

***Neospora caninum* and bovine viral diarrhoea virus infections in dairy cattle**

Investigation of seroprevalences in imported and local crossbreed cows in dairy herds from southern Vietnam

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The present thesis is a partial fulfilment of the requirements for a Master of Science Degree for International Students (MSc) in Veterinary Medicine, at the Swedish University of Agricultural Sciences (SLU), in the field of ruminant medicine

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To my parents

ABSTRACT

The protozoan parasite *Neospora caninum* and bovine viral diarrhoea virus (BVDV) are major causes of abortion in cattle and related with economic losses in many countries. When introduced into a herd, both infections can be expected to remain for a long time if no control measures are taken. The purpose of this study was to investigate the prevalences of *N. caninum* and BVDV infections in dairy cows in South Vietnam. Specific goals were to study the seroprevalences of the infections in dairy cows from state herds and smallholder farms, and if there were differences in seroprevalences between imported Holstein Friesian cows and local crossbreeds.

A total of 345 serum samples, including 215 sera collected from 5 state farms and 130 sera from 97 smallholder herds, from southern Vietnam were analysed for presence of antibodies to *N. caninum* and BVDV. In state herds with imported cows, the *N. caninum* and BVDV seroprevalences varied between 38-53% and 78-93%, respectively. The infection rates were higher in imported cows than in local crossbreeds. The results suggested that horizontal transmission of *N. caninum* was occurring in at least 4 of the 5 herds. Among cows from smallholder herds with only local crossbreeds, the prevalences of *N. caninum* and BVDV were 19% and 18%, respectively. BVDV antigen was not found in any cow. This is the first report on BVDV infection in Vietnam.

It was concluded that *N. caninum* and BVDV infections are present and appear to be widespread in dairy cows in south Vietnam. Given the high prevalence among imported cows found in this study, it seems advisable that only cattle that are pre-tested free from *N. caninum* infection are imported into the country. Further, it is important not to import any persistently infected BVDV cattle or antibody positive cows that may carry infected foetuses.

Key words: *Neospora caninum*, BVDV, prevalence, imported cows, local crossbreeds, Vietnam.

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GENERAL BACKGROUND

1. Introduction

Neospora caninum and bovine viral diarrhoea virus are two infectious agents related to abortion in cattle. *N. caninum* is an obligate intracellular protozoan parasite, closely related to *Toxoplasma gondii*. It is an important cause of bovine abortion and stillbirth in many parts of the world. Infection in dogs can give paralysis. For reviews on *N. caninum* see e.g. Dubey and Lindsay (1996), Anderson et al. (2000) and Dubey (2003a). Bovine viral diarrhoea virus (BVDV) belongs to the genus *Pestivirus* in the family *Flaviviridae*, and is closely related to classic swine fever virus and ovine border disease virus (Donis, 1995). BVDV infection has a worldwide distribution (Houe, 1995). It is a major reproductive pathogen in cattle (Paton et al., 1998; Houe, 1999) and its immunosuppressive effects may predispose for other infections (Baker et al., 1995; Potgieter, 1995; Cox et al., 1998).

2. *Neospora caninum*

2.1 Life cycle

N. caninum belongs to the family *Sarcocystidae* in the Phylum *Apicomplexa* (Dubey et al., 1988). The parasite was first recognized in 1984 and named in 1988 (Bjerkås et al., 1984; Dubey et al., 1988). A redescription of *N. caninum* was published in 2002 (Dubey et al., 2002). It has a two-host life cycle, including definitive hosts in which sexual reproduction of the parasite can take place, and intermediate hosts. Several animal species such as cattle, sheep, dogs and other warm-blooded animals have been reported as intermediate hosts for *N. caninum* (Dubey et al., 2002). The only today known definitive hosts are dogs and coyotes (McAllister et al., 1998; Lindsay et al., 1999; Gondim et al., 2004).

The life cycle is typified by three morphological stages: tachyzoites, tissue cysts containing bradyzoites, and oocysts containing sporozoites. Tachyzoites are rapidly dividing intracellular stages found in different cell types and organs, most often in the brain and spinal cord. They are lunate, ovoid, or globular and measure 3-7 x 1-5 µm, depending on the stage of division (Dubey and Lindsay, 1996; Dubey et al., 2002). The tissue cysts are mostly found in neural tissues. They are round or oval in shape and up to 107 µm in diameter with a up to 4 µm thick cell wall. The enclosed bradyzoites measure about 7 x 2 µm (Dubey et al., 2002). Thin-walled (0.3-1.0 µm) tissue cysts have been reported in muscles of cattle and dogs naturally infected with a *N. caninum*-like parasite (Peters et al., 2001). Oocysts result from sexual reproduction and are shed by definitive hosts of the parasite. Their morphology resembles that of *T. gondii* and *Hammonia hammondi* oocysts in cat faeces and oocysts of *H. heydorni* in dog faeces. *N. caninum* oocysts are shed unsporulated and are 10.6 – 12.4 x 10.6-12.0 µm in size (McAllister et al., 1998; Lindsay et al., 1999). They sporulate and become infective within 3 days in the environment (McAllister et al., 1998; Lindsay et al., 1999, 2001).

