

**Bovine Viral Diarrhoea Virus
and
Other Reproductive Pathogens**

Epidemiological Studies in Peruvian Cattle

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Abstract

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This thesis deals with aspects of the epidemiology of bovine viral diarrhoea virus (BVDV) of relevance to control, with particular focus on the situation in Peruvian smallholder dairy farms. In addition, the importance of *Neospora caninum* and bovine herpesvirus type 1 (BHV-1) is addressed, as these are important differential diagnoses to BVDV.

The prevalence of BVDV and BHV-1 was studied in smallholder dairy farms in a rural area. Using bulk tank milk testing for detection of antibodies it was shown that the prevalence of active BVDV infection was low, and that a large proportion of the herds were free from BHV-1. A study of the BVDV situation in the major dairy region in the country demonstrated a very high level of exposure to BVDV. Individual testing of young stock indicated, however, that many herds might be cleared from BVDV without intervention, and that a long-term reduction in prevalence can be achieved as long as adequate attention is given to systematic measures aimed at preventing re-introduction of infection.

An endemic abortion problem in a dairy herd with concurrent infections with *Neospora caninum* and BVDV was investigated. Using a prospective seroepidemiological approach it was possible to estimate the magnitude of associations between the infections and risk of abortion, and to demonstrate that *Neospora caninum* infection significantly affected the risk within the herd. Evidence of active BVDV infection, on the other hand, was not associated with abortions.

The phylogenetic analysis of BVDV strains isolated from animals detected as persistently infected, demonstrated that all belonged to genotype 1, subtype 1b. The genetic similarity with previously described strains reflected the role of livestock trade for the diversity of BVDV. Imported livestock is the probable source of BVDV strains circulating in the country, and this was supported by the phylogenetic analysis.

Finally, it was demonstrated that a molecular epidemiological approach might be used to trace routes of transmission, and to identify and prevent risky behaviour.

Keywords: BVDV; self-clearance; control; phylogenetic analysis; *Neospora caninum*; BHV-1; Arequipa; Peru

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Resumen

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Esta tesis trata aspectos epidemiológicos del virus de la diarrea viral bovina (BVDV) relevantes para su control y particularmente en la situación de pequeñas granjas lecheras del país. Se incluye además la importancia de *Neospora caninum* y del herpesvirus bovino tipo 1 (BHV-1) como patógenos de importancia diagnóstica diferencial.

Utilizando leche de tanque para detectar anticuerpos contra BVDV y BHV-1, se estudió su prevalencia en animales de áreas rurales. Se constató una prevalencia baja de infección activa con BVDV y que una gran parte de los hatos estaban libres de BHV-1. Un estudio de la situación de BVDV en la región lechera más grande del país, demostró por su parte un alto índice de exposición a BVDV. Pruebas individuales en animales jóvenes indicaron sin embargo, que BVDV se habrá eliminado sin necesidad alguna de aplicar medidas especiales y que una disminución de la prevalencia de la enfermedad a largo plazo, puede lograrse si se aplican medidas sistemáticas con el objeto de evitar la re-introducción de la infección.

Se investigó un problema de abortos endémicos en un hato lechero, con infección doble de *Neospora caninum* y BVDV. Mediante un estudio sero-epidemiológico prospectivo fue posible estimar la magnitud de asociación de esta doble infección y riesgo de abortos y se demostró así mismo que una infección con *Neospora caninum* afecta significativamente el riesgo dentro de un hato. Sin embargo no se evidenció en este estudio una asociación de una infección activa con BVDV y abortos.

Un análisis filogénico de cepas de BVDV aisladas de animales con infección persistente, demostró que el virus pertenece al genotipo 1, subtipo 1b. La similitud genética con cepas antes descritas en otros países, refleja el papel del comercio de ganado para la diversidad de BVDV. Animales importados son la causa probable de la circulación de cepas del virus en el país. Esto fue corroborado por el análisis filogénico.

Finalmente, se demostró que un estudio de epidemiología molecular podría ser utilizado para identificar las rutas de transmisión e identificar y prevenir los posibles riesgos.

Palabras claves: BVDV; self-clearance; control; phylogenetic analysis; *Neospora caninum*; BHV-1; Arequipa; Peru

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To my little gang!

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Papers I-V

The thesis is based on the following papers:

I. Ståhl K., Rivera H., Vågsholm I., and Moreno-López J. (2002). Bulk milk testing for antibody seroprevalences to BVDV and BHV-1 in a rural region of Peru. *Preventive Veterinary Medicine* 56 (3), 193-202.

II. Ståhl K., Lindberg A., Rivera H., Ortiz C., and Moreno-López J. Self clearance from bovine viral diarrhoea virus (BVDV) infections - a prevalent finding in an endemically infected dairy region in Peru. (Manuscript).

III. Ståhl K., Björkman C., Emanuelson U., Rivera H., Zelada A., and Moreno-López J. (2006). A prospective study of the effect of *Neospora caninum* and BVDV infections on bovine abortions in a dairy herd in Arequipa, Peru. *Preventive Veterinary Medicine* (In print).

IV. Ståhl K., Benito A., Felmer R., Zuñiga J., Reinhardt G., Rivera H., Baule C., and Moreno-López J. Genetic diversity of BVDV isolates from Peru and Chile. (Manuscript).

V. Ståhl K., Kampa J., Baule C., Isaksson M., Moreno-López J., Belak S., Alenius S., and Lindberg A. (2005). Molecular epidemiology of Bovine Viral Diarrhoea during the final phase of the Swedish BVD-eradication programme. *Preventive Veterinary Medicine* 72 (1-2) 103-108.

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Abbreviations

BHV-1	bovine herpesvirus type 1
BTM	bulk tank milk
BVDV	bovine viral diarrhoea virus
COD	corrected optical density
cp	cytopathogenic
ELISA	enzyme-linked immunosorbant assay
FCS	foetal calf serum
FMD	foot-and-mouth disease
HR	hazard ratio
IBR	infectious bovine rhinotracheitis
IPB	infectious balanopostitis
IHC	immunohistochemistry
IPV	infectious pustular vulvovaginitis
MD	mucosal disease
nep	non-cytopathogenic
NCR	non-coding region
nt	nucleotide
OD	optical density
OIE	<i>Office International des Epizooties/</i> World Organisation for Animal Health
ORF	open reading frame
PI	persistently infected
PP	percentage positive
RT-PCR	reverse transcriptase-polymerase chain reaction
SENASA	<i>Servicio Nacional de Sanidad Agraria/</i> National Agrarian Health Service
VI	virus isolation
VN	virus neutralization

Background

Smallholder farming systems dominate livestock production in many developing countries, and are of crucial importance to both household and national economy, and thus also to rural development (McDermott, Randolph & Staal, 1999). These systems have, in general, a lower capacity to bear economic risk compared to large-scale commercial farms, and are typically much closer to the survival threshold (McDermott, Randolph & Staal, 1999). This is why smallholder households are particularly affected by endemic infectious diseases that continually threaten the health of livestock, and cause sub-optimal production, reproduction and economic losses (Tisdell, Harrison & Ramsay, 1999).

Smallholder farming systems dominate the dairy production in Peru, and ~95% of the farms have less than 20 animals (Bernet, Staal & Walker, 2001). A major obstacle for these farmers, and for the national dairy production in general, consists of reproductive failures and the mean abortion rate in some dairy areas exceeds 15% (Olivera, 2001). Bovine viral diarrhoea virus (BVDV), endemic in most cattle-raising countries, is considered one of the major causative agents for these failures (Rivera, 2001). The present project deals with aspects of the epidemiology of BVDV of relevance to control, with particular focus on the situation in Peruvian smallholder dairy farms. In addition, the importance of *Neospora caninum* and bovine herpesvirus type 1 (BHV-1) is addressed, because these are likely differential diagnoses to BVDV in herds with reproductive disorders. This project was supported by the Swedish International Development Cooperation Agency, Sida/SAREC.

Introduction

Dairy production in Peru

Peru's milk consumption is low by South American standards (Vera, 2000). However, since the 90s there has been a consistent effort to increase the consumption, and the dairy industry has experienced an average annual increase of almost 5%. In 2003, there were 296 000 agricultural holdings, with a total of 635 000 cows producing 1.2 million tons of milk (Portal Agrario). More than 50% of the national dairy production was produced in three main milk sheds: Arequipa in the south, Lima in the centre, and Cajamarca in the north. Whereas Holstein-Friesian and Brown Swiss are the predominant dairy breeds among specialized dairy producers in the main milk sheds, the local *criollo* breed and cross breeds dominate among the smallest producers in the highlands. Approximately 95% of the specialized dairy producers have less than 20 cows, and an average annual milk yield of between 2000-4500 kg per cow, depending on production system and breed (Bernet, Staal & Walker, 2001).

