4			195.3	0.76	b,c
	Very moist- trop. forest	4.7			-1,000.4	0.26	c
	Rainy-low mount. forest	5.7			1,844.9	0.01	a,b
	Moist trop. forest	20.9	.		0.0	.	b,c

Goodness of fit for normal distribution evaluated by Kolmogorov-Smirnov statistic p=0.15 (Ho: residuals fit to normal distribution).

* Represents the overall effect for each variable.

ⴲ Differences between levels are based on least square means (LSM). Levels showing different letters have statistically different effect estimates (p<0.05).

The calving-conception interval was not significantly affected by the serostatus or parity, but it was affected by breed and ecozone (Table 4).

There was a weak non-significant positive association between serostatus and abortion (OR 1.7, 95% CI: 0.8, 3.9), while there was no association between serostatus and SPC (OR= 0.95, 95% CI 0.7, 1.3).

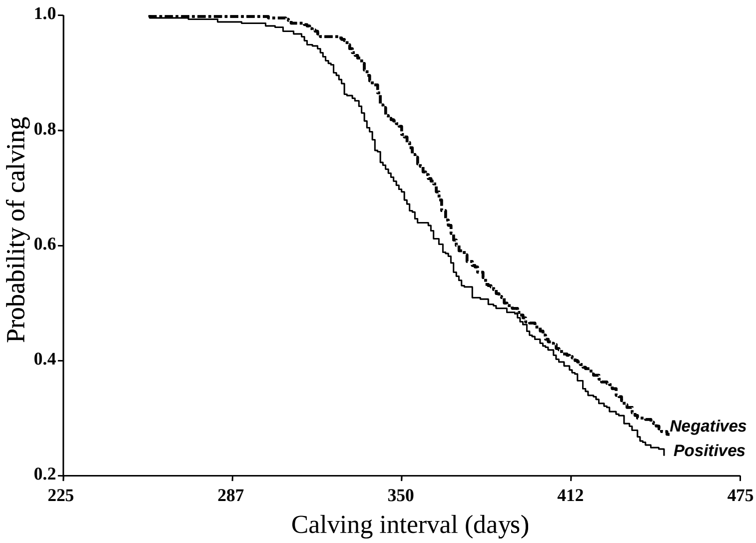

Figure 1. Kaplan-Meier survival curves related to the calving interval for the seropositive group (thin line) and the seronegative group (bold line).

Table 3.

Effect of the serostatus, ecozone, race and parity on calving interval expressed as Hazard ratios with accompanying 95% Confidence Intervals (CI) and p-value as assessed by Cox proportional hazard analysis.

Variable	Level of variable	Frequency %	Hazard ratio	Lower CI 95%	Upper CI 95%	*p-value*
Serostatus	Negative	59.0	1.0	.	.	.
	Positive	41.0	1.2	1.0	1.4	0.09
Parity	**		0.9	0.9	1.1	0.90
Breed	Holstein	61.8	1.0	.	.	.
	Jersey	15.0	1.6	1.3	2.1	0.00
	Holstein*Jersey	7.3	1.1	0.8	1.7	0.54
	Others	15.9	1.3	0.9	1.5	0.37
Ecozone	Moist-low mount. forest	6.4	1.0	.	.	.
	Moist-pre mount. forest	44.2	0.6	0.5	0.8	0.00
	Very moist-low mount. forest	18.8	0.5	0.3	0.9	0.02
	Very moist-pre mount. forest	15.7	0.7	0.5	0.9	0.00
	Rainy-low mountain forest	14.9	0.4	0.3	0.6	0.00

** Parity was included as continuous variable, so the HR represents the increase in hazard when parity increases one unit.

2.4. Discussion

2.4.1. Descriptive statistics for animal and environmental characteristics

It is plausible that the climate (represented in this study as ecozone) influences the seroprevalence at herd and within-herd level; levels are comparable with those reported elsewhere in similar conditions like in Costa Rica (Morales *et al*., 2001; Garcia-Vasquez, 2002). The Costa Rican climate conditions, with warm temperatures and some humidity (Holdridge, 1989), can promote well the maintenance of the oocysts -sporulated or not- in soil and pastures for many months (Dubey, 1999) even with the possibility to contaminate the drinking water from natural springs. Besides, it is possible that in ecozones with high levels of rainfall and topography with hillsides, the oocysts could be washed away being available to cows even far away from where the dog defecated.

Farms vary a lot in size and production performance in Costa Rica, with various levels of intensity in management and herd health practices (Vargas, 2000). A calving interval of 670 d is quite long but not unlikely. An age at first conception of 9 mo is low but not impossible, especially because the Jersey heifers can be inseminated as early as this age, regardless of whether or not it is biologically and technically pertinent. The 305-d milk production is comparable with the production in temperate countries as some farms are highly specialised.

A crucial parameter in the economy and management of the farm is the abortion rate, which is a remarkable problem in Costa Rican dairy farms as is shown in Table 1. The most important infectious abortifacients - brucellosis, leptospirosis, infectious bovine rhinotracheitis (IBR), bovine viral diarrhoea (BVD) and neosporosis - have been diagnosed in Costa Rican dairy cattle. Although their prevalence has not been assessed they are related with endemic abortion (Romero *et al*., 2000).

Our study dealt with serostatus regarding neosporosis as assessed in a cross-sectional design. Therefore, we do not know when the cows were infected, neither if and when they showed an abortion. Therefore, the results are related to *N. caninum* serostatus but not to *N. caninum* induced abortion. Other types of studies (e.g. matched case-control) are needed to assess whether or not *N. caninum* induced abortion affects the production and reproduction figures. To prevent a large gap between sample result and the period in which the performance parameters were realised, the first full lactation after the blood sampling moment was included in the analyses.

Table 4.

Effect of the serostatus, ecozone, race and parity on calving-conception interval expressed as Hazard ratios with accompanying 95% Confidence Intervals (CI) and p-value as assessed by Cox proportional hazard survival analysis.

Variable	Level of variable	Freq. %	Hazard ratio	Lower CI 95%	Upper CI 95%	*p-value*
Serostatus	Negative	59.0	1.0	.	.	.
	Positive	41.0	1.0	0.9	1.2	0.76
Parity	**		0.9	0.9	1.1	0.51
Breed	Holstein	61.8	1.0	.	.	.
	Jersey	15.0	1.6	1.2	2.0	0.00
	Holstein*Jersey	7.3	1.6	1.1	2.2	0.00
	Others	15.9	1.4	1.1	1.8	0.00
Ecozone	Moist-low mount. forest	6.4	1.0	.	.	.
	Very moist-low mount. forest	44.2	0.8	0.7	1.1	0.13
	Very moist-pre mountain. forest	18.8	0.9	0.6	1.4	0.58
	Very moist- tropical forest	15.7	0.9	0.6	1.2	0.30
	Rainy-low mount. forest	14.9	0.6	0.5	0.8	0.00

** Parity was included as continuous variable, so the Hazard Ratio represents the increase in hazard when parity increases one unit.

2.4.2. Effect on productive and reproductive parameters

2.4.2.1. Milk production

A seronegative cow produces around 84.7 Kg more milk (305-d milk production) when it is seronegative to *N. caninum*. However this result was not statistically significant, probably due to the important reduction of data available to this analysis. In this sense, other studies have reported a detrimental effect of *Neospora* infection on milk production in first-lactation dairy cows in amounts such as 3.1 lbs/cow/day (Thurmond and Hietala, 1997) or 2.8 lb/cow/day in cows regardless of parity (Hernandez *et al*., 2001). Furthermore, Hobson *et al*. (2002) reported a herd case-control study involving 3,702 Holstein cows, showing statistical differences in milk production between seropositive and seronegative cows in herds with *Neospora*-related abortions, and in herds with abortions attributable to pathogens other than *N. caninum*. However, as part of the same study, they carried out a cross-sectional study in which they found no statistical differences between seropositive and seronegative animals, similar to our study.

Because of the time lapse between taking the blood sample and the calculation of the 305-d milk production, it is probable that some negative cows became positive in that period, so the milk production originally assigned to a negative cow could correspond to a positive cow (recently seroconverted). This fact is especially relevant because in a longitudinal study (not yet reported) we assessed a cumulative incidence about 0.13 per yr. This could have consequences on the estimation of the effect of the serostatus on milk production; however, due to the high p value for this figure (0.62) it is highly probable that the difference in 305-d production between seropositives and seronegatives remains statistically insignificant.

Because milk production depends of many factors, it is risky to conclude that this parameter is only (or mainly) influenced by *N. caninum* infection. The farm effect, which is the sum of genetics, management and environmental effects, involves many variables that may affect the milk production. Taking only some major parameters into account, like in this and other studies, it is difficult to assess whether or not neosporosis causes a major effect on milk production.

2.4.2.2. Reproductive parameters

In this study, the serostatus towards *N. caninum* did not have a significant effect on reproductive parameters. Although the effect of serostatus affecting the calving interval is almost significant, the association is quite weak; likewise, there were not significant effect on calving-conception interval and SPC.

The direct effect of neosporosis on calving interval has not yet been reported; however, there is a Canadian report of a detrimental effect of neosporosis on open days (Waldner *et al*., 2001) in which the seropositive cows in the spring were more likely to be open in autumn. On the other hand, Jensen *et al*. (1999) did not find differences in interval calving 1st insemination between seropositive and seronegative cows. Björkman *et al*. (1996) found the same result for the number of effective pregnancies. These findings show that there is not a significant effect on open days and the calving interval, like in our study.

The effect of neosporosis on SPC has not frequently been documented. Muñoz-Zanzi *et al*. (2004) found that *Neospora caninum* infection was associated with SPC (OR 1.8, 90% CI: 1.0-3.3), differing substantially from our results (OR= 0.95, 95% CI 0.7, 1.3). The probable reason for the difference could be that Muñoz-Zanzi *et al.* conducted a study with first breeding heifers while we used cows with a wider range of parities, and they used a liberal 90% CI while we used a 95% CI.

It is very well known that reproduction is a very complicated component of dairy practice, because it is influenced by many factors such as genetics, nutrition, artificial insemination efficacy, presence or absence of diseases affecting fertility, etc. This can lead to large variations in reproductive parameters regardless of environmental conditions or general management practices. Therefore, it is hard to estimate the real effect of a specific disease on the reproductive performance in a relatively small scale cross-sectional study, a matched design would be better into that respect. However, due to the random sampling and the significant number of cows sampled it may be expected that these factors are equally distributed over the sero-positive and sero-negative group in our study.

Two circumstances that could lead to a biased estimate of the effect of the *N. caninum* serostatus on reproductive (and productive) parameters: the reduction of the original number of farms (94) to 51 and 39 for milk production and reproduction analysis respectively; and the errors in the record-keeping at farm level, data transmission to the information system (VAMPP in this study) or the definition of events in the information system. In VAMPP, an abortion over 210 d of gestation is considered as parturition when a new milk production period after the abortion is present. This may lead to an average calving interval for aborting animals that is lower than in reality.

In this study it was not possible to establish with high confidence that seropositive cows had an increased probability of abortion; however, a tendency to increase the abortion rate in seropositive cows was observed. This is in agreement with most other studies in which the association between *N. caninum* infection and abortion has been assessed; however, the strength of the association is variable and the confidence intervals are wide (Thurmond *et al*., 1997; Davison *et al*., 1999; Mainar-Jaime *et al*., 1999; Jensen *et al*., 1999; Garcia-Vasquez *et al*., 2002; Vaclaveck *et al*., 2003).

In conclusion, it can be stated that in the herds included in our study sero-positivity towards *N. caninum* induced a small effect on milk production (at average less than 100 kg per lactation and statistically non-significant), but not on reproduction.

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Don't say you don't have enough time. You have exactly the same number of hours per day that were given to Helen Keller, Pasteur, Michelangelo, Mother Teresa, Leonardo da Vinci, Thomas Jefferson, and Albert Einstein.

H. Jackson Brown

Chapter 3

Factors associated with *Neospora caninum* serostatus in cattle of 20 specialised Costa Rican dairy herds.

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Abstract

Twenty-five specialised Costa Rican dairy farms, located in the Poás area, were used to determine neosporosis seroprevalence and the association of seropositivity with environmental and management factors. The farms involved were selected intentionally and all of them use VAMPP 5.1 as management-information system. Holstein-Friesian, Jersey and crosses between them were the most-frequent breeds in these herds. The number of females per farm varied from 41 to 296. Our cross-sectional study had two phases. In the first phase, we determined the presence or absence of seropositivity at herd level. For the second phase, all females in 20 seropositive farms were bled. Serum samples were tested for antibodies to Neospora caninum using an indirect enzyme-linked immunoassay (ELISA). A questionnaire with factors mentioned in the literature was administered to the farmers. Logistic regression (LR), with herd as random effect, was used to assess the relationships of the serostatus at the individual level with characteristics of the cows and environmental factors. In the first phase all herds had > 20% seropositive females; therefore, all herds were eligible for the second phase. In the second phase, the overall prevalence was 39.7% (1191/3002), and within-herd prevalences were between 25.0% and 70.5%. Age, parity of the dam of the cow, breed and purposive sampling to diagnose abortive infectious disease were associated with serostatus; other management and environmental factors did not show significant associations. The lack of association between management and environmental factors with serostatus might be due to the fact that all farms were exposed to a considerable number of potential factors. The fact that all herds of this study were seropositive for neosporosis, and the fact that the within-herd prevalence was considerable, raises questions about how far the infection is spread in other dairy areas of Costa Rica.

Keywords: Neospora caninum; Cattle; Seroprevalence; Factors associated; Costa Rica.

3.1. Introduction

Neosporosis is a recently discovered disease that is considered as the most important cause of infectious abortion in dairy cattle around the world (Anderson et al., 1995; Dubey and Lindsay, 1993; Barr et al., 1997; Dubey, 1999). *Neospora* infection and *Neospora*-induced abortion have been reported in the most important dairy countries in America, Europe, Asia, Africa and Oceania (Anderson et al., 1995; Boulton et al., 1995; Wouda et al., 1997; Paré et al., 1998; Bergeron et al., 2000; Reichel, 2000; Atkinson et al., 2000).

Recently, the life cycle of neosporosis was described (Dubey, 1999); the dog is the definitive host (McAllister et al., 1998). Some other risk factors for *Neospora* infection include: born to a seropositive dam, the presence of poultry on the farm, season and feeding mouldy corn-silage during summer (Thurmond et al., 1995; Bartels et al., 1998; Wouda et al., 1998, Paré et al., 1998, Wouda et al., 1999a).

The diagnosis of *N. caninum* infection is usually done by serum-antibody tests: enzyme-linked immunosorbent assay (ELISA), direct agglutination test (DAT) and indirect fluorescent-antibody test (IFAT). Positive tests are confirmed by histopathology using immunohistochemistry (Lindsay and Dubey, 1989; Dannat et al., 1995; Paré et al., 1995; Barr et al., 1997; Wouda et al., 1997) or by PCR (De Marez et al., 1999; Kim et al., 2000; Spencer et al., 2000).

The percentage of seropositive animals within-herds varies between 4.3% and 70% (Thurmond et al., 1997; Paré et al., 1998; Wouda et al., 1999a). This range might reflects differences in prevalence between countries, but may be also due to the characteristics (sensitivity, specificity) of the test used (Trees et al., 1994; Paré et al., 1997; Paré et al., 1998; Pérez et al., 1998; Wouda et al., 1998; Bergeron et al., 2000; Barling et al., 2000). Herd-level seroprevalence to *Neospora* is estimated to be 58% in Spain (Ould-Amrouche et al., 1999) and 83% in France (Quintanilla-Gonzalo et al., 1999). In Costa Rica, previous studies have confirmed the presence of *Neospora caninum* (Morales et al. 1995; Dubey et al., 1996; Pérez et al., 1998), and it was estimated that *Neospora*-seropositive cows had a 12-times increased risk of abortion compared to seronegative cows (Pérez et al., 1998).