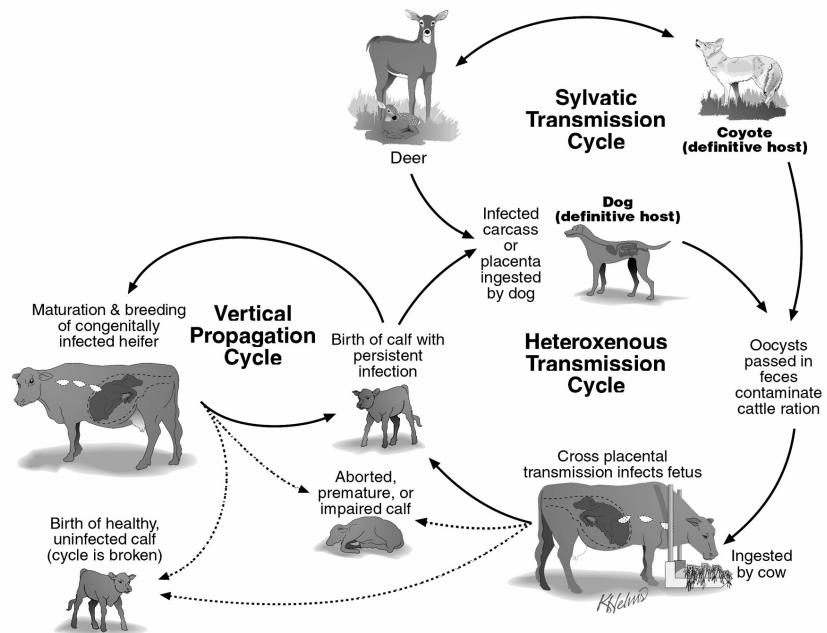


Figure 1. Life cycle of *N. caninum* (Artist Kerry Helms, University of Illinois, College of Veterinary Medicine)

2.2 Transmission

Animals may be infected with *N. caninum* by two main routes, ingestion of organisms (horizontal transmission) or transplacentally (vertical transmission) (Björkman et al., 1996; De Marez et al., 1999; Anderson et al., 2000).

2.2.1 Vertical transmission

Transplacental infection from a mother to her calf seems to be the most common transmission route in cattle (Björkman et al., 1996; Thurmond and Hietala, 1997; Schares et al., 1998; Davison et al., 1999b; Dijkstra et al., 2001). This occurs both in acutely and persistently infected cows (Pare et al., 1996; Davison et al., 1999b; Dubey, 2003a). Vertical transmission is very efficient in cattle, and more than 80% of calves born to infected dams are infected (Anderson et al., 1997; Schares et al., 1998; Davison et al., 1999b). Congenitally infected heifer calves remain persistently infected throughout life and can pass the infection to their own offspring (Anderson et al., 1997). Thus the parasite can persist in a herd for many generations, if not perpetually (Björkman et al., 1996; Pare et al., 1996).

2.2.2 Horizontal transmission

Cattle can also acquire the infection by ingestion of feed or water contaminated with *N. caninum* oocysts (De Marez et al., 1999; Wouda et al., 1999b; Bergeron et al., 2000). The dog is now known to be able to excrete oocysts and epidemiological studies have shown that there is an association between presence of dogs and bovine *N. caninum* infection at a farm (Wouda et al., 1999b; Dijkstra et al., 2001).

et al., 2002a, b). In the Netherlands and Korea, 22-24% of investigated farm dogs have been reported to be *N. caninum*-infected (Wouda et al., 1999b; Kim et al., 2003). However, horizontal transmission does not seem to have a major role in the spread of *N. caninum* in most herds. A low grade of *N. caninum* post-natal infection, less than 9%, has been reported in longitudinal studies (Pare et al., 1996; Davison et al., 1999b; Hietala and Thurmond, 1999).

2.3 Clinical signs

N. caninum causes asymptomatic persistent infection in adult non-pregnant cattle (Buxton et al., 2002). Abortion or birth of weak calves are clinical signs that can be seen in pregnant cattle acutely or persistently infected with *N. caninum* (Thurmond and Hietala, 1997; Wouda et al., 1998; McAllister et al., 2000; Dubey, 2003a). Abortion can occur throughout pregnancy, but is most common in mid- to late gestation (Anderson et al., 1991; Lopez-Gatius, 2003; Dubey, 2003a, b) and *N. caninum* infected cows can abort in successive pregnancies (Anderson et al., 1995; Thurmond and Hietala, 1997). Several studies have shown that seropositive cows have a higher risk of abortion compared with their seronegative herd mates (Thurmond and Hietala, 1997; Lopez-Gatius et al., 2004). Abortions caused by *N. caninum* may show different patterns: epidemic and endemic. If more than 10-12% of the animals at risk abort during a short period of time the abortion pattern is considered as epidemic (Wouda et al., 1999a; Schares et al., 2002). If more than 3% of pregnant cows abort throughout the year without an obvious peak, the abortions are referred to as endemic (Davison et al., 1999a).

Most calves born to infected dams are clinically healthy, but persistently infected with the parasite (Pare et al., 1996). However, live born congenitally infected calves may show clinical signs such as underweight, an inability to rise, flexed or hyperextended hind limbs or/and forelimbs, ataxia, decreased patellar reflexes, loss of conscious proprioception, exophthalmia or an asymmetrical appearance of the eyes and hydrocephalus (Dubey, 2003a, b).

2.4 Diagnosis

2.4.1 Histology and immunohistochemistry

Histopathological examination of an aborted foetus is necessary to obtain a definitive diagnosis of neosporosis (Thurmond et al., 1999; Jenkins et al., 2002). The brain, spinal cord, heart and liver are the best specimens for diagnosis (Dubey, 2003a). The histological lesions in the brain of an aborted foetus caused by *N. caninum* are typical of protozoal infections (Barr et al., 1991; Lindsay et al., 1993; Wouda et al., 1997a). Immunohistochemistry using peroxidase-labelled parasite specific antiserum can be employed to confirm the identity of demonstrated parasites, and distinguish them from related coccidians e.g. *T. gondii* (Lindsay and Dubey, 1989; Otter et al., 1995). The method is highly specific, but laborious and not very sensitive (Gottstein et al., 1998; Gonzalez et al., 1999; Boger and Hattel, 2003; Dubey, 2003a).

