Bovine herpesvirus 4 infections and bovine mastitis

Gerard Wellenberg

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Wellenberg, Gerardus Johannus

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Gerardus Johannus Wellenberg

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Promotor: Prof. Dr. J.T. van Oirschot
Faculty of Veterinary Medicine
University of Utrecht, The Netherlands
Institute for Animal Science and Health
ID-Lelystad, Lelystad

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Voor: Hermien,

Ruud en Tim

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Prof. Dr. E. Thiry

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Contents

CHAPTER 1

INTRODUCTION AND OUTLINE OF THE THESIS

1. The virus

Bovine herpesvirus 4 (BHV4) is a member of the family of *Herpesviridae*, subfamily *Gammaherpesvirinae* (Roizman et al., 1992). Epstein-Barr virus and herpesvirus saimiri are other members of this subfamily (Bublot et al., 1992); BHV4 is more closely related to herpesvirus saimiri than to Epstein-Barr virus (Lomonte et al., 1996). Bovine herpesvirus 4 has an enveloped icosahedral nucleocapsid, with a diameter of 100 nm, while the overall diameter of the virus particle is approximately 150 nm. The BHV4 virion contains a double-stranded DNA of 144 ± 6 kb, and the genome possesses a coding sequence (L-DNA) of approximately 108 kb. The L-DNA is flanked by a (G+C)-rich tandem repeat region, called polyrepetitive DNA (pr-DNA), which are sequential copies of a 1.5 – 3 kb region (Broll et al., 1999). The number of copies of pr-DNA at each end of the linear genome varies, but the average number per genome is approximately 15 (Ehlers et al., 1985). Based on restriction endonuclease patterns, BHV4 can be classified mainly in two groups, as differences between BHV4 isolates can be detected both in the unique L-DNA and in the pr-DNA fragments. Restriction analyses show that almost all American strains belong to the DN-599 group, whereas the Movar 33/63 group comprises the European strains (Bublot et al., 1990). In addition, the restriction patterns of some strains do not fit completely with those belonging to the DN-599 or the Movar 33/63 group, e.g. the LVR140 strain, which has been called the Belgian reference strain (Wellemans et al., 1983; Thiry et al., 1992), and the recently described Taiwan strain which was isolated from bovine vascular endothelial cells (Lin et al., 1999). Cloning and mapping studies indicate further that the size of the pr-DNA of BHV4 varies among isolates, and therefore, restriction endonuclease analyses can be used to differentiate between BHV4 isolates. In addition, BHV4 restriction patterns are sharply different from those of other herpesviruses.

All BHV4 strains are antigenically closely related (Osorio et al., 1985; Castrucci et al., 1986), and indistinguishable by FAT and by neutralisation with a specific antiserum (reviewed by Thiry et al., 1989). The use of a panel of monoclonal antibodies confirmed the close antigenic relationship between BHV4 strains, and only slight antigenic differences were found (Dubuisson et al., 1989a; Duboisson et al., 1989b). There is no antigenic relationship with other bovine herpesviruses (Bartha et al., 1987).

2. Viral replication and host range

The replication cycle of BHV4 in infected cells is slow (McCoy et al., 1985), and expression of viral genes was shown to be dependent on the cell cycle. Vanderplasschen et al. (1995) reported that BHV4 needs dividing cells for effective virus replication, as the S-phase of the cell cycle increased viral DNA replication and protein expression. In contrast to other animal herpesviruses, BHV4 was able to

replicate in human embryonic lung cells and giant-cell glioblastoma cell cultures (Egyed, 1998). The virus has been isolated from different ruminant species, e.g. cattle, sheep (VanOpdenbosch et al., 1988), but also from non-ruminants like cats suffering from urolithiasis (Fabricant 1977; Kruger et al., 2000). It is uncommon that a herpesvirus of ruminants is also able to infect carnivorous species (Egyed, 2000).

3. Clinical signs

Bovine herpesvirus 4 was first isolated and characterised in Hungary in 1963 from calves with respiratory disease and keratoconjunctivitis (Bartha et al., 1966). Bovine herpesvirus 4 was later isolated in the United States by Mohanty et al. (1971) from a steer with signs of respiratory disease. The virus has been isolated from cattle with various clinical signs such as conjunctivitis, pneumonia and inflammations of the upper respiratory tract (Mohanty et al., 1971; Smith et al., 1972), skin lesions (House et al., 1990), mammary dermatitis (Cavirani et al., 1990; Reed et al., 1977), enteritis (Eugster, 1979), postpartum metritis (Parks and Kendrick 1973), and chronic metritis (Wellemans et al., 1983; Wellemans et al., 1984; VanOpdenbosch et al., 1984). An association of BHV4 with abortion has been reported (Czaplicki et al., 1998), and in addition, BHV4, in combination with BVDV, has been recovered from aborted foetuses (Reed et al., 1979). Kendrick et al. (1976) inoculated a strain, isolated from a cow with metritis, into foetuses at various gestational stages. Inoculations were made in 16 cows of gestational ages ranging from 3 – 9 months. The inoculation resulted in the death of some foetuses in the 3rd and 4th month, and all older than 4 months were alive and normal at the time of delivery. One foetus was aborted in an autolysed condition and one foetus was mummified. Pathological changes observed in the foetuses at 3 – 4 months of gestation were confined mostly to lymphoreticular activation. Virus was reisolated from 4 out of 12 examined foetuses.

Studies have implicated BHV4 in orchitis with potential of virus spread in semen (Dubuisson et al., 1987). Reproduction of orchitis and azoospermia was attempted in bulls by using the V-test isolate of BHV4, but no clinical signs were apparent except that a few bulls showed temporary azoospermia and conjunctivitis (Dubuisson et al., 1989c).

Whether BHV4 was the causative agent of the above mentioned clinical signs is not clear, as the virus has also been detected in apparently healthy animals (Luther et al., 1971). Experimental infection failed to elicit any severe or moderate clinical signs in cattle and sheep (Bartha et al., 1966), but induced only mild clinical signs in cattle (reviewed by Thiry et al., 1989). In experimentally infected rabbits BHV4 may accelerate the atherosclerotic process (Lin et al., 2000). This study suggests that rabbits experimentally infected with BHV4 may be a useful model for atherosclerosis. The rabbit may

also be suitable as a model for studying the reproductive tract pathogenesis of BHV4 (Naeem et al., 1991a; Naeem et al., 1991b). After intrauterine inoculation of rabbits with BHV4 some isolates caused abortion, reabsorption and mummification. Infection of the foetus after an intravenous inoculation of rabbits with BHV4 indicates transplacental transfer. Intraurethral inoculation of cats with BHV4, which were given immunosuppressive doses of methylprednisolone-acetate prior to the inoculation of the urinary bladder with BHV4, resulted in dysuria and gross haematuria in one of the 18 inoculated cats. However BHV4 infections in cats may also remain clinically inapparent (Kruger et al., 1990).

4. Epidemiology

BHV4 is distributed worldwide. BHV4 infections have been diagnosed in the United States (Mohanty et al., 1971; Naeem et al., 1989), in several countries of Europe, and also in different countries of Africa. A serological survey study revealed that 70% of the examined cattle in Zaire were BHV4 seropositive (reviewed by Thiry et al., 1989). The BHV4 seroprevalence in cattle has also been determined in many European countries, including the countries surrounding the Netherlands (Metzler and Wyler, 1986). However, no information concerning the seroprevalence of BHV4 in Dutch cattle has been published until yet. In the northern part of Belgium 15%, and in Wallony (Belgium) 29%, of the cattle older than 1 year were BHV4 seropositive (Van Malderen et al., 1987). In the former West Germany, the BHV4 seroprevalence in cattle was 18.4%, while 38% of the bulls used for artificial insemination had BHV4 antibodies (Truman et al., 1986). BHV4 antibodies have also been detected in cats (Kruger et al., 2000), but the epidemiological distribution of BHV4 in cats is unknown.

5. Pathogenesis

Transmission experiments with BHV4 are very scarce. It has been suggested that the natural route of infection may be through the respiratory and alimentary tracts, by inspired air, and recently it has been suggested that cattle may be infected by virus-infected-cells in milk (Donofrio et al., 2000). After infection, BHV4 replicates in the epithelial cells of the upper respiratory tract and of the intestines. The virus may also replicate in peripheral blood leukocytes and may spread throughout the body by these infected cells. At that time BHV4 can be isolated from various tissues and organs, and the association of BHV4 with peripheral blood leukocytes could explain the diversity of tissues and organs from which the virus has been isolated (Egyed et al., 1996; Osorio et al., 1985).

BHV4 establishes latency after experimental and natural infection. At 48 days post-inoculation (pi), BHV4 DNA has been detected in several organs of experimentally infected cattle e.g. in the nasal

mucosa, trachea, lung and spleen, and in a low amount in lymph nodes, kidneys, tonsils and thymus (Egyed and Bartha, 1998; Egyed et al., 1996).

Several cells and tissues have been considered to be sites of viral latency. Splenic mononuclear cells (macrophages), and mononuclear blood cells (Osorio and Reed, 1983; Lopez et al., 1996) have been shown to be sites of BHV4 latency. The ability to infect a bovine macrophage cell line with BHV4 supports the likely site of virus latency in cells of the monocyte-macrophage lineage (Donofrio and Van Santen, 2001). The fact that BHV4 can persist in a latent form in cells involved in the immune system suggests that BHV4 may induce immunosuppressive effects in the host, but clear evidence has not been reported so far (Wellemans et al., 1984; VanOpdenbosch et al., 1984). Besides splenic macrophages and mononuclear blood cells also the nervous system has been shown to be a possible site of BHV4 latency (Naeem et al., 1991b; Egyed and Bartha, 1998). Recently, BHV4 has been isolated from the spinal cord of a cow with astasia. This finding supports the theory that the nervous system is one of the sites for viral latency in natural infection (Yamamoto et al., 2000).

BHV4 can be reactivated from the latent state (Castrucci et al., 1987). Stress and dexamethasone (glucocorticoids) have been reported to reactivate BHV4 in cattle (Thiry et al., 1986). In addition, BHV4 has been isolated from cows showing signs of metritis after parturition (Wellemans et al., 1986), which suggests that parturition may also be a trigger for BHV4 reactivation.

6. Diagnosis

BHV4 infections can be detected by virus isolation. But, despite the tropism of BHV4 for many cell types the virus is relatively difficult to isolate in cell cultures, e.g. Madin Darby bovine kidney (MDBK) cells, which is still the commonly used cell type for BHV4 isolation. After isolation, restriction endonuclease analyses is one of the best ways to differentiate between BHV4 strains (Bublot et al., 1990; Thiry et al., 1992).

For the detection of BHV4 DNA in organs and tissues, DNA hybridisation techniques might be used, but this has not been routinely used as a diagnostic tool (Galik et al., 1992). Recently the complete genome of BHV4 strain 66-p-347, and the thymidine kinase (Lomonte et al., 1992), the glycoprotein B (Goltz et al., 1994), the early genes, and the pr-DNA of some other BHV4 strains, have been sequenced (Broll et al., 1999; Zimmermann et al., 2001). This information offers opportunities for the development of molecular biological tools such as PCR and micro-array.

BHV4 specific antibodies have been demonstrated by complement fixation (Guo et al., 1988), dot immunobinding assay, AGIDT, indirect-FAT (Sass et al., 1974), and iELISA (Edwards and Newman, 1985). Natural and experimental infections do not induce high levels of neutralising antibodies; they

may even remain undetectable (reviewed by Thiry et al., 1989; Thiry et al., 1990). Therefore, the virus neutralisation test (VNT) is not a suitable method for BHV4 antibody detection. The iELISA and the indirect-FAT are the best standardised serological tools for the detection of BHV4 antibodies, but these test are not commercially available and the indirect-FAT is laborious (Edwards and Newman, 1985). Therefore, highly sensitive, specific, rapid and reliable tests are still required for the detection of BHV4 antibodies.

7. Outline of this thesis

The research described in this thesis started with an investigation on the possible role of viruses in the aetiology of bovine mastitis. When we found that BHV4 might be involved in the aetiology of bovine mastitis, the first part of this research mainly concentrated on the development and validation of new tools to detect BHV4 and its antibodies. These newly developed tools were used in the studies that were undertaken to gain further insight into the role of BHV4 in bovine mastitis.

Not many tests are available for the detection of BHV4, its DNA or antibodies in fluids and tissues of infected animals, and in addition, most of these tests have not been validated thoroughly according to modern principles. The detection of BHV4 infections by virus isolation is often difficult due to its slow replication cycle on many cell culture systems routinely used. Therefore, the value of a non-conventional cell type in the isolation of bovine herpesviruses, including BHV4, has been examined and will be discussed in Chapter 2. In Chapter 3, the evaluation and validation of a newly developed immunoperoxidase monolayer assay (IPMA) for the detection of BHV4 antibodies in bovine serum samples is described, and this newly developed IPMA was used to estimate the BHV4 seroprevalence in Dutch cattle at different age categories. Molecular diagnostic tools were developed and validated for the rapid and reliable detection of BHV4 glycoprotein-B and thymidine kinase DNA in bovine milk samples (Chapter 4).

Due to the high percentage of unknown causes of bovine clinical mastitis, still 20 – 35% of the clinical cases of bovine mastitis have an unknown aetiology (Miltenburg et al., 1996; Barkema et al., 1998), we performed a case-control study to examine the role of viruses in the aetiology of bovine clinical mastitis. Newly developed assays for the detection of viral antibodies and genomic sequences, in combination with new ideas concerning the isolation of viruses on non-conventional cell types, might provide new insights in the role of viruses in bovine mastitis. In a first case-control study (Chapter 5), non-conventional cell types, including bovine umbilical cord endothelial (BUE) cells, were used for the detection of viruses in milk from cows with clinical mastitis and in their controls. A second case-control study was performed to extend the first study and to attempt to confirm the results

of the first case-control study (Chapter 6). A simultaneous intramammary and intranasal inoculation of lactating cows with BHV4 was performed to examine whether the virus would induce clinical mastitis under experimental conditions (Chapter 7).

In Chapter 8, the *in vitro* susceptibility of BHV4 was evaluated to a variety of antivirals. This *in vitro* study was performed to examine whether antivirals might offer opportunities for the development of pharmaceutical drugs to inhibit the replication of BHV4.

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CHAPTER 2

SUSCEPTIBILITY OF BOVINE UMBILICAL CORD ENDOTHELIAL CELLS TO BOVINE HERPESVIRUSES AND PSEUDOCOWPOX VIRUS

G.J. Wellenberg [1], E.R.A.M. Verstraten[1], F. Jongejan[2], and J.T. Van Oirschot[1]

[1] *Department of Mammalian Virology, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands.*

[2] *Division of Parasitology and Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80165, 3508 TD Utrecht, The Netherlands,*
and
Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.

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Abstract

The purpose of this study was to determine the susceptibility of bovine umbilical cord endothelial (BUE) cells to bovine herpesvirus (BHV) 1, BHV2, BHV4, and BHV5, and to pseudocowpox virus. Detection limits and growth curves of these viruses in BUE cells were compared with those in Vero or Madin Darby bovine kidney (MDBK) cells. Detection limits were determined by inoculating cell cultures with serial 10-fold dilutions of above-mentioned viruses, and growth curves by titration of virus, harvested at various times after infecting cells at a multiplicity of infection of 0.1. The detection limits of BHV2 and BHV4 were lower in BUE cells than in Vero and MDBK cells, and cytopathic effects were observed earlier in BUE cells. In addition, BHV2 and BHV4 grew to higher titres in BUE cells than in Vero and MDBK cells. BUE cells appeared to be equally susceptible to BHV5, and less susceptible to BHV1.1 and BHV1.2 than MDBK cells. This study shows that the BUE cells are highly susceptible to BHV2 and BHV4, and the use of BUE cells can improve the laboratory diagnosis of these viruses. The use of BUE cells can also improve the isolation and growth of pseudocowpox virus.

1. Introduction

Virus isolation is the most conventional method to detect the presence of the virus in clinical samples. This method is still commonly used, despite the introduction of new advanced molecular biological techniques, such as the polymerase chain reaction. Many studies on the susceptibility of different cell culture systems to human herpesviruses have been reported (Landry et al., 1982; Mayo et al., 1985), but similar studies with animal herpesviruses are scarce. The development of new cell lines, highly susceptible to animal viruses, could improve the detection limits of viruses, and thereby facilitate the diagnosis of virus infections. This development may also lead to higher yields of animal viruses, e.g. vaccine viruses.

Bovine endothelial cells are seldom applied in veterinary virology, and the number of studies on the susceptibility of bovine endothelial cells to bovine viruses is very low. Recently, BHV4 has been isolated from milk samples from cattle with clinical mastitis. BHV4 was isolated on bovine umbilical cord endothelial (BUE) cells, whereas it was not detected on three other cell types (Wellenberg et al., 2000). This report indicated that BUE cells are susceptible to BHV4, but information regarding the replication of BHV4 in BUE cells, or the susceptibility of BUE cells to other bovine herpesviruses have not been published before. Although, the susceptibility of bovine endothelial cells to BHV4 has been described (Lin et al., 1997), these cells were derived from bovine carotid arteries, and the replication cycle and total yields of viruses may vary for different types of endothelial cells used.

We determined the detection limits of BHV1.1, BHV1.2, BHV2, BHV4, and BHV5 on BUE cells, and the susceptibility of these cells to above-mentioned viruses by growth curve analysis. These data were compared with those obtained with commonly used cell cultures.

Pseudocowpox virus was included in this study, because this virus is usually isolated on primary cells, of which the growth is more laborious, e.g. primary foetal lung cells and secondary cells (Thomas et al., 1980; Mayr and Büttner, 1990). Virus isolation has sometimes been unsuccessful even though virus-like particles were observed in lesions (Inoshima et al., 2000). The use of cells that can be maintained for many passages would facilitate the diagnosis of pseudocowpox virus infections.

2. Materials and methods

2.1 Reagents

Cells were grown and maintained in Eagle's minimal essential medium (EMEM) supplemented with 2% normal calf serum (NCS) and 0.5% antibiotic mix (stock mix containing: 10,000 IU penicillin, 11.25 mg streptomycin, 10 mg kanamycin and 5,000 IU nystatin per ml). NCS showed to be free of antibodies against BHV1, BHV2, BHV4, and BHV5. Cell monolayers were washed with Hanks minimal essential medium (HMEM). Rabbit antibodies against Von Willebrand factor (factor VIII) were obtained from Dakopatts, Denmark. All chemicals were of high grade and commercially available.

2.2 Cells

Bovine endothelial cells from the umbilical cord were isolated as described (Van der Wiel et al., 1989; Jongejan, 1991). These cells were pestivirus-free, as determined by virus isolation, and mycoplasma-free, as determined by isolation technique and by using the Mycoplasma Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) and the Immu-Mark Myco-test (ICN Biomedicals, Inc., Ohio, USA). The cells from the umbilical cord were identified as endothelial cells by immunostaining with rabbit antibodies against Von Willebrand factor, and by cell morphology. The endothelial cells appeared as typical "cobblestone" patterned monolayers. Madin Darby bovine kidney (MDBK) cells and African green monkey kidney (Vero) cells, are commonly used cells for virus propagation. Bovine foetal diploid lung (BFDL) cells and embryonic bovine trachea cells are used for the isolation of various viruses, e.g. bovine respiratory syncytial virus. Cells of passages 21 – 32 (BUE), 17 – 42 (MDBK), 214 (Vero), and 15 (BFDL) were used in the experiments.