Our aim was to identify and quantify factors associated with the serostatus at the cow level in 25 specialised dairy herds in Costa Rica.

3.2. Materials and methods

3.2.1. Study population

Twenty-five dairy farms located in the Poás area were used in this study. The Poás area lies between 1500 and 2500 meters above sea level, the temperature ranges between 12.9°C and 28°C and the relative humidity oscillates between 77% and 93%. There are two clearly defined seasons (dry and rainy), with average precipitations of 500mm and 3000mm, respectively. The farms involved in this study were selected intentionally, because since

1986 they participated in a research project about dairy production and herd health, and all of them used VAMPP 5.1 (Veterinary Automated Management and Production control Programme, [Noordhuizen and Buurman, 1984]) as their management-information system. These farms represent 43% (25/58) of all dairy farms in the Poás area.

The most-frequent breeds in these herds are Holstein-Friesian, Jersey and crosses between them. The number of females (cows and calves) per farm varies from 41 to 296.

3.2.2. Study design and data collection

Our cross-sectional study had two phases. In the first phase, blood samples were taken on 25 farms to determine the presence or absence of the disease at herd level. The formula of Cameron and Baldock in the FreeCalc 1.02 program (Cameron and Baldock, 1998) was used to calculate the sample size for each farm. The parameter values used in the hypergeometric exact probability formula were: ∀ and ∃ both 0.05; sensitivity and specificity of the test: 88% and 97% respectively. The sample size per herd varied from 22 to 29. Samples were taken proportionally to the size of the age group. The 4 age categories were 6-12 months, 13-35 months, 36-72 months and ≥ 72 months. Calves < 6 months old were not included due to the likelihood of false-positive results as a consequence of maternal antibodies (Davison et al.,1999b; Tizard, 2000). A simple random sampling, using the random number generator of the Excel computer program (Microsoft® Corp., 2000), was done for the selection of animals within each age category . All farms were bled between 15^{th} of July and 16^{th} of August 2000. Sera were stored at -20°C until being tested.

Sera were tested for antibodies to *Neospora caninum* with an ELISA, based on the water-soluble fraction of sonicated tachyzoites (Paré et al., 1995). This ELISA was standardised in our laboratory using the IFAT as gold standard (Conrad et al., 1993). Serum samples from 220 cows from herds with history of *Neospora*-associated abortion, and 10 serum samples provided by the California Veterinary Diagnostic Laboratory System, School of Veterinary Medicine, University of California-Davis (5 positive and 5 negative controls) were used for the standardisation. IFAT titers for the positive controls were > 1:320; the negative controls had IFAT titers ≤ 1:80. Sensitivity, specificity and predictive values were calculated using a receiver-operating characteristic curve (Hanley et al., 1982) with the WinEpiscope 2.0 program (Thrusfield et al., 2001; in press). The cut-off value was chosen so that the sum of the sensitivity and specificity was maximal (i.e. so that the likelihood ratio was maximal). Then, sensitivity and specificity were 87.8% (CI 95%: 81.3, 94.3) and 96.4% (CI 95%: 93.3,99.5) respectively.

For the second phase of this study, all females in farms defined as seropositive were sampled. A farm was defined as seropositive if two or more reactors for ELISA were found (Cameron and Baldock, 1998). Of the 25 farms, five were withdrawn because the farmers did not want to take part in this phase of the study. Sera were stored at -20°C until being tested. Thirty-four samples, from different farms were lost during the transportation, centrifugation or testing. The same indirect ELISA used in the first phase also was used in this phase to determine the serostatus for each animal.

As part of the second phase, a questionnaire with 23 closed questions was administered to the managers of the farms to determine some environmental and management aspects of the farms and the presence of wild canids -coyotes or wolves- and feral dogs (in or around the farm) that might be risk factors for *Neospora* infection. Control policies to keep away those kinds of animals were also included. This questionnaire was designed taking factors mentioned in literature into account. It was administered once by the first author, by direct interview (in person) to the managers. The time to complete this questionnaire was around 1 hour.

3.2.3. Data analysis

VAMPP 5.1 was used as source of some basic data (animals by farm, identification of the cow, age, breed, date of birth, days of gestation at the sampling, productive and reproductive parameters in the last gestation, and the ecological zone in which the farm is situated). Data were extracted from VAMPP, and merged with a file containing the ELISA results. Descriptive statistics about the farm characteristics and overall prevalence at herd and cow level were performed using SAS/STAT ver. 6.12. To assess the relationships of the serostatus at the individual cow-level with characteristics of the cows and environmental factors, in which the cows are living, logistic regression with herd as random effect was used. The logistic procedure consisted of 2 steps: 1) univariable analysis 2) multivariable analysis. In the second step, all variables with P <0.25 in the univariable analysis were included. A backward building model was followed based on the likelihood ratio test (Hosmer and Lemeshow, 1989). The process of exclusion-inclusion of each variable into the multivariable model tested confounding and interaction by comparison of the estimated coefficients in the new model with the estimated coefficients and likelihood ratio of the old model. Confounding was deemed present if at least one coefficient changed more than 0.1 (if the coefficient had a value between –0.4 and 0.4) or if at least one coefficient changed more than 25% (if the coefficient had a value < –0.4 or > 0.4). Finally, variables that were excluded in the univariable step were checked on collinearity with the variables in the final model to check for potential confounding by calculation of simple correlations. Analyses were carried out in STATA® release 6.0.

3.3. Results

3.3.1. Seroprevalence at herd level

The first phase was meant to determine the serostatus of the herds. It showed that all herds had positive animals. In this first sampling the percentage of seropositive females to neosporosis varied from 20% to 62%. Therefore, all herds were eligible for the second phase.

3.3.2. Seroprevalence within-herds

In the second phase, the overall prevalence was 39.7% (1191/3002). Minimum and maximum values of prevalence within herds varied between 25.0% and 70.5% respectively (Figure 1). The median farm-specific was 35.0%.

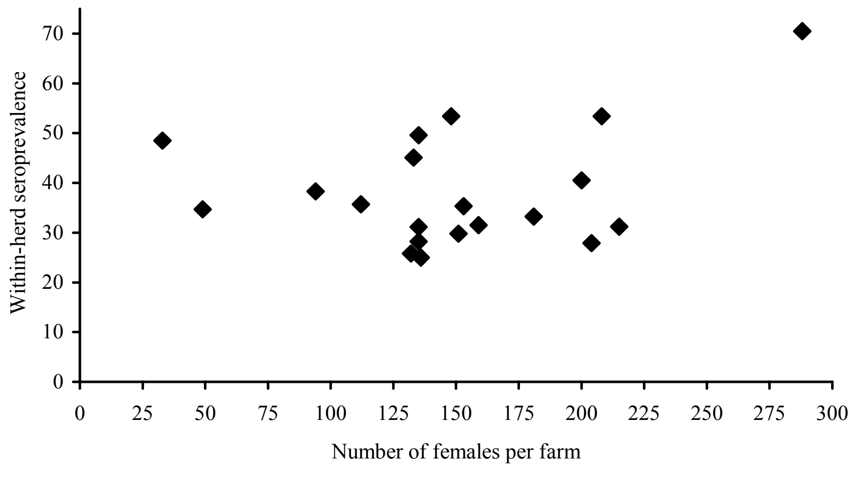

Figure 1. Within-herd seroprevalence for neosporosis (Y-axis) and total number females per farm (X-axis) in the 20 dairy herds included in the second phase of the study.

3.3.3. Descriptive statistics for animal characteristics and environmental factors

All farms included in the study had > 6% of cumulative foetal losses in the last 5 years. Age of animals ranged from 1 week to 12 years old (average 3 years). Holstein-Friesian was the most frequent breed (Table 1). The 20 farms were situated in three ecological zones (Holdridge, 1967): tropical premontane moist forest, tropical premontane wet and tropical lower montane rain forest with 53% of cows in the first one and 24% and 23% in the second and third, respectively. Of the 23 environmental and management factors included in the questionnaire 11 factors had the same answer (yes or no) for all herds (Table 2).

Table 1
Multivariable analysis for factors associated with animal-level seropositivity to bovine neosporosis in 20 specialised Costa Rican dairy farms.

Factor	Level	Nº of cows n =3002	positive (%)	OR	CI 95%	P
Breed	Holstein (ref)	2330	39.7	1.0		-
	Jersey	630	38.6	1.9	1.2 , 3.2	0.01
	Crossbreed (H4*J4)	42	53.7	1.3	0.6 , 2.9	0.57
Age (years)	< 3 (ref)	1833	37.6	1.0		-
	3-6	867	43.6	1.7	1.2 , 2.3	0.01
	7	302	42.3	1.5	1.0 , 2.4	0.06
Parity of the cow's dam at the cows birth	2 (ref)	2336	41.1	1.0		-
	3-5	302	37.0	0.8	0.5 , 1.2	0.32
	6	364	33.5	0.6	0.4 , 0.9	0.02
Serological sampling of aborting cows	Yes (ref)	1336	34.9	1.0		-
	No	1666	43.5	1.9	1.2 , 3.0	0.01

The proportion of the total variance contributed by the panel-level variance component (ρ) was 0.15, where $\rho = \frac{\sigma_v^2}{\sigma_v^2 + 1}$. Log of standard deviation = -1.77, standard deviation (σ_v) = 0.42.

Table 2
Questionnaire (with frequency of positive answers) administered to the managers of farms in the Poás area (n= 25) by direct interview, about environmental and management aspects mentioned in the literature as factors associated to *Neospora* infection.

Topics	% Yes
About the farm	
1. Is there a forest on or around the farm?	100.0
2. Does the farm border on other dairy farms?	100.0
3. Which is the origin of the drinking water?	
Public service	25.0
Natural Springs	75.0
4. Are there natural springs that originated in a neighbour farm?	80.0
5. Is the residual water that was used to clean the stables spread on the paddocks?	100.0
About wild, feral and domestic canids	
6. Are there wild or feral canids into or around the farm?	100.0
7. Have you ever seen those canids in the paddocks?	100.0
8. Do you make some practices to keep these animals away?	0.0
9. How many domestic dogs are there on the farm?	
10. Do they have free access to paddocks, wells and natural spring?	75.0
About the perception and management of abortions	
11. Do you have abortions in outbreaks (Yes) or endemically (No)?	0.0
12. Did the veterinary report mummies in the last 2 years?	75.0
13. Are the aborted foetuses properly buried?	65.0
14. Some of them can not be recovered?	100.0
15. Does the cows give birth in a paddock with other cows?	95.0
About cows with abortions	
16. Are they (serologically or pathologically) tested for the most common agents of abortion?	45.0
17. Are they culled if there is a positive result?	0.0
18. Are they culled only because of the abortion?	0.0
About calves and new-borns	
19. Did the cows have stillborn calves, feeble calves, or calves with any symptom of nervous disease in the last 2 years?	55.0
20. Do they receive the colostrum of their specific dam?	90.0
About the preventive control of infectious reproductive diseases	
21. Do you vaccinate against brucellosis?	100.0
22. Do you vaccinate against IBR and BVD?	60.0
23. Do you vaccinate against leptospirosis?	25.0

3.3.4. Association between serostatus and potential risk factors

Factors with *P* values < 0.25 in the univariable were: breed, age, parity of the dam at the cow's birth, origin of the drinking water, free access of domestic dogs to the paddocks, handling of aborted foetuses, place in which the cows give birth and vaccination against other diseases that can cause abortion. Breed, age, the parity of the dam at the cow's birth and purposive sampling towards neosporosis remained in the analysis after variable reduction. No variables that were deleted in the univariate step showed a correlation higher than 0.6. Management and environmental factors did not show a significant association with seropositivity.

There was only one herd-level factor associated with seropositivity to neosporosis. The cattle belonging to farms in which the aborted cows are not routinely tested (serology, pathology, histopathology) to discriminate the possible cause of abortion had 1.9-times increased odds (CI 95%: 1.26-3.03) to be seropositive than cows belonging to farms that do test their aborting cows. The inclusion of the herd effect showed that 15% of all variation was due to herd-related factors that were not in the questionnaire and therefore could not be analysed.

3.4. Discussion

Based on the results of the first phase of this study, *Neospora caninum* infection might be widespread in dairy herds of the Poás area and likely in other areas from Costa Rica, because all herds were found to be seropositive.

The herd level seroprevalence in this study (100%) is higher than the ones reported in literature by Ould-Amrouche et al. (1999) (64%), Quintanilla-Gonzalo et al. (1999) (83%) and Jensen et al. (1999) (74%). Also, the within-herd seroprevalence varied between 25% and 70% with an overall seroprevalence of 39.7%. We are aware that our study is not conclusive for seroprevalence at herd level because the farms involved were not a product of random sampling, but it is clear that there is a reasonable probability of a wide range of within-herd seroprevalences.

It was impossible for us to assess the association between the presence of dogs in the farms and the seropositivity, because in all farms there was at least one dog. Besides that, dogs wandered around to neighbouring farms frequently. Therefore we used the number of dogs to define risk in both ways: continuous and categorical variable. There was not a factual association, probably due to the number of dogs reported by the manager is not equal to the real exposure of the herd to contact with dogs as there also contacts with wandering and feral dogs. Nevertheless, it is necessary to carry out a study that estimates the real abundance of dogs (domestic and feral) as was done by Barling et al*.*, (2000).

In the Poás area the topography is very irregular with slopes as intense as 40°. Also, the high and constant precipitation (around 3500 mm annual mean) promotes a lot of natural springs that originate in the farms and run down into the paddocks. This water is often used as drinking water for cattle by means of tubing or directly from the crossing of the natural springs between the paddocks. It is possible that dogs (domestic and feral) and cattle drink from the same water source; therefore, cows have the opportunity of drinking water contaminated with *Neospora* oocysts released by dogs. Likewise, the high levels of rainfall plus the hillside terrain could increase the probability of contamination of the water sources by oocysts. The possibility of water contaminated with *Neospora* oocysts being a route of horizontal transmission, is mentioned (but not documented) in the literature (Dubey, 1999; Ould-Amrouche et al., 1999).

Jersey cattle had increased odds of being seropositive compared with Holstein-Friesian. There are no references in the literature that support this finding. In most of the papers dealing with risk factors to neosporosis, breed was not studied or the breeds involved did not correspond with the ones studied here.

Cows older than 3 years had increased odds of seropositivity. Davison et al. (1999b) and Wouda et al. (1999a) found significant variation of seroprevalences between herds, but not between groups of cattle of different ages. On the other hand, in a Danish study, the proportion of seropositive cows increased gradually with the gestation number (Jensen et al., 1999). This finding might be explained in the increased probability of postnatal infection with age as suggested by Jensen et al. (1999), Wouda et al. (1999a) and Dijkstra et al. (2001).

Animals born to dams with 6 or more parturitions had a decreased probability to be seropositive (OR=0.6). This effect could be explained because of an increased humoral and especially cellular immunity in chronically infected cows (Anderson et al., 2000; Hemphill et al., 2000) which attenuates the possibility of recrudescence of the infection and its subsequent transmission to the foetus. Also, selective culling might lead to a reduced number of seropositive dams and so the probability of calves being seropositive is lower.