2.4.2 Nucleic acid based detection methods

N. caninum DNA can be detected by polymerase chain reaction (PCR) methods in formaldehyde fixed or paraffin-embedded foetal brain tissue (Baszler et al., 1999; Ellis et al., 1999). Many different PCR techniques including standard PCR, semi-quantitative PCR, single tube nested PCR and PCR followed by probe hybridisation have been used to detect parasite-DNA in target tissues from aborted foetuses (Jenkins et al., 2002). These techniques are highly sensitive and specific but the stage of autolysis of the foetus, and sampling procedures play key roles in the efficiency of the diagnosis (Jenkins et al., 2002). PCR has also been used to identify *N. caninum* parasite found in the brain from an adult cow (Sawada et al., 2000). Further, PCR methods that can detect *N. caninum* DNA in oocysts in dog faeces have been developed (Hill et al., 2001).

2.4.3 Antibody assays

The presence of antibodies in an individual indicates that it is infected with *N. caninum* (Dubey and Lindsay, 1996; Dubey, 1999). Antibody assays including the indirect fluorescent antibody test, the direct agglutination test, Western blot and different enzyme-linked immunosorbent assays have been developed. For a review see Björkman and Uggla (1999).

Indirect fluorescent antibody test

An indirect fluorescent antibody test (IFAT) was the first *N. caninum* antibody assay to be developed and was for a long time considered a reference test for *N. caninum* serology. The sample is incubated with dried or fixed *N. caninum* tachyzoites on a microscopic slide. In the second step, fluorescein labelled antibodies directed against immunoglobulins of the animal species investigated are added. The reaction is evaluated with a fluorescence microscope (Björkman and Uggla, 1999). IFAT has been used to analyse serum and foetal fluids (Trees et al., 1994; McNamee et al., 1996; Buxton et al., 1997; Otter et al., 1997).

Enzyme-linked immunosorbent assay

Several *N. caninum*-specific enzyme-linked immunosorbent assays (ELISAs) have been described which use whole tachyzoite lysates (Pare et al., 1997), fixed whole tachyzoites (Williams et al., 1997), tachyzoite antigens incorporated into immunostimulating complexes (iscoms) (Björkman et al., 1997), or recombinant tachyzoite antigens (Lally et al., 1996; Louie et al., 1997). All the tests can be used to detect antibodies in serum and some of them have been applied on foetal fluid (Williams et al., 1997; Slotved et al., 1999). The iscom ELISA and p38-*Neospora* ELISA are also used to demonstrate *N. caninum* antibodies in bulk milk samples (Björkman et al., 1997; Chanlun et al., 2002; Schares et al., 2003).

Recently, an avidity-ELISA has been developed to distinguish recent and chronic infections in cows (Björkman et al., 1999; Björkman et al., 2003). The basis for this assay is that the strength of the binding of antibodies to *N. caninum* increases with time after infection.

2.5 Prevention and control

Currently, there is no chemotherapy for treatment of infection or proven vaccine to prevent *N. caninum* abortion in cattle (Innes et al., 2002; Dubey, 2003a). Therefore other control measures to prevent infection and accompanying reproductive problems are needed. A part of the control strategy should be to decrease the vertical transmission in a herd by reducing the number of infected cattle. This could be done by culling seropositive cows and not keeping infected heifer calves for replacement. Farm management practices to prevent horizontal transmission of *N. caninum* should also be applied. Dogs should be prevented to eat bovine placenta aborted foetuses or dead calves. Further, they should not to be allowed to defecate in cattle feed or water (Anderson et al., 2000; McAllister et al., 2000). In case new animals are brought into a herd, they should be pre-tested free from infection of *N. caninum* (Frössling, 2004).

2.6 Economic impact

Up to now, the economic losses in cattle herds infected with *N. caninum* due to e.g., abortion, decreased reproductive performance, reduced milk production and premature culling have not been reported firmly (Trees et al., 1999; Antony and Williamson, 2001). It is estimated that the losses per year due to neosporosis is approximately 35 millions USD per year in California, and more than 72 million USD (100 million Australian dollars) in Australia and New Zealand (Reichel, 2000; Dubey, 2003a).

3. Bovine viral diarrhoea virus

Bovine viral diarrhoea virus (BVDV) is a small enveloped virus with a positive-stranded RNA genome of approximately 12.5 kb, classified within the family *Flaviviridae* (Nettleton and Entrican, 1995). Based on the comparison of sequences from the 5'-untranslated region (5'-UTR) of the viral genome, two genotypes BVDV-1 and BVDV-2 have been defined (Pellerin et al., 1994; Ridpath et al., 1994). BVDV-1 represents the classical strains of BVDV, whereas BVDV-2 was first isolated from outbreaks of severe acute BVDV. BVDV are either cytopathic (cp) or noncytopathic (ncp), as defined by their effect in cultured cells (Baker, 1987).

3.1 Transmission

Persistently infected (PI) calves are the main active vectors of viral transmission within a herd (Houe, 1999; Lindberg and Alenius, 1999). Trade with PI animals or with non-PI dams carrying PI foetuses (PI carriers) or contact with PI on pasture constitute the major routes for transmission of the virus between herds (Lindberg et al., 2001).

Different ways of indirect transmission of BVDV have been demonstrated, e.g. reuse of needles, nose tongs and rectal gloves (Lang-Ree et al., 1994; Niskanen and Lindberg, 2003) and contaminated vaccines (Loken et al., 1991). The probability of indirect transmission is dependent on e.g. duration of contact,

amount of excreted virus and temperature (Gunn, 1993; Lang-Ree et al., 1994; Niskanen and Lindberg, 2003).