2.3 Viruses

The BHV1.1 reference strain Cooper ("IBR-like") and the Dutch field isolate BHV1.2 strain LAB ("IPV-like") (Metzler et al., 1985; Kaashoek et al., 1995) were used. Virus stocks were prepared and titrated on embryonic bovine trachea cells. The BHV2 used was kindly provided by K. Borchers (Institut für Virologie der Freie Universität Berlin, Berlin, Germany). This virus has been isolated from teat lesions (strain BMV; B. Roizman, Chicago, USA). The American BHV4 reference strain DN-599 (Cat. no.: VR 631) was obtained from the American Type Culture Collection, Rockville, USA. The

BHV5 Australian encephalitic strain N569, which is recognised as the prototype BHV5 strain, was kindly provided by M.J. Studdert. The pseudocowpox virus has been isolated from a Dutch cow with teat lesions.
The susceptibility of BUE cells to BHV1, BHV4 and BHV5 was compared with that in MDBK cells, to BHV2 was compared with Vero cells, and to pseudocowpox virus was compared with BFDL cells.

2.4 Detection limit

Serial 10-fold dilutions were prepared from each viral strain, ranging from 100 – 0.01 $TCID_{50}$/ml, in EMEM culture medium. A volume of 1 ml of each dilution was added in triplicate to a 70-80% semi-confluent monolayer of BUE cells, MDBK, BFDL or Vero cells grown on 6-wells cell culture plates (Costar, The Netherlands), followed by an incubation step at 37°C (5% CO_2) for one hour. Thereafter, cells were washed twice with HMEM and a volume of 5 ml of this medium was added to each well. The cell cultures were incubated at 37°C (5% CO_2) for 7 days and examined daily for the presence of cytopathic effect (cpe). After this incubation period the plates were stored at –70°C.
Wells without cpe were checked for the presence of virus by a second inoculation. Therefore, 1 ml of freeze/thawed cell-virus suspension from the first inoculation was added into a new 6-well cell culture plate with a 70-80% semi-confluent monolayer of BUE cells, MDBK, Vero or BFDL cells. Cells were washed after one hour of incubation, and incubated as described above. Cells were inoculated again for 7 days and checked daily for the presence of virus by cpe. Semi-confluent cells, with only EMEM (supplemented with 2% NCS and 0.5% antibiotics) maintenance medium, were used as negative control cells. Negative control cells were washed, incubated and evaluated as described above.

2.5 Growth curves

For growth curve analysis and growth kinetics, cells were plated in 6-wells (35 mm diameter) tissue culture plates and incubated at 37°C (5% CO_2). BUE cells, and other commonly used cell types, were routinely maintained as described before (Wellenberg et al., 2000). Prior to infection, semi-confluent (70-80%) monolayers were counted in duplicate, and the mean number of cells was calculated. Cell culture medium was discarded from the remaining wells that contained semi-confluent monolayers. Bovine herpesviruses and pseudocowpox virus were inoculated onto cell cultures at a multiplicity of infection (m.o.i.) of 0.1, followed by an incubation step at 37°C (5% CO_2) for one hour. Thereafter, cells were washed twice with HMEM to remove non-adsorbed viruses. To each well, 5 ml of EMEM (supplemented with 2% NCS and 0.5% antibiotics) maintenance medium was added, and the cell

cultures were incubated at 37°C (5% CO_2). At various times thereafter, cells with supernatant were harvested, namely on: 0 (directly after washing and the addition of 5 ml of fresh EMEM), 6, 12, 18, 24, 48 and 72 hours post-infection (pi.), and frozen at –70°C. The titre of infectious virus, harvested on various times after infection of cells, was determined to create growth curves. Data of the growth curves were also used to obtain information about the growth kinetics, i.e. the eclipse period, in which the main processes of viral replication are initiated, and the total yield of infectious bovine herpesviruses and pseudocowpox virus on BUE cells.

2.6 Virus titration

The total amount of infectious virus at any given time point (sum of extracellular and intracellular virus) was determined by median tissue culture infectious dose ($TCID_{50}$) assays in 96-wells cell culture plates (Costar, The Netherlands). Therefore, serial 10-fold dilutions of virus were prepared in EMEM maintenance medium. Cells, in suspensions of 150 µl, were inoculated with 50 µl of diluted virus aliquots in 24-fold, and incubated for 7 days at 37°C (5% CO_2). Cell cultures were observed for cpe, and titres were determined according to the method of Reed and Muench and expressed as log10 $TCID_{50}$/ml. Growth curves were obtained by plotting the titres as a function of time.

The influence of cell types on virus titres was first determined by titration of infectious BHV1.2, grown in BUE cells and grown in MDBK cells, on BUE cells and MDBK cells as well. Titration of infectious BHV1.1, BHV2, BHV4, BHV5 and pseudocowpox virus was further performed on the cell type analogous to the cell type used for primary infection, i.e. viruses grown on BUE cells were quantified on BUE cells, and viruses grown on MDBK were quantified on MDBK cells.

3. Results

3.1 Detection limit

The detection limits of BHV1.1 and BHV5 in BUE cells and MDBK cells were comparable, but BUE cells inoculated with BHV5 displayed cpe earlier than MDBK cells (Table I). Lower quantities of BHV1.2 were detectable in MDBK cells (up to 0.01 $TCID_{50}$/ml) than in BUE cells (up to 0.1 $TCID_{50}$/ml), although in both cell types cpe was rapidly observed. In contrast to the BHV1 and BHV5 strains, the detection limits of BHV2 and BHV4 were lower in BUE cells than in Vero and MDBK cells, respectively. Although, BUE cells appeared to be slightly more susceptible in detecting low amounts of BHV2 than Vero cells, cpe was more rapidly observed in BUE cells. After inoculating the cells with 10 $TCID_{50}$ BHV2, cpe was observed in BUE cells on day 1 pi., whereas no cpe was detectable in Vero cells within 7 days pi. In BUE cells, even 10-100 lower levels of BHV4 were detectable than in MDBK cells, and cpe occurred more rapidly in BUE cells than in MDBK. BUE cells, that were inoculated with BHV4, displayed cpe 3-4 days pi., whereas no cpe was detectable in MDBK cells within 7 days pi. The detection limits of pseudocowpox virus in BUE cells and BFDL cells, and the appearance of cpe in both cell cultures, were comparable. None of the negative control cells showed cpe.

3.2 Growth curves

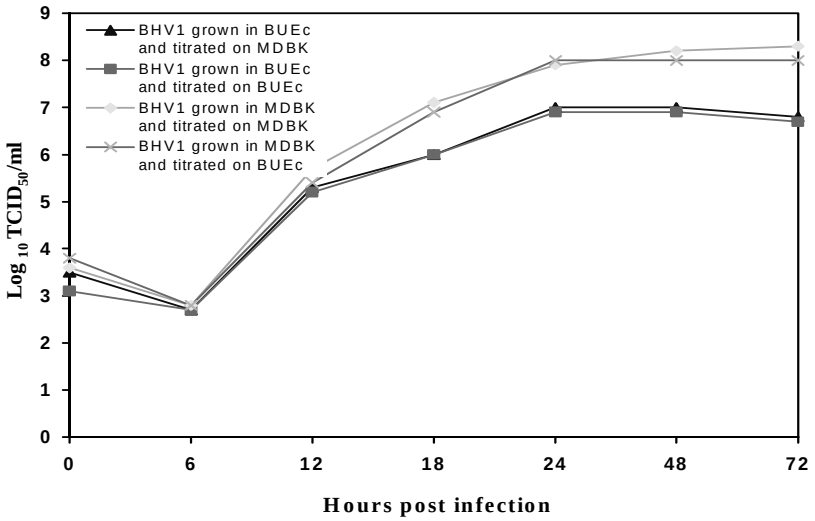

Fig 1. Growth curves of BHV1.2 in BUE cells and in MDBK cells at an m.o.i. of 0.1. Titration of BHV1.2, harvested on various times pi, was performed for both cell types on BUE cells and on MDBK cells.

The growth curve of BHV1.2 propagated in BUE cells was similar when titrated either on BUE cells or on MDBK cells (Figure 1), and a similar growth curve was also recorded of BHV1.2 propagated in MDBK cells and titrated on MDBK cells or on BUE cells. Because of this finding, the titres of the other virus harvests, harvested at various times pi., were determined on the same cells that were used to propagate the virus. The eclipse period for BHV1.1, BHV1.2, and BHV5 in BUE cells and in MDBK cell was between 6 and 12 hours pi. (Figures 1 - 2). Thereafter, virus progeny was produced at a steady rate and production reached a plateau at approximately 48 hours pi. The total yield of BHV1.1 and BHV1.2 was approximately 10 times lower in BUE cells than in MDBK cells, whereas the growth curve of BHV5 in BUE cells was comparable to that in MDBK cells (Figure 2).

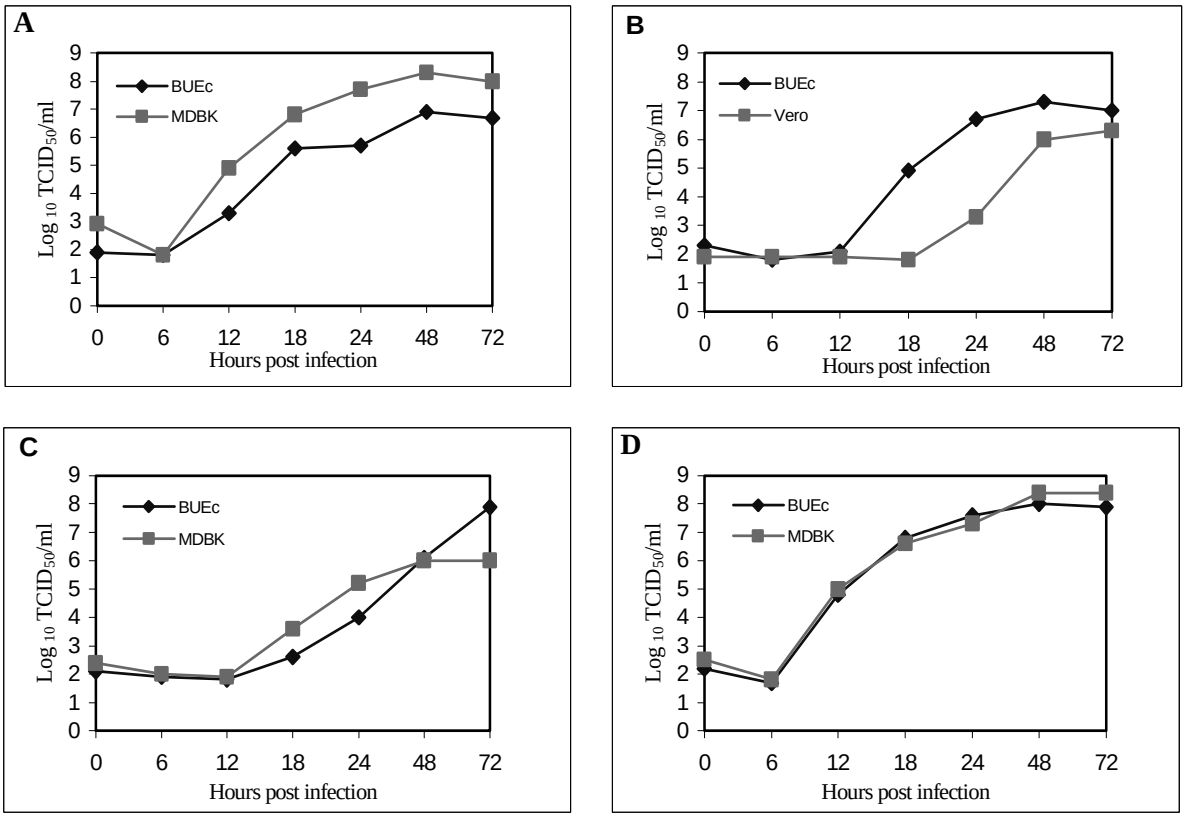

Fig 2. Growth curves of bovine herpesviruses; (A) BHV1.1, (B) BHV2, (C) BHV4, and (D) BHV5 in BUE cells and in MDBK cells or in Vero cells (BHV2) at an m.o.i. of 0.1.

Production of infectious BHV2 in BUE cells was detectable after 12 hours pi., and in Vero cells after 18 hours pi. (Figure 2). A plateau in both growth curves occurred between 48 and 72 hours pi., but at 72 hours pi., the BHV2 titre was approximately 5 times higher in BUE cells than in Vero cells. The

growth curves of BHV4 showed that replication of BHV4 in BUE cells and MDBK cells did not occur within 12 hours pi. Thereafter, virus production increased more rapidly in MDBK cells than in BUE cells. A plateau in the production of BHV4 was reached in MDBK cells around 48 hours pi, whereas this did not occur in BUE cells before 72 hours pi. At 72 hours pi., the BHV4 titre was, however, approximately 100 times higher than in MDBK cells.

The growth curves of pseudocowpox virus in BUE cells and BFDL cells were similar (Figure 3). The eclipse period in both cells was between 6 and 12 hours pi., and thereafter, the titres of pseudocowpox virus increased at a steady rate, but more slowly compared to bovine herpesviruses. The total yields of pseudocowpox virus in BUE cells and in BFDL cells were comparable at 72 hours pi., but no plateau was observed.

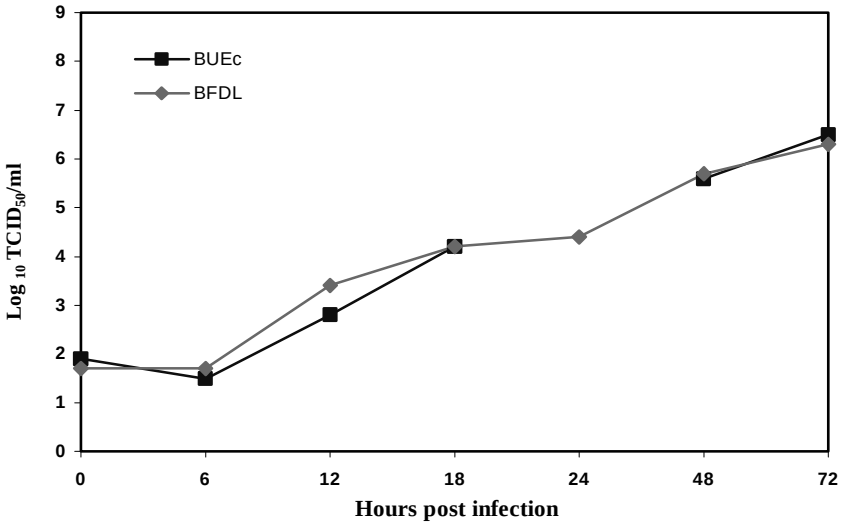

Fig 3. Growth curve of pseudocowpox virus in BUE cells and in BFDL cells at an m.o.i. of 0.1.

Table 1. Detection limits of bovine herpesviruses and pseudocowpox virus inoculated in triplicate on BUE cell cultures, and MDBK, Vero or BFDL, as determined by cytopathic effect (cpe)

Virus	$TCID_{50}$/ml	BUE cells		MDBK, Vero or BFDL cells	
		No. wells with cpe	Cpe first appeared on day pi	No. wells with cpe	Cpe first appeared on day pi
BHV1.1	100	**3/3**	2	**3/3** [(a)]	2
	10	**3/3**	3	**3/3**	3
	1	0/3		**1/3**	4
	0.1 , 0.01	0/3		0/3	
BHV1.2	100 , 1	**3/3**	1	**3/3** [(a)]	1
	0.1	**1/3**	2	**3/3**	4
	0.01	0/3		**2/3**	> 7[(d)]
BHV2	100	**3/3**	1	**3/3** [(b)]	2
	10	**3/3**	1	**1/3**	> 7
	1 , 0.01	0/3		0/3	
BHV4	100	**3/3**	3	**3/3** [(a)]	> 7
	10	**3/3**	3	0/3	
	1	**1/3**	4	0/3	
	0.1 , 0.01	0/3		0/3	
BHV5	100 , 10	**3/3**	1	**3/3** [(a)]	3
	1 , 0.01	0/3		0/3	
Pseudocowpox virus	100	**3/3**	4	**3/3** [(c)]	3
	10	**1/3**	4	0/3	
	1 , 0.01	0/3		0/3	

[(a)] : grown in MDBK cells; [(b)] : grown in Vero cells; [(c)] : grown in BFDL cells; > 7[(d)] : cpe observed first at the second passage

4. Discussion

This study demonstrates that BUE cells are susceptible to bovine herpesviruses and pseudocowpox virus, and therefore, these cells may be used to improve the laboratory diagnosis and/or the *in vitro* growth of these viruses. So far, it has only been reported that bovine arterial endothelial cells can be used for the detection and quantitation of BHV4 (Lin et al., 1997), and that BHV4 has been successfully isolated on BUE cells inoculated with milk samples from cows with clinical mastitis (Wellenberg et al., 2000), indicating that BUE cells might be susceptible to BHV4. Isolation of BHV4 on conventional cells, e.g. MDBK is difficult (Naeem et al., 1991), and therefore BUE cells were used in this preceding study, which was undertaken to gain more insight into the possible role of viruses in bovine clinical mastitis. Our data demonstrate that BUE cells are highly susceptible to BHV4. Ten to hundred times less BHV4 is detectable in BUE cells than in MDBK cells, and in addition, BHV4 can grow to much higher titres in BUE cells than in MDBK cells. Our findings are in agreement with those reported by Lin et al. (1997), although in their study bovine endothelial cells derived from carotid arteries were used to examine the sensitivity of bovine endothelial cells to BHV4 by e.g. a plaque formation assay. Data from both studies strongly suggest that bovine endothelial cells, derived from umbilical cord or from carotid arteries, may be the cells of choice for the isolation of BHV4 from clinical samples and to obtain high yields of BHV4.

Our findings further indicate that the BUE cells are also susceptible to: BHV1.1, BHV1.2., BHV2 and BHV5. However, MDBK cells are preferable above the BUE cells for the isolation of BHV1.1 and BHV1.2 from e.g. clinical specimens, and for the multiplication of these viruses, whereas, for the isolation and multiplication of BHV5 both BUE cells and MDBK cells can be used. However, BUE cells infected with BHV5 display cpe more rapidly than MDBK cells, which could also be a step in the improvement in an early diagnosis of BHV5. Data on detection limits and growth curves of BHV2 in BUE cells and Vero cells demonstrate that BUE cells are more susceptible to BHV2 than Vero cells. Therefore, BUE cells appeared to be preferable above the commonly used Vero cells for the detection of BHV2 in clinical samples, not only because of a slightly lower detection limit, but also because of a marked earlier appearance of cpe. Growth curves showed that the replication rate of BHV2 was faster in BUE cells than in Vero cells, and that BHV2 titres were higher in BUE cells than in Vero cells at various times pi. Normally, BHV2 replicates in a variety of cells, e.g. foetal bovine kidney cells, but the diagnosis of BHV2 infections by virus isolation can be unsuccessful (Farnsworth, 1995). Reasons for false-negative cell culture results could be the application of disinfectants, or the examined lesions are too old. On the other hand, the introduction of a susceptible BUE cell culture could be one of the contributing factors to improve the diagnosis of BHV2 infections.

In general, pseudocowpox virus is difficult to isolate from teat lesions. Virus isolation is sometimes unsuccessful even though virus-like particles are observed in lesions. This study shows that the susceptible BUE cell cultures could offer new opportunities in the diagnosis and isolation of pseudocowpox virus from teat lesions above the commonly used, but more laborious, primary or secondary cells.

Blunden et al. (1995), suggested that viral replication in the endothelium might be of importance in the pathogenesis of viral diseases, or suggests a contributing factor of viruses in the initiation of vessel-wall injuries, as suggested for cytomegalovirus in the initiation of atherogenesis (Melnick et al., 1993; Nicholson and Hajjar, 1998). Others described a novel endotheliotropic herpesvirus in elephants with a highly fatal hemorrhagic disease (Richman et al., 1999), and also the major role of a maternal endothelial cell infection by equine herpesvirus 1 in the pathogenesis of equid abortion has been discussed (Edington et al., 1991). Recently, Lin et al. (2000) reported that experimental infection of rabbits with BHV4 enhances atherosclerosis processes. Further studies are needed to prove that endothelial cells might be the target cells *in vivo* for e.g. other bovine herpesviruses, and whether endothelial cells might act as latent reservoir cells for these viruses as reported for BHV4 (Lin et al., 1999).