Of the large number of herd level factors (see Table 2) analysed in this study, only one of them remained in the final logistic model. Those cows belonging to farms without policies of regular serological testing toward diseases (such as infectious bovine rhinotracheitis, bovine virus diarrhoea, brucellosis, leptospirosis and neosporosis) had increased odds of seropositivity. The explanation might be that the serological testing is used as a criterion of selective culling, so the number of seropositive cows is decreasing in these farms. Besides that, a non-testing policy might lead to an important number of cows infected with infectious reproductive diseases coming into the herds. Some authors have suggested an association between neosporosis and BVD (Bjorkman et al*.*, 2000). BVD can immunosuppress (Howard, 1990; Tizard, 2000). So, it is possible that non-vaccinated immunosuppressed cows acquire the infection, or have a recrudescence of a chronic infection (resulting in seropositivity).

The strengths of the associations were rather low. The most important factors related with the seropositivity to *N. caninum* were intrinsic characteristics of the cows, and the only management factor with a significant association with the serostatus was the absence of serologic testing in aborted cows/foetuses.

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Every really new idea looks crazy at first.

Alfred North Whitehead

Chapter 4

The effect of the dam-calf relationship on serostatus to *Neospora caninum* on 20 Costa Rican dairy farms

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Abstract

An epidemiological study was conduced on 20 dairy herds previously diagnosed as seropositive to *Neospora caninum*. The number of females per farm varies from 41 to 296. All females present in the farms were bled once in the period of July and August 2000. A total of 3002 females were bled. An indirect ELISA was used to determine the serostatus of the animals. The analysis of the data was performed in four steps: 1) descriptive statistics about the serological status and general characteristics of the cattle; 2) calculation of vertical and horizontal transmission; 3) an univariate analysis and, 4) a multivariate logistic regression analysis with herd as random effect. The within-herd seroprevalence varied between 25.0% (34/136) and 70.5% (203/288). Seven hundred and forty-seven dam-daughter pairs were available, involving daughters of any age. Daughters in the specific age-class of 2 and 3 years old had a higher seroprevalence ($p < 0.01$) compared with younger and older age-classes. The risk of being seropositive when being born to a mother that is tested seropositive (prevalence ratio) was 2.8 fold increased which coincides with a 5.3 fold increased odds. The probability of horizontal infection amounts to 0.22. The probability of a seropositive offspring due to vertical transmission was 0.64 (AF_{exp}). The multivariate logistic regression showed a significant 6.0-fold increased odds for being seropositive when born from a seropositive mother. Also the within-herd seroprevalence level was significantly associated to the serostatus of the daughters. In the specific conditions of the dairy herds involved in this study, the serostatus of the cows should be not used as predictor of the serostatus of daughters due to the increased probability of horizontal transmission.

Keywords: Neospora caninum; Vertical and Horizontal Transmission; Serostatus; Dairy cattle; Epidemiology.

4.1. Introduction

Neosporosis is a recently recognised parasitic (protozoan) disease caused by *Neospora caninum*. This disease affects a wide range of animals and has effects on cattle reproduction. It causes foetal losses by resorption, mummification or abortion between the 4th and the 9th month of pregnancy (Dubey, 1999). Since the first report of *Neospora*-associated abortion in cattle (Thilsted and Dubey, 1989), many studies have reported

Neospora caninum as a causative agent of abortion all around the world (Anderson *et al*., 1995, Otter *et al*., 1995; Boulton *et al*., 1995, Dubey *et al*., 1997, Thurmond *et al*., 1997, Venturini *et al*., 1999, Wouda *et al*., 1999a,b, Jenkins *et al*., 2000). In Costa Rica neosporosis has been diagnosed and associated with bovine abortion since 1996 (Pérez *et al*., 1998; Romero *et al*., 2000).

The life cycle of *Neospora* was elucidated in 1998, when the dog was recognised as the definitive host (McAllister *et al*., 1998). Therefore, dogs may play an important role in the introduction and maintenance of the infection in herds (Dubey, 1999). Two means of transmission among cattle are assumed: vertical and horizontal. The vertical mode (transplacentally from the mother to her calf) is the most prevalent means of transmission (Paré *et al*., 1996, Thurmond *et al*., 1997, Schares *et al*., 1998, Venturini *et al*., 1999, Davison *et al*., 1999a). Horizontal transmission has not been documented until recently. Some authors report findings that suggest that horizontal transmission is possible by means of ingestion of feed or water contaminated with *Neospora* oocysts (McAllister *et al*., 1998, De Marez *et al*., 1999, Wouda *et al*., 1999b; Dubey, 1999, Anderson *et al*., 2000; Bergeron *et al*., 2000). Another suggested means of horizontal transmission in newborn calves is by ingestion of colostrum contaminated with tachyzoites, especially in the first hours of life (Uggla *et al*., 1998). Ingestion of infected amniotic fluid or infected placenta have been proposed in other studies as potential routes of infection (Ho *et al*., 1997, Piergili-Fioretti *et al*., 2000, Bergeron *et al*., 2001); however, these suggested means of infection in cows remain to be proven, despite evidence for this in dogs (Dijkstra *et al*., 2002).

It is widely accepted that abortion due to neosporosis is the result of a lethal infection of the foetus following a maternal parasitaemia. However, an intra-uterine infection with *N. caninum* will not always induce foetal loss. Factors such as the timing of the parasitaemia during gestation, the quantity and duration of the parasitaemia, the effectiveness of the maternal immune response and the ability of the foetus to mount an immune response have been recognised as crucial factors that determine the fate of the foetus (Hemphill *et al*., 2000). The acquisition of infection in the third trimester of gestation allows the foetus to develop a sufficient grade of immunocompetency to limit the parasite multiplication, resulting in a calf that will be born clinically healthy (Williams *et al*., 2000).

Several studies in which *N. caninum* antibodies were measured before colostrum intake by calves report that between 81% and 95% of seropositive cows gave birth to seropositive calves (Paré *et al*., 1996, Wouda *et al*., 1998, Davison *et al*., 1999a). On the other hand, in a cross-sectional study there was not a clear relationship between the serologic status of dam and offspring, resulting in proportions of seropositive daughters born from seropositive dams ranging from 0% to 85% (Bergeron *et al*., 2000).

Other cross sectional studies focused on the proportions of seropositive daughters born from seronegative dams in an effort to document postnatal acquisition of *Neospora*. Thus, Dyer *et al*. (2000) using IFAT to detect antibodies for *Neospora* reported that 77.9% (120/154) of seronegative dams had seronegative daughters, while the rest of seronegative dams (22.1%) had seropositive daughters. In the same way, Dijkstra *et al*. (2001), using an ELISA based on a detergent lysate of whole sonicated tachyzoite, reported that 83.5% (207/248) of seronegative dams had seronegative daughters and 16.5% (41/248) had seropositive daughters.

The objective of our study was to assess the mother-calf relationship with regard to serological status to *Neospora* in 20 Costa Rican dairy herds. We investigated whether the serological status of the dam could be used as predictor for the serological status of the calf. If so, this prediction might be used as potential criterion for selective culling.

4.2. Materials and methods

4.2.1. Study population

In this study, 20 dairy farms previously diagnosed as seropositive to *Neospora* were used. The farms were located between 1500 and 2200 meters above sea level, the temperature range were between 12.9°C and 28°C and the relative humidity oscillated between 77% and 93%. There were two clearly defined seasons, which were dry and rainy. The total rain precipitation was approximately 500mm in the dry season and 3500mm in the rainy season. The farms involved in this study were selected intentionally. Since 1986 they participated in a research project focused on dairy production and herd health, and all of them use VAMPP 5.1 (Veterinary Automated Management and Production control Programme, [Noordhuizen and Buurman, 1984]) as their management information system. The most frequent breeds in these herds were Holstein-Friesian, Jersey and crosses between them. The number of females (cows and calves) per farm varied from 41 to 296.

4.2.2. Data collection

All females present in the selected farms were bled once in the period of July/August 2000. Sera were stored at -20°C until assay. An indirect ELISA (Paré *et al*., 1995) with

87.8% of sensitivity (CI 95%: 81.3-94.3) and 96.4% of specificity (CI 95%: 93.3-99.5) for detection of antibodies against *N. caninum* was used to determine the serologic status of the animals (Romero *et al*., 2002).

VAMPP 5.1 was used as the source of basic data (animals per farm, identification of the cow, age, breed, date of birth, days of gestation at the moment of sampling and genealogy of the cows). Dam-daughter pairs were extracted from VAMPP and merged with a file containing the ELISA results.

4.2.3. Data analysis

Descriptive statistics about the general characteristics of the cattle, regarding the serological status, especially for the dam-daughter pairs, were performed using STATA® (StataCorp, 1999).

4.2.3.1. Calculation of vertical and horizontal transmission

The overall probability of horizontal transmission, calculated as the proportion of seropositive daughters born from seronegative dams with its exact binomial 95% confidence interval (95% CI) was assessed using EpiInfo 6.0 (Center for Disease Control and Prevention, USA).

The probability of vertical transmission was calculated in several steps. In order to carry out this calculation we used a contingency (2x2) table in which the seropositivity of the dams was defined as exposure (Table 1).

Table 1.
Contingency (2x2) table to explain the way of calculation of vertical and horizontal transmission of the disease as was performed in this study.

Status of daughters	Status of dam	
	Seropositive	*Seronegative*
Seropositive	a	b
Seronegative	c	d
	a+c	b+d

As it is a cross-sectional study, with plausible evidence of horizontal transmission, we assumed that a proportion of seropositive dams ***(a+c)*** were horizontally infected after the date the calf was born. Therefore the "crude" frequencies were adjusted in the following way. First, the proportion of daughters that was horizontally infected (***b/b+d***) was calculated. Next, the frequencies of ***a, c*** and (***a+c***) were reduced according to this proportion while these subtracted numbers were added to ***b, d*** and ***(b+d)*** respectively. For both the crude and adjusted frequencies the probabilities of vertical transmission using the attributable fraction among exposed (AF_{exp}) were calculated. AF_{exp} represents the proportion of daughters that were seropositive due to being born from seropositive mothers. Confidence intervals for these proportions were calculated with EpiInfo 6.0 (Center for Disease Control and Prevention, USA).

4.2.3.2. Univariate analyses

Age-specific seroprevalences of daughters and mothers were calculated using age categories of one year each, except for calves under 1-year old which were divided in two categories: under 150 days old and between 151 and 365 days old. Cows of more than 7-years old were grouped in the same age-class. Differences between proportions of seropositives by age-class were tested by means of the χ^2 statistic using EpiInfo 6.0 (Center for Disease Control and Prevention, USA).

In order to eliminate the effect of possible false positives by maternal antibodies in daughters (Davison *et al*., 1999b; Tizard, 2000), the age of daughters was categorised in two levels, 150 days or less, and older than 150 days. A new data set keeping pairs with daughters older than 150 days was constructed. Furthermore, to assess the effect of the within-herd seroprevalence on the means of transmission of the disease, herds were divided in 3 categories. Herds with seroprevalence between 25% and 35% were classified as 'low', herds with seroprevalence between 35.1% and 45% were classified as 'medium' and herds with seroprevalence higher than 45.1% were classified as 'high'. A contingency table for pairs in each subset was constructed to calculate the probability of horizontal transmission. The prevalence ratio (PR) as measure of association and the AF_{exp} as measure of effect of the exposure to seropositive dams were calculated using the epidemiological software WinEpiscope 2.0 (Thrusfield *et al*., 2001).

4.2.3.3. Multivariate analysis

Finally, a multivariate logistic regression model with herd as random effect was used to assess the relationship between the serostatus of the daughter and her age, the serostatus of the mother and the within herd seroprevalence (STATA® release 6.0, StataCorp, 1999). The multivariate analysis can only be carried out on unadjusted frequencies, because we do not know which mothers were horizontally infected after the calf was born.

4.3. Results

4.3.1. Seroprevalence

A total of 3002 females were bled on the 20 farms studied. The overall seroprevalence for Neospora was 39.7% (1191/3002), whereas the within-herd seroprevalence varied between 25.0% (34/136) and 70.5% (203/288). Taking the sensitivity and specificity of the test into account, the overall prevalence is 42.8%.

Seven hundred and forty-seven dam-daughter pairs were available for analysis. In the group of daughters, 614 (82.2%) were less than 2 years old, whereas 711 (95.2%) were younger than 3 years old. In the group of dams, 684 (91.5%) were more than 3 years old. Apparent seroprevalences in daughters and dams were 33.3% (249/747) and 38.2% (285/747) respectively. For the age categories of dams the seroprevalence varied between 33.8% and 44.3%, with no statistical differences between age categories (Fig. 1). Daughters in the specific age-class of 2 and 3-years old had a higher seroprevalence ($p < 0.01$) compared with the other age-classes, but not among them. Furthermore, there were no differences in seroprevalence between the other age-classes (Figure 1). In all age-classes of daughters there were seropositive and seronegative daughters that were born from seropositive or seronegative dams. Furthermore, the percentage of seropositive dams (around 38.0%) within the age-categories of daughters was not significantly different ($p > 0.5$) (Fig. 2).

4.3.2. Association between serostatus of daughters and dams.

Results of the 747 pairs are shown in Table 2. The risk of being seropositive is 2.8 fold increased when being born from a mother that is tested seropositive (prevalence ratio) which coincides with a 5.3 fold increased odds; both measures are based on the adjusted numbers.

The probability of horizontal transmission is to 0.22 (110/512). Of seropositive daughters born from seropositive mothers, 63.7% is due to the fact that their mothers were seropositive (AF_{exp}), which is the probability of seropositive offspring due to vertical transmission.

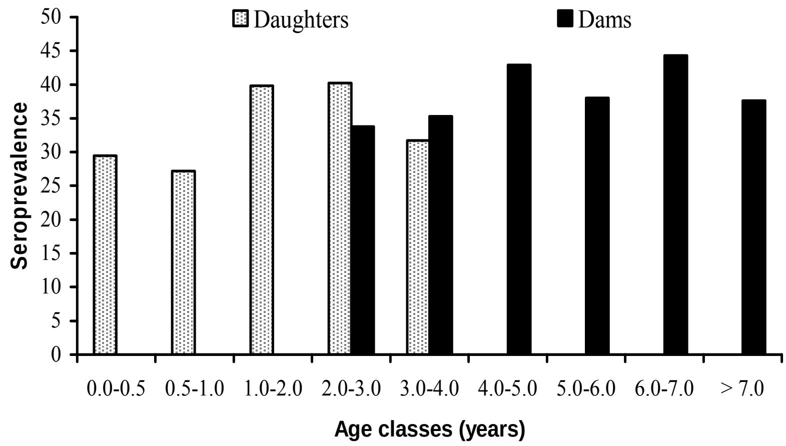

Figure 1. Seroprevalence in dams and daughters by age groups.

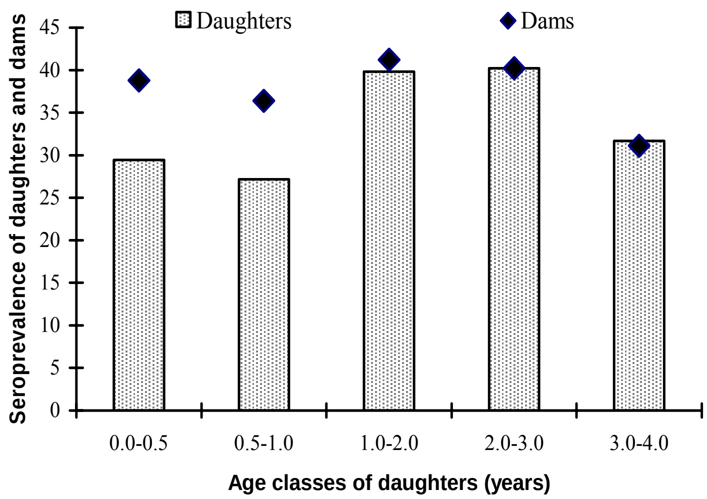

Figure 2. Seroprevalence for daughters and their dams, by age-groups of daughters, at the date of sampling. Seropositive daughters born from seropositive or negative dams and negative daughters born from seropositive or negative dams were present in each age-group.