There are official control and eradication programmes against the major livestock diseases: foot-and-mouth disease (FMD), bovine brucellosis and bovine tuberculosis, run by the National Agrarian Health Service (SENASA). The prevalence of bovine brucellosis and bovine tuberculosis in the dairy population is low, estimated to 0.2% and 0.1%, respectively (SENASA, 2003). Since 1998, Peru has been conducting a plan to eradicate FMD throughout the national territory, and in early 2005, the southern region (including Arequipa) was approved by the World Organization for Animal Health (OIE) as free of FMD without vaccination (Fairfield, 2006).

Bovine viral diarrhoea virus

The virus

BVDV is the denomination a heterogeneous group of viruses in the family *Flaviviridae*, genus *Pestivirus*, which primarily infects ruminants. The virion is spherical, 40-60 nm in diameter, and consists of a lipid envelope surrounding a spherical nucleocapsid. The genome is a single-stranded positive-sense RNA molecule with a standard size of approximately 12.5 kb, and contains a single long open reading frame (ORF) flanked by 5' and 3' non-coding regions (NCR). The ORF is translated to a polyprotein of about 4000 amino acids, which is co- and post-translationally processed into the 11-12 mature proteins by viral and cellular proteases. The first third of the ORF encodes an autoprotease (N^{pro}) and four structural proteins (C, E^{ms}, E1, E2), whereas the remaining part of the ORF encodes for the non-structural proteins (p7, NS2-3, NS4A, NS4B, NS5A, NS5B) (reviewed in: Ridpath, 2005a).

Biotypes

BVDV can be divided into two biotypes: cytopathogenic (cp) BVDV and non-cytopathogenic (ncp) BVDV (Meyers & Thiel, 1996). This division does not correlate with virulence *in vivo*, but is based on the ability of the virus to induce cytopathic effects in cell cultures. Whereas cpBVDV induces apoptosis of the infected cell, ncp strains inhibit interferon synthesis and thus cell death (Schweizer & Peterhans, 1999, 2001; Perler *et al.*, 2000). The molecular background of the different properties of the two biotypes concerns the processing and expression of the non-structural protein NS2-3 within the infected cells, and whereas ncp strains only express NS2-3, cp BVDV also expresses the non-structural protein NS3 (Brownlie, 1990; Kummerer *et al.*, 2000). This processing of NS2-3 in cp strains is associated with genetic alterations, and insertions, duplications or deletions are often found within the genome (Meyers & Thiel, 1996; Becher, Orlich & Thiel, 1998, 2001). In nature ncpBVDV predominate, and have the ability to establish lifelong persistent infections. Cytopathogenic BVDV strains, on the other hand, do not establish persistence, and are mainly isolated from cases of mucosal disease, a fatal form of BVDV that will be described later.

Genotypes

Traditionally, pestiviruses were classified based on host species of origin and three species were recognized: BVDV, Classical Swine Fever Virus, and Border Disease Virus. This classification, however, proved problematic when it was shown that some pestiviruses, particularly BVDV, could cross the species barrier (Paton *et al.*, 1995a). Today classification of pestiviruses is based on the genetic relatedness between isolates, including genetic similarity with the type virus of the species (Heinz *et al.*, 2000). Furthermore, because pestiviruses are serologically cross-reactive, species demarcation considers antigenic relationships based on binding assays with monoclonal antibodies or cross-neutralisation assays with polyclonal antisera (Paton *et al.*, 1995b; Hamers *et al.*, 2001; Becher *et al.*, 2003). Today the genus pestivirus consists of four accepted species, because it has been shown that isolates previously classified as BVDV are genetically and antigenically diverse, and can be divided into two different genotypes: BVDV-1 and BVDV-2 (Pellerin *et al.*, 1994; Ridpath, Bolin & Dubovi, 1994). Recently, additional tentative species have been proposed, isolated from wildlife and from foetal calf serum (Becher, *et al.*, 2003; Schirrmeier *et al.*, 2004; Vilcek *et al.*, 2005).

The two genotypes of BVDV can be further subdivided, and at least 11 subtypes of BVDV-1 and 2 of BVDV-2 have been identified (Vilcek *et al.*, 2001; Flores *et al.*, 2002; Vilcek *et al.*, 2004). This heterogeneity seen among field isolates is characteristic of RNA viruses, and is a result of the high mutation rate during viral replication due to the error prone viral RNA polymerase (Bolin & Grooms, 2004). Despite this, BVDV strains isolated at different points in time, from different animals within a herd show high degree of homology, suggesting that BVDV is genetically stable after transmissions within the herd. This has led to the concept of herd-specific strains of BVDV, and is thought to be related to the role of immunotolerant, persistently infected (PI) animals within the herd (Paton, *et al.*, 1995a; Hamers *et al.*, 1998; Vilcek *et al.*, 1999).

Practical consequences of BVDV diversity

Whereas BVDV-1 is the dominant genotype found worldwide, BVDV-2 appears to prevail mainly in the United States and Canada, even though it has been reported sporadically also in Europe, Asia and South America (Beer, Wolf & Kaaden, 2002; Flores, *et al.*, 2002; Park *et al.*, 2004; Cranwell, Jones & Wakeley, 2005; Barros *et al.*, 2006; Pizarro-Lucero *et al.*, 2006). BVDV-2 was originally associated with outbreaks of severe acute BVDV or haemorrhagic syndrome (Corapi *et al.*, 1990); today however, it is well accepted that most BVDV strains are low virulent, and exhibit the same range of pathogenicity regardless of genotype or subgenotype (Ridpath, 2005b). The practical implication of BVDV diversity is thus not primarily related to differences in clinical consequences, but rather to biologically significant differences in antigenic determinants between genotypes and subtypes (Fulton *et al.*, 2003a,b; Ridpath, 2005b), and several studies indicate that these differences may contribute to BVDV vaccine failure (Zimmer *et al.*, 2002; Fulton *et al.*, 2005). Genetic and antigenic diversity should also be considered when designing diagnostic assays because it is essential that assays used for BVDV control purposes are sufficiently broadly reactive to detect all circulating strains.

Clinical manifestations

Contrary to what might be expected from its name, BVDV can be associated with pathology in most organ systems, including the respiratory, gastroenteric and reproductive systems. The outcome of a postnatal infection depends to a certain extent on viral factors, i.e. genetic and antigenic properties of the infecting strain (Ridpath *et al.*, 2000). More important for the outcome, however, are two main factors associated with the infected animal: whether it is pregnant or not, and whether or not it has been exposed previously (reviewed in: Baker, 1995; Evermann & Barrington, 2005). Immunocompetent animals that have been exposed previously have long-lasting immunity (Fredriksen *et al.*, 1999).

Infection in seronegative non-pregnant animals may result in severe disease, depending on the infecting strain (Corapi, *et al.*, 1990), but is in most cases subclinical. Typical clinical symptoms are fever, transient leucopenia, inappetence and mucosal erosions, and in calves, also respiratory and gastrointestinal signs. These signs can be a direct consequence of the BVDV infection, but also a result of secondary or concurrent infections, because BVDV is known to induce immunosuppression and increase susceptibility to other pathogens (de Verdier Klingenberg, Vagsholm & Alenius, 1999).

The main economic impact of BVDV infections, however, is probably caused by infections in the seronegative pregnant animal that result in transplacental transmission and foetal infection (reviewed in: Grooms, 2004). The specific outcome of the foetal infection depends primarily on the stage of gestation, and thus on the gestational age of the early conceptus or foetus, and a wide range of reproductive disorders can be seen in infected herds. These include embryonic

deaths, abortions, malformations, birth of stillborn or weak calves, or birth of PI calves. Abortions are most common during the first trimester but may occur at any time during pregnancy. Foetuses infected from around 30 days in gestation, and until the foetus becomes immunocompetent at around 120 days in gestation may be born PI with BVDV. PI calves are immunotolerant to the infecting strain, and are in general seronegative. If exposed to a heterologous strain, however, they may develop an antibody response (Bruschke *et al.*, 1998; Fulton, *et al.*, 2003b). PI calves shed large quantities of virus throughout their lives and are the key transmitters of the infection. They are often born weak and undersized, but many appear normal at birth. Due to an impaired immune system they are particularly susceptible to other infections, which partly explains the high mortality during young age, compared to non-infected calves (Houe, 1999). Some PI animals remain clinically unaffected and may breed satisfactorily (McClurkin, Coria & Cutlip, 1979). They will then transmit the infection to the foetus, and the offspring will always be PI.