In conclusion, this study shows that the BUE cells are highly suitable for the isolation and growth of BHV2, BHV4 and pseudocowpox virus. This BUE cell culture is easy to maintain, and all examined viruses rapidly induce cpe. The use of BUE cell cultures might facilitate the diagnosis of above mentioned bovine herpesviruses and pseudocowpox virus infections. For e.g. diagnostic purposes, the susceptibility of BUE cells to more (bovine) viruses should be determined.

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CHAPTER 3

EVALUATION OF NEWLY DEVELOPED IMMUNOPEROXIDASE MONOLAYER ASSAYS FOR DETECTION OF ANTIBODIES AGAINST BOVINE HERPESVIRUS 4

G.J. Wellenberg[1], E.M.A. Van Rooij[1], J. Maissan[1] and J.T. Van Oirschot[1]

[1] Department of Mammalian Virology, Institute for Animal Science and Health (ID-DLO), P.O. Box 65, 8200 AB, Lelystad, The Netherlands.

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Abstract

This study describes the evaluation of immunoperoxidase monolayer assays (IPMAs) for detection of antibodies against bovine herpesvirus 4 (BHV4) DN-599 or BHV4 LVR140 in sera of cattle. We compared the quality of these IPMAs with the quality of a BHV4 indirect enzyme-linked immunosorbent assay (ELISA). In addition, a preliminary serological survey on BHV4 antibodies was carried out to estimate the seroprevalence of BHV4 in Dutch cattle at different ages. The specificities of both BHV4 IPMAs were 1.00. The geometrical mean titers (detection limit) of the BHV4 IPMAs were twice as high as that of the BHV4 indirect ELISA. In experimentally infected cattle, BHV4 antibodies were detectable by IPMAs 16 to 18 days post-infection, which was almost 2 weeks earlier than in the indirect ELISA The reproducibility of the BHV4 DN-599 IPMA (κD-value: 0.92) and of the BHV4 LVR140 IPMA (κD-value: 0.87) were good. For field sera the overall agreement between the BHV4 indirect ELISA and the two BHV4 IPMAs, DN-599 and LVR140, was 95% and 96%, respectively. The serological-survey study showed that the estimated seroprevalence of BHV4 in Dutch cattle was 16-18%, and that the percentage of BHV4-positive animals varied by age category (between 6% and 43%). In summary, the two BHV4 IPMA formats have several advantages that make IPMA a useful alternative to the BHV4 indirect ELISA for detecting BHV4 antibodies in cattle.

1. Introduction

Bovine herpesvirus 4 (BHV4), a group of virus strains belonging to the *Gammaherpesvirinae* (17), is distributed worldwide, and BHV4 strains have been isolated from cattle with a variety of clinical symptoms (8, 12, 14, 20, 23, 24). For antibody detection, a sensitive test is required because the humoral immune response of cattle to BHV4 infections is characterized by a low neutralizing antibody response, or even by absence of neutralizing antibodies (21). Other methods have been developed to detect BHV4 antibodies in serum, such as complement fixation (9), indirect fluorescent-antibody test (IFAT) (18), and indirect enzyme-linked immunosorbent assay (iELISA) (7). According to Guo et al. (9), the complement fixation test was less sensitive than the iELISA for the detection of immunoglobulin G (IgG) to BHV4. The IFAT and the iELISA are the most common tests to be used for detection of BHV4 antibodies, but the IFAT is laborious, particularly if quantitative results are required (7). Also cross-reactions between BHV4 and other herpesviruses have been recorded by IFAT (11, 16). Edwards and Newman (7) described that the iELISA appeared to be as sensitive as the IFAT, but these results were only based on sera collected from experimentally infected cattle. Until now, no thorough validation of any BHV4 ELISA has been published. Because there is a need for an easy test to detect BHV4 antibodies, we developed an immunoperoxidase monolayer assay (IPMA). IPMAs have proven to be an easy method and a valuable tool for the diagnosis of several infectious diseases (19), for instance it is a common method for the detection of antibodies against porcine reproductive and respiratory syndrome virus in pigs (10).

The purpose of this study was to evaluate newly developed BHV4 IPMAs which are based on the American BHV4 reference strain, DN-599 (13), and on LVR140, the Belgian BHV4 reference strain (26), which belongs to the European Movar 33/63-like group (22). The results of the IPMAs were compared with the results of a BHV4 iELISA. Data on the prevalence of BHV4 antibodies in Dutch cattle are be presented.

2. Materials and methods

2.1 BHV4 IPMA

2.1.1 Preparing BHV4 IPMA plates

Trypsinized embryonic bovine trachea cells (EBTr) were resuspended in Earle's minimal essential medium (EMEM) cell culture medium, containing 10% horse serum and 0.5% antibiotic mix (antibiotic stock solution containing; 10^7 IU of penicillin G, 8.6 x 10^6 IU of streptomycin, 1% kanamycin and 5 x 10^6 IU of nystatin per l liter aquadest), and seeded into wells of a 96-wells cell culture plate (Greiner). Each well contained 100 µl of an EBTr cell suspension of approximately 7.5 x 10^4 cells/ml. The plates were placed in a humidified incubator at 37°C with 5% CO_2. After 3 to 4 days, when the monolayer was 100% confluent, the cell culture medium was discarded and the cells were infected with 25 µl (containing 10^2 50% tissue culture infective doses/ml) of the BHV4 DN-599 strain (ATCC VR631) or the BHV4 LVR 140 strain (kindly provided by E. Thiry). After 1 hour at 37°C (5% CO_2), a volume of 75 µl EMEM (containing 2% horse serum and 0.5% antibiotic mix) was added to each well. When cytopathogic effect started to appear the incubation was stopped. The virus-cell culture medium was discarded and a volume of 150 µl of 4% formaldehyde in phosphate-buffered-saline (PBS) was added to the wells for 10 minutes at 18 to 25°C. The fluid was poured off, and again a volume of 150 µl of 4% formaldehyde in PBS (fixative) was added to the wells. Plates were sealed and stored at 4°C.

2.1.2 Performance BHV4 IPMA

Prior to use, the fixative was removed and the plates were washed once with 100 µl/well of a 2% Triton X-100 solution (Sigma) in PBS. To reduce nonspecific reactions, the plates were first incubated with a volume of 100 µl of 2% Triton X-100 in PBS for 60 minutes at 37°C. After the Triton X-100 solution was discarded, plates were incubated with 75 µl of PBS (containing 10% horse serum)/well for 60 minutes at 37°C. Serum test samples were diluted 1:20 in IPMA buffer (38.5 g of NaCl, 2% Tween-80, 0.1%NaN_3 and 10% horse serum per 1 liter of PBS). After the pre-incubation, the PBS-horse serum solution was discarded and a volume of 75 µl of the 1:20 pre-diluted serum sample were added to the wells and incubated at 37°C for 1 h. The plates were washed six times in wash solution (PBS, containing 1% Tween-80 [Merck]). A volume of 75 µl of the conjugate solution was added to the wells (1:200 dilution of horseradish peroxidase labeled rabbit anti-bovine immunoglobulin

[Dakopatt] in IPMA buffer [without NaN_3]), and incubated at 37°C for 1 h. The plates were washed five times as described above, and 75 µl of substrate-chromogen (H_2O_2/3-amino-9-ethylcarbazole) was added and incubated at 18 to 25°C for 30 min. Without stopping the reaction, the results were read under a light microscope by at least two persons, independently from each other. The serum samples were retested if the interpretations of the two persons were not the same. Positive reactions were characterized by an intense red-brown staining of mainly the nucleus of the EBTr cell, indicating binding of antibodies to BHV4 DN-599 or BHV4 LVR 140 antigens (Fig. 1). The results were interpreted as either negative (a titer of < 20) or positive (a titer of ≥20).

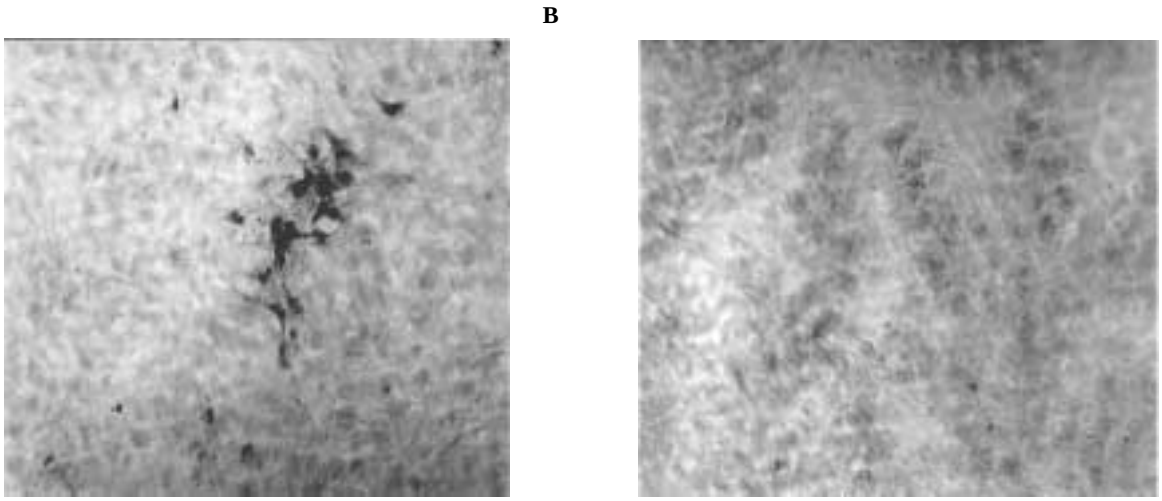

Fig. 1. (A) BHV4 IPMA positive staining reactions, which are characterized by red-brown staining mainly of the nuclei of BHV4 infected EBTr cells, indicate the binding of serum antibodies to BHV4 antigens. (B) Negative reactions are characterized by the absence of color.

2.2 BHV4 iELISA

De BHV4 iELISA (BIO-X, Brussels, Belgium) was performed as described by the manufacturer; it follows the principle of an iELISA. Briefly, the odd wells (antigen wells) of the ELISA microplate are coated with a BHV4 LVR 140-cell suspension lysate, while the even wells (control wells) are coated with a lysate of cell suspension. Volumes of 100 µl (each) of 1:100 diluted serum samples, including the positive reference serum sample of the kit, were added to the antigen and the control well and incubated at 18 to 25°C for 1 h. The plates were washed six times, and a volume of 100 µl of conjugate (horseradish peroxidase-labeled anti-bovine IgG1 monoclonal antibody) was added to each

wells. Plates were incubated at 18 to 25°C for 1 h, and washed again (six times). After the washing, a volume of 100 µl of the substrate-chromogen (H_2O_2-tetramethylbenzidine) was added to each well. After 10 min the color reaction was stopped by the addition of 50 µl of 1M phosphoric acid. The optical density (OD) value was determined by an ELISA plate reader at 450 nm. The interpretation of the results was as follows: the OD value of the control well was subtracted from the OD value of the antigen well. The net OD value was compared with the net OD value of the positive reference serum sample by dividing the net OD value of the serum sample by the net OD value of the positive reference serum sample. Finally, this value was multiplied with the coefficient of the iELISA batch, as noted for each BHV4 iELISA batch. Serum samples with values < 0.2 were classified as negative, and serum samples with values ≥0.2 were classified as positive for BHV4 antibodies.

2.3 Controls

To guarantee the quality of the performed tests, control serum samples were incorporated into ELISA test plates and into each IPMA test run. Therefore a strong positive, a dilution of positive (weak-positive) and a negative serum sample were used (15). The BHV4-positive serum samples were kindly provided by E. Thiry and by G. Czaplicki (Belgium), while the negative control was provided by E. VanOpdenbosch (Belgium). The results of test runs were accepted if the controls were correct.

2.4 Specificity

The specificities of the BHV4 tests were determined by testing monospecific serum samples containing high antibody levels directed against BHV1 (number of serum samples; [n] = 5), BHV2 (n = 1), bovine respiratory syncytial virus (n = 3), parainfluenza virus type 3 (n = 3), bovine leukemia virus (n = 2), bovine immunodeficiency virus (n = 2), bovine viral diarrhea virus (n = 3), rotavirus (n = 4), and coronavirus (n = 4). The specificities of the BHV4 IPMA's and the BHV4 iELISA were also determined by testing 69 individual serum samples collected from 69 specified-pathogen-free (SPF) cattle (cattle born by caesarean section, held in isolation stables, and raised colostrum free).

2.5 Detection limit

For the determination of the detection limits for both BHV4 IPMAs, and the BHV4 iELISA, the

following sera were used; the positive bovine BHV4 antiserum of the Central Veterinary Laboratory (Weybridge, England), one serum sample collected from one experimentally infected SPF calf (4797), and randomly chosen positive serum samples of cattle from the field study (n = 8). The sera were titrated in serial twofold dilutions, starting at a dilution of 1:20. The detection limit was defined as the reciprocal of the highest dilution giving a positive reaction.

2.6 Experimental infection

Two SPF calves (4797 and 4798), 3 weeks of age, were intranasally inoculated with 10 ml of $10^{6.5}$ 50% tissue culture infective doses of BHV4 DN-599 (ATCC VR631; bovine viral diarrhea virus and *Mycoplasma* free)/ml. Pre- and post-inoculation serum samples were collected and tested for BHV4 antibodies. The serum samples were tested in the BHV4 IPMAs and the BHV4 iELISA in a 1:20 dilution. Positive serum samples were titrated in serial twofold dilutions, starting at a dilution of 1:20.

2.7 Reproducibility

The reproducibility of the BHV4 tests was calculated from the results of duplicate tests by use of the Kappa test (2). Kappa (κD) was defined as the quotient of the observed proportion of agreement beyond chance and the maximal proportion of agreement. Therefore, serum samples (n = 150) of six randomly chosen BHV4-positive herds of the field study were tested twice on two different occasions, at least 2 months apart, using new IPMA reagents and ELISA kits with different lot numbers.

2.8 Field sera

Field sera were used to compare the newly developed BHV4 IPMAs with the iELISA and to estimate the BHV4 seroprevalence in Dutch cattle. For that purpose, a total amount of 750 serum samples were collected at random from 30 randomly chosen Dutch herds (25 serum samples per herd). These herds had participated in a field study for BHV1 marker vaccine efficacy (3). The date of birth of each animal was recorded to estimate the BHV4 seroprevalence in Dutch cattle at different ages.

2.9 Statistical analyses and data processing

Statistical analysis was performed on the data in Table 1 using the Friedman nonparametric two-way analysis of variance test, followed by the Wilcoxon signed-rank test (one-sided test) for pairwise comparison of both IPMAs with the iELISA. The data were processed with Statistix for Windows,

version 2.0.

3. Results

3.1 Evaluation of the BHV4 IPMAs

3.1.1 Specificity

No positive reactions were observed in the BHV4 IPMAs and the BHV4 iELISA with the monospecific serum samples containing high antibody levels directed against BHV1, BHV2, bovine respiratory syncytial virus, parainfluenza virus type 3, bovine leukemia virus, bovine immunodeficiency virus, bovine viral diarrhea virus, rotavirus and coronavirus. In addition, all 69 serum samples collected from the SPF cattle reacted negative in the three BHV4 tests, indicating test specificities of 100%.

3.1.2 Detection limit

The geometrical mean titers (reciprocal of the mean logarithmic titers) of the BHV4 DN-599 IPMA, the BHV4 LVR 140 IPMA, and the BHV4 iELISA were 640, 686, and 343, respectively. Hence, the detection limits of both BHV4 IPMAs were twice as high as that of the BHV4 iELISA (Table 1). Statistical analyses by the Friedman test showed that the differences in BHV4 antibody titers in both BHV4 IPMAs and those in the BH0V4 iELISA were statistically significant (P value, 0.048). The one-sided P value, calculated by the Wilcoxon signed-rank test, was 0.031 for BHV4 DN-599

3.2 Experimentally infected cattle

The BHV4 IPMAs first demonstrated a BHV4 antibody response around 16 to 18 days after experimental infection; the iELISA first demonstrated a response around 30 days after experimental infection (Table 2). In the commercial BHV4 iELISA, serum samples of experimentally infected cattle were screened for BHV4 antibodies in a 1:20 dilution, instead of a 1:100 dilution. For the screening of serum samples for BHV4 antibodies, a 1:100 serum dilution has been recommended by the manufacturer. At this dilution, no BHV4 antibody responses were detectable at day 37 (calf 4797), or even at day 44 (calf 4798), post-infection.

3.3 Reproducibility

The reproducibilities of the BHV4 tests were good. The overall agreement of duplicate test results were as follows: BHV4 DN-599 IPMA, 0.96 [(83⊕ + 62θ)/150], BHV4 LVR 140, 0.94 [(75⊕ + 66θ)/150]; and BHV4 iELISA, 0.97 [(87⊕ + 59θ)/150]. Based on these results the κD-values of the BHV4 tests were as follows: DN-599 IPMA, 0.92; BHV4 LVR 140 IPMA, 0.87; and BHV4 iELISA, 0.96.

Table 1. Titer of antibody against BHV4 in sera of cattle as determined in the BHV4 IPMAs and the BHV4 iELISA (n = 10)

Serum	BHV4 antibody titer		
	BHV4 DN-599 IPMA	BHV4 LVR 140 IPMA	BHV4 LVR 140 iELISA
CVL[a] serum	320	320	320
4797 24/5	320	320	40
Herd A			
Cow 1	640	640	320
Cow 2	1280	640	1280
Herd B			
Cow 1	640	1280	640
Cow 2	640	1280	160
Herd C			
Cow 1	640	640	160
Cow 2	1280	1280	1280
Herd D			
Cow 1	640	320	320
Cow 2	640	1280	640
Geometric mean:	640	686	343

[a] CVL, Central Veterinary Laboratory

3.4 Field sera

Of the 750 field serum samples, 120 (16.0%), in the BHV4 DN-599 IPMA and 135 (18.0%) in the

BHV4 LVR 140 IPMA scored positive for BHV4 antibodies, while in the BHV4 iELISA, 134 (17.9%) of the 750 field serum samples scored positive (Table 3). For field sera, the overall agreement of the BHV4 DN-599 IPMA and the BHV4 LVR 140 IPMA with the BHV4 iELISA were 95% and 96%, respectively. The overall agreement between the BHV4 DN-599 IPMA and the BHV4 LVR 140 IPMA was 98% (n = 750).

Table 2. Antibody responses against BHV4 in experimentally infected calves as determined in the BHV4 IPMAs and the BHV4 iELISA.

Day post-infection	BHV4 antibody titer					
	IPMA				iELISA	
	DN-599		LVR 140			
	4797[a]	4798	4797	4798	4797	4798
0	< 20	< 20	< 20	< 20	< 20	< 20
7	< 20	< 20	< 20	< 20	< 20	< 20
11	< 20	< 20	< 20	< 20	< 20	< 20
14	< 20	< 20	< 20	< 20	< 20	< 20
16	< 20	< 20	< 20	20	< 20	< 20
18	20	40	20	40	< 20	< 20
21	80	80	80	80	< 20	< 20
23	160	80	320	160	< 20	< 20
30	320	160	320	320	40	20
37	640	320	320	320	80	20
44	640	320	320	320	160	40

[a] Calf number

3.5 Serological survey

3.5.1 Dutch seroepidemiological field study

Based on the results of the three BHV4 tests (Table 3), 16 to 18% of the 750 Dutch field sera tested reacted positive for BHV4 antibodies. In 17 of the 30 herds examined (57%), BHV4 antibodies were detected in the BHV4 IPMAs. The herd prevalence of BHV4 antibodies varied between 0 and 76%, and within positive herds the seroprevalence of BHV4 antibodies varied by age category (Fig. 2). Cattle 5 years old and older showed the highest BHV4 seroprevalence: 43% of the sera contained antibodies to BHV4. The lowest level of BHV4 antibodies (6 to 7%) was found in the group of 0.5- to 2-year-old cattle. In young calves (between 0 and 6 months old) the BHV4 seroprevalence was 31%. Figure 2 shows only the results of the BHV4 LVR 140 IPMA for BHV4 seroprevalence in Dutch cattle at different ages; the same patterns were observed with the BHV4 DN-599 IPMA and the BHV4 iELISA.