From the general data set containing 747 pairs, a subset with 542 pairs was selected excluding daughters younger than 150 days old at the time of bleeding. In this data set, 93.2% of daughters were younger than 3-years old, while 92.3% of dams were more than 3-years old.

The seroprevalences among daughters and dams in this subset were 34.9% (189/542) and 37.8% (205/542), respectively. An increased risk of being seropositive was observed in daughters that were born from seropositive dams (PR=2.6, 95% CI: 2.1-3.3). The proportion of daughters horizontally infected was 0.23 (95% CI: 0.19-0.28). In this subset the probability of vertical transmission of the disease was 0.62 (95% CI: 0.60-0.63) (Table 2).

In herds with high, medium and low within-herd seroprevalence, the probability of horizontal transmission increases as the within-herd seroprevalence increases while the probability of vertical transmission decreases as the within herd seroprevalence increases. (Tables 2 and 3).

The multivariate logistic regression with herd as random effect showed a significant 6.0-fold increased odds for being seropositive when born from a seropositive mother. Also the within-herd seroprevalence level was significantly associated to the sero-status of the daughters (Table 3). The inclusion of the herd effect showed that 18% of the variation is due to herd related factors that were not included in the analysis.

4.4. Discussion

4.4.1. General

In this study, we were able to estimate both vertical and horizontal transmission of *Neospora caninum,* because of the presence of seropositive daughters born from seropositive dams, as well as seropositive daughters born from seronegative dams. We are aware that the results of this study can be partially biased because the study design used is not an optimal one for making accurate and high quality estimates about the impact of vertical and horizontal transmission. For example, a dam that is tested seropositive at the moment of bleeding might have been negative at the moment the calf was born. Indeed, she might have acquired the infection after parturition in a horizontal way. In a cross-sectional study this bias will lead to an underestimation of the horizontal transmission and subsequently in an overestimation of the vertical transmission. We adjusted for this at least partly by assuming that the level of horizontal transmission was equal in dams and daughters. A cohort study, as has been carried out by Davison *et al.* (1999), in which both dam and daughter are bled at the moment of parturition would give better information about the sero-status at the moment of birth, and when seroconversion occurred.

Table 3.
Multivariate analysis of the relation between seropositivity to *N. caninum* for daughters with serostatus of the dam at sampling, age of the daughter and the within-herd seroprevalence.

Factor	Level	Frequency %	Prevalence (daughters)	ß (95% CI)	OR (95% CI)	P
Serostatus of the dam at sampling	Seronegative	61.8	0.18	-	1.0	-
	Seropositive	38.2	0.59	1.79 (1.62 , 1.97)	6.0 (5.1 , 7.2)	0.000
Age of the daughter at sampling	> 150 days	72.6	0.35	-	1.0	-
	≤ 150 days	27.4	0.28	-0.72 (-1.34 , -0.10)	0.5 (0.3 , 0.9)	0.023
Within-herd seroprevalence	High	15.7	0.62	-	1.0	-
	Medium	28.4	0.40	0.49 (0.09 , 0.89)	1.6 (1.1 , 2.4)	0.021
	Low	55.9	0.25	1.21 (0.75 , 1.52)	3.4 (2.1 , 4.6)	0.000

The proportion of the total variance contributed by the herd-level variance component (ρ, rho) was 17.8, where $p = \sigma_v^2 / (\sigma_v^2 + 1)$ log of standard deviation = -1.52, standard deviation (σ_υ) =0.46.

Other sources of misclassification can be: variation in antibody titers throughout pregnancy (Conrad *et al*., 1993; Hemphill *et al*., 2000; Stenlund *et al*., 1999; Maley *et al*., 2001), a transient rise of antibody titres in daughters without a true *Neospora* infection (Hietala and Thurmond, 1999) and the limitation of an imperfect diagnostic test with a relatively low sensitivity (0.88) compared to the specificity (0.97). However, it is very unlikely that those potential sources of bias affect the results to a large extent (e.g. true prevalence in both daughters and dams hardly change after correction for imperfect SE and SP).

In addition to fluctuations in antibody titers that could cause false negative results, it might be possible that chronically infected cattle show weaning antibody titres over time (Hemphill *et al*., 2000; Maley *et al*., 2001). Also, there is still a lack of information about the behaviour of the infection and the course of antibody titres over time in naturally infected cattle.

4.4.2. Vertical and horizontal transmission

Under the assumption that horizontal transmission in dams and daughters is equal, we adjusted for the horizontal infection in dams. Then, the proportion of animals infected vertically reduces from 0.70 to 0.64, while the proportion of horizontal infection increases from 0.18 to 0.22 (Table 2, all pairs). When excluding animals that might still have colostral antibodies, the proportion of vertical transmission reduces from 0.64 (all pairs) to 0.62 (542 pairs). As both proportions are quite similar it can be concluded that presence of colostral antibodies has little influence on the outcome of the proportion of animals vertically or horizontally infected. Hietala and Thurmond (1999) determined that colostral anti-*Neospora* antibodies decayed at 128 days from parturition. In our study, seropositive daughters fewer than 150 days could be positive due to intra-uterine infection and/or horizontal infection and/or colostral antibodies. It is possible that in those daughters under 150 day of age, antibodies titres were not increased much by colostral uptake, and the presence of antibodies was mainly due to intra-uterine infection and/or postnatal acquired infection, as in the group older than 150 days. The probabilities of vertical infection that were found in this study were lower than in other studies that used blood samples before ingestion of colostrum. Those studies reported probabilities of vertical infection over 0.80 (Paré *et al*., 1996; Shares *et al*., 1998; Davison *et al*., 1999a; Hietala and Thurmond, 1999). However in two other cross sectional studies, carried out in Spain (Mainar-Jaime *et al*., 1999) and Canada (Bergeron *et al*., 2000), the rates of vertical transmission, calculated as AF_{exp}, were lower than 60.0%. The authors of those studies conclude that the low vertical transmission rate could be due to differences related to environmental conditions resulting in an increased efficiency of horizontal transmission. Secondly, the low probability of vertical transmission found in our study may be due to a continuous stimulation of the maternal immune system in conditions of endemicity. Under these conditions, dams might be frequently exposed to environmental sources of the parasite, leading to a continuous stimulation of the immune system sufficient to prevent the exposure of the foetus to an infective dose of tachyzoites (Paré *et al*., 1996). However, there is no conclusive evidence about the role of the maternal immune system to prevent the vertical transmission of neosporosis. Thirdly, antibody concentrations may decline to a level below the detection limit when the infection occurred a long time ago. This might explain some cases in which mothers were negative while daughters were positive. This fluctuation in antibody titres for *Neospora* was demonstrated by Maley *et al*. (2001) in experimentally infected cattle. We do not have firm evidence that indicates a different behaviour in naturally infected cows.

In our study we observed an increased probability of horizontal transmission as the within-herd seroprevalence increased, whereas the probability of vertical infection decreased at the same time. This seroprevalence-related probability of horizontal transmission might be explained by an increased exposure to environmental sources of infection.These could include infected placentas or amniotic fluid (Ho *et al*., 1996; Ho *et al*., 1997; Bergeron *et al*., 2001) or food and water contaminated with *Neospora* oocysts from naturally infected dogs (Dubey, 1999).

4.4.3. Age-specific results

A higher seroprevalence for daughters in the age-class between 1 and 3 years old was observed (Fig. 1). This was also observed by Dijkstra *et al*. (2001). The differences in seroprevalence by age-classes in daughters might be due to a point source of infection as suggested by others (McAllister *et al*., 1996; McAllister *et al*., 2000; Dijkstra *et al*., 2001). Furthermore, there might have been unrecognised host and/or environmental factors, just previously to the reproductive age, which promotes the horizontal acquisition of the infection. This strongly suggests that horizontal transmission is present in the studied dairy herds, but that the level of it may not be constant over time. Other studies reported no difference in seroprevalence by age-classes, suggesting that the horizontal transmission plays a minimal role in the transmission of the disease (Davison *et al*., 1999a,b). However, by means of mathematical models, it was estimated that in presence of imperfect vertical transmission, without horizontal transmission, the prevalence of the disease has to decline over time (French *et al*., 1998; French *et al*., 1999). Our study supports this finding. The seroprevalences for the dams in all age-classes of the daughters were not different but seroprevalences do differ between age categories of daughters (Fig. 2), which might indicate that the serostatus of the daughters was not solely due to vertical infection.

In Costa Rica, veterinary practitioners have used the serostatus of an animal to predict the serostatus of its mother or its off-spring. This attitude was based on studies showing vertical transmission rates of over 80% (Paré *et al*., 1996; Shares *et al*., 1998; Davison *et al*., 1999a; Hietala and Thurmond, 1999). However, under the specific conditions of the dairy herds involved in this study, the serostatus of the mother should be not used as predictor of the serostatus of her daughter(s), because of the much lower probability of vertical transmission.

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Table 2

Probabilities of vertical (AF_{exp}) and horizontal transmission of *N. caninum*. This table includes the overall probabilities using all pairs, the probabilities when daughters younger than 150 days were excluded and the probabilities of the latter dataset stratified to high, medium and low within-herd seroprevalence. Prevalence ratio (PR) is included as measure of association. Numbers and output between *[]* correspond to values adjusted for the proportion of dams infected horizontally.

		Dams		% VT (95% CI)	% HT(95% CI)	Prev. Ratio (95% CI)
	Daughters	**Positive**	**Negative**			
General (747 pairs)	Positive	168 *[139]*	81 *[110]*	0.70 (0.69 , 0.71)	0.18 (0.14 , 0.21)	3.4 (2.7 , 4.2)
	Negative	117 *[96]*	381*[402]*	*[0.64 (0.62 , 0.66)]*	*[0.22 (0.18 , 0.25)]*	*[2.8 (2.3 , 3.3)]*
Older than 150 days (542 pairs)	Positive	122 *[105]*	67 *[84]*	0.67 (0.65 , 0.68)	0.20 (0.16 , 0.25)	3.0 (2.3 , 3.8)
	Negative	83 *[71]*	270 *[282]*	*[0.62 (0.60 , 0.63)]*	*[0.23 (0.19 , 0.28)]*	*[2.6 (2.1 , 3.3)]*
High (85 pairs)	Positive	40 *[37]*	13 *[16]*	0.43 (0.41 , 0.46)	0.42 (0.25 , 0.61)	1.8 (1.1 , 2.8)
	Negative	14 *[13]*	18 *[19]*	*[0.38 (0.36 , 0.41)*	*[0.46 (0.29 , 0.63)]*	*[1.6 (1.1 , 2.4)]*
Medium (154 pairs)	Positive	37 *[35]*	24 *[26]*	0.60 (0.58 , 0.62)	0.25 (0.17 , 0.35)	2.5 (1.7 , 3.7)
	Negative	22 *[21]*	71 *[72]*	*[0.58 (0.56 , 0.60)]*	*[0.27 (0.18 , 0.36)]*	*[2.4 (1.6 , 3.5)]*
Low (303 pairs)	Positive	45 *[43]*	30 *[32]*	0.71 (0.69 , 0.72)	0.14 (0.09 , 0.19)	3.4 (2.3 , 5.1)
	Negative	47 *[45]*	181 *[183]*	*[0.70 (0.68 , 0.71)]*	*[0.15 (0.10 , 0.20)]*	*[3.3 (2.2 , 4.8)]*

In theory, there is no difference between theory and practice. But, in practice, there is.

Jan L.A. van de Snepscheut

Chapter 5

Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions.

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Abstract

A standard field trial was carried out to assess the effect of a commercial *Neospora*-vaccine based on whole killed tachyzoites (Bovilis-Neoguard, Intervet®) on the abortion rate. Eight-hundred and seventy-six cows, over 2.5 months pregnant, belonging to 25 Costa Rican dairy herds, were used in the analysis. For each cow vaccinated, a cow of the same herd, breed and age category, was selected as control. The period of administration of treatments extended from June to November of 2000. The treatments were administered in two, 5 ml doses 1 month apart, the first dose given between day 75 and 90 of gestation. The incidence of abortion among all treated cows was of 16.0% (140/876). The treatment specific incidence was 11.2% (49/438) and 20.8% (91/438) for the vaccinated and the placebo group, respectively. The prevented fraction by vaccination amounted to 0.46 (95% CI: 0.26; 0.61), and the cumulative incidence ratio for the vaccinated group was 0.54. The Cox hazard ratio was 0.51(95% CI: 0.37; 0.72), meaning that the force of abortion is reduced twice in the vaccinated group. The results of this study, the first one following this type of design, shows that the killed whole *Neospora caninum* tachyzoite preparation had a reasonable effect on the abortion rate in Costa Rican dairy cattle.

Keywords: *Neospora caninum;* Vaccine, Efficacy; Field trial; Dairy cattle; Costa Rica.

5.1. Introduction

Neospora caninum is a protozoan parasite (phylum Apicomplexa) discovered in 1984 by Bjerkas *et al*. and is recognised as one of the most important causes of foetal losses in both dairy and beef cattle around the world. It affects several species of mammals including cats, dogs, wild canids, camels, water buffaloes, rodents and cattle Dubey (1999).

Previously, neosporosis was misdiagnosed as toxoplasmosis (*T. gondii*) due to close structural similarities and clinical signs (Dubey *et al*., 1988; Speer *et al*., 1999). Also some other aplicomplexan parasites should be taken into account as differential diagnosis of neosporosis, especially parasites of the *Hammondia* genera (Mugridge *et al*., 1999).

The life cycle of the parasite has been widely studied. The biology of the parasite is better known since the discovery that the dog is the definitive host of *N. caninum* (McAllister *et al*., 1998; Lindsay *et al*., 1999). The vertical way of transmission has been documented very well (Bjorkman *et al*., 1996; Paré *et al*., 1996; Thurmond *et al*., 1997;

Schares *et al*., 1998; Davison *et al*., 1999a,b). Besides, several studies provide epidemiological evidence of the existence of horizontal transmission (McAllister *et al*., 2000; Dijkstra *et al*., 2001).

The economic impact of bovine abortion on dairy industry has encouraged the development of effective vaccines against Bovine Herpesvirus (BHV-1), Bovine Diarrhoea Virus (BVDV), *Brucella abortus* and *Leptospira* sp. In the specific case of *N. caninum*, there is an increasing knowledge about some basic topics that are necessary to develop an effective vaccine and vaccination strategy against it such as: the molecular biology of the parasite, the role of the immune system of the host, the possible ways of acquisition of the disease and the critical periods during pregnancy in which the foetus may acquire congenital infection (Hemphill *et al*., 2000; Andrianarivo *et al*., 1999; Innes *et al*., 2001).

Experimental studies carried out in mice, described the effectiveness of a *N. caninum* crude lysate vaccine (Lidell *et al*., 1999) and of a recombinant vaccinia virus expressing the surface proteins NcSRS2 and NcSAG1. These vaccines rise the level of specific IgG antibodies, complement, interleukin-12 and IFN-γ which conferred protection against vertical transmission to the offspring (Nishikawa *et al*., 2000; Nishikawa *et al*., 2001a,b). Experimental studies in cattle under laboratory and field conditions, have shown the effectiveness of several vaccinia preparations, based on killed tachyzoites with adjuvants, to elicit a response at both cellular and humoral level (Andrianarivo *et al*., 2000; Choromanski and Block, 2000). However, their efficacy to prevent *Neospora*-infection in cattle is not well demonstrated until now. Moreover, the effect of vaccination on the probability of bovine abortion and/or its effect on prevention of infection in susceptible animals have not yet been very well documented.