Foetuses infected during later stages of gestation are in general able to develop an effective immune response and clear the virus. Despite this, many congenitally infected non-PI calves are weak at birth, and may be at more risk of experiencing serious postnatal health effects (Munoz-Zanzi *et al.*, 2003).

Mucosal disease

Mucosal disease (MD) is a fatal form of BVDV that develops when a PI animal is superinfected with a cp strain of the virus (Brownlie, 1990). The source of the superinfecting cp strain is either endogenous, i.e. a result of a mutation in the non-structural part of the genome of the resident ncp strain within the PI animal, or exogenous, e.g. from a live BVDV vaccine. MD is characterized by fever, anorexia, and massive mucosal erosions throughout the gastrointestinal tract, with profuse diarrhoea and death within a few days in the acute form of the disease. In the more protracted form, so-called late-onset MD, the animal may survive several months before the onset of disease.

Epidemiology

Prevalence

Infections with BVDV are widespread in most cattle-raising countries, with herd-level seroprevalences typically ranging between 70-100% in endemic areas, with 60-75% of the cattle being antibody carriers, and with an estimated prevalence of PI animals of 1-2% (Houe, 2005). Given the assumption that presence of antibodies reflects exposure to the pathogen, it can be concluded that BVDV is present worldwide. However, considering the persistence of BVDV specific antibodies (Fredriksen, *et al.*, 1999), serosurveys may give positive results years after last exposure to the virus, and the prevalence of herds with indications of current infection shows considerable variation, both on a regional and on a national level (Paton *et al.*, 1998; Graham *et al.*, 2001; Mainar-Jaime *et al.*, 2001; Viltrop *et al.*, 2002; Kampa *et al.*, 2004). In non-vaccinated populations with endemic BVDV, the within-herd seroprevalence is mainly dependent on whether

PI animals are present in the herd or not. In general, the seroprevalence is high in herds with PI animals as a result of the efficient spread of virus from the PIs to the surrounding group (Lindberg & Alenius, 1999). The prevalence and age distribution of seropositive animals in herds without PI animals, on the other hand, reflects the time that has passed since the elimination of the last PI (Houe, 1992), and theoretically, all individuals born after the elimination of the last PI animal are seronegative. This means that the within-herd seroprevalence will decrease at a pace dependent on the replacement rate, as long as the replacing animals are seronegative and non-PI.

Transmission

PI animals are the main source of infection within the infected herd, because they shed virus in very high concentrations in all bodily fluids throughout their life. Transiently infected animals may be a source of horizontal infection, and a few reports suggest that BVDV may persist in a herd in absence of PI animals (Moerman *et al.*, 1993; Moen, Sol & Sampimon, 2005). However, because they shed comparably lower amounts of virus and only for a few days during acute infection (Brownlie *et al.*, 1987), their importance for viral transmission and persistence of the infection within the herd is limited (Niskanen, Lindberg & Traven, 2002; Lindberg & Houe, 2005).

The conventional way of spread of BVDV between herds is through trade or contact with infected animals (particularly PIs, but also transiently infected animals), or through trade with dams carrying PI foetuses (PI carriers). In addition, infection can be introduced to a susceptible herd by indirect means, through the use of contaminated biological by-products such as live vaccines (Falcone, Tollis & Conti, 1999; Barkema *et al.*, 2001), or through contaminated or infected embryos or semen (Givens & Waldrop, 2004). Wildlife reservoirs have been suggested as a possible source of transmission of BVDV (Anderson & Rowe, 1998; Pizarro-Lucero *et al.*, 2005), but even though persistent infections in non-bovine hosts have been confirmed (Vilcek *et al.*, 2000; Pizarro-Lucero, *et al.*, 2005), the importance of this source is unclear (Lindberg & Houe, 2005).

Impact

Production losses and damage caused by BVDV can be considerable due to the broad effect on reproduction and general health in infected herds. Total costs due to the infection may include losses due to reduced milk production, reduced reproductive performance, growth retardation, and increased mortality and culling rate, as well as extra expenses for treatments of diseased animals. To quantify long-term losses associated with BVDV is difficult due to the insidious nature of the disease. Most estimates have been based on case studies from individual herd outbreaks, and these estimates are very variable and range between \$25-410/cow-year (Houe, 2003). Losses in any single case depend on factors as initial herd immunity, number of cows in early pregnancy, and virulence of infecting strain, and new infections in naïve herds can be associated with very high, but transient losses. In a recent study, long-term losses due to ongoing BVDV infection within a typical French dairy were estimated at between €75-133/cow-year, depending on

the severity of the infection. The total costs due to BVDV infection were comparable with costs due to mastitis (Fourichon *et al.*, 2005). At the national level, most estimates of the losses due to BVDV lie in the range of \$10-40 million per million calvings (reviewed in: Houe, 2003).

Control

For long, attempts to control BVDV infections were limited to prophylactic vaccination practices, implemented primarily to reduce or prevent clinical disease on a herd basis. Today, however, it is well established that the benefit of preventing clinical disease in transiently infected animals is negligible when considering the overall prevention and control of the disease (Brock, 2004). It is also well established that the PI animal is the key to the evolutionary success of BVDV, and that prevention of foetal infection is the key to BVDV control (Lindberg & Alenius, 1999). Consequently, modern vaccination programmes are designed not only to prevent clinical disease, but also to protect against viremia and to prevent foetal infection (reviewed in: Kelling, 2004; Fulton, 2005). Several challenge studies indicate that inactivated as well as live vaccines may prevent foetal infection under controlled experimental conditions (Cortese *et al.*, 1998; Frey *et al.*, 2002; Patel *et al.*, 2002). However, the efficacy of these vaccines to protect foetuses against infection under field conditions have been questioned (van Oirschot, Bruschke & van Rijn, 1999; O'Rourke, 2002; Graham *et al.*, 2003), and field observations, where PI calves have been born in vaccinated herds, support this concern (Van Campen *et al.*, 2000; Gaede *et al.*, 2004; Graham *et al.*, 2004). The antigenic diversity among BVDV field strains may, as previously mentioned, partly explain these vaccine failures. However, difficulties associated with reaching complete foetal protection under field conditions are not only related to vaccine and viral properties, but also to human factors, as inconsistent use of the vaccines, combined with the risk of conveying a false sense of security, with subsequent biosecurity¹ breaches. Moreover, because 100% efficacy and coverage is needed to prevent the infection from being established, if it is introduced, vaccination has, despite the widespread use, failed to reduce the incidence and prevalence of BVDV (Lindberg & Houe, 2005).

Another strategy to control BVDV has evolved during the last decades within eradication programmes in Europe, and particularly in the Scandinavian countries. This has been based on an initial determination of herd BVDV status, followed by implementation of systematic zoo-sanitary measures at a regional or national scale (without the use of vaccines) to prevent introduction of BVDV in non-infected herds, and to reduce the prevalence of infected herds by identification and elimination of PI animals (Lindberg & Alenius, 1999; Greiser-Wilke, Grummer & Moennig, 2003; Sandvik, 2004). These programmes have been very successful, and all of the Scandinavian countries are currently either free, or almost free from

¹ Biosecurity in this context means reduction of risk factors for introduction and spread of the infection.

BVDV (Nyberg, Østerås & Plym-Forshell, 2004; Hult & Lindberg, 2005; Rikula *et al.*, 2005).

Today, after the success of the control programmes in Scandinavia, it is well established that BVDV control must comprise the necessary elements: biosecurity, virus elimination and monitoring. The role of vaccination, on the other hand, is more controversial. According to a general model proposed by Lindberg and Houe (2005), vaccination of non-PI animals without natural immunity, could, in addition to the three necessary elements, constitute an optional element in areas where the risk of re-introduction into free herds is perceived as very high, and such a model is currently underway in Germany (Moennig *et al.*, 2005).

Self-clearance

Reduction of the prevalence of infected herds can, as previously mentioned, be achieved through systematic identification and elimination of PI animals. Under some circumstances, however, infection may be eliminated from an infected herd without intervention, i.e. through self-clearance. This is an important phenomenon that works in favour of control, and that occurs when PI animals do not succeed in establishing additional persistent infections before they are removed from the herd due to death, trade or culling (Lindberg & Houe, 2005).