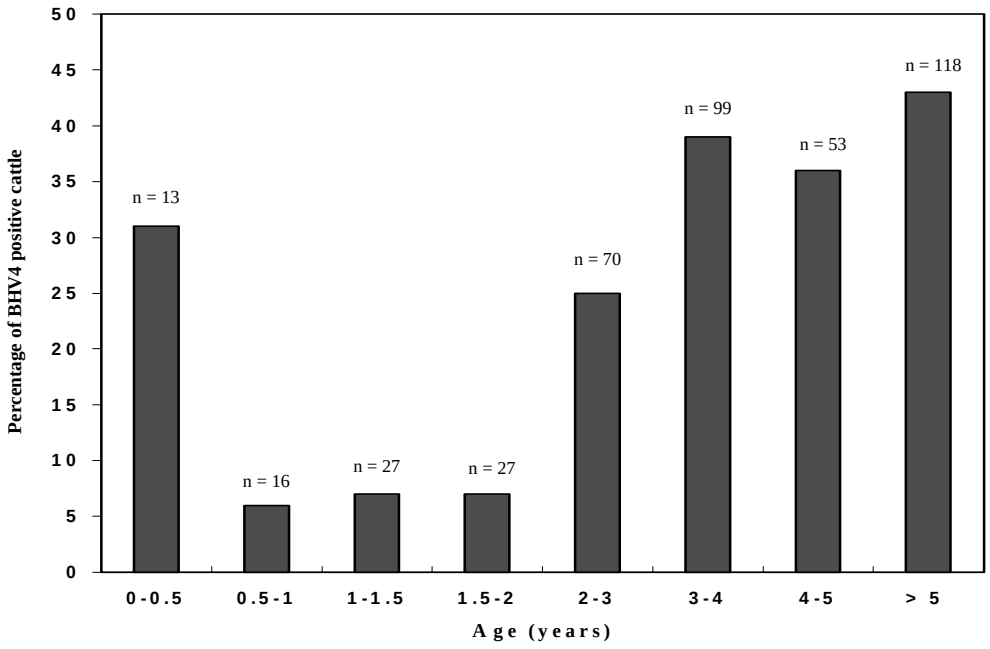

Fig 2. BHV4 seroprevalence in Dutch cattle at different ages as assessed by the BHV4 LVR 140 IPMA

4. Discussion

Our study shows that the BHV4 DN-599 and the BHV4 LVR 140 IPMAs can be used for the screening of herds for BHV4 antibodies as an alternative to the BHV4 iELISA. Based on the specificity, detection limit, results from early-infection sera collected from calves, and reproducibility, the newly developed BHV4 IPMAs are reliable practical tests for the detection of BHV4 antibodies. In this evaluation study, the BHV4 IPMA specificities (100%) are comparable with those of the BHV4 iELISA (100%). The data on the detection limits (analytical sensitivity) and the experimental infection sera (diagnostic sensitivity) of both BHV4 IPMAs are even better than those of the BHV4 iELISA. The geometrical mean antibody titers (detection limits) of the BHV4 IPMAs were twice as high as the geometrical mean antibody titer of the BHV4 iELISA, and the differences between the BHV4 antibody titers of both BHV4 IPMAs and those of the BHV4 iELISA were statistically significant (P value, <0.05). The BHV4 IPMAs were able to detect BHV4 antibody responses 16 to 18 days post-experimental infection. Even when sera were tested in the commercial BHV4 iELISA in a 1:20 dilution, instead of a 1:100 dilution, no BHV4 antibody response was detectable within 30 days post-infection.

Table 3. Concurrence of BHV4 IPMAs and BHV4 iELISA results for field sera (n = 750)

		No. of samples			
IPMA	Results[a]	BHV 4 iELISA[b]		BHV4 DN-599 IPMA[c]	
		+	-	+	-
BHV4 DN-599 [c]	+	107	13		
	-	27	603		
BHV4 LVR 140 [c]	+	119	16	120	15
	-	15	600	0	615

[a] +, positive; - , negative ; [b] Titer of <100 was considered negative; titer of ≥100 was considered positive

[c] Titer of <20 was considered negative; titer ≥20 was considered positive

One of the explanations for the inefficient detection of BHV4 antibodies could be that the commercial BHV4 iELISA detects only bovine IgG1. The immunoglobulin-specific conjugate, used in the BHV4 IPMAs to detect antibodies in the bovine serum samples, is known to have affinity for all bovine immunoglobulin subclasses (IgM, IgG1, IgG2, etc). The iELISA described by Edwards and Newman (7) was based on the detection of bovine IgG (IgG1 and IgG2) and detected BHV4-specific antibodies in experimentally infected calves 21 days after primary infection. The calves (infected with Movar 33/63) showed a response to the primary inoculation, which consisted mainly of IgG1. However, the detection of IgG2 and IgM could have been underestimated due to competitive inhibition by high levels of IgG1 (7). Although other parameters, e.g., buffer solutions and substrate, could also influence the difference in sensitivity of IPMA versus iELISA, the choice of the anti-bovine Ig conjugate is probably the most relevant factor.

The reproducibilities of the BHV4 DN-599 IPMA (κD value, 0.92) and the BHV4 iELISA (κD value, 0.96) are good, while the reproducibility of the BHV4 LVR 140 is somewhat lower but still acceptable (κD value, 0.87).

The seroepidemiological field study shows that the estimated BHV4 seroprevalence in Dutch cattle is 16 to 18%. The BHV4 seroprevalences in cattle determined in countries surrounding the Netherlands are comparable with those in Dutch cattle. In the Northern part of Belgium 15%, and in Wallony (Belgium) 29%, of the cattle older than 1 year were BHV4 seropositive (24). In former West Germany, the BHV4 seroprevalence in cattle was 18.4%, while 38% of the bulls used for artificial insemination contained BHV4 antibodies (23). Young calves (< 6 months) had antibodies directed against BHV4. These antibodies are probably of maternal origin and not the result of a BHV4 infection. According to VanOpdenbosch et al. (25), 38% of veal calves in Belgium (< 3 months) had maternal antibodies against BHV4, and only 3% of the BHV4-negative veal calves seroconverted within a period of 3 months. In this study, an increase in BHV4-seropositive animals was recorded within the group of 2- to 3-year-old cattle. Hence, it is likely that most cattle become infected after introduction in the dairy herd. Whether there is a relation between this finding and that of Czaplicki and Thiry (4), who reported an association between BHV4 seropositivity and abortion in cows, is unknown.

Although the group of BHV4 comprises a collection of antigenically closely related isolates (1), and no major antigenic differences between BHV4 isolates have been demonstrated by cross-serological analysis with polyvalent antisera (5), the evaluation of the newly developed BHV4 IPMA was based on two different BHV4 strains. The IPMAs were performed with the American BHV4 DN-599 reference strain, and the Belgian LVR 140 reference strain, which belongs to the European Movar 33/63-like group (22), because the use of monoclonal antibodies showed some differences in the

antigenic patterns of BHV4 isolates (6), and BHV4 strains can be isolated in many countries all over the world. Dubuisson et al. (6) confirmed the close antigenic relationships between BHV4 by comparison of field isolates with BHV4 monoclonal antibodies, but some monoclonal antibodies recognized all BHV4 isolates, while others allowed differentiation among them. Our data support the finding that there could be some minor differences in the antigenic patterns of BHV4 isolates, because the results, obtained with field sera, showed some slight differences between the BHV4 DN-599 IPMA and the BHV4 LVR 140 IPMA. Fifteen field serum samples reacted positive in the BHV4 LVR 140 IPMA, while these sera did not react in the BHV4 DN-599 IPMA. Thirteen of these 15 sera also reacted positive in the BHV4 iELISA, in which the plates were coated with a BHV4 LVR 140-cell suspension lysate. Differences in affinity of certain immunoglobulins of cattle for the different BHV4 strains could be an explanation, but another explanation could be the differences in antigenic expression or differences in the exposure of certain BHV4 antigens. Probably the antigenic expression and antigen exposure of Dutch BHV4 strains are more related to BHV4 LVR 140 than to those of BHV4 DN-599.

The BHV4 IPMA has several advantages compared to the BHV4 iELISA, e.g., this study shows better analytical sensitivity results and the IPMA can be more easily adapted to the BHV4 strain of choice (for example, the BHV4 strain which has been isolated in the area of interest) while the commercial BHV4 iELISA is based on the use of BHV4 LVR 140. This study also shows that, in comparison to the BHV4 iELISA, the BHV4 IPMA, which is based on the detection of all bovine immunoglobulin subclasses, is the test of choice for the early detection of BHV4 antibodies in bovine serum samples. A disadvantage of the IPMA is that it depends on subjective readings of test results whereas the iELISA is more objective because of the use of automatic readers. IPMA test results need to be interpreted by at least two trained persons to reduce the subjectivity of readings of test results (as indicated in Materials and Methods).

In conclusion, the BHV4 IPMA is a reliable practical test for the screening of cattle for BHV4 antibodies, and it is a useful alternative to iELISA for detecting BHV4 antibodies in cattle.

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CHAPTER 4

DETECTION OF BOVINE HERPESVIRUS 4 GLYCOPROTEIN B AND THYMIDINE KINASE DNA BY PCR ASSAYS IN BOVINE MILK

G.J. Wellenberg[1], E.R.A.M.Verstraten[1], S. Belák[2], S.B.E. Verschuren[1], F.A.M. Rijsewijk[1],R. Peshev[3], and J.T. Van Oirschot[1]

[1] *Division of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands*

[2] *Department of Virology, The National Veterinary Institute. Biomedical Centre. P.O. Box 585, SE-75123, Uppsala, Sweden*

[3] *Central Veterinary Research Institute, Department of Virology, P. Slaveikov 15a, Sofia, Bulgaria*

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Abstract

A polymerase chain reaction (PCR) assay was developed to detect bovine herpesvirus 4 (BHV4) glycoprotein B (gB) DNA, and nested-PCR assay was modified for the detection of BHV4 thymidine kinase (TK) DNA in bovine milk samples. To identify false-negative PCR results, internal control templates were constructed, added to milk samples, and co-amplified with viral DNA using the same primers for both templates. Specificity, sensitivity, and reproducibility of the two PCR assays were examined. In both PCR assays, all 31 BHV4 strains examined were scored positive, whereas 14 unrelated viruses scored negative. Sensitivity studies showed that two - ten copies of BHV4 DNA were detectable by the gB-PCR, while one - three copies could be detected by the TK-PCR. For the detection of BHV4 in milk samples, the gB-PCR amplification was found to be ten-times, and the TK-PCR was found to be 55-times more sensitive than virus isolation. BHV4 DNA was detected by gB-PCR and TK-PCR in 93 and 95%, respectively, of 61 milk samples collected from cows infected intramammarily with BHV4, while only 61% were positive by virus isolation. Four out of 48 cows with clinical mastitis were positive for BHV4-gB and BHV4-TK DNA, whereas no BHV4 DNA was detected in milk from control cows. Considerable agreement was seen between the results of the two PCR assays, and both methods were considered as rapid and reliable tests for the screening of BHV4 DNA in bovine milk. The less laborious gB-PCR might be the recommended test of choice for screening large amounts of milk samples for the presence of BHV4.

1. Introduction

Bovine herpesvirus 4 (BHV4), a member of the *Gammaherpesvirinae* subfamily, has been isolated from cows with various diseases all over the world. Recently, BHV4 has been isolated from milk samples of cows with clinical mastitis, and not from milk samples of the matched control cows (Wellenberg et al., 2000). More studies need to be carried out to clarify whether BHV4 might play a role in the aetiology of bovine clinical mastitis. Therefore, a rapid, sensitive and reliable method is required to screen large numbers of milk samples from cows with mastitis and their controls for the presence of BHV4. A nested-PCR for the detection of thymidine kinase (TK) genomic sequences has been described by Egyed et al. (1996). This assay has been shown to be suitable to study the *in vivo* distribution of BHV4 in its host, but it has never been validated as diagnostic tool for the detection of BHV4 DNA in milk samples, nor has this TK-PCR been used with the addition of an internal control template. Internal control templates can be used to identify failures in DNA extractions, in PCR amplifications, and allow semi-quantitative estimations of the amount of viral DNA (Van Engelenburg et al., 1993). In this study, we report on the modification of the TK-PCR, and on the development of a single PCR for the detection of BHV4 glycoprotein B (gB) DNA. Both PCR assays were validated for the detection of BHV4 DNA in bovine milk samples. For that purpose, we tested milk samples from cows infected intramammarily with BHV4, and from cows with clinical mastitis and from their matched controls.

2. Materials and methods

2.1 Cell cultures

Bovine umbilical cord endothelial (BUE) cells were used for BHV4 isolation and propagation (Wellenberg et al., 2000). These cells were grown and maintained in Dulbecco's minimal essential medium (DMEM) (Gibco Laboratories, Life Technologies Inc., USA) supplemented with 10% fetal bovine serum (Gibco Laboratories, Life Technologies Inc., USA), and 0.5% antibiotic mix (stock mix contained: 10,000 IU penicillin, 11.25 mg streptomycin, 10 mg kanamycin and 5,000 IU nystatin per ml). Other virus stocks were propagated in cell cultures of the Madin Darby bovine kidney, embryonic bovine trachea, or bovine diploid lung cells (Van der Poel et al., 1996).

2.2 Virus strains

The American BHV4 DN-599 reference strain was obtained from the American Type Culture Collection, Manassas, VA (Cat. No. VR 631). BHV4 Movar 33/63, the European reference strain, and BHV4 LVR 140 were kindly provided by Dr. E. Thiry. In total, 28 other BHV4 strains, from various European countries, and 14 viruses not belonging to the *Gammaherpesvirinae*, were used to determine the specificity of both PCR assays. The BHV4 strains were: five Dutch strains (including strain Tolakker), two strains from Italy (strains 415 and 418), five strains from Hungary (strains 458, 461, 463, 488, and 497 were kindly provided by Dr. L. Egyed), nine strains from Belgium (strains LVR260, AVR1478, 31788, BVR318, 99/3114, 99/2823, 00/2768, 00/2776, and 00/3847 were kindly provided by Dr. P. Kerkhofs), and seven Bulgarian strains (strains Momchilgrad, Nikolovo, Svoboda, Levski, Kazichene, Movar 90, and 453). BHV4 strain Tolakker, which was used for experimental infection, was isolated from a Dutch cow with signs of clinical mastitis (Wellenberg et al., 2000). Ovine herpesvirus 2 (OHV2 DNA from bovine lymphocytes) and virus stocks, containing $>10^4$ tissue culture infective doses $(TCID)_{50}$/ml, of bovine herpesviruses (BHV)1.1, BHV1.2, BHV2, BHV5 (N569), equine herpesvirus (EHV) 1, EHV4, suid herpesvirus 1 (SHV1), herpes simplex virus (HSV) 1, HSV2, (bovine) para-influenza 3 (PI-3) virus, bovine adenovirus 1, bovine viral diarrhoea virus (BVDV-1) and bovine respiratory syncytial virus (BRSV) were also used to determine the specificity of both PCR assays.

2.3 BHV4 DNA cloning

BHV4 DNA was cloned to prepare internal control templates and to determine the number of the DNA copies that can be detected by both PCR assays. Therefore, the gB-primers gB1 (5'-CCCTTCTTTACCACCACCTACA-3') and gB2 (5'-TGCCATAGCAGAGAAACAATGA-3') (Eurogentec, Belgium) and the TK-primers, 5'-GTTGGGCGTCCTGTATGGTAGC-3' and 5'-ATGTATGCCCAAAACTTATAATATGACCAG-3' (Egyed et al., 1996), were used to amplify parts of the gB and the TK gene of BHV4 Movar 33/63. The amplification products were extracted from agarose gels, and cloned into plasmid pCR 2.1 using the TA cloning kit (Invitrogen, USA). After cloning, plasmids were purified using the Wizard^{R} *Plus* Minipreps DNA purification system (Promega, USA). DNA concentrations were determined by comparison with known quantities of DNA on agarose gels, and 10-fold dilutions of plasmids pCR 2.1 with inserts of BHV4-gB (pB34) or BHV4-TK (pB19) (Fig. 1) were prepared in milk and stored at –20°C.

2.4 Construction and use of internal control templates for the gB-PCR and TK-PCR

The internal control templates were constructed by digestion of plasmid pB34 (gB) with restriction enzyme *Ssp*I, and plasmid pB19 (TK) with restriction enzyme *Bsm*I (Fig. 1). Both templates were digested briefly with *Bal*31 exonuclease. After a ligase treatment, the resulting plasmids, containing the shortened BHV4-gB insert (pB88) and the BHV4-TK insert (pB48), were cloned, isolated, and the DNA concentration was determined. The resulting plasmids were used as internal control templates and amplified under PCR conditions identical to those used to amplify BHV4 DNA. The internal control products were distinguished from BHV4 viral DNA PCR products by agarose gel electrophoresis.

2.5 DNA extraction

DNA extractions from defatted milk samples, and extractions from viruses used to examine the specificity, were carried out by column chromatography using the QIAamp Blood and Tissue kit (QIAGEN, Westburg, The Netherlands). For DNA extraction of defatted milk samples a volume of 1000 µl was used. Prior to DNA extraction, the defatted milk samples were centrifuged at 14,000 rpm for 3 min. A volume of 800 µl was discarded and the cell pellet was resuspended in the remaining 200 µl. Prior to DNA extraction, both internal control templates, plasmid pB88, and plasmid pB48, were added to the defatted milk samples. For the DNA extraction of virus strains, 200 µl of virus stocks

were used. Digestion with protease, DNA precipitation and elution of DNA was carried out according to the description of the manufacturer. The DNA extraction products were stored at –20 °C.

2.6 gB and TK-PCR assays

For the amplification of BHV4-gB gene, primers gB1 and gB2 were used. The primers showed 100% homology with the sense strand, on nucleotide positions –38 to –17 (gB1), and with the anti-sense strand, on nucleotide positions 555 to 576 (gB2) of the BHV4-gB sequence published by Goltz et al. (1994). Optimisation of the gB-PCR was performed for various PCR buffers, concentrations of magnesium chloride, annealing temperatures, numbers of cycles, types of DNA polymerase, addition of dimethylsulfoxide, glycerol, and primer concentrations. The optimised PCR assay conditions were as follows: amplification of BHV4-gB DNA was performed by adding 5 µl of DNA extract in a final volume of 50 µl containing, 1x PCR buffer minus magnesium chloride (Gibco BRL, Life Technologies, USA), 5 mM dNTP-mix (containing 1.25 mM of each dATP, dCTP, dGTP, and dTTP), 2 mM magnesium chloride (Roche), 2 U Ampli *Taq* Gold polymerase (Perkin Elmer/Applied Biosystems, USA) and 30 pmol of primers gB1 and gB2. The amplification was performed on a GeneAmp PCR System DNA Thermal cycler (Perkin Elmer Cetus, Norwalk, USA). The program started with one step at 95 °C for 10 minutes, followed by 45 cycles of, 94°C for 60 sec, 58°C for 60 sec, and 72°C for 90 sec. The amplification was completed by an ultimate elongation step at 72°C for 7 min.

The amplification of BHV4-TK DNA templates was based on the nested TK-PCR as described by Egyed et al. (1996), but with some modifications. DNA templates were amplified in the PCR mix as described for the nested TK-PCR, but with 2 mM magnesium chloride (Roche), and 2 U Ampli *Taq* Gold polymerase (Perkin Elmer/Applied Biosystems). A volume of 5 µl of PCR product of the first PCR round was amplified further in a nested PCR round, using the same PCR mix as described for the first round, but with the nested primers 3 and 4 (Egyed et al., 1996). In the first and the nested PCR round, the following program was used to amplify BHV4-TK templates: one step at 95 °C for 10 minutes, followed by 30 cycles of 94°C for 60 sec, 60°C for 60 sec, and 72°C for 90 sec. The amplification was finalised by an ultimate elongation step at 72°C for 7 min. The amplification products were stored at –20 °C. Eight microlitres of PCR products were analysed by horizontal agarose (1%) gel electrophoresis. Gels were stained with 1 µg/ml ethidium bromide and photographed under UV light.