3.2 Primary infections with BVDV

3.2.1 Infection in non-pregnant cattle

Factors such as immunocompetence and the BVDV genotype influence the clinical outcome of infection with BVDV (Baker, 1987). The majority of acute infections are subclinical, but are still important as BVDV may act as an immunosuppressive agent or a potentiator for other diseases (Houe, 1995; Taylor et al., 1997). Cattle undergoing a subclinical infection may demonstrate a mild elevation in body temperature, leukopenia and decreased milk production. Young animals infected with BVDV may also develop diarrhoea, respiratory signs and extensive oral and digestive tract erosions (Baker, 1995; Flores et al., 2000). Subclinical infections account for the high prevalence of seropositive cattle (Houe, 1996).

3.2.2 Infection in pregnant cattle

The clinical signs associated with BVDV infection during pregnancy are complex (Rufenacht et al., 2001). Depending on stage in gestation, infection in pregnant cows can result in abortion, mummification or birth of persistently infected (PI), weak or malformed calves. Infection in early gestation, before 30 days, may decrease conception rates and give embryofoetal losses (McGowan and Murray, 1999). If infection occurs during 30-120 days of gestation, the foetus may become PI. These foetuses have not yet established immunocompetence against BVDV and a permanent immunotolerance to the infecting virus is therefore achieved. Moreover, the inability of ncpBVDV to induce IFN- α in the foetus is also one of the major immune evasion mechanisms that allow BVDV to establish persistence (Charleston et al., 2002). Typically, there is no detectable antibody response to the virus and the calf remains PI throughout life (McClurkin et al., 1984). Pregnant cattle infected with BVDV during 125 to 175 days of gestation may abort or give birth to antibody positive calves with congenital abnormalities such as alopecia, pulmonary hypoplasia, retarded growth, thymic aplasia and ataxia (Dubovi, 1994). Pregnant cattle infected with BVDV later in gestation often give birth to normal calves with high levels of precolostral antibodies. However, abortions and abnormalities in this late stage have also been reported (Moennig and Liess, 1995).

3.2.3 PI animals

PI animals can appear with or without symptoms (Houe, 1993; Baker, 1995). One study reported that 44% of PI animals remained clinically normal until slaughter (Houe, 1993). However, many PI calves are born undersized and have retarded growth rate (Houe and Meyling, 1991). Some calves have a curly hair coat (Larsson et al., 1991) or other skin defects (Bielefeldt-Ohmann, 1995). PI animals appear to be more susceptible to other infections and it is quite common that they die or are culled before they reach adult age (Houe, 1995, 1999).

3.2.4 Mucosal disease

Mucosal disease occurs only in PI animals. Both cp and ncp BVDV can be isolated from cattle with mucosal disease. The infected animals develop clinical signs including depression, high fever, anorexia, profuse diarrhoea, salivation, dehydration, emaciation and death. Animal with chronic mucosal disease are characterized by anorexia, alopecia, hyperkeratinization, erosive lesions, laminitis or interdigital erosions (Baker, 1995).

3.3 Diagnosis

3.3.1 Detection of virus and antigen

Virus isolation from clinical specimens including culture and identification on primary cell cultures is still considered as the gold standard technique for BVDV diagnosis. Tissues from lymphoid organs such as the spleen, peyer's patches from the small intestine, mesenteric lymph nodes, and thymus or mononuclear cells extracted from whole blood are the best samples for virus isolation. The sensitivity of the cell culture system and the inoculation method affect the ability to culture BVDV. Fluorescent antibody (FA) staining or other immunologic staining methods are required to detect the presence of ncp strains (Saliki and Dubovi, 2004).

For detection of BVDV antigen, two kinds of methods are used: immunologic staining of fresh or formalin fixed paraffin embedded tissue sections and antigen capture ELISAs. Detection of BVDV antigen is quicker and cheaper, but it lacks the reliability of virus isolation. They are often not used as the final rule in/rule out tests, but as screening methods (Saliki and Dubovi, 2004).

3.3.2 Nucleic acid based detection methods

Several reverse-transcriptase polymerase chain reaction (RT-PCR) techniques have been developed for detection of BVDV ribosomal nucleic acid (RNA) (Belak and Ballagi-Pordany, 1991; Vilcek et al., 1994; Ridpath et al., 2002). Testing protocols have been refined and validated for many kind of samples e.g. serum, sperm, whole blood, buffy coat cells, skin and fresh and formalin-fixed tissues (Saliki and Dubovi, 2004). RT-PCR can also be used to detect viral RNA in bulk milk somatic cells, and thereby indicate the presence of one or more PI lactating cows in a dairy herd (Radwan et al., 1995; Drew et al., 1999). Bulk milk samples are easily available and enable cost-effective screening for herds exposed to the virus. However, this technique requires rigorous precautions to avoid contamination in the laboratory and thus false positive test results (Belak and Ballagi-Pordany, 1991).

3.3.3 Detection of antibodies (virus neutralization test and ELISA)

The virus neutralization (VN) assay is the accepted reference test for detection of antibodies to BVDV (Edwards, 1990). The strain of virus and the cells used in the assay are however two important factors to consider when interpreting the test result (Saliki and Dubovi, 2004).

For detection of antibodies in milk and serum samples, indirect and blocking ELISAs have been used (Howard et al., 1985; Junnti et al., 1987; Niskanen et al., 1991). The advantages of the ELISA techniques are that they are easy to perform, can be applied for mass screening and give reliable and quick results (Niskanen et al., 1989).