Diagnostics

In general, diagnostic assays for detection of BVDV infections can be divided into serological methods, i.e. methods aimed at detection of virus-specific antibodies as an indication of exposure to the pathogen, and methods aimed at detection of infectious virus or viral components (antigen or nucleic acids), as an indication of current infection (reviewed in: Sandvik, 2005).

Serological methods

In the context of BVDV control, the main objectives of serological methods are to differentiate between exposed and non-exposed herds, to monitor progress and drawbacks within an ongoing control programme, and to investigate immune status in individual animals in infected herds, to identify possible PI animals (Lindberg & Alenius, 1999). A PI animal is, as previously mentioned, generally seronegative, unless it has persisting maternal antibodies, or has been exposed to and infected with a heterologous strain. Serological methods can also be used to diagnose acute infection by detection of seroconversion in paired samples.

Virus neutralization

Virus neutralization (VN) is the reference test for antibody detection. VN is sensitive and specific, but cell culture dependent and labour demanding and will typically take 5-6 days to perform. Thus, it is mostly used as a reference test for back-up and calibration purposes (Sandvik, 2005).

Antibody ELISA

For testing of large series of samples, enzyme-linked immunosorbent assays (ELISAs) have many advantages over VN. They are rapid, relatively inexpensive both to establish and run, and are suitable for automation. Two principally different ELISA formats are common in use for antibody detection: indirect or blocking (competitive) assays. In the indirect format, specific antibodies are trapped by immobilized viral antigen, and subsequently detected using enzyme-conjugated species-specific antiglobulins. A positive reaction is recognized by colour development in the substrate solution, which is read optically and reported as optical density (OD) values. In blocking ELISAs, virus-specific antibodies in the sample block the binding of conjugated virus-specific antibodies to fixed viral antigen. Thus, a positive sample causes a reduction of the OD, which is expressed as a percent inhibition, relative to the OD of a negative reference serum. Antibody ELISAs are widely used within BVDV control programmes, either as herd-level tests aimed at detecting antibodies in pooled samples of milk or serum, or at the individual level.

Detection of virus

Two main objectives for the application of virus detection assays can be distinguished: firstly, to identify PI animals within infected herds, for subsequent removal, and secondly to certify the non-infectious status of individual animals, semen or embryos, to prevent introduction of infection into non-infected herds or populations. Commonly used methods include virus isolation, different immune based antigen detection assays, such as ELISA or immunohistochemistry (IHC), and RT-PCR.

Virus isolation

Virus isolation (VI) in bovine cell cultures, followed by identification of the viral isolate by immunoperoxidase or immunofluorescence staining is considered the standard reference test for detection of infectious virus. Although VI demands time, resource and skill, it is a reliable and widely used method. Presence of antibodies, or cell toxic substances, or both in the sample, however, can yield a false negative result. Furthermore, because BVDV is a common contaminant of biological by-products of bovine origin such as foetal calf serum (FCS) and, consequently, bovine cell cultures grown in FCS-supplemented media, the cell culture system must be monitored to ensure freedom from contamination with virus or antibodies (Bolin, Matthews & Ridpath, 1991).

Detection of antigen

Assays for detection of viral antigen rely on existing BVDV-encoded antigens in the sample material, i.e. there is no amplification of the target, as is the case for VI and RNA detection protocols. This minimises the risk of cross contamination of samples, and also favours detection of PI over acutely infected animals (Sandvik, 2005).

Several formats of ELISAs are commercially available for detection of viral antigens. The basic principle consists of the use of virus-specific monoclonal

antibodies to capture viral antigens, followed by detection of antigen-antibody complex with enzyme-conjugated antibodies. Antigen ELISAs are widely used for identification of PI animals, and can be used for detection of virus in serum, buffy coat cells or skin biopsies (e.g. ear notch samples). Like VI, antigen ELISAs may yield false negative results if antibodies are present in the sample. This should be considered when testing young animals that might have persisting maternal antibodies (Zimmer *et al.*, 2004).

Immunohistochemical detection of antigen in skin biopsies, particularly ear notch samples, is another method that is used for identification of PI animals (Brodersen, 2004). This method is considered suitable for mass screening of young calves both because ear notch samples can be obtained with relative ease and IHC can be applied for detection of viral antigen in young PI calves without risk of interference with persistent maternal antibodies.

RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) is a rapid and sensitive method for detection of viral RNA that has the advantage of being insensitive to toxic substances in the specimen. A general RT-PCR protocol includes four different steps: extraction of RNA, reverse transcription to complementary DNA, primer-directed amplification, and detection of amplified products. In the classical protocols, these steps are carried out separately, which is time-consuming, and which increases the risk of cross-contamination of samples. More recent real-time RT-PCR systems combine the last three steps in a single tube, and by eliminating the need for gel electrophoresis, the risk of carry-over contamination with previously amplified DNA yielding false positive results is greatly reduced (McGoldrick *et al.*, 1999). By use of separate sets of primers and probes, BVDV-1 and -2 can also be discriminated between in the same assay (Letellier & Kerkhofs, 2003). Furthermore, by direct sequencing of the PCR products, phylogeny studies can be performed for rapid and exact identification of virus variants.

Genotyping

The genetic typing of BVDV has most frequently been based on sequence comparison of partial sequences of the 5' NCR, Npro or E2 regions (Vilcek, *et al.*, 2001; Becher, *et al.*, 2003; Nagai *et al.*, 2004; Toplak *et al.*, 2004). Analysis of the 5' NCR, a highly conserved region of the genome, has shown to be a reliable and reproducible method for genetic characterization of BVDV isolates (Ridpath, 2005b) even though the genetic resolution obtained is not as high as that obtained with more variable regions as Npro or E2. Furthermore, it is the target region for most PCR-based diagnostics, and as such a suitable target for direct sequencing from the PCR product.

Neospora caninum

Neospora caninum is an intracellular protozoan parasite, closely related to *Toxoplasma gondii*, which is increasingly recognized as a major cause of bovine abortions (reviewed in: Anderson, Andrianarivo & Conrad, 2000; Dubey, 2003). Abortions associated with the infection occur from month 3 of gestation to term

with a peak in incidence during month 4-6. The main transmission route in cattle is vertical, i.e. by transmission of the parasite from the cow to her foetus during gestation. In addition, horizontal transmission may occur, and refers to cattle being infected postnatally by ingestion of oocysts excreted from a definitive host (McAllister *et al.*, 2000; Dijkstra *et al.*, 2002). Currently, dogs and coyotes are the only species verified as being definitive hosts of the parasite, and presence of dogs at the farm has been identified as an important risk factor associated with *Neospora caninum* related abortions as well as high prevalence (Bartels, Wouda & Schukken, 1999; Mainar-Jaime *et al.*, 1999). *Neospora caninum* infected individuals remain carriers of the infection for life, and may pass the infection to their offspring (Björkman *et al.*, 1996). However, not all calves born from infected cows are congenitally infected, and what decides the outcome of pregnancy in an infected cow is still unknown (Innes *et al.*, 2002).

For laboratory diagnosis of *Neospora caninum* in the live animal, serological assays are generally applied, because presence of *Neospora caninum* specific antibodies, in general, indicates that the animal is infected with the parasite. Several assays have been developed for detection of antibodies in serum and milk, and indirect-fluorescent antibody tests (IFAT) and ELISAs are among the most commonly used (Björkman & Uggla, 1999).

BHV-1

Bovine herpesvirus type 1 belongs to the family *Alphaherpesvirinae*, and is an economically important pathogen of cattle, with worldwide distribution (reviewed in: Kahrs, 1977; Straub, 2001). It is the causative agent of respiratory and genital tract infections such as infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/infectious balanopostitis (IPV/IBP), and abortion. As with other herpesvirus, BHV-1 can establish latency, during which the agent is harboured in the sensory ganglia as viral DNA (Ackermann, Peterhans & Wyler, 1982). Reactivation from the latent state and re-excretion of virus can occur (after stimuli, such as stress or corticosteroid treatment) with the risk of transmission to susceptible animals (Pastoret & Thiry, 1985; Thiry *et al.*, 1987). Upon introduction of the virus into a susceptible herd, the infection typically spreads rapidly and completely, resulting in clinical or subclinical disease and seroconversion (Hage *et al.*, 1996).