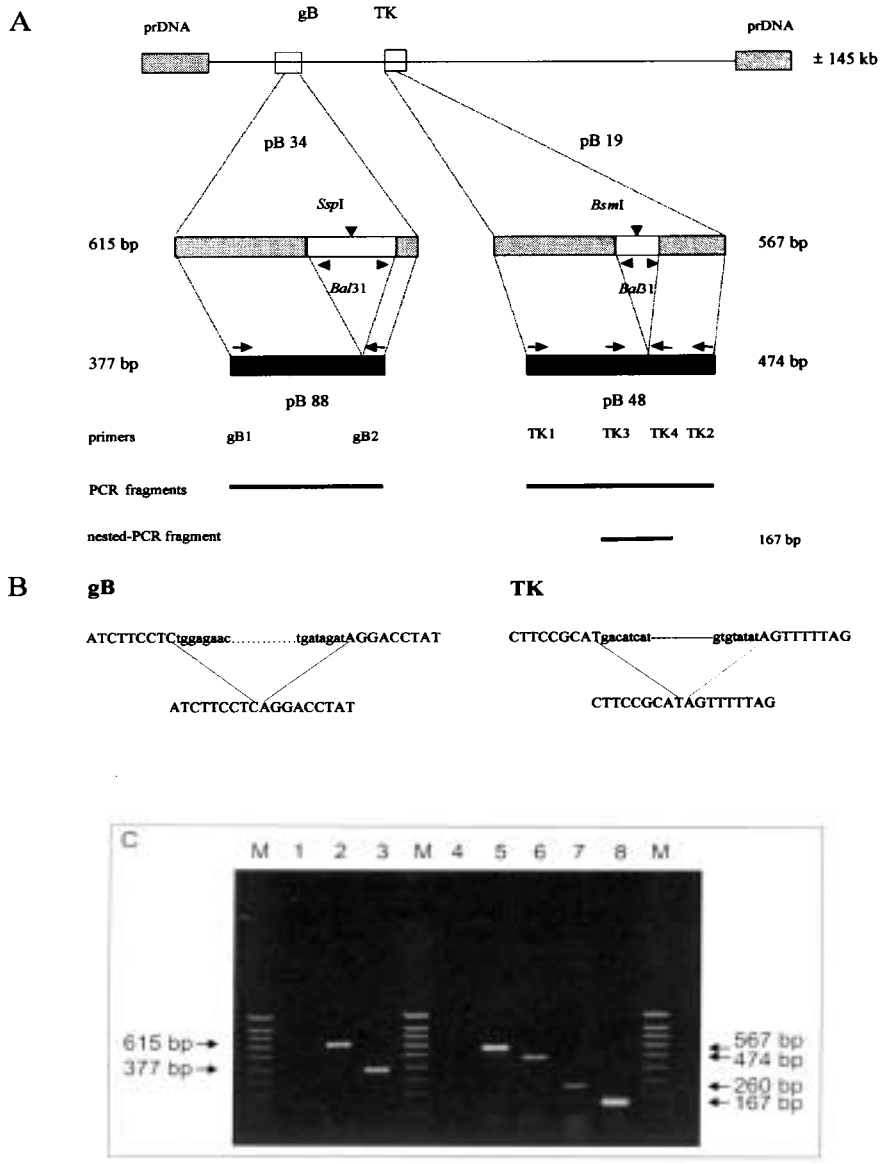

Fig 1. Construction of internal control templates for the gB-PCR and TK-PCR assays. (A) Schematic representation of the BHV4 Movar 33/63 genome indicating the positions of the gB and TK genes and the cleavage positions of the restriction enzymes *Ssp*I and *Bsm*I (arrowheads). The *Bal*31 exonuclease digested segments in pB34 and pB19 are indicated as white boxes that are removed in pB88 and pB48, respectively. During PCR, fragments of 377 bp and 167 bp (n-PCR product) were amplified from pB88 and pB48, respectively. The positions of the primers are indicated by arrows. (B) The *Bal*31 exonuclease digested segments in pB34 and pB19 analysed on nucleotide level. The *Bal*31 cleaved nucleotides (lower case) are flanked on both sides by remaining nucleotides within the internal control templates (upper case) pB88 and pB48. (C) PCR products obtained after amplification of pB34 (lane 2) and pB88 (lane 3) by gB-PCR, and pB19 (lane 5) and pB48 (lane 6) by TK-PCR after the first PCR round, and pB19 (lane 7) and pB48 (lane 8) by the nested TK-PCR. Lanes M, 100-bp marker; lane 1, negative control gB-PCR; lane 4, negative control nested TK-PCR.

2.7 Specificity of PCR products

The specificity of the gB-PCR and TK-PCR products was examined by evaluating their size, their pattern after restriction enzyme analysis and by sequencing. For the digestion of gB-PCR products the *Ssp*I restriction enzyme was used and for TK-PCR products the *Bsm*I restriction enzyme.

Nucleotide sequences of BHV4-gB and BHV4-TK internal template constructs and the PCR products were determined by the fluorescent dideoxy chain termination method using an automated sequencer (model 373A; Applied Biosystems Inc., Foster City, USA).

2.8 Controls for PCR assays

In addition to the use of internal template controls, positive and negative controls were incorporated into the total DNA extraction step, and into each step of the amplification, i.e. into the first (or single) PCR round and into the nested (second) PCR round. A dilution, containing two $TCID_{50}$ of BHV4 Movar 33/63 in milk, was incorporated as positive control into each test run. Also negative controls (without DNA), consisting of PCR mix buffer and DNAse-free H_2O, were incorporated into each amplification step. Special care was taken to identify false-positive PCR results, and therefore, a BHV4-free negative milk control (free of BHV4 and BHV4 antibodies) was added to each set of 4 test milk samples. The negative controls were processed together with the test samples, starting at DNA extraction level. To avoid false-positive results, classical precautions for PCR were strictly followed in the laboratory (Kwok and Higuchi, 1989; Crespin et al., 1998).

2.9 Validation of the PCR assays

2.9.1 Specificity of the PCR assays

To determine the specificity of both PCR assays, DNA extracts were prepared from virus stocks (see Section 2.2), and tested whether they were amplified by PCR with the selected primers.

2.9.2 Sensitivity of the PCR assays

Three methods were used to determine the sensitivity of both PCR assays. The sensitivity, defined as the detection limit of the PCR assay, was first determined by an end-point titration assay using the BHV4-gB and BHV4-TK internal control templates. Serial tenfold dilutions of plasmids pB88 and pB48 in BHV4-free milk were used for DNA extraction, and the end-point dilution was determined.

The amount of DNA copies in this dilution was considered to be the detection limit. The sensitivity was also evaluated by preparing serial tenfold dilutions of the BHV4 reference strains DN599 ($10^{7.0}$ $TCID_{50}$/ml) and Movar 33/63 ($10^{5.1}$ $TCID_{50}$/ml) in milk. DNA extractions of these dilutions were prepared with and without the addition of the internal control templates and amplified by both PCR assays (see Section 3.5). The highest dilution giving a positive reaction was defined as the detection limit. Thirdly, the sensitivity of both PCR assays was determined for BHV4 infected milk samples and compared with virus isolation. Therefore, four milk samples from cows, intramammarily inoculated with BHV4, were diluted in BHV4-free milk samples up to 10,000 times, and the internal control templates were added to each dilution prior to DNA extraction. Detection limits of both PCR assays were compared with the detection limits as determined by virus isolation. PCR assays and virus isolations were performed in duplicate.

2.9.3 Reproducibility of the PCR assays

The reproducibility of both PCR assays was determined by analysing 24 selected milk samples, from which BHV4 was isolated, of four cows experimentally infected with BHV4 strain Tolakker, and by analysing 26 bovine milk samples from a case/control study shown to be BHV4-free by virus isolation. All milk samples were examined twice by both PCR assays for BHV4 DNA by the same technician on two different days. Internal control templates were added to the milk samples prior to DNA extractions.

2.10 Virus isolation

Milk samples were defatted by centrifugation at 1500 x g for 10 min at 4°C, and stored at –70°C. After thawing, a volume of 500 µl of de-fatted milk sample was examined for the presence of BHV4 by virus isolation on BUE cells using six- and 24-wells cell culture plates. One hour after inoculation, the supernatant was replaced by new cell culture medium and the cell culture was incubated at 37°C with 5% CO_2. After 4 days of incubation, the virus/cell suspension was frozen at –70°C and passed into a new six- or 24-wells cell culture plate with semi-confluent monolayers of BUE cells. Cells were observed for cpe after an incubation period of 5 days at 37°C (5% CO_2).

2.11 Bovine milk samples collected under experimental and field conditions

Bovine milk samples were collected from cows experimentally infected with BHV4 and from cows in

a case/control study to compare both PCR assays as diagnostic tool in detecting BHV4 in milk with virus isolation.

2.11.1 Experimental infection

Two quarters of four dairy cows each were inoculated intramammarily with BHV4 strain Tolakker. On day 14 post-infection (pi.), the same two quarters of two out of four cows were inoculated with *Streptococcus uberis*. Milk samples were collected from infected quarters between day 0 and day 28 pi., and stored at –70°C after collection. Prior to virus isolation and DNA extraction, milk samples were thawed, and a volume of 2-5 ml of milk was defatted by centrifugation at 4°C (10 min at 1500 x g). Internal control templates were added prior to DNA extraction. Lipid components in milk can inhibit *Taq* polymerase which results in a failure to amplify low levels of viral DNA from native milk samples (Hamprecht et al., 1998).

2.11.2 Field conditions

In a case/control study, milk samples were collected from Dutch dairy cows with clinical mastitis and from matched control cows as described previously (Wellenberg et al., 2000). Milk samples have been collected according to the recommendations of the National Mastitis Council (Harmon et al., 1990). Samples for virus isolation and DNA extraction were stored directly at –70°C. Prior to virus isolation and DNA extraction, milk samples were de-fatted as described above, and internal control templates were added.

3. Results

3.1 Construction of internal control templates

Internal control templates for the gB-PCR and the TK-PCR were constructed by deleting 238-bp of the 615-bp PCR product of BHV4-gB, and 93-bp of the 567-bp PCR product of BHV4-TK (Fig. 1). The resulting plasmids, pB88 and pB48, were amplified under PCR conditions identical to those used for the amplification of BHV4 viral DNA. After amplification of the internal control template pB88 by the gB-PCR, a fragment of 377 bp was amplified. In the nested TK-PCR, the BHV4 viral DNA product was 260 bp, whereas the internal control template product had a length of 167 bp.

Restriction enzyme analysis of PCR products showed that the internal control template fragments were not cleaved by *Ssp*I and *Bsm*I. gB-PCR products of BHV4 viral DNA were cleaved by *Ssp*I and resulted in restriction enzyme digestion products of 441 bp and 174 bp, while *Bsm*I cleaved TK-PCR products from BHV4 viral DNA in restriction products of 171 bp and 89 bp (Fig. 2).

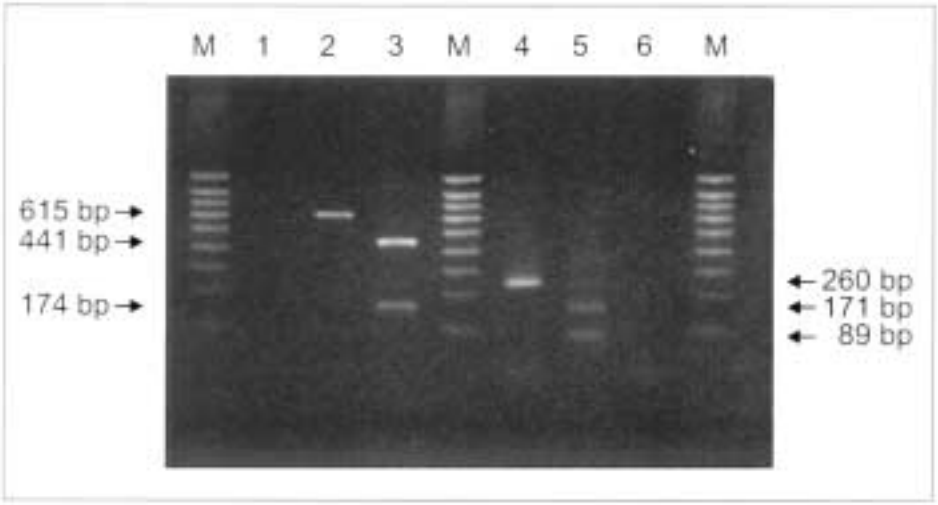

Fig 2. gB-PCR and TK-PCR amplification fragments of BHV4 DNA positive milk. Specificity of the PCR products was checked by restriction enzymes *Ssp*I and *Bsm*I. Lanes M, 100 bp marker; lane 1, negative milk control BHV4-gB PCR; lane 2, BHV4-gB positive milk (PCR fragment of 615 bp); lane 3, BHV4-gB PCR fragment cleaved by restriction enzyme *Ssp*I (restriction products of 441 and 174 bp); lane 4, BHV4-TK positive milk (PCR fragment of 260 bp); lane 5, TK-PCR fragment cleaved by restriction enzyme *Bsm*I (restriction products of 171 and 89 bp); lane 6, negative milk control BHV4-TK PCR.

3.2 Specificity of both PCR assays

Both PCR assays yielded fragments of the predicted size using DNA extracts from BHV4 strains DN-599, Movar 33/63, and LVR 140, and from the other 28 BHV4 strains. The obtained PCR products were cleaved into restriction products of the correct sizes by restriction enzymes *Ssp*I and *Bsm*I. No specific PCR products were obtained after amplification of DNA or RNA extracts from BHV1.1, BHV1.2, BHV2, BHV5, OHV2, EHV1, EHV4, SHV1, HSV1, HSV2, PI3, bovine adenovirus 1, BVDV, and BRSV. Only after the amplification of SHV1 DNA, weak PCR products were obtained in the gB-PCR but the lengths of these two bands were approximately 200-bp and 480-bp, hence not of the correct size.

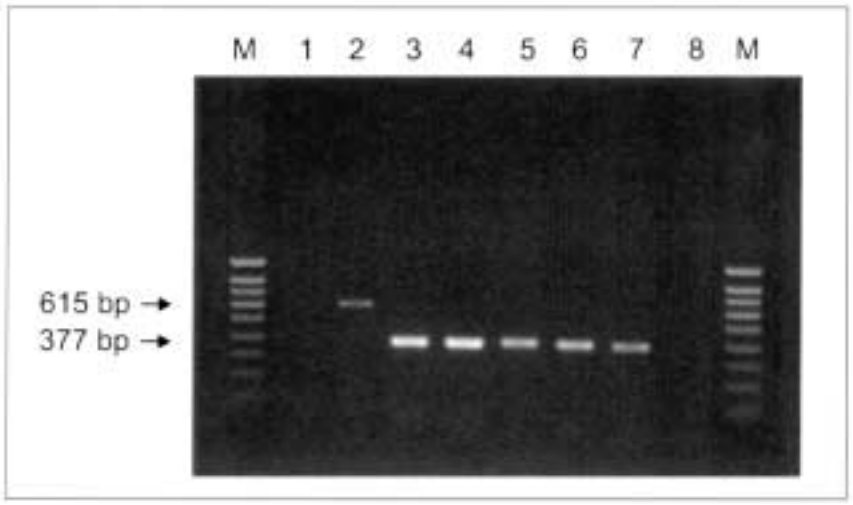

Fig 3. The sensitivity of the gB-PCR, as determined by serial 10-fold dilutions of internal control template DNA (plasmid pB88). Lanes M, 100-bp marker; lane 1, negative control (not spiked); lane 2, positive PCR control Movar 33/63 (2 $TCID_{50}$); lanes 3 – 8, serial 10-fold dilutions of internal control template plasmid pB88, containing 20,000 – 0.2 copies/µl.

3.3 Sensitivity of the PCR assays

The estimated sensitivity of the gB-PCR, as determined with plasmid DNA of the gB gene (pB88), was two - ten molecules (Fig. 3), whereas one – three molecules, as determined with plasmid pB48 containing DNA of the TK gene, was detected by the TK-PCR. In the TK-PCR, amplification products were detectable of 10-fold dilutions of plasmid pB48 up to dilutions containing one molecule

(data not shown). PCR assays, as performed on DNA extracts of 200 µl of serial dilutions of BHV4 strains DN599 and Movar 33/63 (infectious viruses without the addition of internal control templates) showed that between 2 – 0.2 $TCID_{50}$ were still detectable by gB-PCR, and 0.2 $TCID_{50}$ by the TK-PCR. The sensitivity of both PCR assays was also determined by serial 10-fold dilutions of four milk samples obtained from cows after an intramammary inoculation with BHV4 (Fig. 4). Results of the detection limits of both PCR assays and virus isolation are presented in Table 1. The gB-PCR was one - ten times more sensitive in detecting low amounts of BHV4 in milk samples than virus isolation, while the TK-PCR was 1 - 55 times more sensitive in detecting low amounts of BHV4 than virus isolation.

Table 1. Comparison of the detection limits of the gB-PCR, the TK-PCR, and virus isolation by using serial tenfold dilutions of milk from cows intramammarily inoculated with BHV4

	Highest dilution positive for BHV4			Sensitivity ratio [a]	
Milk sample	BHV4-gB	BHV4-TK	Virus isolation	BHV4-gB	BHV4-TK
1	10^1 ,10^1	10^1 ,10^1	10^1	1	1
2	10^2 ,10^2	10^2 ,10^3	10^2	1	5
3	10^2 ,10^2	10^3 ,10^2	10^2	1	5
4	10^1 ,10^1	10^2 ,10^1	10^0	10	55

[a]: The sensitivity ratio is determined by dividing the detection limit of the PCR assay by that of the virus isolation method

3.4 Reproducibility

All 24 milk samples that were collected from cows infected experimentally with BHV4 were positive for BHV4 DNA by both PCR assays, and the 26 milk samples of the control cows were BHV4 DNA negative. All duplicate analyses, as obtained by the two PCR assays on a second occasion, revealed the same results.