Costa Rica is a country with an important dairy industry, with a sufficient production to supply the internal necessities and to export it to some countries in Latin America (FAOSTAT, 2000). Recently, it has been described that *N. caninum* is present in cattle of the most important Costa Rican dairy regions (Perez *et al*., 1998; Romero *et al*., 2000). A serological study reported 100% of seropositive herds and within herd seroprevalences ranged between 25% and 70% in the Poás area (Romero *et al*., 2002). Besides, the horizontal transmission rate was estimated to be higher than reported elsewhere (Romero and Frankena, 2003). Furthermore, in a previous case-control study it was assessed that seropositive cows had a 12 times increased probability to abort compared to seronegative cows (Perez *et al*., 1998).

The objective of this study was to assess the effectiveness of a commercially available killed whole *Neospora caninum* tachyzoite preparation on the crude abortion rate under field conditions, in dairy cows from the most important dairy regions in Costa Rica.

5.2. Materials and methods

5.2.1. Study population

Nine hundred and thirty-one pregnant dairy cows from 25 Costa Rican dairy herds were included. The herds were located in five ecological zones (Holdridge, 1967): moist-low montane forest, moist-pre montane forest, very moist-low montane forest, very moist-pre montane forest and rainy-low montane forest, of three dairy areas: Poás, Cartago and San Carlos, were used in this study. The farms involved were selected intentionally because all of them use VAMPP 5.1 (Veterinary Automated Management and Production control Programme (Noordhuizen and Buurman, 1984) and were disposed to collaborate in this study. Besides, these farms have a veterinary practitioner who is responsible for the herd health program. Abortive diseases such as bovine herpesvirus (BHV-1), bovine diarrhoea virus (BVDV) and leptospirosis were previously diagnosed in the farms by serologic testing at least once but not as part of a serological survey (or serological sampling), but not as part of this study. Prevalence or incidence of these diseases for each farm was unknown. The within-herd seroprevalence for brucellosis did not reach 2% in those herds in which it was present, but most of the herds were free of brucellosis (source: Ministry of Agriculture and Livestock). The incidence of abortions ranged from 10 to 33% in the 2 years previous to this study, with a global rate of 19.0% (source: CRIPAS project [Centro Regional de Investigación en Producción Animal Sostenible]) of the Escuela de Medicina Veterinaria of the Universidad Nacional, Costa Rica (Baaijen and Perez, 1996).

5.2.2. Study design

This study was designed as a standard field trial based on a cohort study. The number of cows selected from a herd was proportional to the farm size. All females over 2 months in pregnancy (confirmed by rectal palpation by a veterinary practitioner) were eligible for inclusion. For each cow treated with the active component, a control animal of the same herd, breed and age category was selected. Four age categories were considered: parity 0 (heifers), parity 1, parity 2 and 3, parity 4 or higher. The sample size was calculated using the formula to estimate a difference between percentages in WinEpiscope 2.0 (Thrusfield *et* *al.*, 2001) with an expected percentage of abortion of 8% in vaccinated and 15% in controls, which is the global percentage of abortion as described above. With a confidence level of 95% and a power of 90%, the sample size calculation resulted in 433 animals per group.

Cows with a previous positive diagnosis of any abortive disease such as BHV-1, BVDV, brucellosis, leptospirosis and neosporosis were not included. The serological status towards abortive diseases for the rest of the selected cows was unknown but due to the large number of animals it is expected that cows with an unknown history of potentially abortive infections are evenly distributed over the treatment groups. Selected cows that were culled during the trial before parturition and without abortion were dropped from the database.

Treatments were applied during the herd health routine visits of the veterinarians. These visits had an interval of 2 to 4 weeks between each other. Because of the large number of cows involved in this trial, the period of application of treatments was almost 5 months, beginning at June 2000. A single blind design was used to reduce bias; in this way, farmers and veterinarians were blinded regarding to application of vaccine and placebo. To reach this objective, vaccine and placebo were packed up in identical bottles.

Veterinarians followed all treated cows from the first application of the treatment until birth or foetal loss. There were two ways for detecting foetal loss 1) direct observation of the aborted foetus, 2) cow diagnosed as empty by rectal palpation after it had been found pregnant previously during the biweekly or monthly routine herd health visit of the veterinarian. The cows were routinely checked at the 4th and 7th month of gestation, and/or after the notice of vaginal discharge emerging from the vagina or by repetition of heat. If the veterinarian reports the cow as empty or the cow returns to heat, VAMPP assigns a date of abortion 21 days before the reported day of the vet check or heat. In the case of cows with vaginal discharge, VAMPP interrupts the gestation at the day the farmer made the report when the veterinarian confirms the foetal loss.

5.2.3. Treatments

The vaccine administered was a commercial preparation (NeoGuard, Intervet®), based on inactivated *N. caninum* tachyzoites (3 x 10^6 per ml at harvest), 10% of adjuvant, 5% of stabilisers and 5% of Phosphate Buffered Saline. The placebo consisted of isotonic saline infusion specially prepared for this trial. Thus, the placebo had an appearance like the vaccine and both were packed in identical bottles.

The first treatment (vaccine or placebo) was administered between day 75 and 90 of gestation. A booster was administered 4 weeks later. Vaccines and placebos were stored at 4° Celsius in our laboratory. Veterinarians transported the bottles from our laboratories to the farms in an icebox, keeping the recommended storing temperature. A dose of 5 ml of vaccine was injected subcutaneously (in the neck with a disposable needle 18 x 1.5"), following instructions of the manufacturer.

5.2.4. Data collection

Characteristics of the cows such as age, parity, breed, gestational age at the end of the pregnancy, previous diagnosis for other diseases, etc., were taken from the VAMPP central database. The farmer captures the data about the events at the farm using a notebook. Then, this information is transferred to VAMPP by him/her self, or by technicians that give technical assistance to the farms. The staff of the CRIPAS project periodically collects the VAMPP information.

The outcome variable had a dichotomous nature indicating the type of end of the gestation; 0) parturition of a healthy calf after day 270 of gestation or 1) any event apart from a normal parturition such as: mummification, abortion, neonatal death or stillbirth with neurological disorders. The abortion was detected as described above. Mummification was diagnosed by rectal palpation.

5.2.5. Data analysis

A description of proportions of treatments administered by farm, breed, ecological zone and parity was performed. The prevented fraction (Rothman and Greenland, 1998) was used to assess the efficacy of the vaccine. This measure was calculated with the formula:

$$PF_{vaccine} = 1 - \frac{incidence_{vaccine}}{incidence_{placebo}}$$

Also, the cumulative incidence ratio (CIR) with its 95% confidence interval was calculated using WinEpiscope 2.0 .

A survival analysis was performed to compare the failure curves of both treatment groups and to assess the relation between treatment and survival time. This analysis was performed using a Kaplan-Meier survival graph and a Wilcoxon test using SAS/STAT release 8.0 (SAS Institute Inc, 1990a,b). The survival functions for vaccinated and placebos were also compared using Cox proportional hazard regression while taking the herd effect and the pair effect into account (frailty model) using S-PLUS 2000 release 3 (S-PLUS 2000, MathSoft Inc.).

5.3. Results

The total number of treatments administered was 931 (464 in the vaccine group and 467 in the placebo group. Major breeds were Jersey (616), Holstein (262) and Holstein x Jersey cross breed (53). Regarding to parity, 200 were heifers, 286 were first calving, 233 were of parity 2 or 3 and 212 cows had 4 or more parities. The distributions of cows toward ecological zone were 287 from the moist-low montane forest, 128 from moist-pre montane forest, 253 from very moist-low montane forest, 209 from very moist-pre montane forest, and 54 from rainy-low montane forest. A total of 438 pairs were available for analysis. Exclusion of animals and pairs was due to culling (17 animals), missing pair mate (30 animals) or administrative mistakes (4 pairs). The number of matched pairs per farm varied between 2 and 49 (average = 18, median = 16).

The incidence of abortion among all cows in the trial was 16.0% (140/876). The treatment specific incidence was 11.2% (49/438) in the vaccinated group and 20.8% (91/438) in the placebo group (Table 1). The incidence of abortion for Holstein and Jersey were 15.8% (38/240) and 15.7% (92/587) respectively. The incidence per parity number and ecological zones are shown in Table 2.

Table 1
Contingency (2x2) table for calculation of the prevented fraction and the cumulative incidence ratio for abortion in placebo and vaccine treated groups.

	Exposure		
End of gestation	Placebo	Vaccine	Total
Abortion	91	49	140
No abortion	347	389	736
Total	438	438	876

There were no reports of weak calves born in the farms during the study. Regarding to the safety of the vaccine, there were also no reports of adverse effects at the place of the application of the vaccine, or other side effects like decreased milk yield or changes in the behaviour of the cows.

The prevented fraction by vaccination amounted 0.46 (95% CI: 0.26, 0.61). The cumulative incidence ratio for the vaccinated group compared to the placebo group was 0.54 (95% CI: 0.39, 0.74). The effect of the vaccine was not uniform in all herds, a positive effect was observed in 15 herds, no effect in 4 and a (small) negative effect in 6 herds. There is not a large correlation between the efficacy (prevented fraction) of the vaccine and the global abortion rate within the herd during the previous two years (*corr.* = 0.31, p = 0.14).

Table 2
Overall frequencies of abortion by treatment, breed, parity and ecological zone (n=876).

Variable	Level of variable	% of abortions
Vaccination	Vaccine	11.2 (49/438)
	Placebo	20.9 (91/438)
Breed	Holstein	15.8 (38/240)
	Jersey	15.7 (92/587)
	Cross-breeds	19.6 (10/49)
Parity number	0	14.8 (28/188)
	1	17.4 (46/269)
	2 and 3	16.2 (35/220)
	> 3	15.5 (31/199)
Ecological zone	Moist-low montane forest	18.8 (51/270)
	Moist-pre montane forest.	20.6 (25/121)
	Very moist-low montane forest	15.0 (35/238)
	Very moist-pre montane forest	10.2 (20/196)
	Rainy-low montane forest	18.9 (9/51)

The Kaplan-Meier failure graph for both groups shows that the difference between both groups arises in the period between day 150 and 200 of gestation (Figure 1). After day 200, the number of abortions in both groups is equal (23). The Wilcoxon test resulted in a chi-square value of 16.32 ($p < 0.001$), which indicates that both survival functions are different.

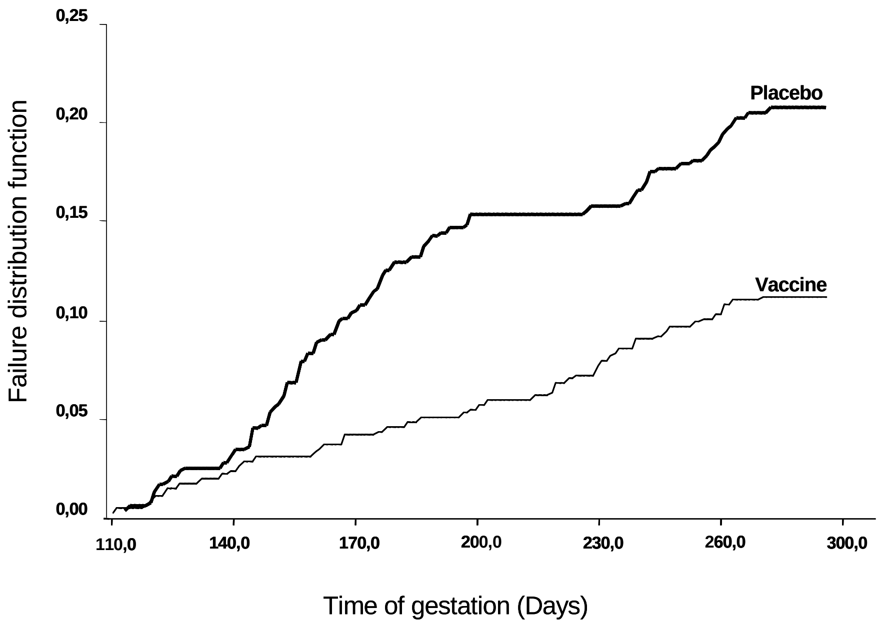

Figure 1. Kaplan-Meier abortion curves in the placebo and vaccine treated groups.

A further analysis of the time until abortion using the Cox proportional hazard regression showed that there was no effect of parity, breed or herd on abortion, which was expected due to the matched design. Also, in a more design-based analysis, the effect of pairs within-herds was insignificant, reducing the statistical model to a model with treatment only. The Cox hazard ratio, was 0.51 (95% CI: 0.36, 0.72), meaning that the force of abortion in the vaccinated group is half of that in the placebo group.

5.4. Discussion

This is the first report of a large multi-herd comparative trial of a N. caninum vaccine under field conditions, carried out in cattle of specialised dairy farms. Only one paper has been found that reported a decreasing number of abortions after a year of vaccination using this vaccine in a Minnesota dairy herd (reducing from 27 abortions in the two previous years to 4 during the year of study (Choromanski *et al.*, 2001). This result should be interpreted carefully as no simultaneous control group was present and considering that bovine abortion is multi-causal in origin. Another vaccination field trial was carried out in 5 seasonally calving, commercial dairy farms in New Zeeland (Heuer *et al*., 2003). The efficacy (prevented fraction) to prevent abortion was variable between farms (0.7%, 39.0%, 54.2%, 31.4%, and –5.2% respectively). These authors suggest that *Neospora caninum* may induce abortion not only in about mid gestation but also at an earlier stage of gestation when the foetus is exceptionally vulnerable to *N. caninum* infection, and is unlikely to survive. In our study, this period was excluded as the treatments were administered during pregnancy and hence an underestimation of abortion rates and of efficacy may have occurred. Concerning the time of vaccination, Williams *et al*. (2000) and Innes *et al*. (2001) reported that experimental inoculation with tachyzoites before pregnancy did not result in transmission of infection to their calves. So, it is probable that immunization before insemination or in earlier stages of gestation than used in our study could provide an even higher effect on the observed crude abortion rate; however, the effect of neosporosis during the first trimester of pregnancy is still unclear.

The methodology followed in this trial allowed assessing the effect of the vaccine on the crude abortion rate under field conditions. The systematic error was minimised by using a paired design where a pair consisted of 2 animals of the same parity and breed and originating from the same herd. Sample size calculations indicated that over 400 animals per group were to be included. Due to these large numbers, other potential sources that may affect the abortion rate, like abortion due to other abortive diseases are assumed to be equally distributed over both treatment groups. Factors such as herd, parity and breed were controlled for in the design. Therefore a difference between both treatment groups can be assigned to the vaccine.

The crude analysis indicated that vaccination was associated with a 46.2% decrease of the abortion rate. In other words, almost half of the abortions in the unvaccinated group could have been prevented using the vaccine. The design-based analysis using survival techniques showed that the force of abortion in the vaccinated group was half that of the placebo treated group indicating that the design (pairs and pairs within-herd effects) had no significant effect on the crude outcome. This reduction is very significant when considering that abortion can be induced by several infectious and non-infectious causes, not only being neosporosis. Between 30 and 50% of abortions are attributable to a non-infectious cause (Kirkbride, 1992), meaning that infectious causes might contribute for 50% to 70% to the abortion rate. The level of *Neospora* infections in Costa Rica is higher than measured elsewhere, and therefore the vaccine effect on the crude abortion rate might be considerably high. Romero *et al*. (2002) found within-herd seroprevalences between 25% and 70% in a specific dairy area in Costa Rica. Another preliminary study (Romero *et al*., unpublished) showed seroprevalences at herd level close to 95% and within-herd between 20% and 90%. Besides, a case-control study carried out by Perez *et al*. (1998) estimated that cows seropositive to neosporosis had 12 times increased odds to abort compared with seronegative cows, while the odds were not increased for seropositives to brucella, BHV-1, leptospires and BVDV.