The conventional way of controlling BHV-1 in most parts of the world is through vaccination. In a few countries within Europe (including Switzerland and the Scandinavian countries), however, BHV-1 has been eradicated through culling of seropositive animals. Most BHV-1 vaccines reduce the severity of the disease, and also virus replication and transmission, but are not able to prevent infection and the establishment of latency (Ackermann & Engels, 2006).

As for BVDV, diagnostic assays for detection of BHV-1 infections can be divided into serological methods, and methods aimed at detection of virus. Various antibody ELISAs are commonly used, and seropositive cattle are considered to be latently infected. Modern ELISAs, developed together with gene-deleted marker

vaccines, can be used to differentiate vaccinated animals from naturally infected animals. This is considered critical for trade within Europe (van Drunen Littel-van den Hurk, 2006). For virus detection, VI and PCR are commonly used (Straub, 2001).

Aims

The overall aim of this project was to address questions regarding the epidemiology of BVDV of relevance to control, with particular focus on the situation in Peruvian smallholder dairy farms. In addition, the ambition was to transfer knowledge and experience gained within the Swedish BVDV control programme to relevant institutions in Peru.

More specifically, the following aims were:

- To introduce appropriate and rapid methods for herd-level testing, and to use them to estimate the current status of BVDV, *Neospora caninum*, and BHV-1 in smallholder dairy herds in Peru (I-II).
- To estimate the probability of self-clearance of BVDV infections, and to investigate possible herd and management associated with it (II).
- To investigate the association between BVDV and *Neospora caninum*, and endemic abortions in a dairy herd with reproductive disorders (III).
- To describe the genetic diversity of BVDV strains circulating in Peru (IV).
- To characterize BVDV strains that circulate in Sweden, and to investigate whether a molecular epidemiological approach may serve to trace routes of transmission (V).

Methodological considerations

Detailed descriptions of materials and methods applied are given in each paper.

Study populations

Our first study (Paper I) was carried out in the Mantaro Valley; an agricultural region in the central highlands where mixed small-scale animal and crop farming is the dominant system. Traditionally, this was an important dairy region, but the political circumstances in the country during the 1980s and early 1990s severely affected the region, and hampered the expansion of the dairy sector (Fernandez-Baca & Bojorquez, 1994). Difficult access, due to the poor conditions of the road system that links the highlands to the coast, also contributed to the regression and isolation of the sector. Today, however, with political stability in the country and infrastructural investments being made, the dairy sector in the region has the potential to expand. The estimated annual milk production in 2003 was 19000 tons (Portal Agrario). At the time this study was performed, none of the major dairy companies were present in the region, and there was a lack of information on livestock demography. The selection of the 60 herds included in the study was therefore based on convenience.

The following studies (Papers II-III) were carried out in the region of Arequipa in southern Peru. This is the most important milk shed in country, with an annual production in 2003 of 270 000 tons, i.e. 22% of the national production (Portal Agrario). Unlike Mantaro Valley, the dairy sector in Arequipa has been expanding, and during the last decades thousands of dairy cattle have been imported to the region, from North America, Europe, and other countries within South America. Furthermore, due to the presence of major dairy companies in the region, the infrastructure is good. Today, the number of dairy producers in the region is 7000-8000, with 50000- 60000 cows in production. Cattle density is high, reaching 150-200 animals/km².

Study design

Paper I-II

In Paper I we selected 60 herds in the Mantaro Valley, and visited them in October- November 1998 for BTM sampling. In Paper II we selected 221 herds for BTM sampling, and a subset of 55 herds for data collection and individual blood sampling from a number of young animals (spot tests). BTM was collected at the milk collection plant in Majes, Arequipa, on two occasions, in August 2003 (n=192) and in February 2004 (n=195), and the 55 herds were visited for spot testing and data collection in December 2003.

We used BTM tests and spot tests to determine the BVDV status of the herds, i.e. to predict absence or presence of current infection. In addition, BTM samples were used for determination of herd-level status of *Neospora caninum* (Paper II) and BHV-1 (Paper I). The prevalence of herds with evidence of BVDV exposure,

but without indication of current infection, was then used as an indirect estimate of the probability of self-clearance (Paper II). Basic herd data and information on farm biosecurity were gathered through a short questionnaire, and a logistic regression model was used to investigate possible associations between herd and management factors, and the probability of self-clearance. However, as many of the farmers did not keep written records only very basic and robust information could be included in the analysis.

Paper III

In Paper III we selected a herd with a history of endemic abortions, to investigate the association between abortions, and BVDV or *Neospora caninum* infections or both. All animals >6 months were tested for antibodies to BVDV and *Neospora caninum* at the start of the study. Animals that were seronegative to BVDV were then tested for antigen, and those identified as PI animals were eliminated. The group of animals tested at the initial screening were followed for two years, and calvings, abortions and cullings were recorded. During this time, changes in BVDV status were monitored by consecutive samplings of young stock, and PI animals were identified and eliminated. The herd was considered free from BVDV infection one year after the start of the study. In the analysis, the results from the initial screening were used to classify animals as *Neospora caninum* infected, or not. Regarding BVDV, on the other hand, seropositivity in individual animals is not an indication of ongoing infection, but of long-lasting immunity after previous exposure to the agent (Fredriksen, *et al.*, 1999). Therefore BVDV status was assessed at the herd level and included as a seasonal effect.

Paper IV

BVDV isolates from animals identified as PI in Papers I-III, were genetically characterized in paper IV, and used to describe the genetic diversity of BVDV in Peru. In addition, we characterized a number of strains from major dairy regions in Chile.

Paper V

In this study a molecular-epidemiological approach was applied within the Swedish BVDV control programme. The control programme has been successfully running since 1993 and is in its final phase, and by August 2005, 97.8% of all herds were officially free from BVDV. Nevertheless, new infections are being detected despite the strict rules of the programme. Since 2002 there have been established routines to trace sources of new transmission and thereby identify risky behaviour. Still, in about 40% of the cases where new infections are detected in previously free herds, the route of transmission remains unidentified. The first results from this study were published in Paper V, but the project is continuing with the purpose to characterize BVDV strains that circulate in Sweden and to build up a bank of BVDV isolates from all remaining herds detected as infected within the programme. The purpose is also to apply the molecular-epidemiological approach to trace routes of infection and to survey the national BVDV situation.

Comments on herd-level tests

The cost-efficiency of BVDV control, as implemented in Scandinavia has been dependent on the use of rapid and cheap herd-level tests as BTM tests or spot tests for screening and monitoring (Lindberg & Alenius, 1999). BTM is particularly convenient because it is easy to collect and handle, and covers the entire lactating population within one sample, which facilitates screening of large populations. However, results from a single BTM test should be interpreted cautiously since it excludes non-lactating individuals. Thus, a herd may be falsely considered non-infected (or infected) if a large proportion of the seropositive (or the seronegative) cows are dry or low producing at the day of the sampling. This problem is probably larger in small herds, because milk from an individual animal will then represent a larger proportion of the BTM. By using repeated sampling, however, one can reduce the probability of such misclassification and increase the usefulness of the test.

Herds with current infection will generally have high within-herd seroprevalence of BVDV, and, consequently, high levels of BVDV antibodies in BTM (Niskanen, 1993). On the other hand, herds with previous exposure to BVDV, but without current infection, may also have high levels of antibodies in BTM due to high and long-lasting antibody titres in previously infected animals. Generally, the prevalence of seropositive animals, and consequently the antibody levels in BTM, will decrease at a pace dependent on the replacement rate, as long as the replacing animals are seronegative and non-PI. In smallholder dairy systems, replacement rate is typically slow because cows are kept longer than in larger commercial dairy systems, and this should be kept in mind when interpreting the results. In regions where the use of vaccines (particularly live vaccines) is widespread, the vaccination status of the herd should be considered, because the potential presence of vaccine-induced antibodies in vaccinated herds creates difficulties in the interpretation of the results.

A spot test is a herd-level test based on individual samples of serum or milk from a small number of animals of a certain age group, which can be used to predict presence or absence of current BVDV infection in herds with high antibody levels in BTM due to previous exposure (or vaccination). This principle, used within BVDV control programmes in Scandinavia, is based on the high probability of seropositivity in groups of animals where PI animals are present because of the efficient spread of virus from PI animals to the surrounding group (Lindberg & Alenius, 1999). A negative spot test in a (non-vaccinated) herd with a positive BTM test indicates that the herd has been exposed to BVDV, but that the infection has been eliminated. Given that the infection has been eliminated without intervention, a negative spot test can be interpreted as an indication of self-clearance (Paper II). As with other herd-level tests, results from single spot tests should be interpreted cautiously and should not be used to rule out infection, because herds where PI animals have recently been introduced might be missed. To be reliable and useful for monitoring within a control programme spot tests must therefore be repeated at regular intervals.