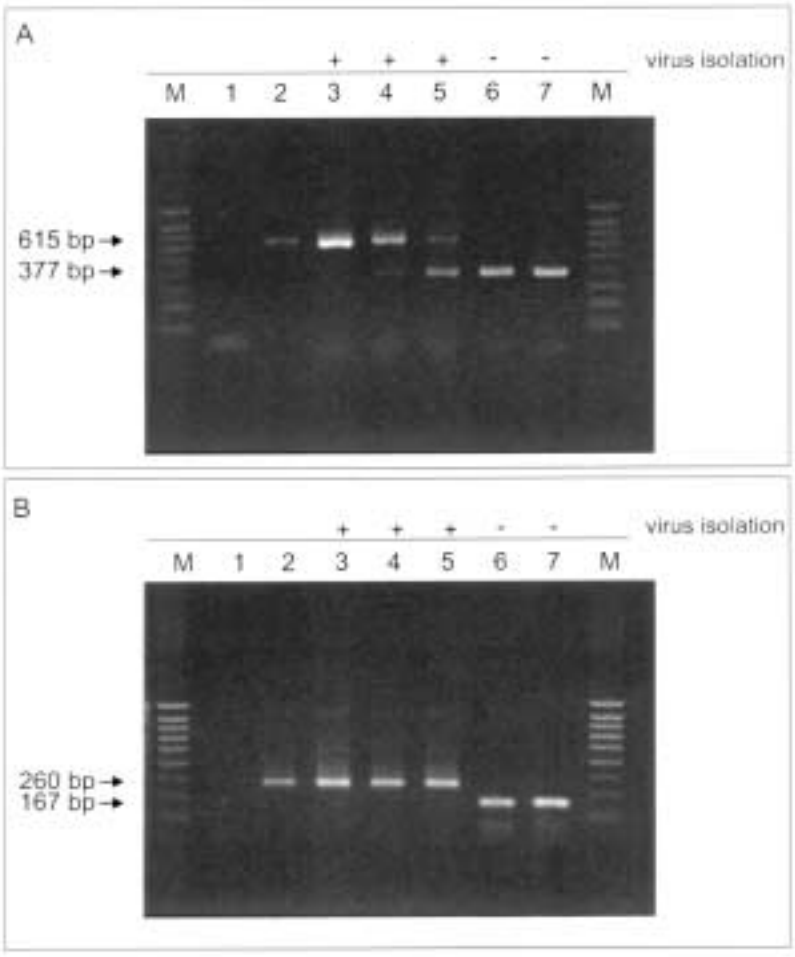

Fig 4. Comparison of the sensitivity of the gB-PCR and the TK-PCR assays and virus isolation by serial tenfold dilution of milk sample 2, obtained from a cow intramammarily inoculated with BHV4, spiked with internal control templates pB88 and pB48. (A) Amplification products of the gB-PCR of viral DNA (615 bp) and internal control template (377 bp). (B) Amplification products of the TK-PCR of viral DNA (260 bp) and internal control template (167 bp). For A and B: lanes M, 100-bp marker; lane 1, negative milk control (not spiked); lane 2, positive PCR control Movar 33/63 (2 $TCID_{50}$); lanes 3 - 7, serial tenfold dilution (1 – 10,000 times) of milk sample 2. Above the lanes, the results of the virus isolation from each dilutions of milk sample 2 are indicated.

3.5 Use of internal control templates

An essential requirement of an internal control template is that it is amplified with the same efficiency as the viral template, and that the influences of the addition of internal control templates on the sensitivity of the PCR assays are negligible. Therefore, the amount of internal control templates must be as low as possible, but always detectable after addition to BHV4-free test samples prior to DNA

extraction. Different amounts of copies of the internal control templates pB88 and pB48 were added to native defatted milk prior to DNA extraction. After DNA extraction and amplification, the amount of copies of each internal control template to be added to each test sample was calculated. In this study, test samples were spiked, prior to DNA extraction, with 2.5 copies/µl of the internal control template pB88 and six copies/µl of the internal control template pB48. This means that in each PCR run, 12.5 copies (5 µl x 2.5) of the internal control template pB88, and 30 copies (5 µl x 6) of the internal control template pB48 were co-amplified with the viral DNA.

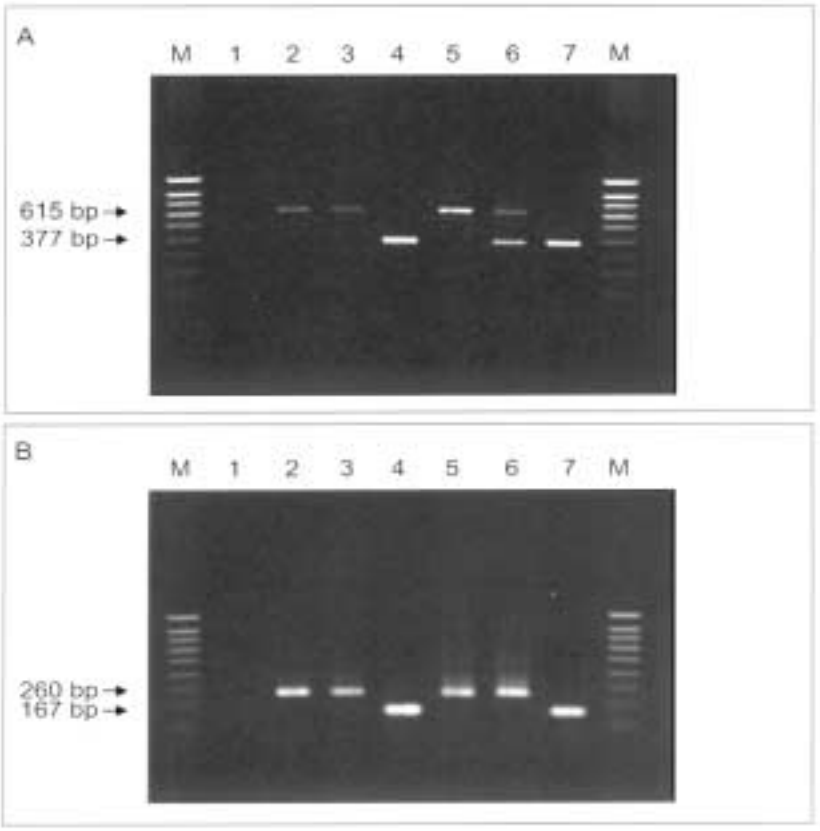

Fig 5. Determination of the detection limits of the gB-PCR and TK-PCR by amplification of serial tenfold dilution of BHV4 DN-599 (stock: $10^{7.0}$ $TCID_{50}$/ml) and Movar 33/63 (stock: $10^{5.1}$ $TCID_{50}$/ml) DNA spiked with internal control templates pB88 and pB48. (A) Amplification products of the gB-PCR of viral DNA (615 bp) and internal control template (377 bp). (B) Amplification products of the TK-PCR of viral DNA (260 bp) and internal control template (167 bp). For A and B: lanes M, 100-bp marker; lane 1, negative milk control (not spiked); lanes 2 - 4, 10^{-5} - 10^{-7} dilution of DN-599; lanes 5 - 7, 10^{-3} - 10^{-5} dilution of Movar 33/63.

To test the influence of the addition of internal template controls on the sensitivity of both PCR assays, we added fixed amounts (as described above) of molecules of the internal template controls pB88 and

pB48 to serial tenfold dilutions of the BHV4 reference strains Movar 33/63 and DN-599, and compared the data on detection limits with those obtained without the addition (see Section 3.3.). For both BHV4 reference strains, the detection limit of the gB-PCR was 2 $TCID_{50}$, while 2 – 0.2 $TCID_{50}$ was detected by the TK-PCR (Fig. 5).

3.6 Comparison of BHV4 detection in milk samples by PCR assays and virus isolation

3.6.1 Milk samples collected from cows inoculated intramammarily with BHV4

In total 61 milk samples were examined for BHV4 by PCR assays and by virus isolation. From 37 out of 61 milk samples BHV4 was isolated. In all those 37 BHV4 positive milk samples, BHV4 DNA was detectable by both PCR assays. From the 24 milk samples, in which no BHV4 was detected by virus isolation, 21 were positive for BHV4-TK DNA and 20 for BHV4-gB DNA. In total, 7 randomly selected PCR products, including 3 PCR products obtained from milk samples that were negative by virus isolation, were examined by restriction enzyme analyses or sequence analysis for specificity. After restriction analyses of gB-PCR and TK-PCR product by restriction enzymes *Ssp*I and *Bsm*I, respectively, the expected restriction fragments were obtained, confirming the specificity of the PCR products. The sequences of PCR products were identical to parts of the BHV4-gB and the BHV4-TK gene. After a TK-PCR on a DNA extract from one milk sample (collected on day 23 pi.), no PCR product of the internal template control was detected, nor amplification products of the viral DNA were observed. BHV4-gB DNA was detected in this milk sample by gB-PCR.

3.6.2 Milk samples from clinical mastitis and control cows

In total, milk samples from 48 clinical mastitis cases and 48 control cows were tested for BHV4 by both PCR assays and by virus isolation. From milk samples of 4 out of 48 clinical mastitis cows, BHV4 specific amplification products were obtained by both PCR assays (Table 2), whereas all control cows were negative. In three clinical mastitis cases BHV4 was isolated from the milk, whereas no BHV4 was isolated from their control cows (Wellenberg et al., 2000). In milk from two of these three clinical mastitis cases, BHV4-gB and BHV4-TK DNA were detected. Statistical analyses, using the Fisher's exact test, demonstrated that BHV4-gB and BHV4-TK DNA were detected more frequently in milk from cows with clinical mastitis than in control cows ($P = 0.059$).

3.7 Comparison between the two BHV4 PCR assays in detecting BHV4 DNA in milk samples from cows intramammarily inoculated with BHV4 and from the case- and control cows

In total, 61 milk samples from cows inoculated intramammarily with BHV4 and 48 milk samples from clinical mastitis cases and 48 milk samples from control cows were examined for BHV4 DNA by the two PCR assays. BHV4-gB DNA was detected in 61 (57 (= 20 + 37) + 4 + 0) milk samples while BHV4-TK DNA was detected in 62 (58 (= 21 + 37) + 4 + 0) of these 157 milk samples. Sixty milk samples were scored positive by both PCR assays, while 94 milk samples were negative. The overall agreement between the two PCR assays was 98% with a $_{K}$D-value of 0.960.

Table 2. Detection of BHV4 in milk samples from clinical mastitis cases (n=48) and matched control cows (n=48) from 10 herds by virus isolation, the gB-PCR and the nested TK-PCR

Herd	Case / control pairs per herd *(M / C)*[a]	BHV4 isolation (milk) *(M / C)*	BHV4 -gB (PCR) *(M / C)*	BHV4 -TK (nPCR) *(M / C)*
1, 2, 4, 5 ,6, 7	30 / 30	-	- / -	- / -
3	3 /3	-	1 / -	1 / -
8	3 / 3	1 / -	1 / -	1 / -
9	5 / 5	1 / -	- / -	- / -
10	7 / 7	1 / -	2 / -	2 / -
Total:	48 / 48	3 / -	4 / -	4 / -

[a] M, mastitis cows; C, control cows

4. Discussion

This study demonstrates that the newly developed gB-PCR and the adapted TK-PCR, can reliably be used for the detection of BHV4 DNA in bovine milk samples. We have chosen to develop a PCR to detect gB sequences because the gene for gB is one of the most conserved among the members of the family of *Herpesviridae*. For most if not all members of the *Herpesviridae,* gB is essential for viral infectivity (Little et al., 1981), and for BHV4, Lomonte et al. (1997) reported that gB is a major component of the virion. These reports imply that the gB gene is probably present in all BHV4 strains. For the development of a gB-PCR, the choice of primers was critical, because: (1) their nucleotide sequence must be within a conserved region of the gB gene of BHV4 to detect all BHV4 variant strains; and (2) on the other hand the primers must not react with regions of the gB genes of other herpesviruses. The selected gB-primers, based on the oligonucleotide sequence of the gB gene as published by Goltz et al. (1994), were shown to be specific as demonstrated by amplification of DNA extracts from all 31 BHV4 strains by the gB-PCR, and the absence of specific amplification products from other animal viruses and human herpesviruses.

The primers, selected for the amplification of regions of the BHV4-TK gene, have been shown to be specific (Egyed et al., 1996), and we confirmed these findings. We chose to target also the BHV4 TK-coding gene for PCR amplification, to detect BHV4 DNA in milk, for three reasons: (1) to evaluate and compare the sensitivity of the gB-PCR with the sensitivity of the nested TK-PCR; (2) to confirm the presence of BHV4 DNA in milk by a second PCR in case BHV4 DNA was detected by gB-PCR (false-positive reactions may occur in PCR assays); and (3) to validate the TK-PCR that we adapted by the addition of an internal control template and in the presence of milk as background. Prior to the validation of the two PCR assays, internal control templates were constructed for each PCR. False-negative PCR results can be traced by using an internal control (Van Engelenburg et al., 1993). Lipid components in milk samples have been reported to inhibit *Taq* polymerase, which can lead to false-negative PCR results (Hamprecht et al., 1998). In addition, milk collected from cows with clinical mastitis show often abnormal morphology, high cell counts, and bacteria, and therefore may contain inhibitors resulting in false-negative PCR results. The influence of these factors on the sensitivity of PCR assays can be determined by the use of an internal control template. Although this control competes with the BHV4 DNA template for e.g. primer binding and enzymes, it did hardly not influence the detection of BHV4 DNA in milk samples. The sensitivities of both PCR assays, with or without the addition of internal control templates, were comparable. The use of an internal control template has certain advantages above the use of an extra PCR to check for quality of DNA extraction and inhibition of amplification. An additional PCR, e.g. for the amplification of β-actin DNA, has to

be performed for each test sample, and therefore this approach is more expensive, laborious, less rapid, and more often other PCR conditions are used. Moreover, the extra PCR cannot give any semi-quantitative indication. Boerner et al. (1999) described a PCR for the detection of BHV4-DNA in combination with a PCR for the amplification of β-actin as test control, but their method seems less useful for screening large numbers of samples by PCR than the use of an "internally controlled" PCR.

In the initial phase of this study the sensitivity, defined as detection limit, of the two PCR assays was determined. Data on detection limits, as obtained by: (1) serial tenfold dilutions of purified BHV4 DNA plasmids; (2) serial tenfold dilutions of the two BHV4 reference strains DN-599 and Movar 33/63 in milk; and (3) serial tenfold dilutions of BHV4 infected milk, showed that both assays were very sensitive. The sensitivity of the gB-PCR, as determined by the detection of low amounts of BHV4 DNA copies, was somewhat lower than the sensitivity of the TK-PCR. Two - ten copies of BHV4 DNA were detectable by the gB-PCR, while one - three copies of BHV4 DNA were detected by the TK-PCR. Boerner et al. (1999) described a PCR for the detection of BHV4 DNA in bovine tissue samples. This PCR, in combination with a Southern blot assay, detected six copies of BHV4 DNA.

This study also demonstrates that the two PCR assays are superior to virus isolation for detecting BHV4 in bovine milk samples collected under experimental and field conditions. BHV4 DNA was detected by gB-PCR and TK-PCR in 93% and 95%, respectively, of the milk samples collected from cows infected intramammarily with BHV4, whereas only 61% were positive by virus isolation. The presence of BHV4 DNA was confirmed by successful amplification of two different regions of the viral genome, supporting the correctness of the PCR results. The fact that many milk samples, which were positive in both PCR assays, were negative by virus isolation could be due to: (1) a low amount of infectious virus (beneath the detection limit of the virus isolation method); (2) the presence of cell toxic components produced by bacteria, e.g. *Streptococcus uberis*, which could influence with virus isolation; and (3) the presence of non-infectious virus particles. A potential advantage of the PCR assays over virus isolation is that BHV4 DNA can be detected in milk samples despite the presence of BHV4 antibodies. BHV4 antibodies have been detected in milk samples of cows infected with BHV4 (unpublished observations). These considerations suggest that PCR assays will improve the diagnosis of BHV4 in milk. Both PCR assays were also able to detect BHV4 DNA in milk samples containing *Streptococcus uberis* and large amounts of cellular DNA (somatic cell count of > 1,000,000/ml). In only one case, a milk sample collected on day 23 pi., the TK-PCR was probably inhibited by components in the milk. No amplification products of the internal control template or any viral templates were detectable.

The value of the two PCR assays, as diagnostic tools for detecting BHV4 in milk, was also

corroborated in a case/control study. BHV4 DNA was detected in four out of 48 cows with clinical mastitis and not in control cows. The data also demonstrate that BHV4 is more frequently detected in mastitis cases than in control cows (P = 0.059). Taken together, these observations and the data recently published by Wellenberg et al. (2000) indicate that BHV4 might play a role in bovine mastitis, although more research on this subject is required. The use of one of the validated PCR assays for the detection of BHV4 DNA in milk could facilitate these research programs. In only one occasion, BHV4 was detected in a milk sample by virus isolation while no BHV4 DNA was detected by both PCRs (herd 9). We have tested this milk sample in triplicate for BHV4 DNA by both PCRs, and in only one occasion BHV4-TK DNA was detected. Taking all PCR data together, this sample had to be presented as "negative" for BHV4 DNA. In addition, herpesvirus-like particles, which were further characterised as BHV4, were detected in inoculated BUE cell cultures only after one passage, while no cpe was observed in the BUE cells inoculated with this milk sample. Based on the data, obtained from virus isolation and both PCRs, we may conclude that the amount of BHV4 in this milk sample was low, and for both methods just around the limit of detection.

Data on the experimental infection and the case/control study, also demonstrate that the results of the single gB-PCR were comparable to those obtained by the nested TK-PCR (overall agreement of 98%, and a high $_{K}$D-value of 0.960). PCR has become an important diagnostic tool for veterinary virologists, and a single PCR has several advantages above nested-PCR assays, e.g. less laborious, less expensive, more rapid, and is less prone to contamination. For practical considerations, we recommend the use of the single gB-PCR for the screening of milk samples for BHV4 DNA, although the nested TK-PCR was slightly more sensitive.

In summary, the gB-PCR and the adapted TK-PCR are specific, sensitive, reproducible and rapid PCR assays for the detection of BHV4-gB and BHV4-TK DNA in bovine milk samples. Both PCR assays have several advantages above virus isolation for the screening of large numbers of bovine milk samples to demonstrate the presence of BHV4.

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CHAPTER 5

BOVINE HERPESVIRUS 4 IN BOVINE CLINICAL MASTITIS

G.J. Wellenberg[1], W.H.M. van der Poel[2], T.J.K. van der Vorst[1], P.H.R. Van Valkengoed[3], Y.H. Schukken[4], F. Wagenaar[5], and J.T. Van Oirschot[1]

[1] *Department of Mammalian Virology, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands.*

[2] *Microbiological Laboratory for Health Protection (MGB), National Institute of Public Health and the Environment (RIVM), P. O. box 1, 3720 BA Bilthoven, The Netherlands.*

[3] *Department of Ruminant Health, Animal Health Service, P.O. Box 9, 7400 AA, Deventer, The Netherlands.*

[4] *Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, 14853 USA.*

[5] *Department of Avian Virology, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands.*

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Abstract

This study describes the first isolation of bovine herpesvirus 4 (BHV4) from milk samples collected from cows with clinical mastitis. Serum and milk samples were collected in 10 different herds from 58 dairy cows with clinical mastitis in the acute and the convalescent phase of the disease, and from 58 healthy matched control cows. Milk samples were examined for presence of viruses, using 4 different types of cell cultures, and for bacterial pathogens. Bovine herpesvirus 4 was isolated, only in bovine umbilical cord endothelial cells, from milk from 3 mastitis cows within 3 different herds, whereas no virus was isolated from their matched control cows. Two of the 3 mastitis cows, from which BHV4 was isolated, developed antibodies against BHV4 concomitant with the infection, whereas none of their matched controls did. In total, 16% of the mastitis cows and 10% of the controls developed antibodies against BHV4. In one herd, 4 cattle developed BHV4 antibodies at the time mastitis occurred, whereas no bacterial pathogens were isolated from the milk. *Streptococcus uberis* was also isolated from the milk from 2 of the 3 cows from which BHV4 was isolated. This association between BHV4 and *Streptococcus uberis* infections was statistically significant. The above findings strongly suggest that BHV4 may play a direct or indirect role in causing bovine mastitis.

Clinical mastitis has the largest economic impact on the dairy cattle industry. Despite intensive bacteriological research, 20 to 35 per cent of clinical cases of bovine mastitis have an unknown aetiology (Miltenburg and others 1996, Barkema and others 1998). Although virus infections have occasionally been associated with bovine mastitis (Siegler and others 1984, Yoshikawa and others 1997), it is generally considered that viruses do not play a role in the aetiology of bovine mastitis (Watts 1988, Radostits and others 1994). This study was undertaken to gain more insight into the possible role of viruses in bovine clinical mastitis, due to the high percentage of unknown causes of clinical mastitis.

In a case/control study, serum and milk samples were collected from 58 dairy cows with clinical mastitis in 10 different Dutch herds at the acute (day 0) and the convalescent phase (day 21) of the disease. Serum and milk samples were also taken from 58 healthy, matched control cows. The control cows were from the same herd, did not show mastitis symptoms, were of the same age as the mastitis cows and were in the same stage of lactation as the mastitis cows. Milk samples from the matched control cows were collected on the same day from the same quarters as the affected quarters of the corresponding clinical mastitis cases. The milk samples were collected as described by the National Mastitis Council (Harmon and others 1990). Samples for virus isolation were stored directly at –70°C, while milk samples for the screening of bacterial agents were stored at 2 to 4°C, and usually cultured within 24 hours. Blood samples were obtained from the median sacral vein of the tail, and centrifuged at 2000 *g* for 10 minutes. Sera were stored at –20°C.