3.4 Prevention and control

BVDV has worldwide distribution and affects cattle, sheep as well as several domestic and wild ruminant species (Baker, 1995). In most cattle populations, infection with the virus is common, often with a seroprevalence of 60 to 85% (Houe, 1999). The main reason for the high seroprevalence is contact with PI individuals. The implementation of a programme to control the infection must be based on, first, the identification and protection of herds which are free from the infection, and secondly, control measures to prevent transmission of the virus between herds, and third, the clearance of virus shedders from the infected herds (Lindberg and Alenius, 1999). For dairy herds, the level of antibodies to BVDV in bulk tank milk, measured by the ELISA, has been recognised as a valuable tool for estimating the prevalence of positive animals in a herd (Niskanen, 1993). Bulk milk testing can be used to identify dairy herds which are free of BVDV and those which may be suspected of harbouring an active infection (Niskanen et al., 1991; Bitsch and Ronsholt, 1995). Such ELISA tests are currently applied to bulk milk samples to identify and monitor herds in the control and eradication programmes for BVDV implemented in Scandinavian countries (Lindberg and Alenius, 1999).

Despite the use of BVDV vaccines for more than 40 years this control option has not been proven effective. There are at present considerable problems concerning both the safety and efficacy of the existing BVDV vaccines. However, safe and effective marker vaccines, that can be used in control programs, might be developed in the future (van Oirschot et al., 1999).

3.5 Economic impact

The economic losses in dairy herds infected with BVDV include those caused by reduced milk production, reduced conception rate and increased incidence of respiratory disorders as well as abortions, congenital defects and growth retardation (Baker, 1995; Bielefeldt-Ohmann, 1995; Houe, 1995). However, the calculations of the economic losses are complex because the losses depend on the initial herd immunity, pregnancy status of the cows at the time of the infection and the virulence of the infecting virus strain (Houe, 1999). The calculated losses in individual herd outbreaks have accordingly varied from a few thousand up to 100,000 USD per herd (Houe, 2003).

4. Dairy production in Vietnam

Since 1990s, the demand of milk and dairy products for consumption has rapidly increased in Vietnam. Milk production is low in the country and about 90% of dairy products are imported. In order to meet the demand, a nationwide program

for dairy development in Vietnam was issued by the Government in 2001. Many dairy cows and heifers were imported to Vietnam. The population of dairy cattle was 50,000 heads in year 2002 and is expected to have risen four-fold until 2010 (Tuyen and Giao, 2002). The dairy cattle are kept either in smallholder herds or in large state farms. In the state farms all cattle are kept in open stalls. The dry cows, heifers and young stock are often kept in separate pens in the same stall as the lactating cows. The calves are housed separately until they are 3 to 4 months old. The cows are milked twice a day. All animals except the young calves are free ranging together in a feed yard for some hours each day. Dairy production is a new sector in Vietnam, and there is limited experience of management and feeding of dairy cattle. Also, little is known about the reproductive diseases present in the country.

INTRODUCTION TO THE RESEARCH REPORT

N. caninum and BVDV are infections that cause financial losses to the dairy industry all over of the world. Little is known about the prevalence and importance of these infections in dairy cattle in Vietnam, although prevalence data for the diseases have been established in many other countries. *N. caninum* is a protozoan parasite that has been recognized as one of the most important infectious causes of bovine abortion throughout the world and it has been associated with sporadic, endemic and epidemic abortion (Dubey and Lindsay, 1996; Wouda et al., 1997b; Moen et al., 1998). The only study on *Neospora* infection in Vietnam revealed a 5.5% seroprevalence in cattle from the South part of the country (Huong et al., 1998). BVDV is a major reproductive pathogen in cattle (Houe, 1999; Fray et al., 2000) and its immunosuppressive effects may predispose for other infections (Baker et al., 1995; Potgieter, 1995; Cox et al., 1998). No studies on BVDV infection in dairy cattle have been performed in Vietnam. This study was initiated to increase the knowledge about these infections that may cause reproductive and health problems among dairy cows in Vietnam.

AIMS OF THE STUDY

The general aim of this study was to investigate how common *N. caninum* and BVDV infections are in dairy cows in South Vietnam.

The specific goals were to investigate:

- the seroprevalences of the infections in dairy cows from state herds and smallholder farms.
- if there were differences in seroprevalences between imported Holstein Friesian cows and local crossbreeds.

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RESEARCH REPORT

Prevalences of *Neospora caninum* and bovine viral diarrhoea virus in dairy cows in southern Vietnam

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Abstract

The aim of this study was to investigate the prevalences of *Neospora caninum* and bovine viral diarrhoea virus (BVDV) in dairy cows in Vietnamese state and smallholder farms. A total of 345 serum samples including 215 sera collected from 5 state farms and 130 sera from 97 smallholder herds were tested. The sera were analysed for presence of antibodies to *N. caninum* and BVDV. An avidity ELISA was used for determining of the duration of *N. caninum* infection in dairy herds. Further, all BVDV antibody negative animals were tested for presence of BVDV antigen. Moderate to high prevalences of *N. caninum* and BVDV seropositive cows were found in this investigation performed in Vietnamese dairy cattle. This is the first report on BVDV infection in Vietnam. In state herds with imported cows, the *N. caninum* and BVDV seroprevalences varied between 38-53% and 78-93%, respectively. The infection prevalences were higher in imported cows than in local crossbreeds. The prevalences of *N. caninum* and BVDV were 19% and 18%, respectively, among cows from smallholder herds with only local crossbreeds. BVDV antigen was not found in any cow. Given the high prevalence among imported cows found in this study, it seems advisable to test for *N. caninum* infection in cattle imported into the country. Further, it is important not to import any persistently infected BVDV cattle or antibody positive cows that may carry infected foetuses.