Survival analysis

In Paper III, Cox proportional-hazards models were used to investigate the association between *Neospora caninum* and BVDV infections, and the endemic abortions. These are semi-parametric survival models that ignore the distribution of the survival-time (i.e. time-to-abortion in this case) and that assume that the hazard ratio, i.e. the effect of a unit change in a predictor on the frequency of the outcome, is constant. By using survival models in the analysis, we could utilize information from all observations, even from those lost to follow-up (e.g. cows that were sold or culled) while at risk of abortion. This way we could also model the herd-level effect of BVDV as time-dependent, because the BVDV status of the herd changed during the study. The preliminary data suggested that the assumption of proportional hazards was violated and that the effect *Neospora caninum* infections on the abortions was dependent on when the abortions occurred. This observation was supported by previously published results (Lopez-Gatius, Pabon & Almeria, 2004). Therefore, two outcomes for the models were defined: early abortions (between day 42 and day 100 in gestation), and late abortions (after day 100). In the models, we considered the possible confounding by parity and animal origin. Furthermore, because data from repeated pregnancies were included, we also considered possible lack of independence between observations, and included frailty effects. The coefficients for the frailty effects were not significantly different from zero, however, and were excluded from the final models.

PCR and genotyping

In Paper IV and V, standard protocols were used to amplify and characterize a segment of the 5'NCR of included BVDV strains. This included routine precautions and safety measures to avoid cross contamination of the samples (Belak & Ballagi-Pordany, 1993). For the RT-PCR we used a fully automated one-step protocol, which further reduced the handling of the samples and, consequently, the risk of contamination. The target region was then sequenced directly from the PCR product. For the phylogenetic analysis we used a 237 nucleotide fragment of the 5'NCR, corresponding to position 135-371 of BVDV SD-1 (Deng & Brock, 1992). Reference strains and previously described field isolates representing different subtypes of BVDV-1 were obtained from the GenBank and included for comparison. Phylogenetic trees were constructed using neighbour-joining, and bootstrap resampling was used to evaluate the robustness of the method.

Results and discussion

A detailed description and discussion of the results is given in each paper.

Prevalence of BVDV infections and self-clearance (Papers I and II) *BTM*

The results from the BTM testing clearly demonstrated that most of the herds in Mantaro Valley (92%) as well as Arequipa (100%) had been exposed to BVDV. However, whereas >95% of the herds in Arequipa had high levels of antibodies in BTM, suggesting current or recent infection, most of the herds in the Mantaro Valley (73%) had low or moderate levels (Fig.1). This discrepancy reflects the differences of the study populations. The dairy sector in Arequipa is strongly influenced by the presence of the major dairy companies and the easy access to the national dairy market in Lima (Bernet, Pradel & Walker, 2001). The expansion of the sector has led to high cattle density, and to introduction of large numbers of livestock, in most cases with insufficient, or lacking health documentation. Given an approximate prevalence of PI animals of 2% (including PI carriers), the risk (P) of introducing BVDV when buying 100 cattle with unknown BVDV status is $P=1-0.98^{100} = 87\%$ (Houe, 1999). In the Mantaro Valley, on the other hand, the relative isolation has prevented massive introduction of livestock, and this has resulted in a reduced risk of introducing BVDV.

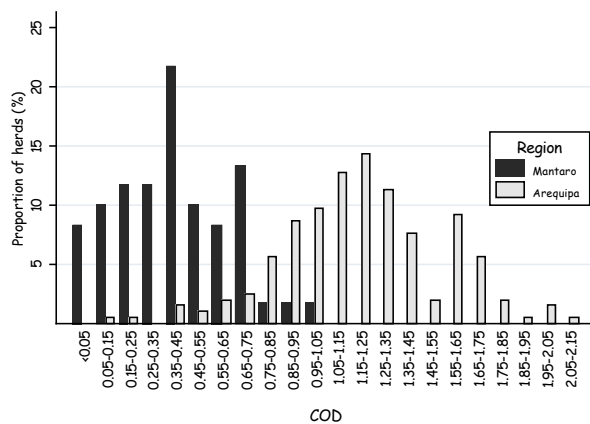


Figure 1. Distribution of BVDV corrected optical density values (COD) in bulk tank milk (BTM) as measured in an indirect ELISA (SVANOVA, Biotech, Uppsala, Sweden). BTM samples were collected in dairy herds in the Mantaro Valley (n=60) and Arequipa (n=195), Peru, in 1998 and 2004, respectively.

Spot tests

From the 55 herds we collected serum samples from 267 animals, i.e. 4-5 from each herd. Of these, 152 animals tested negative, and 115 positive. The spot tests from 35 herds (64%) had ≤ 2 positive samples, and were considered negative.

Consequently, despite the high infection pressure and the high cattle density in Arequipa, almost two thirds of the herds had spot test results suggesting absence of current infection. Given that these herds previously had been infected with BVDV, which was suggested by the high antibody levels in BTM, these results suggested a high probability of self-clearance.

Self-clearance

According to the results from the logistic regression model, the probability of self-clearance was not associated with herd size or vaccination practices. Self-clearance is theoretically more likely to occur in smaller herds, but a generally higher herd biosecurity standard in the larger herds, and an increased risk for early death in PI animals due to more intensive production and harder rearing conditions, could possibly explain this result. Regarding vaccination, any effect could be hard to detect, because the number of farmers that used BVDV vaccines was limited. However, it was noteworthy that among the seven farmers that practiced vaccination (using the same inactivated vaccine), at least six different regimes were used, clearly reflecting the fact that vaccination in general is implemented on a herd-to-herd basis leaving the decision to the farmer or responsible veterinarian.

BVDV and *Neospora caninum* infections and abortions (Paper III)

Between January 2002 and March 2004, 1094 pregnancies were confirmed in 538 cows. Of these, 137 pregnancies (13%) in 121 cows ended in abortion and 207 cows were lost from follow-up. Of the abortions, 94 (69%) were registered after day 100 in gestation. The seroprevalences of *Neospora caninum* and BVDV in the group of animals tested at the initial screening were 47% and 97%, respectively. One heifer was identified as being persistently infected at the initial screening, and another three calves at the testing in January 2003. All PI animals were eliminated, and the herd was considered free from BVDV infection after the 1st of April 2003.

According to the survival analysis, current BVDV infection was not associated with the abortion problem in the herd. BVDV infections can cause abortion at any time during gestation, but only in dams not previously exposed to the infection (Grooms, 2004). Thus, in this study any effect of BVDV infection could only be expected in the group of susceptible animals. With a BVDV seroprevalence of 97% at the initial screening, the size of this group was too small to enable us to detect any effect.

Neospora caninum infections, on the other hand, significantly affected the risk of abortions, but only those occurring after day 100 in gestation. The magnitude of the effect was most prominent in heifers, and decreased with parity. In heifers, the hazard of late abortion was >6-fold higher in infected animals compared to non-infected. In first-lactation and multiparous cows, the hazard ratios for infected animals were 3.7 and 1.9, respectively. This observation, i.e. that the hazard of late abortions associated with *Neospora caninum* infections decreased with parity, suggested that protective maternal immunity to the parasite might increase with age.

Genetic diversity of BVDV (Paper IV)

The phylogenetic analysis of 15 BVDV strains isolated from animals detected as PI during the studies in Arequipa and the Mantaro Valley, demonstrated that all belonged to genotype 1, and could further be subdivided into subtype 1b. In addition, it showed that strains circulating in Peru were genetically very similar to previously described strains from South America, USA and Europe. This was the first study of the genetic diversity of BVDV in Peru. Despite the limited number of strains included in the study, and despite the fact that most of the strains originated from Arequipa, a few interesting observations could be made. Several studies have addressed the question of the geographical distribution of BVDV genotypes or subtypes within a country, and in these a low regional variation has been found (Tajima *et al.*, 2001; Hurtado *et al.*, 2003; Mishra *et al.*, 2004). This lack of variation is probably the result of uncontrolled livestock trade within a country and an efficient spread of prevalent strains. Thus, we could expect that our results were valid also for the other major dairy regions in the country. According to the phylogenetic analysis all strains were BVDV-1b, the predominant BVDV subtype in many countries all over the world, including USA (Ridpath, 2005b). In spite of this, most vaccines on the market are produced with prototype BVDV-1a strains, sometimes in combination with BVDV-2 strains. Four BVDV vaccines are currently available in Peru; all are based on inactivated BVDV-1a, in one recently launched vaccine, in combination with an inactivated BVDV-2 strain. The genetic similarity with previously described strains was also noteworthy, and clearly reflected the role of livestock trade for the diversity of BVDV. Imported livestock is the probable source of BVDV strains circulating in the country, and that is supported by the phylogenetic analysis.