After sampling on day 0, cows with clinical mastitis were treated with antibiotics; the matched controls were not treated. Convalescent samples were taken at least two weeks after the last antibiotic medication. The number of lactating cows in the 10 different herds varied between 31 and 57, and the number of case/control pairs between one to 22 per herd (Table 1). Milk samples were examined for presence of viruses, using 4 different types of cell cultures: embryonic bovine trachea cells (EBTr; a semipermanent cell line developed in the authors' laboratory, ID-Lelystad); bovine epithelial udder cells (including fibroblasts) (Schmid and others 1983); bovine umbilical cord endothelial (BUE) cells (Van de Wiel and others 1989); and bovine alveolar lung macrophages obtained from specified-pathogen-free (SPF) cattle (Schrijver and others 1995).

The milk samples were thawed, defatted by centrifugation at 1500 *g* for 10 minutes, and 0.5 ml of the defatted milk was used for virus isolation. For the virus isolation on macrophages, 100 µl of defatted milk samples was pipetted into the wells of a 96-wells cell culture plate, containing 3 to 5 x 10^4 cells per well. The inoculated cells were incubated for five to seven days at 37°C, with 5 per cent carbon dioxide for the macrophages. After a freeze/thaw cycle, a second passage was performed. During the first and the second passages, cell cultures were observed every day for cytopathogenic effect (cpe). Four controls were included in each culture run. Two controls, containing 10^1 and 10^3

median tissue culture infective dose ($TCID_{50}$) of bovine herpesvirus 1 (BHV-1) per millilitre of milk, served as positive controls. A milk sample without viruses and a plain cell culture control, served as negative controls. After the second passage, a haemadsorption reaction, for the detection of, for example, *Orthomyxoviridae* and *Paramyxoviridae*, was performed on EBTr cells with 0.2 per cent of guinea pig erythrocytes, and incubated at 37°C for one hour. An EBTr cell culture inoculated with parainfluenza type-3 virus was used as positive control. Electron microscopy (EM) was performed after the second passages for all four cell types. A 400 mesh carbon-coated nickel grid with a collodion film was floated on a drop of the inoculated cell cultures for five minutes, drained onto filter paper and stained with 2 per cent phosphotungstic acid (pH 6.8). The grids were examined by transmission EM after drying.

Serum samples collected from mastitis cows were examined for antibodies against BHV-1 by ELISA (Kramps and others 1994), against bovine herpesvirus 2 (BHV-2) by a 24-hour virus neutralisation test (Bushnell and Edwards 1988), against bovine herpesvirus 5 (BHV-4) by ELISA (Wellenberg and others 1999), against bovine respiratory syncytial virus (BRSV) by ELISA (Westenbrink and others 1985), against bovine viral diarrhoea virus (BVDV) by ELISA (Westenbrink and others 1986), against bovine leukaemia virus (BLV) by ELISA (Pourquier, Montpellier, France), and against adenovirus type-3 by ELISA (BIO-X). In case blocking percentages, ELISA coefficients or optical density values indicated a significant increase in antibody titre, the serum samples were titrated by serial two-fold dilution steps. The control cows were only examined for antibodies against BHV-4, because only a few cows with clinical mastitis seroconverted (where seroconversion is defined as a seronegative acute serum and a seropositive convalescent serum) against viruses other than BHV-4. Serum samples containing antibodies against BHV-4, were titrated by serial two-fold dilution steps. A four-fold (two dilution steps) higher antibody titre in convalescent serum compared with acute serum is defined as a significant increase.

Bacteriological culture of milk samples was performed according to standards of the National Mastitis Council (Harmon and others 1990). Milk samples (0.01 ml) were inoculated on 6 per cent blood agar plates (both aerobically and anaerobically), on TCT medium (Thallium sulphate, Crystal Violet, *Staphylococcus* β-toxin; Merck) and on MacConkey number 3 agar (Oxoid). The plates were incubated at 37°C and bacterial growth was evaluated after both 24 and 48 hours. Bacterial colonies were identified as described by the National Mastitis Council. Bacteria were considered to be pathogenic or non-pathogenic, on the basis of the description by Barkema and others (1998) which, in some cases, depended on the number of colonies isolated.

Table 1. Isolation of bovine herpesvirus 4 (BHV-4) and pathogenic bacteria from milk samples, including BHV-4 antibodies in sera from clinical mastitis cases (n = 58) and control cows (n = 58) in 10 herds

Herd	Case / control pairs per herd	BHV4 isolation (milk) (M / C)*	BHV4 antibody at day 0 (M / C)	Pathogenic bacteria in milk (day 0)	
				(M)	(C)
1	1	-	1 / -	*Escherichia coli*	-
2	7	-	1 / 3	*E. coli* (x2), *S. uberis* (x2)	-
3	3	-	3 / 1	-	-
4	22	-	2 / 1	CNS (x2)**, *E. coli* (x2), *S. aureus* (x2),*S. uberis* (x3)	-
5	1	-	- / -	CNS	-
6	1	-	1 / 1	-	-
7	2	-	1 / -	*S. aureus*, *S. aureus* + *A. pyogenes*	*S. aureus*
8	3	1 / -	1 / 1	*S. uberis*, *S. dysgalactiae*	-
9	10	1 / -	- / 1	*E. coli*, *S. aureus* (x2), *E. coli* + *S. uberis*, CNS (x2), *S. dysgalactiae*, *S. uberis*.	*S. aureus* (x2)
10	8	1 / -	2 / 4	*A. pyogenes*, *C. bovis* (x2), *S. uberis*.	*C. bovis* (x3)
Total	58	3 / -	12 / 12	31	6

*) M mastitis cows, C control cows,

**) CNS Coagulase-negative staphylococci.

No cpe was observed during the first and second passages in EBTr cell cultures, in bovine udder epithelial cells, or macrophages that were inoculated with milk samples from the cows with clinical mastitis. No virus particles were detectable in these cell cultures by EM. The haemadsorption reaction, performed on EBTr cell cultures after the second passage, was negative for all these samples. The BUE cell cultures inoculated with milk samples from the clinical mastitis cow 49 (day 0), and cow 400 (day 21) of herd 8 and herd 10, respectively, showed cpe six to seven days after inoculation. Herpesvirus

particles were detected by EM in the BUE cell cultures inoculated with milk from cow 12 of herd 9 (day 0 and 21). No virus particles were detected in all the other milk samples from cows with clinical mastitis and the matched control cows by virus isolation on BUE cells, or by EM. The results of virus isolation on herd level and on an individual level are given in Tables 1 and 2, respectively. The three virus isolates were characterised as herpesviruses by EM. The virus isolates and control BHV-4 reference strains DN-599 and LVR 140, were partly neutralised with monospecific antiserum against BHV-4, and not with monospecific antisera against BHV-1 and BHV-2. In an immunoperoxidase monolayer assay, the three virus isolates and the BHV-4 reference strains DN-599 and LVR 140 reacted with monoclonal antibody 123 which is directed against glycoprotein 1 of BHV-4 (Dubuisson and others 1992), while BHV-1, BHV-2 and bovine herpesvirus 5 (BHV-5) did not react with monoclonal antibody 123. Restriction enzyme analyses of DNA of the virus isolates, and the BHV-4 reference strains DN-599, Movar 33/63 and LVR 140, showed that the patterns of the virus isolates were comparable to those of the BHV-4 reference strain Movar 33/63 (Fig 1). Statistical analysis, using the sign test, indicated that BHV-4 was more frequently isolated from mastitis cows than from controls (P = 0.125).

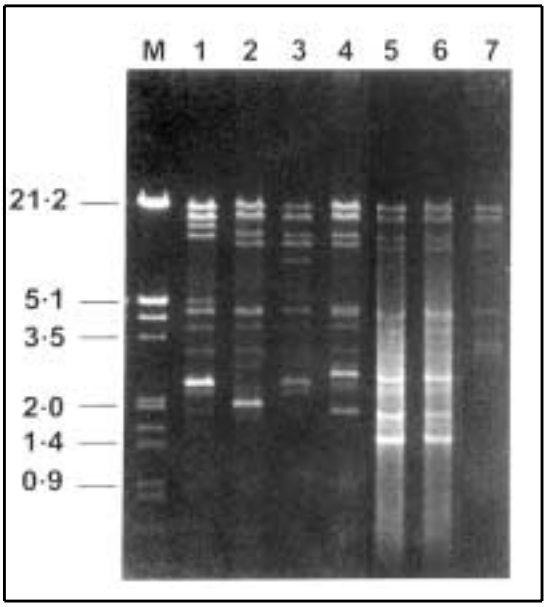

Fig 1. Restriction endonuclease analysis of viral DNA digested with *Eco*RI. Lane 1 bovine herpesvirus 4 (BHV-4) DN-599, Lane 2 BHV-4 Movar 33/63, Lane 3 BHV-4 LVR 140, Lane 4 BHV-4 strain from herd 8 (day 0), Lane 5 BHV-4 strain from herd 9 (day 0), Lane 6 BHV-4 strain from herd 9 (day 21), Lane 7 BHV-4 strain from herd 10 (day 21), M Marker. Molecular mass standards (in kb) are identified on the left.

Table 2. Isolation of bovine herpesvirus 4 (BHV-4) and pathogenic bacteria from milk, and antibody development against BHV-4 in sera from clinical mastitis cows and the matched control cows on an individual level

			BHV4			
Herd	Cow	Case / control	Virus isolation from milk	Antibody development (serum) * acute phase	convalescent phase	Pathogenic bacteria in milk (day 0)
1	24	case	-	-	-	*E. coli* (sporadic)
		control	-	-	800	*C. bovis* (day 21)
4	16	case	-	-	800	-
		control	-	-	-	-
	33	case	-	-	400	-
		control	-	-	400	-
	37	case	-	-	200	-
		control	-	-	400	-
	51	case	-	100	400	-
		control	-	-	-	-
	54	case	-	100	400	*S. uberis* (sporadic)
		control	-	-	-	-
8	49	case	+	100	400	*S. uberis*
		control	-	-	-	-
	52	case	-	-	-	*S. dysgalactiae*
		control	-	200	800	-
9	28	case	-	-	800	*S. uberis* and *E. coli*
		control	-	-	-	-
	22	case	-	-	-	*S. aureus*
		control	-	-	200	*S. aureus* (day 21)
	12	case	+	-	-	*E. coli*
		control	-	-	-	-
10	275	case	-	-	100	*A. pyogenes*
		control	-	200	800	-
	400	case	+	-	200	*S. uberis*
		control	-	-	-	-

Antibody titres expressed as the reciprocal of the highest serum dilution giving a positive reaction; - Not detected, + Detected

Two of the three cows from which BHV-4 was isolated, developed antibodies against BHV-4 (cow 49 and cow 400), while no antibodies against BHV-4 were detectable in cow 12 within 21 days. No development in BHV-4 antibodies was detected in the three corresponding control cows of these three cows (Table 2). In herd 4, which had a large number of clinical mastitis cases, even five cows with clinical mastitis developed antibodies against BHV-4 at the time mastitis occurred. In total, 16 per cent of the mastitis cows and 10 per cent of the controls developed antibodies against BHV-4. However, development of antibodies against BHV-1, BRSV or adenovirus type 3 was detected in only four cows with clinical mastitis, but none of these viruses was isolated from the milk. In the sera of these four cows, no antibodies against BHV-4 were detected. No development of antibodies was detected against BHV-2, BVDV or BLV in the sera of all 58 cows with clinical mastitis.

Pathogenic bacteria were isolated in 31 (53 per cent) of the 58 milk samples from clinical mastitis cows that were collected on day 0 (Table 1). *Streptococcus uberis* was also isolated from the milk samples of cow 49 in herd 8 on day 0 (day 21; not determined), and cow 400 in herd 10 both on day 0 and 21 (Table 2). *Escherichia coli* was isolated from the milk of cow 12 (herd 9) on day 0 and day 21. Fisher's exact test showed that there was a significant positive association between BHV-4 isolation and *S. uberis* isolation ($P = 0.02$).

This is the first report on the isolation of BHV-4 from milk from cows with clinical mastitis. Although, BHV-4 DN-599 strains have been isolated from udder lesions from lactating dairy cows with mammary pustular dermatitis (Reed and others 1977), and BHV-4 has been isolated from the cellular fraction of a milk sample collected from a cow with chronic ulcerative mammary dermatitis (Cavirani and others 1990), BHV-4 has never been identified as a possible cause of bovine mastitis. The three cows from which BHV-4 was isolated did not show mammary dermatitis or teat lesions. BHV-4 DN-599 strain was also recovered from milk samples after experimental inoculation via the teat channel, showing that viral multiplication can occur after injection into the mammary gland (Osorio and Reed 1983).

The following findings further support a role of BHV-4 infection in bovine mastitis: in four of the 10 herds of this study there was an ongoing BHV-4 infection at the same time as clinical mastitis occurred; in herd 4, where more than 50 per cent of the dairy cows showed clinical mastitis problems per year, five (23 per cent) mastitis cows showed seroconversion or a significant increase in antibody titre against BHV-4, compared with two (9 per cent) controls (Table 2); in four of the five mastitis cows of this herd no pathogenic bacteria were isolated from milk, only a few colonies of *S. uberis* were isolated from the milk of the fifth cow; of all 58 mastitis cows, 16 per cent developed antibodies against BHV-4, and 10 per cent of the 58 control cows did so, but because subclinical mastitis was not

included in the case population, the latter relatively high percentage might partially be explained by the fact that some control cows had subclinical mastitis. Support for this explanation is the finding that in milk samples of two control cows that developed antibodies against BHV-4, *Corynebacterium bovis* (herd 1) or *Staphylococcus aureus* (herd 9) were isolated, both on day 21 only (Table 2); in nine of the 10 herds studied, antibodies against BHV-4 were prevalent, whereas only 57 per cent of 30 randomly chosen Dutch herds had antibodies against BHV-4 (Wellenberg and others 1999).

Whether BHV-4 may play a direct and/or indirect role in the pathogenesis of bovine mastitis remains to be clarified. The finding that four cows develop antibodies against BHV-4 at the time of clinical mastitis and that no pathogenic aerobic or anaerobic bacteria were isolated from these cows, suggests BHV-4 as a primary cause. On the other hand, the concomitant isolation of BHV-4 and of *S. uberis* or *E. coli*, both known to cause severe clinical mastitis (Barkema and others 1998), suggest that BHV-4 infection of the udder may lead to a higher susceptibility for bacterial infections. VanOpdenbosch and others (1984) reported that BHV-4 could induce immunosuppression favouring the development of secondary infections in cattle. Infected splenic mononuclear cells and peripheral blood leukocytes persistently infected with BHV-4 (Osorio and Reed 1983) could reduce the phagocytic function of these cells. This enhances the possibility for bacterial infections to run a more severe course which, in turn, may result in prolonged bacterial infections. On the other hand, cases of clinical mastitis, that is, induced by *S. uberis*, could lead to reactivation of latent BHV-4 infection, which in turn also may enhance the severity of mastitis cases.

The fact that this is the first report on the isolation of BHV-4 in milk samples from cows with clinical mastitis may be explained by the use of BUE cells which are seldom used in bovine virology. These cells appear to be much more susceptible to BHV-4 than other bovine cells, for example, several BHV-4 strains reached 20- to 3000-fold higher titres in BUE cells than in EBTr cells (data not shown). The susceptibility of endothelial cells to BHV-4 has recently been reported by Lin and others (1997).

In conclusion, these results demonstrate that BHV-4 may play a role in clinical mastitis in dairy cows. Whether BHV-4 plays a primary or secondary role in the pathogenesis of bovine clinical mastitis needs to be elucidated. The authors are currently performing experimental infections with the BHV-4 isolate to attempt to reproduce bovine mastitis, and to fulfil Koch's postulates.

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CHAPTER 6

DETECTION OF BOVINE HERPESVIRUS 4 IN MILK FROM COWS WITH MASTITIS: A SECOND CASE-CONTROL STUDY

G.J. Wellenberg[1], O. Sampimon[2], J. Sol[2], and J.T. Van Oirschot[1]

[1] *Division of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands*

[2] *Department of Ruminant Health, Animal Health Service, P.O. Box 9, 7400 AA, Deventer, The Netherlands*

Abstract

A second case-control study was performed to investigate whether bovine herpesvirus 4 (BHV4) plays a role in the aetiology of bovine mastitis. Serum and milk samples were collected in 16 different herds from 54 dairy cows in the acute and convalescent phases of mastitis and from 54 healthy matched control cows. Milk samples were examined for the presence of BHV4 by a BHV4 glycoprotein-B (gB) PCR and by virus isolation, and for the presence of bacterial udder pathogens. The milk somatic cell counts (SCC) were determined, and serum samples were screened for BHV4 antibodies. Bovine herpesvirus 4 gB DNA was detected by PCR in 3 milk samples from 2 clinical mastitis cows. BHV4 was isolated on bovine umbilical cord endothelial cells from the same 3 milk samples, whereas no BHV4 was detected by gB-PCR and virus isolation from their matched control cows. One of the cows, from which BHV4 was isolated, developed antibodies against BHV4 concomitant with the BHV4 infection, whereas no increase in BHV4 antibodies was detectable in the second cow within 21 days. No BHV4 antibodies were detected in both matched control cows. *Staphylococcus aureus* and a few colonies of *Staphylococcus sp.* were isolated from 2 out of 3 milk samples from which BHV4 was isolated, whereas only BHV4 was isolated from the third milk sample (day 21). The somatic cell count of this latter milk sample was 305.000 cells/ml.

This study confirmed the finding that BHV4 may play a role in the aetiology of bovine mastitis, although, it probably does not play an important role as primary agent in the aetiology of bovine clinical mastitis.

1. Introduction

Bovine herpesvirus 4 (BHV4), a member of the subfamily of the *Gammaherpesvirinae*, has been isolated from cows with various clinical signs, but also from apparently healthy cows (Thiry et al., 1989). In a case-control study, BHV4 has been isolated from milk of cows with clinical mastitis, but not from control cows (Wellenberg et al., 2000). A simultaneous intramammary and intranasal inoculation of lactating cows with BHV4 resulted in subclinical mastitis in 2 out of 4 inoculated lactating cows (Wellenberg et al., 2001a). Others, Zadoks et al. (2001), reported a positive association between the BHV4 seropositivity of cows and the incidence of bovine mastitis caused by *Staphylococcus aureus*, which may suggest that a BHV4 infection makes cows more susceptible for mastitis especially when caused by *Staphylococcus aureus*. BHV4 has also been isolated from the cellular fraction of milk from cows with antibodies against BHV4. Unfortunately, no data were reported whether these milk samples were collected from cows with clinical or subclinical mastitis (Donofrio et al., 2000).

We performed this second case-control study to get additional insight in the role of BHV4 in the aetiology of bovine mastitis, and to examine whether results from the first case-control study could be confirmed.

2. Materials and methods

2.1 Design of the study and collection of samples

This second case/control study was started in 21 dairy herds with cows that suffered from clinical mastitis. Clinical mastitis cases were defined as: abnormal morphology of the milk, painful udder, swelling of the udder, or a combination of these signs. The farmer notified the local veterinarian when observing these signs. At the first visit, the local veterinarian selected matched control cows in the same herd that**:** (a) did not show signs of mastitis; (b) were of the same age; and (c) were in the same stage of lactation as the mastitis cows. Samples were collected by the veterinarians and veterinary technicians from dairy cows with clinical mastitis in the acute (day 0) and the convalescent phase of the disease (day 21). At the same time, milk samples (from the same quarter as the affected quarter of the corresponding case) were collected from their matched control cows. Milk samples were collected according to the procedure described by the National Mastitis Council (Harmon et al., 1990).