Until now, most of studies carried out with preparations based on killed tachyzoites showed variable effects in the humoral and cell-mediated response in the hosts. However, most of them showed increasing Ab titers (especially of IgG) from 2 weeks after the application of the formulation. Besides, it was shown that IFN and IL were produced after administration of different doses of preparation based on tachyzoites (Andrianarivo *et al*., 2000, 2001). This multiple way of reaction of the immune system of the host could lead to a clearance of parasites in early stages of infection or protection against the intracellular infection at the late stage of infection (Nishikawa *et al*., 2001b).

A study carried out by Innes *et al*. (2001) showed that experimental infection of cows with *N. caninum* tachyzoites prior to mating was protective against vertical transmission when the animals were challenged with *N. caninum* at mid gestation. In their study, the cell-mediate and humoral responses were higher in the group infected and/or challenged at mid gestation compared with the non-infected and not challenged group. This finding suggests that in dairy herds with high seroprevalences towards *N. caninum,* which may indicate high levels of natural infection, an administration of a preparation based on tachyzoites with adjuvant might elicit an immune response to prevent the *Neospora*-induced abortion. Paré *et al*. (1997) and Piergili-Fioreti *et al*. (2000) showed that seropositive cows with high antibody levels at the third trimester of gestation were less likely to abort than cows with low antibody levels at that time. This might be due to a combined effect of the antibody level in the dam and the immune-competence of the foetus at this stage of pregnancy (Osburn, 1986; Piergili-Fioreti *et al*., 2000). However, maternal antibodies to *N. caninum per se* do not prevent foetal infection (Barr *et al*., 1993). The fate of the foetus depends on the timing of parasitaemia, the parasitic load and the gestational age of the foetus (Williams *et al*., 2000). Therefore, it is possible that the vaccine we investigated sufficiently promotes the immune response so that the minimal dose to cause foetal death is not reached. Unfortunately, we do not have evidence of reduced *in uterus* transmission because pre-colostral samples from the calves were not taken as in general calves are born in the field without any assistance.

A criticism on this trial might be that that the placebo did not consist of adjuvant, which might have had some effect on the immune status. Andrianarivo *et al.* (2000) have shown that the adjuvant was able to induce the production of considerable amounts of interferon which most likely plays a role in the defense to this parasite, together with the humoral defense. However, this study assessed the efficacy of the preparation –in the formulation as used in practice- under field conditions, regardless whether this effect was due to the tachyzoites preparation or due to the adjuvant. Another criticism might be that animals were not tested for other abortive diseases but due to the large sample size we believe that such effects have levelled out. Also no testing of aborted foetuses has been done, as in most cases the aborted foetus will either be absorbed or will not be found due to the year round pasturing.

In conclusion, our study shows that the killed whole *Neospora caninum* tachyzoite preparation had a reasonable efficacy and can be used as one of the tools (next to improved management towards other abortifacients) to reduce the abortion rate in herds with a high abortion incidence. Also, this study gives more evidence of the importance of neosporosis as abortifacient in Costa Rican dairy herds.

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GENERAL DISCUSSION

It is very common that epidemiologic knowledge regarding animal diseases, which is generated in temperate countries, is adopted and adapted by developing or third world countries because of the impossibilities to generate their own knowledge or know-how. However, country specific information is often needed to develop efficient control strategies. The studies described in this thesis focused on major aspects of the epidemiology of neosporosis (*Neospora caninum*, Aplicomplexa) in Costa Rican dairy herds in order to obtain information for a more successful intervention. This disease has been reported worldwide as one of the most important causes of abortion in both dairy and beef cattle. Besides, abortion is one of the major causes of economical losses in the dairy sector and results primarily from both infectious and non-infectious diseases (Brand *et al*., 1996).

The main question underlying the studies in this thesis was “how prevalent is neosporosis in Costa Rican dairy cattle and what is the best control strategy to reduce neosporosis?” To answer this (broad) question several studies were carried out. Based on data of a herd health programme (VAMPP) together with a bank of sera from cattle of the most important dairy areas, the serostatus towards neosporosis, at both farm and cow level was assessed and the impact of *Neospora caninum* infection on (re)production has been quantified (Chapter 2). Also, a cross-sectional study was performed to estimate the seroprevalence and factors associated to *Neospora caninum* seropositivity in one of the most important dairy areas of Costa Rica (Chapter 3). The transmission routes (horizontal and vertical) have been quantified in a serological study (Chapter 4). Finally a clinical trial has been performed to evaluate the efficacy of a vaccine in reducing the abortion rate (Chapter 5). Additionally, a study was carried out towards the *Neospora*-serostatus of dogs from rural, urban and peri-urban areas and other study to assess the incidence of neosporosis in a specialised dairy area.

Therefore, main goals of the research described in this thesis were to estimate the current level of *Neospora caninum* infection in Costa Rica and its impact on (re)production, to identify and quantify factors associated to *Neospora caninum* infection and, based on that, to develop prevention and control strategies.

1. Main findings

The cross-sectional study described in Chapter 3 showed that all 25 herds included in the study were seropositive with within-herd seroprevalences ranging between 25% and 70% in the Poás and Vara Blanca area. Besides, almost 95% of farms tested seropositive

(89/94) in a national sampling involving the most important dairy areas (Chapter 2). The percentages of seropositivity found in our studies at cow level are quite similar to those reported in investigations carried out world wide (Dubey, 2003). A complementary study on 239 animals from 15 herds (results not reported in this thesis), with two samplings 14-16 months apart, determined a cumulative incidence of 16.3% (39/239). The same study showed that 23 out 112 (20.5%) initially positive cows became negative. This conversion occurred in samples that were slightly positive (0 to 10% over the cut-off value of the ELISA - which is 40%) at the first sampling. The high prevalence at both the herd and animal level makes a test and cull strategy unfeasible.

Age, parity of the cow's dam, breed and the farmers' attitude to test his/her animals for abortive infectious diseases were associated with serostatus while other management and environmental factors reported in other studies did not show significant associations (Chapter 3). The increased risk of being seropositive for older animals and for animals born from older dams may be related to the increased probability of being infected post-natally (as a consequence of horizontal transmission) which was supported by the finding that the infection due to horizontal transmission in our farms could reach almost 40% of all infections in herds with a high within-herd seroprevalence (Chapter 4). In the complementary study mentioned above the horizontal infection rate is estimated as 0.163 per 14-16 months or about 0.13 per year.

No significant effect of *Neospora* serostatus on the (re)productive performance was detected; however, it was estimated that a sero-positive cow produces 85 litres milk less per lactation (p = 0.62). The logistic regression model with farm as random effect, showed a weak positive association between abortion and serostatus (OR 1.66, 95% CI: 0.79, 3.88). No effect was observed on the length of the calving interval and the calving conception interval (Chapter 2).

A commercial vaccine (Bovilis-Neoguard®, Intervet®) tested by a standard clinical trial on field conditions, using 876 cows (438 pairs) from 25 dairy farms, reduced the crude abortion rate with about 50% (Chapter 5).

2. Impact on (re)production

Reproduction is a very complicated component of the dairy practice, because of the influence of factors such as genetics, nutrition, artificial insemination efficacy, presence or absence of diseases affecting fertility, etc; so it is difficult to estimate the real effect of a disease on the reproductive performance in dairy cows.

The effect of neosporosis on reproduction has been widely studied but specifically as cause of abortion. Then, the effect of *Neospora* infection at different times of gestation and at different infective doses has been very well described. Besides, a broad range of clinical manifestations caused by *Neospora* are known, and some estimations of economical losses due to direct and indirect costs by *Neospora*-related abortions have been reported (Barr *et al.*, 1997; Chi *et al.*, 2002, Dubey, 2003). However, no specific studies have been done to measure the effect of *Neospora* serostatus on primary reproductive parameters like calving interval, age at first pregnancy or number of services per conception. Some studies have measured the effect of neosporosis on parameters that are part of the calving interval. In this sense, there is a report of detrimental effect of *Neospora* serostatus on open days (Waldner *et al.*, 1998).

There are no conclusive findings about the biological mechanism that induces seropositive cows to produce less milk. Thurmond and Hietala (1997) hypothesized that a reduced milk production by seropositive cows could be the result of 1) residual pathologic lesions acquired *in utero* which affect - negatively - the organ system function at mature age (and subsequently milk production may be reduced), 2) recrudescence of an existing infection or 3) a new infection. Hernandez *et al*. (2001) agreed with Thurmond and Hietala and suggested, additionally, that repeated infection during subsequent pregnancies may cause persistent pathologic changes in infected cows.

There is no agreement between studies about the effect of neosporosis on milk production because some of them report a detrimental effect (Thurmond and Hietala, 1997; Hernandez *et al*., 2001). Regarding this issue, Hobson *et al*. (2002) conducted two studies simultaneously in Ontario: a case-control and a cross-sectional study. They reported a detrimental effect of neosporosis on milk production in the case-control study, which was done in 3,702 cows from 83 herds; however, in the cross-sectional study, done in 3,162 cows from 57 herds, there was no effect. Probably, the type of study is the explanation for the disagreement between both studies. Also, Jensen *et al*. (1999) did not find differences in interval calving-1st insemination and services per conception between seropositive and seronegative cows; while Björkman *et al*. (1996) reported the same result for the number of services for effective pregnancies. These findings were similar to what we found in our study in Costa Rica in which there was no significant effect on open days and the calving interval. However, one should discriminate between neosporosis and *Neospora caninum* seropositivity. Abortion due to infection may have a large impact while abortion does not occur in all seropositive animals.

3. Identification and management of risk factors

The understanding of risk factors for diseases is crucial to set up strategies and programmes for control and prevention. Cross-sectional studies are not the best to make inferences about causal relationships between exposure to risk factors and serostatus as one does not know whether or not the exposure started before the time of seroconversion, except for factors that do not change frequently over time (like housing) or factors that are permanent characteristics of the animals (like breed). Nevertheless, cross-sectional studies can give valuable information for generating hypotheses that should be further tested in experiments or cohort studies.

Several studies have documented risk factors for neosporosis. The most frequently reported are: 1) the presence of dogs in the farms, 2) increased age of the cows, 3) seasonal calving patterns, 4) allowing wildlife having access to the weaning supplement, 5) self-reared replacement heifers and 6) lack of purposive sampling to diagnose abortive infectious disease at herd and/or individual level (Thurmond *et al*., 1995; Paré *et al*., 1998; Barling *et al*., 2001; Romero *et al*., 2002).

Neosporosis, and specifically horizontal infection, has been related with the presence of dogs in the farms (Paré *et al*., 1998; Bartels *et al*., 1999; Ould-Amrouche *et al*., 1999; Wouda *et al*., 1999). In our investigation (Chapter 3) it was not possible to determine the precise role of the presence of dogs as a causal risk factor because at least 2 (up to 14) dogs were present in each of the farms involved in the study. However, we found a trend that the within-herd seroprevalence increases with an increasing number of dogs on the farm. Additionally, coyotes (*Canis latrans*) - recently proven to be definite hosts of *Neospora caninum* (Gondim *et al.*, 2004) - were noticed to access properties of all farms as well. Both dogs and coyotes come to the paddocks looking for food, especially small, weak or death calves, or tissues like placentas. As result of this, it is highly probable that coyotes and dogs may acquire the parasite and/or shed oocysts in pastures that subsequently become a source of infection to cattle. Besides that most paddocks have streams from which the wild and feral animals drink water. Also the all year round grazing of cattle in Costa Rica and the climatic conditions promoting the maintenance of the oocysts in the pastures - in analogy of the survival of oocysts of the structurally almost similar *Toxoplasma gondii* (Dubey, 1998, Lindsay *et al*., 2002) - may contribute to a high level of horizontal infection and a high level of seropositivity towards *Neospora caninum*.

In our studies we observed an increased risk of being seropositive for older animals and for animals born from older dams, which most likely is related to an increased exposure time to post-natal infection as was described in Chapter 4; in agreement with that some other studies have reported the age of cows as risk factor (Davison *et al*., 1999; Wouda *et al*., 1999; Jensen *et al.*, 1999). This factor can be taken into account in strategies to control neosporosis; however, a cow should not be removed only due to her age but especially by factors related to productive and reproductive performance.

Some studies have reported an association of neosporosis with BVD (Björkman *et al*., 2000; Romero *et al*., 2000; de Melo *et al*., 2004). BVD virus is known to have an immunosuppressive effect (Howard, 1990; Tizard, 2000); and therefore BVDV infected animals may have an increased chance of acquiring a *Neospora* infection, or have a recrudescence of a chronic infection.

4. Implications for prevention and control strategies

Policies of control, prevention and intervention directed to a specific disease need to be based on the knowledge of its ecology and the pathogenesis. Nowadays, the ecology of *Neospora* infection is very well known, as the ways of transmission within and between species have been documented. Because of the high within-herd seroprevalence of *Neospora* infection in a high percentage of Costa Rican farms, a simple test and cull strategy is not feasible for all of them, solutions should be sought in either management of risk factors, in vaccination strategies or in a culling strategy focussing on seropositive animals that will also reduce horizontal transmission to dogs as less infected tissue will become available.

A strategy to control neosporosis in farms with low and medium seroprevalence, as proposed by Thurmond and Hietala (1995) and Dubey (1999), is based on a specific sampling strategy combined with preventive measures. The sampling strategy is as follows:

1. Sample all cows that are suspect (e.g. because they aborted) and make a random sampling of the rest for presence/absence.
2. Determine if abortion is attributable to neosporosis. An equal number of cows that aborted and that did not show abortion should be tested. The proportion of *Neospora*

caninum seropositivity in cows showing abortion should be (significantly) higher than in non-aborting cows.

3. Estimate the extent to which neosporosis may have contributed to the abortion problem by calculating risk ratios and measures of effect such as risk difference and/or attributable fraction.

4. Establish if *Neospora*-induced abortion is endemic or epidemic. This step is central in definition of control strategies assuming that *Neospora* differs in mode of transmission for the two patterns of occurrence.

5. Estimate the extent of congenital infection. To be sure about this condition, it will be necessary to test both calves and cows, but calves have to be tested prior to colostrum intake.

When all previous points have been assessed, and the situation of neosporosis in the farm is clearly stated, strategies to prevent new infections can be implemented. The next section follows the structure used in the paper of Thurmond and Hietala (1995) incorporating the new knowledge about the disease produced worldwide and locally in Costa Rica especially based on the findings described in this thesis. Note that in the following parts the terms horizontal and vertical transmission (rates) are replaced with horizontal and vertical infection (probabilities) in order to prevent confusion with transmission rates. Transmission rates are measures for the extent to which infectious animals infect susceptible animals (De Jong, 1995). Hence, transmission rates can only be estimated when for case (the susceptible animal) it is also know at which rate contacts have occurred with infectious animals (be it dogs or cattle). .