Molecular epidemiology of BVDV in Sweden (Paper V)

Between October 2002 and August 2005, more than 500 individuals from 130 herds were identified as PI within the Swedish BVDV control programme. The 5'NCR of at least one isolate from each of these infected herds have been sequenced, and it is estimated that these isolates represent >60% of all strains circulating in the country. Phylogenetic analysis has shown that all Swedish BVDV strains belong to genotype 1, and that subtypes 1b (~25%) and 1d (>70%) are the most prevalent. In a previous study from Sweden, Vilcek *et al.* (1999) demonstrated a strict herd-specific genetic clustering of BVDV, i.e. that animals within a herd were infected with one specific strain. Moreover, they observed that isolates from each of the herds included in the study were unique for that herd, based on analysis of the 5'NCR. Our analysis confirmed the herd-specificity of BVDV (data not shown), but showed that, in many cases, isolates from different herds could not be distinguished based on this conserved region of the genome. In total, we could distinguish 73 different strains, and among them 22 groups, in which isolates from between 2 and 16 different herds shared 5'NCR sequences. We assumed that this genetic similarity was an indication of a connection between the herds of origin. The results were compared with reports on suspected transmission routes, and we were in many cases able to confirm or rule out suspicions. In this way we could also identify and prevent risky behaviour.

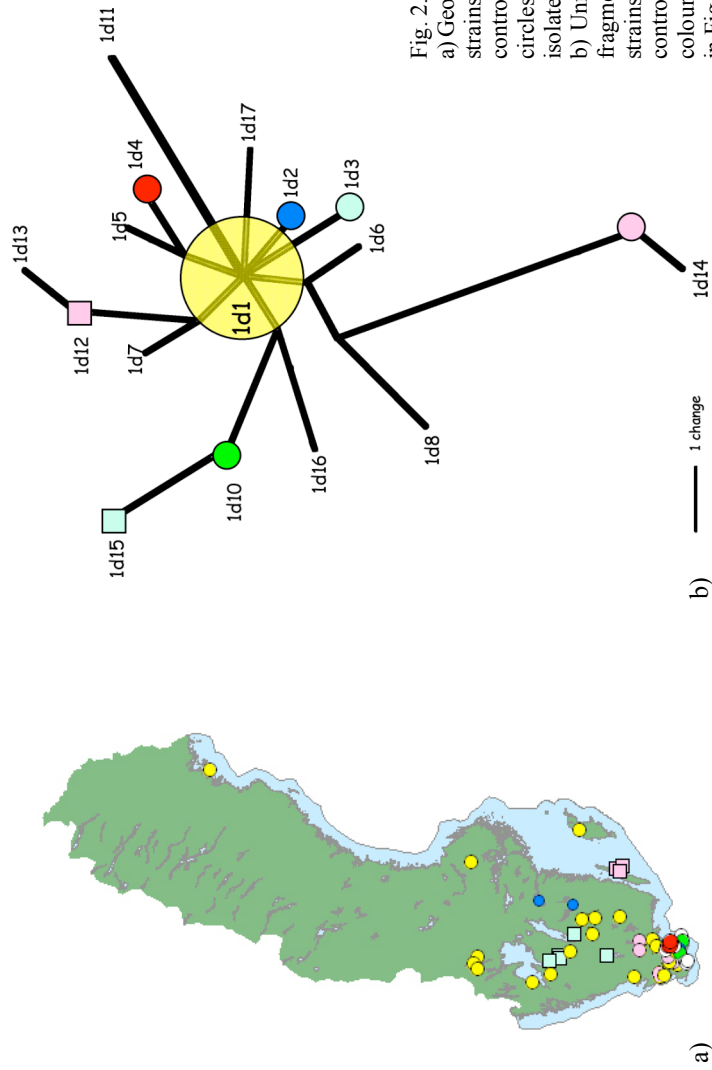


Fig. 2.
 a) Geographical distribution of BVDV-1d strains isolated during the Swedish BVDV control programme, 2002-2005. Coloured circles and squares represent groups of isolates with identical 5'NCR sequences.
 b) Unrooted phylogram based on a 237 nt fragment of the 5'NCR of 17 BVDV-1d strains isolated during the Swedish BVDV control programme, 2002-2005. Strains with coloured circles and squares are represented in Fig. 2a.

The geographical distribution of selected BVDV-1d strains, suggested as expected, a certain clustering of genetically similar isolates, with one clear exception. The strain, called 1d1, that had been isolated from 16 different herds was widely spread (Fig. 2a). Phylogenetic analysis of the 5'NCR of the same selected strains indicated that strain 1d1 was the most probable common ancestor of the other strains (Fig. 2b). The geographical distribution of strain 1d1 and the phylogenetic analysis taken together suggested that a large portion of BVDV strains currently circulating in the country originate from one single introduction, possibly of a 1d1 strain. Thus, this strain has been circulating for many years and has been able to spread over the country.

These results indicated that BVDV might circulate for many years and remain genetically unaltered. The heterogeneity of BVDV is believed to be due to accumulation of mutations generated during acute rather than persistent infections because acute infections favour survival of viral variants that are able of escaping from the immune response (Bolin & Grooms, 2004). The selective pressure and the rate at which the virus mutates under Swedish conditions, however, seems to be low. A low cattle density with restricted livestock movements and low prevalence of other bovine pathogens might, together with a non-vaccination policy, be factors that have contributed to a low selective pressure and low mutation rate. This, however, is only a speculation.

***Neospora caninum* in Arequipa** (Paper II)

The results from BTM testing in Arequipa demonstrated that 97% of the tested herds had been exposed to *Neospora caninum*, a very high level of exposure even in an international comparison (reviewed in: Dubey, 2003). Possibly, this is a result of the introduction of livestock from countries with high prevalence of the infection such as USA, Germany and the Netherlands (Bartels *et al.*, 2006). However, it also suggests a high rate of horizontal spread and between-herd transmission in the region. Dogs, which are known as the main risk factor for horizontal spread, are present on most of the farms, and many are free roaming. Animals are kept outdoors, and, typically, nothing is done to prevent the access of dogs to the cattle area. There, they may get infected by ingestion of naturally infected placentas or aborted foetuses, and then transmit the infection to the susceptible cattle through faecal contamination of water or feed (De Marez *et al.*, 1999; Dijkstra *et al.*, 2001). High cattle density may also have contributed to this situation and has previously been shown to be associated with high *Neospora caninum* prevalence (Barling *et al.*, 2000). In addition, a few studies have indicated an impact of climatic factors (Schaes *et al.*, 2004; Rinaldi *et al.*, 2005). Sporulation of oocysts is favoured by elevated temperatures, and it is speculated that rapid sporulation, shortly after shedding, might increase the chance of infecting cattle, if the contaminated feed or water is ingested immediately after contamination (Schaes, *et al.*, 2004). If so, the sunny subtropical climate in Arequipa would be favourable, and would, consequently, contribute to a high rate of horizontal spread.

BHV-1 in the Mantaro Valley (Paper I)

The results from the BTM testing in Mantaro demonstrated a moderate level of exposure to BHV-1, and approximately half of the herds tested positive. This might indicate that the infection is circulating in the dairy population, and that it has an impact on production and reproductive performance in affected herds. However, latently infected animals remain seropositive for life, and, in small sized herds, even a single seropositive animal may result in the BTM becoming positive (Kampa, *et al.*, 2004). Consequently, in smallholder dairy systems where animals are kept longer and replacement rate is low, BTM tests may be positive many years after last exposure to the virus. In a recent study from Thailand, a herd level prevalence of BHV-1 of 67% was estimated, based on testing of BTM from 220 dairy herds (Kampa, *et al.*, 2004). However, when testing individual animals in a subset of 11 herds, it was demonstrated that none of the animals younger than four years had seroconverted to BHV-1. This suggested that the risk for reactivation of latent BHV-1 infections in smallholder dairy systems with low-intensive production, is low, and indicated that progressive self-clearance might occur. It is not unlikely that this is the case also in the Mantaro Valley. Regarding the situation in Arequipa, we tested 285 lactating animals in the herd described in Paper III, for antibodies to BHV-1. In total, 30% of the animals tested positive. However, based on the distribution of COD values, it was evident that there had not been any virus circulation during the last four years (Fig. 3). This observation suggested that the risk for reactivation of latent BHV-1 might be low also in larger herds.