Introduction

Neosporosis and bovine viral diarrhoea are diseases with worldwide distribution among domestic animals which result in severe economic losses to the cattle industry (Houe, 1999; Dubey, 2003). *Neospora caninum* is an apicomplexan parasite and is considered a major cause of abortion in dairy cattle in many countries (Anderson et al., 1991; Barr et al., 1991; Wouda et al., 1997c; Kashiwazaki et al., 2001). Cows infected with *N. caninum*, both acutely and chronically, have up to 5.7 -18.9 higher risk of abortion than non-infected cows (Thurmond and Hietala, 1997; Lopez-Gatius et al., 2004). *N. caninum* has a two-host life cycle, including intermediate hosts (cattle, sheep, dogs and other warm-

blooded animals) and definitive hosts (dogs, coyotes) (Dubey and Lindsay, 1996; McAllister *et al.*, 1998; Gondim *et al.*, 2004). In cattle, transplacental transmission from infected dams to their offspring appears to be the major natural route of infection, and congenitally infected calves remain persistently infected and can pass the infection to their offspring (Anderson *et al.*, 1997). However, the point source exposure to *N. caninum*, e.g. through oocyst-contaminated fodder or drinking water, is regarded as the most probable cause of infection in some herds (McAllister *et al.*, 2000; Schares and Conraths, 2001; Dijkstra *et al.*, 2002). Presence of *N. caninum* specific antibodies indicates that an animal is infected with the parasite (Björkman and Uggla, 1999) and an IgG avidity ELISA can be used to discriminate between chronic and acute *N. caninum* infection (Björkman *et al.*, 1999).

Bovine viral diarrhoea virus (BVDV) may cause repeat breeding, embryonic death, abortion, stillbirths and congenital defects in infected pregnant cattle (Moennig and Liess, 1995). In non-vaccinated herds, the seroprevalence differs among areas or countries, ranging between 20% and 90% (Bolin *et al.*, 1985; Alenius *et al.*, 1986; Houe, 1995). Between 1-2% of all cattle are persistently infected (PI) with BVDV in most countries without any control program against BVDV (Houe, 1999). The main route of infection of herds is through the introduction of infected animals or by close contact with infected herds (neighbourhood, common pastures, etc.) (Houe, 1995; Valle *et al.*, 1999). Other less likely sources of infection could be contaminated semen and virus transmission through workers' clothing or instruments (Lindberg and Alenius, 1999; Niskanen and Lindberg, 2003). BVDV can induce immunosuppression and contribute to the severity of infections by other pathogens (Baker, 1995).

Because of a high local demand for dairy products for consumption, and a support for dairy cattle development from the Government, dairy cattle population has been increasing during the last few years in Vietnam. In year 2002, there were approximately 50,000 dairy cows in Vietnam (Tuyen and Giao, 2002) in smallholder farms or state farms. Until now, there are no reports of BVDV infections in dairy cattle in Vietnam. The only investigation that has been done on *N. caninum* showed a 5.5% seroprevalence in cattle (Huong *et al.*, 1998). The aim of this study was to investigate the prevalence of *N. caninum* and BVDV in dairy cows in Southern Vietnam.

Materials and methods

Animals

The present study comprises 345 blood samples collected from dairy cows in the Southern part of Vietnam. They were kept either on state farms (herds 1-5, n = 215) or on smallholder farms (97 herds; n = 130).

The state farms were situated in the Mekong delta in Southwest Vietnam. The herd size varied from 53 to 98 animals. Herds 1, 2 and 5 were located in An Giang province and herds 3 and 4 in Can Tho province. Herds 1 and 2 consisted of cows

that had been imported in 2002. Herds 3 and 4 comprised both cows that had been imported in 2002 and local crossbreeds between Holstein Friesian and Lai Sind dairy cattle, whereas the cows in herd 5 were all local crossbreeds. Some young calves from herds 1-2 were moved into herd 5 for 5-6 months before our sampling. The state farms had similar management practices. All cattle were kept in open stalls; the dry cows, heifers and young stock were kept in separate pens in the same stall as the lactating cows. The calves were housed separately until 3-4 months old. The cows were milked twice a day. All animals except the young calves were free ranging together in a feed yard for 4 hours each day. The cows were vaccinated against foot and mouth disease and septicemic pasteurellosis, but no other vaccines were used. Dogs were also kept on the farms.

The smallholder farms were located in the Ho Chi Minh city area. The cows were local crossbreeds of Holstein Friesian and Lai Sind breeds. The herd size varied between 2 and 7 animals.

Sample collection, treatment and storage

Blood samples were collected between January and July 2003. Approximately 50-60% of the cows in each state herd were sampled. In each smallholder herd, at least one blood sample was collected.

The samples were collected from the coccygeal vein into sterile tubes without anticoagulant. The samples were left at room temperature (29-33 °C) for 1-2 hours. Sera were harvested and transported on ice to the laboratory where they were stored at -20 °C. They were then transported to Sweden where they were inactivated at 56 °C for 90 minutes, refrozen, and stored at – 20 °C until analysis.

Antibody detection

The iscom ELISA described by Björkman et al. (1997) and Frössling et al. (2003) was used for demonstration of *N. caninum* antibodies. The sera were diluted 1:100 in PBS-T before analysis. All optical density (OD) values were correlated to a positive control serum with a mean OD value of 1.00. Sera with corrected OD \geq 0.20 were considered positive. To detect BVDV antibodies, a commercial indirect ELISA-kit (SVANOVA Biotech AB, Uppsala, Sweden) was used. The test was performed according to the instructions of the manufacturer. Sera with corrected OD values $>$ 0.25 were deemed positive. Both positive and negative control sera were included in each assay.

Neospora avidity ELISA

Sera with *N. caninum* Elisa OD values \geq 0.40 were tested by avidity ELISA using described procedures (Björkman et al., 1999). Briefly, sera were diluted in 5-fold serial dilutions (initial dilution, 1:100) and applied in duplicate microtitre plate wells. After incubation with serum, 1 well for each sample was treated with urea to release low avidity antibodies from the antigen-antibody complex. Absorbance was measured in untreated and treated wells after incubation with conjugate and substrate. The IgG avidity value was calculated using the formula:

$$\text{IgG avidity} = (\text{end point titre with urea}/\text{end point titre without urea}) \times 100$$

To interpret the result of the IgG avidity value, IgG avidity values > 40 were considered indicative of chronic *N. caninum* infection.