4.1. Preventing vertical infection:

a. Reduction of the infection level in the herd

Removing seropositive cows is the simplest way to reduce the probability of vertical infection under the rationale that a positive cow has a high probability of giving birth to an infected calf. However, because of the high probability of vertical infection (>60%), the persistence of the disease for long periods (several generations of cow-lives) is practically guaranteed. Then selective culling of seropositive animals can diminish the within-herd seroprevalence in a short time; however, this can only be applied in herds with low seroprevalence but is not feasible in herds with high seroprevalence as no sufficient replacement stock will be available.

Selective culling can be applied gradually when the within-herd seroprevalence is low assessed by screening cows toward *Neospora* seropositivity. A good approach is to combine serology with the history of abortion of the cow, or in its familial lines. Depending on the economics of the losses due to neosporosis, the serostatus should not be the exclusive criterion to culling.

A side effect of this selective culling is the reduction of tissues potentially contaminated with the parasite and thus a reduced probability of definitive hosts to become infected.

b. Minimizing the infection level of replacement animals

There are two ways to reduce the probability of using infected replacements: 1) by serological testing of the entire stock of heifers, removing the positives ones and keeping only the negative ones in the herd and, 2) by maintaining the heifers born from negative cows and test them negative prior to insemination. This double testing (mother-daughter) is necessary due to the relatively high probability of horizontal infection in Costa Rican dairy farms.

c. Vaccination

Until now, there are no conclusive studies published that assess the effectiveness of a vaccine to avoid the infection of the offspring *in utero*. Some studies reported different grades of efficacy of the vaccine preventing *Neospora*-induced abortion (Andrianarivo *et al*., 2000; Choromanski and Block, 2000; Barling *et al.*, 2003; Estill, 2004; Heuer *et al*., 2004; Barajas-Rojas *et al*., 2004); but don’t provide evidence of prevention for vertical infection (Estill, 2004). However, Innes *et al.* (2001) documented that inoculation of cows 6 weeks prior to mating and then challenged at mid gestation produced an immune response that protected calves to vertical transmission. Other studies did not report similar results, so the effect on the foetuses in other conditions than this experiment makes it impossible to conclude that the use of a vaccine, even prior to mating, can prevent vertical infection.

4.2. Preventing horizontal infection

a. Minimizing transmission from definitive hosts

The most important environmental risk factor to become infected with *N. caninum* is the presence of definitive hosts in or around the farm, which is a special problem in Costa Rican dairy farms. Though the management of this risk factor is very hard, the following measures can be taken:

- reduction of the number of dogs in the farms, and/or the access to the paddocks,
- prevention of access of coyotes to the paddocks bordering with the forest,
- prevention of access of these canids to the drinking water, concentrates and supplementary foods,
- promotion of calving in a special calving pen near the milking yard,
- reduction of the number of infected cattle (and dogs) by means of serological testing and selective withdrawal (in farms with low seroprevalence).

b. Withdrawal of dogs from the farm is difficult due to the close relationship between the farmers and the dogs. In addition, the clearance of the parasite in dogs - at least in its intestinal form - by chemical treatments is still not available. Even more, the plausibility that the dog remains infected despite the treatment due to the presence of tissues cysts, makes this therapy not 100% effective and reduces the usefulness of this strategy.

c. Minimizing transmission from infected tissues or fluids

<u>By ingestion of infected placentas or aborted fluids</u>

Several studies have demonstrated the presence of *Neospora caninum* in bovine placental tissues (Bergeron *et al*., 2001; Davison *et al*., 2001; Malcadowie *et al*., 2004). Consumption of placentas and amniotic fluid from other cows (Pinheiro-Machado *et al*., 1997) has been thought a probable way of horizontal infection (Modrý *et al.*, 2001; Schares and Conraths, 2001). However, Davison *et al*. (2001) did not find evidence of *Neospora caninum* infection in two calves and two cows that were fed placental tissues from *Neospora caninum*-infected cows.

If placentophagia is a way for horizontal transmission, the specific weight of this on the total amount of the horizontal acquisition of infection remains to be assessed; meanwhile, a control strategy could be the calving in an individual calving pen.

By ingestion of infected colostrum or milk replacer

Even though autogenic infection has been demonstrated by Uggla *et al.* (1998) and Davison *et al.* (2001) based on experimental addition of tachyzoites to the colostrum and/or milk replacer, this way of horizontal infection has not been proven in naturally infected cows. Until now, there are no reports of colostrum or milk that is naturally contaminated with tachyzoites or any form of *Neospora*; so, this way of transmission is very unlikely to occur naturally and no special control strategies need to be implemented with regard to this topic.

By use of semen from infected bulls

Studies carried out in fresh and frozen semen from naturally infected bulls have detected the presence of DNA of the parasites in the cell fraction of semen (Ortega-Mora *et al*., 2003; Caetano-da-Silva, *et al*., 2004); however, there are no reports of cows becoming infected by infectious semen. Given that there are reports of sporadic contamination of semen with *Neospora caninum,* the potential role of semen - by artificial insemination or natural mating - as way of transmission of the disease needs to be determined. Companies that produce semen for artificial insemination should test their bulls towards *Neospora* infection to be sure that the presence of *Neospora caninum* in the semen straws is unlikely or null. The same strategy needs to be taken by farmers using bulls for natural mating.

4.3. Purchase of seronegative cows

A very plausible way to introduce neosporosis in a farm or increase its within-herd seroprevalence is by the purchase of infected cows. These could give birth to vertically infected offspring resulting in a slowly increasing prevalence over time.

It is possible to determine the serostatus of cow toward *N. caninum* prior to purchase; then the farmers have to include neosporosis within the protocols of traceability and acquisition of cows from other farms, especially because it is a disease that is not under surveillance of the animal health services. Since the discovery of neosporosis in Costa Rica, and as product of the information originated from the studies carried out locally and abroad, the practice of testing towards neosporosis as part of a “purchase-exam” is increasing over time.

5. Concluding remarks

The studies described in this thesis were carried out to acquire information about the epidemiology of *N. caninum* infection under Costa Rican conditions and to compare this with other countries. Clearly, the level of horizontal infection is higher than reported elsewhere which is most likely due to the abundance of dogs and wild canids combined with year round pasturing and thus exposure levels are high. It was also found that the herd level prevalence is relatively high; hence control strategies like test and cull are unfeasible. Therefore a preventive strategy like vaccination would be desirable and we found that a commercial vaccine reduced the abortion rate with 50%. This certainly will reduce the costs related to *Neospora* induced abortion, but it is still unknown whether the vaccine on its own will lead to eradication of the infection. Further research is needed to investigate the levels of horizontal and vertical transmission both in unvaccinated and vaccinated animals.

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SUMMARY

The aim of this thesis was to describe the most important features of neosporosis in Costa Rican dairy cattle, so the main questions underlying the studies in this thesis were “how prevalent is neosporosis in Costa Rican dairy cattle and what is the best (local) control strategy to reduce neosporosis?”.

In order to achieve this goal, specific objectives were: **1)** to have an overview of neosporosis around the world - including reports from Costa Rica - by means of extensive literature review about neosporosis, **2)** to assess the *N. caninum* serostatus at farm and cow level, **3)** to quantify the effect of *N. caninum* serostatus on the (re) productive performance of the cows, **4)** to identify and quantify factors associated with *N. caninum* serostatus, **5)** to assess the vertical and horizontal transmission rates, and **6)** to determine the effect of a commercial vaccine on the crude abortion rate.

In **chapter 1,** a broad literature review about neosporosis is given. It deals with the history and evolution of the knowledge on neosporosis; also, a description of the agent and the ecology of the disease - life cycle and ways of transmission – was included. A section describes the host-parasite relationship specifically on clinical signs, lesions and immune response of the host. Further, the most important ways of diagnosis were pointed out as well as the existing - and documented - treatments to the disease around the world. Some general aspects on control and prevention of neosporosis were also included. Finally, the most relevant aspects on the epidemiology of the disease were summarised in a section.

C**hapter 2** describes a cross-sectional study to assess the effect of neosporosis on (re)productive parameters. A selection of 2,743 cows from 94 farms located in the most important dairy areas in Costa Rica was used in the study. The serostatus of the animals towards *N. caninum* was determined by an indirect indirect enzyme-linked immunoassay (ELISA). The effect of *N. caninum* serostatus on milk production, calving interval, calving-conception interval and risk of abortion was assessed by various statistical models. Out of 2,743 animals, 1,185 (43.3%) were seropositive to *N. caninum*. Eighty-nine out of 94 (94.7%) farms were classified as *Neospora*-seropositive. It was estimated that a cow produces 84.7 litres milk more per lactation when it is seronegative to *Neospora* ($p = 0.6$). The serostatus did not show a significant effect on the length of the calving interval (Hazard ratio= 1.2, 95% CI: 0.9, 1.4) and the number of services per conception (OR= 0.95, 95% CI: 0.7, 1.3) while a weak positive association between serostatus and abortion was found (OR 1.7, 95% CI: 0.8, 3.9). In this study no significant effects of *N. caninum* serostatus were detected on (re)productive performance.

In **chapter 3**, 25 specialised Costa Rican dairy farms, located in the Poás area, were used to determine *N. caninum* seroprevalence and the association of seropositivity with environmental and management factors. The farms involved were selected intentionally and all of them used VAMPP as management-information system. Holstein-Friesian, Jersey and crosses between them were the most-frequent breeds in these herds. In the first phase of the study, the *N. caninum* status at herd level was determined using a limited number of blood samples. In the second phase, all females in 20 seropositive herds were bled. Serum samples were tested for antibodies to *N. caninum* using an indirect ELISA. A questionnaire with factors mentioned in the literature was administered to the farmers. All herds had > 20% seropositive females; therefore, all herds were eligible for the second phase where the overall prevalence was 39.7% (1,191/3,002), and within-herd prevalences varied between 25.0% and 70.5%. Age, parity of the dam of the cow, breed and purposive sampling to diagnose abortive infectious disease were associated with serostatus; other management and environmental factors did not show significant associations. The lack of association between management and environmental factors with serostatus might be due to the fact that all herds were exposed to a considerable number of potential factors. The fact that all herds of this study were seropositive for neosporosis, and the fact that the within-herd prevalence was considerable, raises questions about how far the infection is spread in other dairy areas of Costa Rica.

In **chapter 4**, an epidemiological study that aimed at the quantification of horizontal and vertical transmission is presented. The study was conducted on 20 dairy herds previously diagnosed as seropositive to *N. caninum*. All females present in the farms were bled once in July or August 2000 (n=3,002). An indirect ELISA was used to determine the serostatus of the animals. The within-herd seroprevalence varied between 25.0% and 70.5%. 747 dam-daughter pairs were available, involving daughters of any age. Daughters in the specific age-class of 2 and 3 years old had a higher seroprevalence ($p < 0.01$) compared with younger and older age-classes. The risk of being seropositive when being born from a mother that is tested seropositive (prevalence ratio) is 2.8 fold increased. The probability of horizontal infection amounts to 0.22. The probability of seropositive offspring due to vertical transmission is 0.64 (AF_{exp}). The multivariate logistic regression showed a significant 6.0 fold increased odds for being seropositive when born from a seropositive mother. Also the within-herd seroprevalence level was significantly associated to the serostatus of the daughters. Due to the specific conditions of the dairy herds involved in this study, the serostatus of the cows should be not used as predictor of the serostatus of daughters due to the increased probability of horizontal transmission.

In **chapter 5**, a standard field trial was carried out to assess the effect of a commercial *Neospora*-vaccine based on whole killed tachyzoites (Bovilis-Neoguard, Intervet®) on the abortion rate. Eight-hundred and seventy-six cows, over 2.5 months pregnant, belonging to 25 Costa Rican dairy herds, were used in the analysis. For each vaccinated cow, a cow of the same herd, breed and age category, was selected as control (placebo). The period of administration of treatments extended from June to November of 2000. The treatments were administered in two, 5 ml doses 1 month apart, the first dose given between day 75 and 90 of gestation. The incidence of abortion among all treated cows was of 16.0% (140/876). The treatment specific incidence was 11.2% (49/438) and 20.8% (91/438) for the vaccinated and the placebo group, respectively. The prevented fraction by vaccination amounted to 0.46 (95% CI: 0.26; 0.61), and the cumulative incidence ratio for the vaccinated group was 0.54. The Cox hazard ratio was 0.51 (95% CI: 0.37; 0.72), meaning that the force of abortion is reduced twice in the vaccinated group. The results of this study, the first one following this type of design, shows that the killed whole *Neospora caninum* tachyzoite preparation had a major effect on the abortion rate in Costa Rican dairy cattle.

SAMENVATTING

Het onderzoek beschreven in dit proefschrift had als doel om een aantal belangrijke aspecten van de epidemiologie van neosporose in Costa Rica te beschrijven. De kernvragen die beantwoord dienden te worden waren: “wat is de prevalentie van neosporose onder Costa Ricaans melkvee en welke is de beste strategie om neosporose te bestrijden?”

Om dit doel te bereiken werden de volgende specifieke stappen gezet: **1)** een overzicht maken van het vóórkomen van neosporose wereldwijd door middel van een uitgebreid literatuuroverzicht, **2)** het bepalen van de serostatus tegen *N. caninum* op dier- en bedrijfsniveau, **3)** het kwantificeren van het effect van een positieve *N. caninum* serostatus op (re)productiekenmerken, **4)** het identificeren en kwantificeren van factoren geassocieerd met *N. caninum,* **5)** het bepalen van de mate van horizontale en verticale transmissie en **6)** het bepalen van het effect van een commercieel vaccin op de mate van abortus.

Een uitgebreid literatuuronderzoek is beschreven in **hoofdstuk 1.** Dit geeft informatie over de kennisontwikkeling van neosporose middels een beschrijving van de parasiet en zijn levenscyclus en wijze van transmissie. Een paragraaf is gewijd aan de gastheer-parasiet relatie, in het bijzonder de klinische symptomen, de veroorzaakte laesies en de immuunreactie van de gastheer. Daarnaast wordt ingegaan op de diagnostiek en bestaande wijze van behandeling. Algemene aspecten van bestrijding en preventie worden behandeld en het hoofdstuk wordt afgesloten met een samenvatting betreffende de meest belangrijke kenmerken van de epidemiologie van neosporose.

Hoofdstuk 2 beschrijft een dwarsdoorsnede studie om het effect van het seropositief zijn met betrekking tot *N. caninum* te bepalen op re(productie)kenmerken. Een selectie van 2743 koeien op 94 bedrijven uit de meest belangrijke melkveegebieden in Costa Rica vormde de basis van deze studie. De serostatus van de dieren werd bepaald door middel van een indirecte enzyme-linked immunoassay (ELISA). Het effect van de serostatus op melkproductie, tussenkalftijd, interval afkalven-conceptie en risico op verwerpen werd bepaald met behulp van diverse statistische technieken. Van de 2743 dieren waren er 1185 (43.3%) positief en 89 van de 94 (94.7%) bedrijven werden geclassificeerd als zijnde besmet. Het bleek dat seronegatieve dieren 84.7 liter meer melk produceerden per lactatie ($p = 0.6$). De serostatus was niet significant gerelateerd aan de melkproductie, tussenkalftijd (Hazard ratio= 1.2, 95% CI: 0.9, 1.4) en het aantal inseminaties per conceptie (OR= 0.95, 95% CI: 0.7, 1.3). Alhoewel niet significant, werd een zwak positieve relatie gevonden met het risico op verwerpen (OR 1.7, 95% CI: 0.8, 3.9). In deze studie werd dus geen significant effect van *N. caninum* serostatus op (re)productie kenmerken gevonden.