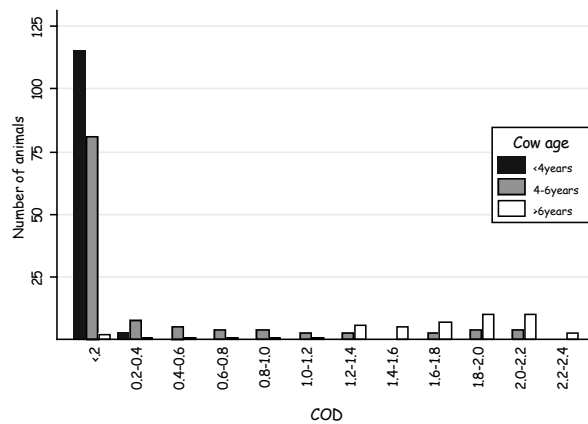


Figure 3. Distribution by age of BHV-1 corrected optical density values (COD) in milk, as measured in an indirect ELISA (SVANOVA Biotech, Uppsala, Sweden), from 285 cows in a dairy herd in Arequipa, Peru, 2002. Samples with $COD \geq 0.2$ were considered positive.

BVDV and reproductive performance (Papers II and III)

Assessment of the impact on production and/or reproduction of endemic diseases is important for the motivation of farmers as well as authorities to establish measures to reduce prevalence and incidence of the diseases in question. However, even to achieve an estimate of the level of an individual producer requires reliable information. Smallholder dairy producers in Peru typically do not keep written records. Consequently, although the focus of this project was the situation in smallholder dairy systems, we decided to perform the study described in Paper III in a larger commercial dairy farm. We believe that there is no reason that the results cannot be extrapolated to the smaller herds.

Reproductive losses due to BVDV are typically most obvious during the first period after introduction of the infection into a naïve population. As the proportion of immunized animals increases, the portion of animals that possibly can be affected decreases. The impact of the infection changes in nature, from mainly affecting reproduction to primarily affect calf health. Consequently, with a BVDV seroprevalence of 97% at the start of the study, it could be concluded that the high-risk period for reproductive losses had already passed. However, during the study three PI calves were identified, corresponding to approximately 0.75% of all calves born during the first year. Assuming a constant level of BVDV exposure to dams from insemination and throughout gestation, it could be expected that a number of infections also resulted in failure of fertilization, early embryonic deaths, or birth of congenitally infected calves that were not considered in the study. In an American study, it was demonstrated that, in endemically infected herds with a prevalence of PI calves of 0.5%, the proportion of congenitally infected calves might amount to 10% (Munoz-Zanzi, *et al.*, 2003). These congenitally infected calves might, besides an increased risk of experiencing serious postnatal health effects, also have lower fertility compared to non-infected individuals (Munoz-Zanzi, Thurmond & Hietala, 2004). Therefore, although we were unable to detect any association between BVDV and the endemic abortions in the studied herd, we can assume that reproduction as well as production was affected by the infection. Given the level of exposure in the dairy population in Arequipa (Paper II), it can also be assumed that BVDV affects reproductive performance in the dairy population in general, and that the infection contributes to the reproductive failures that severely affect the smallholders in the region.

Control

A general model for BVDV control has been described (Lindberg & Houe, 2005), and should, theoretically, be applicable also in Peru, either on a national or on a regional scale, and it is beyond the scope of this project to form recommendations on an alternative strategy. However, several of the results do relate to BVDV control in general, and under the specific circumstances in smallholder dairy systems in Arequipa, in particular.

Vaccination

Today in Arequipa, efforts aimed at controlling BVDV are restricted to the use of inactivated vaccines, typically implemented in herds affected by severe reproductive failures, to prevent abortions. However, in many cases the abortion problem continues, in spite of the implemented vaccination programme (Olivera, SENASA, personal communication). A few reasons for BVDV vaccine failures have already been mentioned, and include antigenic differences between field strains and vaccine strains (Paper IV), and inconsistent use (Paper II). When it comes to prevention of abortions, however, other factors must also be considered. The cause of abortions is in many cases not thoroughly investigated, and the decision to vaccinate is, in general, based only on serological evidence of BVDV. Because BVDV seropositivity in individual animals is not an indication of ongoing infection, but of long-lasting immunity after previous exposure, a serological response cannot be used to infer causality between BVDV and abortions. In a study from Brazil, the prevalence of *Neospora caninum* infections in dairy herds in which vaccination against BVDV and BHV-1 was used, was compared with that in non-vaccinated herds (de Melo, Leite & Lobato, 2004). Vaccinated herds had serious abortion problems despite the use of vaccines, and it was observed that the prevalence of *Neospora caninum* in these herds were significantly higher than in the non-vaccinated herds. Although not mentioned by the authors, this strongly suggested that the abortion problems were associated with *Neospora caninum* infections, and that the decisions to implement vaccination programmes were based on incorrect diagnoses. Considering the high level of exposure to *Neospora caninum* in the dairy population (Papers II and III), it is likely that this could be the case also in Arequipa. However, massive vaccination of cows is questionable also in herds with reproductive losses caused by BVDV. These herds are likely to have a high within-herd prevalence of seropositive cows, i.e. of cows with protective immunity after natural exposure. BVDV vaccination of immune animals is never economically justified, particularly not in regions with limited economic resources.

In addition, although all available BVDV vaccines are inactivated, the most commonly used vaccine is multivalent and contains live virus components. The risk of spreading BVDV through contaminated vaccines should therefore also be considered (Barkema, *et al.*, 2001).

Biosecurity and virus elimination

The ability to prevent introduction of BVDV depends mainly on the level of implemented biosecurity measures. The main risk factors and routes of transmission are well established, and can, as demonstrated by the European BVDV control programmes, be reduced. However, this requires a high degree of involvement and awareness of farmers, industry and authorities (Lindberg & Houe, 2005). By using molecular epidemiological tools (Paper IV and Paper V), biosecurity breaches and transmission routes can be identified, and the results can be used in a pedagogic way to communicate to the stakeholders, the possible outcome of insufficient biosecurity and risky behaviour.

The importance of the PI animal for within-herd transmission and persistence is well established, and the process of identification and elimination of PI animals is

required for systematic BVDV control. In Paper III, a BVDV infected herd was cleared from the infection through active intervention, i.e. through identification and elimination of PI animals, according to the principles described by Lindberg & Alenius (1999). Today, three years later, the herd remains free. However, the results from Papers I and II, strongly suggest that many herds may be cleared from BVDV even without intervention, and that a long-term reduction in prevalence probably can be achieved as long as adequate attention is given to systematic measures aimed at preventing re-introduction of infection.

Concluding remarks

- The level of BVDV exposure in the studied population is very high in a national and international comparison, partly explained by high cattle density and uncontrolled livestock trade. It is assumed that BVDV contributes to the reproductive disorders that severely affect the smallholders in the country.
- A large proportion of infected herds may be cleared from BVDV without intervention even in regions with high cattle density, high BVDV prevalence, and regardless of vaccination practices and herd size. Consequently, self-clearance is a process that should be taken into account when a control programme is under consideration.
- BVDV strains circulating in Peru belong to genotype 1, and can further be subdivided into subtype 1b. Peruvian strains are genetically similar to previously described strains from South America, USA and Europe, reflecting the role of livestock trade for the diversity of BVDV.
- A molecular epidemiological approach may be used to trace routes of BVDV transmission, and to identify and prevent risky behaviour within a BVDV control programme. A single introduction of BVDV into a susceptible population may result in widespread infection, and an introduced strain may circulate for many years and remain genetically unaltered.
- The level of exposure to *Neospora caninum* is high and the infection is an important differential diagnosis to BVDV in the studied population. *Neospora caninum* is associated with abortions occurring after day 100 in gestation in herds with endemic abortions. The magnitude of the effect of infection is most prominent in heifers and decreases with parity, suggesting that protective maternal immunity to the parasite may increase with age.
- The level of exposure to BHV-1 in the studied population is moderate. However, it is suggested that the risk of reactivation of latent infection is low.

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