BVDV antigen detection

A BVDV antigen ELISA test kit (HerdCheck: BVDV Ag/Serum, IDEXX laboratories, INC) was used for analysis of presence of BVDV antigen in all serum samples negative for antibody to BVDV. This test is based on the detection of the envelop associated glycoprotein E_{ms} (gp48). The analysis was performed according to the instructions of the manufacturer.

Statistical analysis

Infection rates of herds or in cows of different origin were compared by Chi-Square test. Student's t-test was used to compare avidity values in imported cows and local crossbreeds. Data processing was done using the Minitab, release 13.31 software (Minitab, PA, USA).

Results

N. caninum

In the state herds, the overall seroprevalences of *N. caninum* was 41% (88/215). The infection rates varied between the herds ($\chi^2 = 14.1$, df = 4, p ≤ 0.001) and was higher in herds 1-4 having imported cows than in herd 5 with only local crossbreeds (Table 1). Sixty nine of the *N. caninum* seropositive samples had OD values ≥ 0.4 and were tested for IgG avidity (Figure 1). In herds 1-4 the majority of the seropositive cows had IgG avidities > 40.

In the two herds with both local crossbreed and imported cows, the seroprevalence was significantly higher in the imported cows ($\chi^2 = 3.8$, df = 1, p ≤ 0.05). There was no difference in mean avidity values between imported and local crossbreeds. Six cows had avidities < 40 indicating that they were recently infected.

Of the 130 sera collected in smallholder farms, 25 (19%) had antibodies to *N. caninum*. This overall seroprevalence was significantly lower than found in any of the state herds (1-4) having imported cows (p < 0.05). Thirteen of the *N. caninum* seropositive samples were tested for IgG avidity. Eight of them (62%) had avidity values > 40.

BVDV

The BVDV seroprevalence in the state herds varied between 58% and 93% (Table 1). The prevalence in herd 5 (58%) having only local crossbreeds was lower than that in the other herds ($\chi^2 = 22.4$, df = 4, p ≤ 0.001). A significant difference was also found between local crossbreeds and imported cows in the state herds 3 and 4 (χ^2

=15.9, p<0.001). In smallholder herds, the prevalence of BVDV among the sampled cows was 18% (23/130) and lower than that in the state herds ($\chi^2 = 136.7$, p<0.001).

The BVDV prevalences by age groups are shown in Table 2. No significant difference was found. Herd 1 and 2 had only cows ≤ 4 years and were not included in the comparison.

When all antibody negative sera (n = 146) from the state farms and smallholder herds were analysed for presence of BVDV antigen by ELISA, they were all negative.

Association of BVDV and N. caninum

In the smallholder herds, 11 samples of the 25 (44%) *N. caninum* positive animals also had antibodies to BVDV and there was a strong association between seropositivity to *N. caninum* and BVDV ($\chi^2 = 8.9$, p=0.003). No such association was seen in the state farms.

Discussion

Moderate to high prevalences of *N. caninum* and BVDV seropositive cows were found in this investigation performed in Vietnamese dairy cattle. This is the first report on BVDV infection in Vietnam.

The within herd seroprevalences of BVDV in the state herds 1-4 with imported cows were 78-93%. Such high infection rates are usually found in unvaccinated dairy herds with ongoing active BVDV-infections (Houe, 1995). Persistently infected animals are frequently found among the young calves in such herds. Also the milking cows can be PI but the prevalence of mature PI animals is often very low (Houe, 1995). Higher seroprevalences were found in herd 1-4 than in herd 5 with only local crossbreed cows. Also, an overall higher BVDV infection rate was seen in imported cows compared to crossbreed cows from state herds and smallholder herds. Taken together, these results imply that some of the imported cows may have been PI or PI carriers and therefore had spread the infection in the herds. Even though all samples from BVDV antibody negative samples were also negative for BVDV antigen, it should be noted that only adult cows were sampled. Any PI animals among the imported cows may have died or been traded before our sampling. Therefore, PI animals could still be suspected to be present among the young animals and additional investigations of calves in the state farms are required.

Even though the prevalence in herd 5 which had only local crossbreed cows was lower, 58%, than in the other state herds it was considerably higher than the 18% found among the cows from smallholder herds. Herd 5 had received calves from state herds (herds 1 and 2) 5-6 months before the sampling. If any of these calves were PI they could have infected the cows with BVDV, thus explaining the

comparatively high infection rate. There might also be other reasons for the difference in prevalence between herd 5 and the cows from smallholder farms, e.g. herd size, population density and management factors (Houe, 1995).

The fairly low seroprevalence among the cows from smallholder herds indicates that BVDV is not a major problem in these herds. This finding is consistent with reports from other investigations that large herds often have higher infection rates than small herds (Loken et al., 1991; Houe, 1995; Mockeliuniene et al., 2004). Further, a recent study in Thailand has shown that the majority of smallholder dairy herds do not have ongoing active BVDV infections despite the fact that the proportion of seropositive cows is relatively high (Kampa et al., 2004). Also in the Thai herds, the majority of the BVDV seropositive cows were imported and no antigen positive animals were found.

The within herd prevalences of *N. caninum* in the state herds were 16-53%. This can be compared with the 31-35% that has been found in Australian and New Zealand dairy herds (Atkinson et al., 2000; Reichel, 2000). From Thailand, infection rates up to 46% have been reported (Chanlun et al., 2002). The present study showed that *N. caninum* infection was more common in state herds with imported cows than in the herd that only had local crossbreeds and in the cows from smallholder herds. The only previous study on bovine *N. caninum* infection in Vietnam reported a 5.5% seroprevalence (Huong et al., 1998). Although no details about herd origin were available on these cows, they were all local crossbreed dairy cattle (Dr L. T. T. Huong personal communication).