In **hoofdstuk 3** wordt een studie beschreven waarin gebruik is gemaakt van 25 gespecialiseerde Costa Ricaanse melkveebedrijven uit de Poás regio. In deze studie werd de *N. caninum* seroprevalentie bepaald en tevens de relatie tussen die seroprevalentie en omgevings- en managementfactoren geanalyseerd. De bedrijven waren niet random geselecteerd omdat het gebruik van VAMPP als managementsysteem een randvoorwaarde was. De meest voorkomende rassen op deze bedrijven waren Holstein-Frisians, Jerseys en kruisingen tussen beide rassen. In de eerste fase werd de *N. caninum* status van de bedrijven bepaald via een indirecte ELISA test op een beperkt aantal bloedmonsters. Op alle bedrijven werden meer dan 20% van de vrouwelijke dieren als seropositief getest en alle bedrijven waren dan ook geschikt voor de tweede fase van het onderzoek. In die tweede fase werd op 20 positief bevonden bedrijven bloed afgenomen van alle vrouwelijke dieren en getest op antistoffen tegen *N. caninum* met diezelfde ELISA. Tevens werd, samen met de veehouder, een enquête met op literatuuronderzoek gebaseerde potentiële risicofactoren ingevuld. In deze tweede fase was 39.7% (1191/3002) van alle geteste dieren positief terwijl de binnen-bedrijfsprevalentie varieerde van 25.0% tot 70.5%. Leeftijd, pariteit van de moeder, ras en gerichte bemonstering voor abortusgerelateerde infectieuze aandoeningen waren significant geassocieerd met de serostatus; overige management- en omgevingsfactoren vertoonden geen significante relatie. Dit laatste kan veroorzaakt zijn doordat alle koppels dieren aan vele factoren blootgesteld waren.

Het feit dat alle koppels seropositief waren en het feit dat de binnen-bedrijfsprevalentie hoog is doet de vraag rijzen in hoeverre de infectie is verspreid in andere melkveegebieden in Costa Rica.

In **hoofdstuk 4** is een epidemiologische studie beschreven met als doel om de mate van horizontale en verticale transmissie te bepalen. Het onderzoek werd uitgevoerd op 20 bedrijven waarvan bekend was dat ze seropositief zijn ten opzichte van *N. caninum*. Van alle vrouwelijke dieren werd eenmalig een bloedmonster genomen in juli of augustus 2000 (n=3,002). Met een indirecte ELISA werd de serostatus van de dieren bepaald. De binnen-bedrijfsprevalentie varieerde van 25.0% tot 70.5%. 747 moeder-dochter paren waren beschikbaar voor de analyse van de wijze van transmissie. Dochters in de leeftijdscategorie van 2 tot 3 jaar hadden een hogere seroprevalentie ($p < 0.01$) vergeleken met oudere dieren. De kans op seropositiviteit van een dochter was met een factor 2.8 verhoogd als de moeder ook positief werd getest (prevalentie ratio). De kans op horizontale transmissie werd berekend als zijnde 22%.

De kans om seropositief te zijn als gevolg van verticale transmissie is geschat op 64% (AF_{exp}). De multivariate logistische regressie liet een 6-voudige toename zien in de odds om seropositief te zijn wanneer de moeder ook positief was.

Ook de binnen-bedrijfsprevalentie was significant gerelateerd aan de serostatus van de dochters. Echter, gezien de specifieke omstandigheden van de bedrijven in deze studie is het niet aan te bevelen om de serostatus van moeders te gebruiken als voorspeller van de serostatus van dochters vanwege het hoge percentage horizontale transmissie.

In **hoofdstuk 5** wordt een veldproef beschreven waarin het effect van een commercieel verkrijgbaar *Neospora*-vaccin gebaseerd op gedode tachyzoiten (Bovilis-Neoguard, Intervet®) op de mate van verwerpen beschreven. De veldproef werd uitgevoerd op 25 Costa Ricaanse melkveebedrijven in de periode van juni tot november 2000. In totaal waren 876 koeien in de proef opgenomen, allen meer dan 2.5 maanden drachtig. Voor iedere gevaccineerde koe werd een controle dier (placebo) geselecteerd van hetzelfde bedrijf, van hetzelfde ras en van dezelfde leeftijd. De vaccinatie bestond uit het toedienen van 2 maal een 5 ml dosis van het vaccin met een interval van 1 maand. De eerste dosis werd toegediend tussen dag 75 en 90 van de dracht. De incidentie van verwerpen over alle koeien bedroeg 16% (140/876). De behandelingsspecifieke incidentie van verwerpen was 11.2% voor de vaccingroep en 20.8% voor de placebo groep. De cumulatieve incidentie ratio voor de gevaccineerde groepten opzichte van de placebo groep was 0.54. Door vaccinatie werd de incidentie van verwerpen met 46% (95% CI: 0.26; 0.61) teruggebracht. De Cox hazard ratio bedroeg 0.51 (95% CI: 0.37; 0.72), hetgeen betekent dat de 'force of abortion’ met een factor 2 werd gereduceerd. Het resultaat van deze studie, de eerste grootschalige veldproef, geeft aan dat het *Neospora caninum* tachyzoiten vaccin een groot effect had op de mate van verwerpen onder Costa Ricaanse omstandigheden.

RESUMEN

El objetivo de esta tesis fue describir los aspectos más importantes de la neosporosis en el ganado lechero de Costa Rica; de ese modo, las principales preguntas que dieron origen a los estudios de esta tesis fueron: "cuán prevalente es la neosporosis en el ganado lechero costarricense?" y, "cuáles son las mejores estrategias de control (locales) para reducir la neosporosis?".

Para alcanzar este amplio objetivo, los objetivos específicos planteados fueron: **1)** realizar una sinopsis de la neosporosis bovina alrededor del mundo -incluyendo reportes de Costa Rica- por medio de una extensa y meticulosa revisión de literatura, **2)** determinar el estatus serológico respecto a *Neospora caninum* tanto a nivel de vaca como a nivel de hato, **3)** cuantificar el efecto de la seropositividad a *N. caninum* sobre los parámetros productivos y reproductivos de las vacas lecheras, **4)** identificar y cuantificar los factores asociados con el estatus serológico a *N. caninum*, **5)** determinar el efecto de una vacuna comercial -basada en taquizoitos de *N. caninum*- sobre la tasa cruda de abortos.

En el **capítulo 1** se presenta una amplia revisión de literatura acerca de la neosporosis bovina. Esta incluye los aspectos más relevantes de la historia y evolución del conocimiento acerca de la neosporosis; también incluye una descripción del agente y de la ecología de la enfermedad –ciclo de vida y formas de transmisión-. Adicionalmente, en otra sección se describen diversos aspectos de la relación hospedador-parásito, específicamente: signos clínicos, lesiones y la respuesta inmune en el hospedador. Además, se presentan los métodos diagnósticos y los tratamientos existentes -documentados- para la enfermedad alrededor del mundo. Asimismo, se incluyen aspectos acerca del control y la prevención de la neosporosis. Finalmente, se ofrece un resumen de los aspectos más relevantes de la epidemiología de la enfermedad.

En el **capítulo 2** se presenta un estudio transversal para determinar el efecto de la seropositividad a *N. caninum* sobre los parámetros productivos y reproductivos de vacas lecheras costarricenses. Se seleccionaron 2,743 vacas de 94 fincas ubicadas en las tres áreas de producción lechera más importantes de Costa Rica (Cartago, Poás-Vara Blanca y San Carlos). El estatus serológico a *N. caninum* fue determinado por medio de un Ensayo Inmunoenzimático Ligado a Enzimas (ELISA) indirecto. El efecto de *N. caninum* sobre la producción de leche, el intervalo entre partos, el intervalo parto-concepción, el riesgo de aborto y el número de servicios por concepción fue determinado mediante varios modelos estadísticos. De las 2,743 hembras muetreadas, 1,185 (43.3%) resultaron seropositivos a *N. caninum*. Ochenta y nueve (94.7%) de las 94 fincas muestreadas fueron clasificadas como seropositivas a *Neospora*. Se estimó que una vaca seronegativa produjo 84.7 litros de leche más -por lactancia- que una vaca seropositiva (p=0.6). El estatus serológico a *N. caninum* no mostró algún efecto significativo sobre la longitud del intervalo entre partos (Hazard ratio= 1.2, 95% CI: 0.9 - 1.4) y el número de servicios por concepción (OR= 0.95, 95% CI: 0.7 - 1.3), mientras que se observó una débil asociación positiva entre el estatus serológico y el aborto (OR 1.7, 95% CI: 0.8 - 3.9). En resumen, en este estudio no se encontró efectos significativos del estatus serológico respecto a *N. caninum* sobre el rendimiento productivo y reproductivo de vacas en hatos lecheros especializados de Costa Rica.

En el **capítulo 3** se determinó la seroprevalencia a *N. caninum* y la asociación de la seropositividad con factores ambientales y de manejo a nivel de las fincas, utilizando para ello 25 fincas lecheras especializadas localizadas en el área de Poás-Vara Blanca (zona norte de Heredia y Ajalueja). Las fincas involucradas fueron seleccionadas intencionalmente de modo que todas ellas utilizaran el sistema de información de manejo de fincas VAMPP. Las razas más frecuentes en el estudio fueron Holstein-Friesian, Jersey y los cruces entre ellas. En la primera parte del estudio se determinó el estatus serológico a *N. caninum* a nivel de finca utilizando un número limitado (suficiente) de muestras de sangre -determinado mediante la fórmula para detectar presencia-ausencia de enfermedad-. En la segunda fase fueron sangradas la totalidad de hembras presentes en 20 hatos seropositivos. Las muestras fueron procesadas mediante un ELISA indirecto para determinar la seropositividad a nivel de vaca. Al mismo tiempo del sangrado, se aplicó un cuestionario que incluía los principales factores asociados con neosporosis mencionados en la literatura. Todos los hatos tuvieron al menos un 20% de hembras seropositivas, lo que las hizo elegibles para la segunda fase del estudio. La seroprevalencia global fue de 39.7% (1,191/3,002), y las seroprevalencias dentro de cada hato variaron entre 25.0% y 70.5%. Los factores asociados con la seropositividad fueron: la edad, el número de parto de la madre de la vaca muestreada, la raza y el hecho de realizar muestreos con la intención de diagnosticar enfermedades infecciosas abortivas en las fincas; mientras que otros factores de manejo y ambientales no mostraron asociación significativa. La falta de asociación entre factores de manejo y ambientales con el estatus serológico a *Neospora* podría deberse a la situación de que todos los hatos estuvieron expuestos a un considerable número de potenciales factores de riesgo a la enfermedad. El hecho de que todos los hatos del estudio fueron seropositivos a *N. caninum*, además de que la prevalencia dentro de hatos fuera considerable, plantea preguntas acerca de cuán extensa es la distribución de esta enfermedad en otras áreas ganaderas de Costa Rica.

En el **capítulo 4** se presenta un estudio orientado a cuantificar las tasas de transmisión vertical y horizontal de la neosporosis en condiciones de campo. El estudio fue conducido en 20 fincas lecheras previamente diagnosticadas como seropositivas a *N. caninum*. Todas las hembras presentes en las fincas fueron sangradas una vez en Julio o Agosto del año 2000 (n=3,002). Se utilizó un ELISA indirecto para determinar el estatus serológico de las vacas a *N. caninum*. La seroprevalencia dentro de los hatos varió entre 25.0% y 70.5%. Un total de 747 parejas *madre-hija*, involucrando hijas de cualquier edad, resultó disponible para el estudio. Las hijas en las edades específicas de 2 y 3 años de edad presentaron la mayor seroprevalencia ($p < 0.01$) comparadas con las más jóvenes o con las de las clases más adultas. El riesgo de una vaca de ser seropositiva cuando la madre resultó seropositiva al muestreo fue de 2.8 veces el riesgo que cuando la madre fue seronegativas. La probabilidad de infección horizontal ascendió a 0.22, mientras que la probabilidad de una cría seropositiva debido a transmisión vertical fue de 0.64 (Fracción atribuible entre los expuestos (FA_{exp})). El modelo de regresión logística multivariado mostró que las hembras nacidas de vacas seropositivas tenían 6.0 veces el riesgo de ser seropositivas, comparadas con las hembras nacidas de vacas seronegativas. Asimismo, la seroprevalencia dentro de los hatos fue asociada significativamente al estatus serológico de las hijas. Debido a las condiciones específicas de los hatos lecheros involucrados en este estudio, el estatus serológico de las vacas no puede ser utilizado como predictor del estatus serológico de las hijas en vista de la alta probabilidad de transmisión horizontal.

En el **capítulo 5** se presenta un ensayo de campo estándar (ensayo clínico controlado) dirigido a determinar el efecto de una vacuna comercial basada en taquizoitos de *N. caninum* (Bovilis-Neoguard, Intervet®) sobre la tasa cruda de abortos en hatos lecheros especializados de Costa Rica. Para este estudio fueron utilizadas 876 vacas que tuvieran al menos 2.5 meses de gestación comprobada mediante palpación rectal realizada por un veterinario pertenecientes a 25 fincas. Para cada vaca vacunada fue seleccionada una vaca control (placebo) que fuera del mismo hato, de la misma raza y de la misma categoría de edad. El período de administración de los tratamientos se extendió de Junio a Noviembre del 2000. Los tratamientos fueron administrados mediante la aplicación de una primera dosis de 5 ml/SC entre los días 75 y 90 de gestación, más un refuerzo aplicado un mes posterior a la primera dosis. La incidencia global de abortos en todos los animales tratados

fue de 16.0% (140/876). La incidencia específica fue de 11.2% (49/438) y 20.8% (91/438) para las vacas vacunadas y las del grupo control, respectivamente. La fracción prevenida por la vacunación alcanzó 0.46 (IC 95%: 0.26 - 0.61), y la razón de incidencia acumulativa para el grupo vacunado fue de 0.54. La razón de riesgos de Cox (Cox hazard ratio) fue 0.51 (IC 95%: 0.37 - 0.72), indicando que la fuerza del aborto se redujo dos veces en el grupo vacunado. Los resultados de este estudio, el primero llevado a cabo mediante este tipo de diseño, mostró que la preparación vacunal basada en taquizoitos de *Neospora caninum* tuvo un efecto razonable sobre la tasa cruda de aborto en las vacas lecheras de Costa Rica.

When you do the common things in life in an uncommon way, you will command the attention of the world."

George Washington Carver

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- Juan José -

Make everything as simple as possible, but not simpler.

Albert Einstein

CURRICULUM VITAE

Juan José Romero Zúñiga was born on August 28th 1969 in the city of Turrialba, Costa Rica. His parents were Efraín Romero Aguilar† and Anabell Zúñiga Pereira†. His elementary studies were completed at Escuela Jenaro Bonilla Aguilar in Turrialba (1980) and Colegio Técnico Profesional Agropecuario de la Suiza (1987). His undergraduate studies in Veterinary Medicine were performed at Escuela de Medicina Veterinaria, Universidad Nacional, Costa Rica (1995). He obtained a Master of Science degree in Animal Reproduction at Posgrado Regional en Ciencias Veterinarias Tropicales (PCVET), Universidad Nacional, Costa Rica (1997). Since 1997 he works in the department of Reproduction and Herd Health of the Universidad Nacional de Costa Rica. In 1998 he was awarded a scholarship by RESAP (Regional Center for Training and Research on Sustainable Animal, Universidad Nacional, Costa Rica) with funds from SAIL (Stichting Samenwerkings Verband IO-instellingen, Wageningen Universiteit, The Netherlands) to perform a PhD program on Epidemiology and Reproduction at Wageningen University and Research Centre. Since 2002 he co-ordinates the Master of Science program on Epidemiology in PCVET.

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