By eluting weakly bound IgG antibodies produced early on after the initiation of infection, the *N. caninum* IgG avidity ELISA effectively distinguishes between acute and chronic infection (Björkman et al., 1999; McAllister et al., 2000). Most of the cows in this study had avidity values >40 indicating that they had been infected for at least 3 months. *N. caninum* infection seems to be lifelong and persistently infected cows give birth to infected offsprings during consecutive pregnancies (Björkman et al., 1996; Schares et al., 1998). Thus, once introduced into a herd, *N. caninum* can be expected to remain for a long time (Anderson et al., 1997; Davison et al., 1999). The IgG avidity pattern can be used to estimate the relative importance of transplacental versus horizontal transmission in infected herds (Björkman et al., 2003; Frössling, 2004). The pattern found in the state herds suggests that horizontal transmission was occurring in herds 1, 3, 4 and 5.

In this study, there was a positive association between being seropositivity to *N. caninum* and BVDV in cows from the smallholder herds, but not in cows from the state farms. This observation is hard to explain. Such an association has been reported in Swedish dairy cattle by Björkman et al. (2000), whereas He et al. (2004) found no association between positive tests for *N. caninum* and BVDV antibodies in a study performed in Australia.

Given the high prevalences of *N. caninum* and BVDV among imported cows found in this study, it seems advisable to test for *N. caninum* infection in cattle

imported into the country. Further, it is also essential not to import any PI BVDV cattle or antibody positive cows that may carry infected foetuses. Today there is, to our knowledge, no requirement to test for either *N. caninum* or BVDV before cows are exported.

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Table 1: Results of *N. caninum* and BVDV antibody analysis of serum samples collected from dairy cows in state and smallholder herds in South Vietnam

Herd number	Total number of cows	Number of sampled cows (imported/ local cows)	<i>Neospora caninum</i> Positive samples (%)		BVDV Positive samples (%)		Origin of cows	Province
			Total	Imported cows	Total	Imported cows		
1	78	46 (46/0)	21 (46)	-	43 (93)	43 (93)	-	Imported An Giang
2	98	53 (53/0)	26 (49)	26 (49)	45 (85)	45 (85)	-	Imported An Giang
3	53	38 (33/5)	20 (53)	19 (58)	35 (92)	31 (94)	4 (80)	Mixed Can Tho
4	67	40 (23/17)	15 (38)	10 (43)	31 (78)	18 (78)	13 (76)	Mixed Can Tho
5	70	38 (0/38)	6 (16)	-	6 (16)	22 (58)	-	22 (58) Local An Giang
Total	215 (155/60)	88 (41)	76 (49)	12 (20)	176 (82)	137 (88)	39 (65)	
Smallholder herds	Herd size 2-7 97 herds	130 (0/130)	25 (19)	-	25 (19)	23 (18)	-	23 (18) Local Ho Chi Minh

Table 2: Distribution of BVDV seroprevalence by age in state herds and smallholder herds in south Vietnam

Herd number	Number of sampled cows (2-4y)	Number of sampled cows (> 4y)	BVDV-positive samples (%)	
			2-4y	> 4y
1	46	-	43 (98)	-
2	53	-	45 (85)	-
3	36	2	33 (92)	2 (100)
4	25	15	19 (76)	12 (80)
5	28	10	20 (71)	2 (20)
Total	188	27	160 (85)	16 (59)
Smallholder herds	92	38	19 (21)	4 (11)

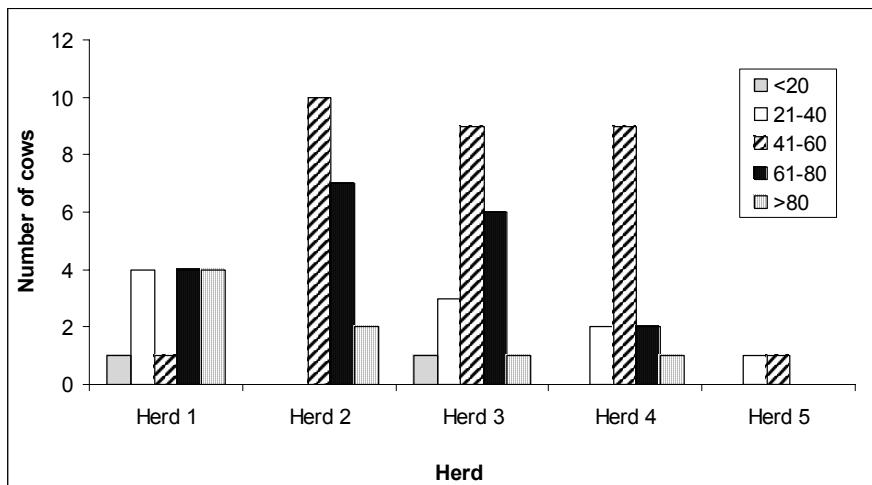


Figure 1: *N. caninum* IgG avidity in seropositive cows in 5 state herds in south Vietnam

GENERAL CONCLUSIONS

The main conclusions of this research report are:

- *N. caninum* and BVDV infections are present and appear to be widespread in dairy cows in south Vietnam. This is supported by the presence of specific antibodies to these agents in the sampled cows.
- Seroprevalences of *N. caninum* and BVDV varied among the investigated state herds and were higher in imported cows than in local crossbreeds.
- Seroprevalences of these infectious agents were low among cows from smallholder herds with only local crossbreeds.
- There were very high BVDV seroprevalences in the state herds indicating presence of PI animals. No such animals were found among the sampled cows, but additional investigations of the calves in these herds are required.
- It is desirable that only *N. caninum* and BVDV free animals are imported to the country in the future.

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