In this study, from the 21 dairy herds, milk samples were collected from 90 cows with mastitis and 90 controls. Unfortunately, in 36 out of the 90 control cows an increased milk somatic cell counts (SCC) of ≥250,000 was recorded on day 0 or on day 21, which is an indication of subclinical mastitis (Dohoo and Leslie, 1991; Smith, 1996). These case-control pairs were not included in this study, and thus milk from the remaining 54 cases and control cows on 16 herds were used for further examination (Table 1).

Milk samples for BHV4-gB DNA detection and virus isolation were stored directly at –70 °C, while milk samples for the screening of bacterial agents were stored at 2-4°C, and cultured within 24 hours. Milk samples used for the detection of SCC were also stored at 2-4°C, and the milk SCC was determined within 24 hours. Blood samples were obtained from the median sacral vein of the tail, and centrifuged at 1,500 x g for 10 minutes. Sera were stored at -20°C. After sampling on day 0, cows with clinical mastitis were treated with antibiotics; the matched controls were not treated. Convalescent blood and milk samples were taken by the local veterinarians 21 days ± 2 days after the last antibiotic medication.

2.2 Detection of BHV4

2.2.1 DNA extraction and BHV4-gB PCR

DNA extractions from defatted milk samples, and from inoculated cell cultures, were performed by column chromatography using the QIAamp Blood and Tissue kit (QIAGEN, Westburg, The Netherlands). For DNA extraction of defatted milk samples a total volume of 1000 µl was used per cow. Prior to DNA extraction, the defatted milk samples were centrifuged at 14,000 rpm for 3 min. A volume of 800 µl was discarded and the cell pellet was resuspended in the remaining 200 µl. Cell lysis, digestion with protease, DNA precipitation and elution of DNA were performed according to the description of the manufacturer. The DNA extraction products were stored at –20 °C. A gB-PCR assay, which has proven to be a valuable tool in detecting BHV4-gB DNA in milk, was performed on DNA extractions (Wellenberg et al., 2001b).

2.2.2 Virus isolation

Milk samples were thawed, defatted by centrifugation at 1,500 x g for 10 minutes, and a volume of 0.5 ml of the defatted milk was used for virus isolation. Bovine umbilical cord endothelial (BUE) cells (Van de Wiel et al., 1989) were used for BHV4 isolation and propagation. BUE cells were grown and maintained in Dulbecco's minimal essential medium (DMEM) (Gibco Laboratories, Life Technologies Inc., USA) supplemented with 10% normal calf serum (Gibco Laboratories, Life Technologies Inc., USA), and 0.5% antibiotic mix (stock mix contained: 10,000 IU penicillin, 11.25 mg streptomycin, 10 mg kanamycin and 5,000 IU nystatin per ml). Normal bovine calf serum was shown to be free of BHV4 antibodies. Cells were inoculated with 0.5 ml of defatted milk. Inoculated cells were washed after an incubation of 1 hour at 37°C, and incubated for 5-7 days at 37°C (5% CO_2). After a freeze/thaw cycle a second passage was performed by inoculating semi-permanent BUE cell cultures with 1 ml freeze/thawed virus/cell suspension. Inoculated cell cultures were incubated again for 5-7 days at 37°C (5% CO_2). During the first and the second passage, cell cultures were observed for cytopathic effect (cpe). Cell cultures showing cpe were examined for the presence of virus particles by electron microscopy (EM). The immunoperoxidase monolayer assay (IPMA) and the gB-PCR were then performed on these cell cultures to identify BHV4. In each cell culture run, 5 controls were incorporated. Three controls, containing 10^1, 10^2 and 10^3 $TCID_{50}$ BHV4/ml milk, served as positive controls. A BHV4-free milk sample, and a non-inoculated cell culture, served as negative controls.

2.2.3 Electron microscopy

Electron microscopy was performed on BUE cell cultures showing cpe. Therefore, a 400 mesh carbon coated nickel grid with a collodion film was floated on a drop of the inoculated cell cultures for 5 minutes, drained onto filter paper and stained with 2% phosphotungstic acid (pH 6.8). The grids were examined by transmission electron microscopy after drying.

2.2.4 Immunoperoxidase monolayer assay

The IPMA, to identify BHV4, was basically performed as described before (Wellenberg et al., 1999). Briefly, virus infected monolayers of BUE cells were washed with phosphate buffered salt solution, dried for 45 minutes at 37°C, and fixed with 150 µl of 4% paraformaldehyde. After Triton X-100, and 10% horse serum pre-adsorption treatments, cells were incubated with a monospecific BHV4 antiserum. Plates were washed and incubated with a rabbit anti-bovine immunoglobulin-HRPO conjugate (Dakopatts, Glostrup, Denmark). After staining with a chromogen/substrate solution (AEC/H_2O_2), the results were read by light microscope. BHV4 DN-599 and non-inoculated BUE cells served as positive and negative controls.

2.3 Somatic cell counts (SCC)

Somatic cells in quarter milk samples were counted by means of a Fossomatic (Foss Electric, Hillerød, Denmark). Mastitis was defined as subclinical in cases of quarter milk SCC of ≥250,000 cells/ml (Dohoo and Leslie, 1991; Smith, 1996), without any visible abnormality of the milk or the udder. Clinical mastitis cases showed increased milk SCC of ≥250,000 cells/ml and with visible abnormality of the milk sample or the udder (International Dairy Federation, 1987).

2.4 BHV4 antibody detection

Serum samples were examined for antibodies against BHV4 by IPMA (Wellenberg et al., 1999). Sera were screened in dilutions of 1:20, 1:40 and 1:200. Serum samples, showing possible increases of BHV4 antibodies, were further titrated by serial 2-fold dilution steps starting at a dilution of 1:100. A 4-fold (2 dilution steps) higher antibody titre in convalescent serum than in acute serum was defined as a significant increase.

2.5 Bacterial analyses

Bacteriological culture of milk samples was performed based on standards of the National Mastitis Council (Harmon et al., 1990). Briefly, milk samples (0.01 ml) were inoculated on 6% blood agar plates (both aerobically and an-aerobically), on TCT medium (Thallium sulphate, Crystal Violet, Staphylococcus β-toxin; Merck) and on MacConkey no. 3 agar (Oxoid, Basingstoke, UK). Plates were incubated at 37° and bacterial growth was evaluated after 24 and after 48 hours. Bacterial colonies were identified as described by the National Mastitis Council. A quarter was considered to have an intramammary infection when ≥500 cfu/ml of minor udder pathogens were cultured from the milk, or when ≥100 cfu/ml of a major udder pathogen was cultivated from a quarter with clinical signs of mastitis (Lam et al., 1997; Barkema et al., 1999). If three or more bacterial species were cultivated from a sample, the sample was considered to be contaminated.

3. Results

In 3 milk samples, collected from mastitis cows, BHV4 was detected by gB-PCR and by virus isolation, namely in milk collected on day 0 and in the convalescent milk sample from cow No. 6600, and in a milk sample from cow No. 2789 on day 21 (Tables 1 and 2).

Table 1. Detection of BHV4 by gB-PCR and virus isolation from milk samples, and BHV4 antibody detection in sera from clinical mastitis cases (n=54) and matched control cows (n=54) in 16 herds

Herd	Case / control pairs per herd	BHV4-gB PCR (milk) (M / C)*	BHV4 isolation (milk) (M / C)	BHV4 antibody at day 0 (M / C)
1	1	- **	-	- / -
2	4	-	-	- / -
3	2	-	-	- / -
4	1	-	-	- / -
5	4	-	-	2 / 2
6	2	-	-	- / -
7	2	-	-	- / 2
8	9	-	-	3 / 4
9	1	-	-	- / -
10	6	-	-	- / -
11	3	-	-	1 / 2
12	2	-	-	- / -
13	6	-	-	4 / 4
14	2	1 / -	1 / -	2 / 1
15	2	-	-	- / 1
16	7	1 / -	1 / -	2 / 2
Totals	54	2 / -	2 / -	14 / 18

*): M = mastitis cows / C = control cows; **): - = Not detected

The BUE cell cultures inoculated with milk samples from mastitis cows No. 6600, collected on day 21, showed cpe on day 6 post-inoculation (first passage), whereas the BUE cell cultures inoculated with the other 2 milk samples showed cpe in the second passage. Electron microscopy revealed the presence of virus particles, possessing the typical herpesvirus morphology, in cell cultures inoculated with the milk sample of cow No. 6600 (day 21). The presence of BHV4 antigens and viral DNA in cell cultures, inoculated with the 3 milk samples, was confirmed by IPMA and gB-PCR, respectively. No BHV4 antigens or BHV4-gB DNA was detected in cell cultures, inoculated with milk from their matched control cows. In addition, BHV4 was not detected in milk from the other control cows by gB-PCR or virus isolation.

BHV4 antibodies were detected in cows from 8 (50%) of the 16 examined herds. Both cows, from which BHV4 was isolated, had BHV4 antibodies in their first collected blood sample. A significant increase in BHV4 antibodies was detected in cow No. 2789 (Table 2), and no increase in BHV4 antibody titre was detectable in cow No. 6600 within 21 days. In addition, no BHV4 antibodies were detected in the 2 corresponding control cows of these 2 mastitis cows (Table 2).

Seroconversion was detected in sera from 2 other control cows. These control cows did not develop clinical or subclinical mastitis and their SCC were <250,000 cells/ml (Table 2).

Pathogenic udder bacteria were isolated from 40 (74%) out of 54 milk samples collected from mastitis cows on day 0 (data not shown). *Staphylococcus aureus* was isolated from the milk from cow No. 6600 on day 0 (SCC of >1,000,000 cells/ml), whereas, no bacteria were isolated on day 21 (SCC of 305,000 cells/ml) (Table 2). A few colonies of *Staphylococcus sp*. were isolated from the milk of cow No. 2789 from which BHV4 was isolated too (the SCC was >1,000,000 cells/ml). No bacteria were isolated from the milk of the matched control cows and the SCC in their milk samples were <250,000 cells/ml.

Table 2. Isolation of BHV4 and pathogenic bacteria from milk, antibody development against BHV4 in serum, and quarter SCC in milk from clinical mastitis cows and their matched control cows presented on an individual level

			BHV4				
Herd	Cow	Case / control	Virus isolation from milk	Antibody development (serum) (acute)	(convalescent)	Pathogenic bacteria in milk (day 0)	Milk SCC/ml (x 1000)
8	1327	case	-*	-	-	-	3781
		control	-	-	200	*C. bovis* (day 21)	32
14	2789	case	- (day 0) + (day 21)	400	3200	*S. dysgalactiae* *Staphylococcus sp.* (a few colonies)	>1,000 >1,000
		control	-	-	-	-	203
15	1120	case	-	-	-	*S. aureus*	4990
		control	-	-	200	*S. aureus*	4
16	6600	case	+ (day 0) + (day 21)	800	800	*S. aureus* -	>1,000 305
		control	-	-	-	-	3

* - Not detected

4. Discussion

In this second case-control study, which was performed on other farms, in another region of the Netherlands and 3-4 years later than the first case-control study, BHV4 was detected in milk from 2 (4%) out of 54 cows with clinical mastitis, and not from control cows. This finding agrees with one of the findings of a previous study in which BHV4 was isolated from milk of 3 (5%) out of 58 cows with clinical mastitis (Wellenberg et al., 2000). In addition, BHV4-gB DNA was detected in 4 (8%) out of 48 mastitis cows from the same case-control study (Wellenberg et al., 2001b).
Concomitant with the isolation of BHV4, an increase in BHV4 antibodies was detected in 1 of the 2 clinical mastitis cows. In the first case-control study, an increase of BHV4 antibodies was detected in 2 out of 3 clinical mastitis cows, from which BHV4 was isolated from the milk. These data support the finding that there was an ongoing BHV4 infection at the same time as clinical mastitis occurred. On the other hand, an increase of BHV4 antibodies was not only detected in 1 (2%) of the mastitis cows, but also in 2 (4%) control cows without clinical or subclinical mastitis. This is in contrast to data obtained from the first case-control study, in which more mastitis cows developed BHV4 antibodies (16%) than controls (10%). Based on data of the first study, we concluded that the relatively high percentage of controls, that developed BHV4 antibodies, might partially be explained by the fact that some control cows developed subclinical mastitis, as the quarter milk SCC was not determined during that study. However, the controls in the second case-control study, which developed antibodies against BHV4, did not develop mastitis and their milk SCC stayed <250,000 cells/ml. This confirms that dairy cows can undergo a BHV4 infection that is not associated with mastitis as was also evident from the literature (Thiry et al., 1989; Egyed, 2000).

Another finding in the first case-control study that supported a possible role of BHV4 in bovine clinical mastitis was that in 9 (90%) of the 10 herds studied, antibodies against BHV4 were prevalent, whereas, only 57% of 30 randomly chosen Dutch herds had antibodies against BHV4 (Wellenberg et al., 1999). In this study, in only 8 (50%) of the 16 examined herds antibodies against BHV4 were detected. However, the number of BHV4 positive herds might have been higher in case more than 1 or 2 cows within one herd were examined for antibodies against BHV4 as was the case in 9 examined herds.

In the first case-control study, BHV4 was isolated from milk in combination with known udder pathogens, such as *Streptococcus uberis* and *Escherichia coli*. In the present study, in two of the three cases BHV4 was accompanied again by bacteria, although other bacteria than noted in the first case-control study. BHV4 was isolated from milk from cow No. 2789 (day 21) in combination with a few colonies of *Staphylococcus sp*. (however no *Staphylococcus aureus*), and from milk of cow No. 6600

in combination with the known udder pathogen *Staphylococcus aureus* (day 0). The presence of known bacterial udder pathogens, besides the presence of BHV4 in milk, makes it even more difficult to understand which primary role BHV4 plays in the aetiology of bovine clinical mastitis. In only one occasion, BHV4 was isolated from milk (cow No. 6600; day 21) while no udder pathogens were detected, and the quarter milk SCC was still increased (305,000 cells/ml).

In conclusion; both case-control studies, in which BHV4 and/or BHV4-gB DNA were detected in milk of 4.5% of the 112 examined mastitis cows and never in milk from matched control cows, support the hypothesis that BHV4 may play a role in the aetiology of bovine mastitis. Based on data found in the 2 case-control studies and the experimental infection (Wellenberg et al., 2001a), we may conclude that this agent does not play an important role as primary agent in the aetiology of bovine clinical mastitis, although it could be involved in subclinical mastitis cases. The data of an epidemiological study indicate that BHV4 may also play an indirect role in the aetiology of bovine mastitis, because BHV4 seropositive cows have a higher susceptibility for mastitis especially when caused by *Staphylococcus aureus* (Zadoks et al., 2001).

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CHAPTER 7

A SIMULTANEOUS INTRAMAMMARY AND INTRANASAL INOCULATION OF LACTATING COWS WITH BOVINE HERPESVIRUS 4 INDUCED SUBCLINICAL MASTITIS

G.J. Wellenberg[1], C.J.M. Bruschke[1], H.J. Wisselink[1], H.W. Barkema[2], and J.T. Van Oirschot[1].

[1]Division Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands.

[2]Animal Health Service, P.O. Box 361, 9200 AA, Drachten, The Netherlands.

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Abstract

In this study we examined whether an experimental bovine herpesvirus 4 (BHV4) infection can induce bovine mastitis, or can enhance bovine mastitis induced by *Streptococcus uberis* (*S. uberis*). Four lactating cows were inoculated intramammarily and intranasally with BHV4, and four lactating control cows were mock-inoculated. After 14 days two of four cows from each group were inoculated intramammarily with *S. uberis*. No clinical signs were recorded in cows inoculated only with BHV4, and their milk samples showed no abnormal morphology, despite the fact that BHV4 replicated in inoculated quarters. Somatic cell count increased significantly in milk from 3 of 6 BHV4 inoculated quarters, compared to the non-inoculated quarters of the same cows (within-cow) and the quarters of mock-inoculated cows (control group) on days 8, 9 and 11 post-inoculation (pi). BHV4 was isolated from nasal swabs between days 2 and 9 post-inoculation (pi). Clinical mastitis was observed in all four cows intramammarily inoculated with *S. uberis*. A preceding BHV4 infection did not exacerbate the clinical mastitis induced by *S. uberis*. *Streptococcus uberis* infections appeared to trigger BHV4 replication. From one quarter of each of two cows inoculated with BHV4 and *S. uberis*, BHV4 was isolated, and not from quarters inoculated with BHV4 only.

In conclusion, BHV4 did not induce bovine clinical mastitis after simultaneous intranasal and intramammary inoculation. However, the BHV4 infection did induce subclinical mastitis in 50% of the cows and the quarters.

1. Introduction

Mastitis is a disease with large economic impact on dairy cattle industry (Miller and Dorn, 1990; Schakenraad and Dijkhuizen, 1991). Bacteria and non-bacterial pathogens play a role in clinical mastitis (Radostits et al., 1994; Watts, 1998). Despite intensive implementation of control programs, still around 20-35% of clinical cases of bovine mastitis have an unknown etiology (Miltenburg et al., 1996). The percentage of bacteriological culture-negative milk samples of both clinical and subclinical mastitis cases is still approximately 25% (Barkema et al., 1998). Recently, in a case-control study, bovine herpesvirus 4 (BHV4) was isolated from milk samples collected from cows with clinical mastitis, whereas no virus was isolated from matched controls (Wellenberg et al., 2000). Concomitant development of BHV4 antibodies in cows supported the hypothesis that BHV4 may play a role in mastitis in dairy cows. In the same study, a positive association was found between BHV4 isolation and the isolation of *S. uberis* from milk samples.

This study was performed to examine whether: a) mastitis was induced after a simultaneous intramammary and intranasal inoculation of lactating cows with BHV4; b) a preceding BHV4 infection exacerbated clinical mastitis induced by *S. uberis*; and c) a *S. uberis* infection triggered the replication of BHV4.

2. Materials and methods

2.1 Cell culture, virus and bacteria

A pestivirus-free and mycoplasma-free bovine umbilical cord endothelial (BUE) cell line was used for virus isolation and multiplication (Van de Wiel et al., 1989). BUE cells were grown and maintained in Dulbecco's minimal essential medium (DMEM) (Gibco Laboratories, Life Technologies Inc., USA) supplemented with 10% foetal bovine serum (Gibco Laboratories, Life Technologies Inc., USA), and 0.5% antibiotic mix (stock mix contained: 10,000 IU penicillin, 11.25 mg streptomycin, 10 mg kanamycin and 5,000 IU nystatin per ml). The BHV4 strain Tolakker, which has been isolated from a cow with clinical mastitis, was used for inoculation (Wellenberg et al., 2000). After isolation, this virus was passaged once in BUE cells to obtain a virus stock. The stock virus batch, used for animal inoculation, contained $10^{7.3}$ median tissue culture infective doses ($TCID_{50}$)/ml of BHV4 strain Tolakker, and was free of other herpesviruses, pestiviruses, mycoplasmas, and bacteria. *Streptococcus uberis* strain O140J has originally been isolated from a cow with mastitis, and was kindly provided by P. Milner (Milner et al., 1996). Prior to inoculation, the *S. uberis* strain O140J was cultivated in Todd-Hewitt broth for 24 hours at 37°C, and was found to be pure.

2.2 Animals and experimental design

Eight cows were selected from three different farms two weeks before the start of the study. Inclusion criteria for selection were: a) no clinical or subclinical mastitis, (b) the same parity (first or second parity), c) no history of *Streptococcus (uberis*) infections, d) first half of lactation, and e) no treatment with antibiotics during the last month. In addition, milk samples from each udder quarter and blood samples were collected from these eight cows to examine whether: (a) these cows contained no antibodies against BHV4, (b) milk samples of the four quarters were free of udder pathogens, and c) cows were BVDV-free. The eight cows were randomly allotted to a BHV4 and a control group. The BHV4 and the control group were housed in two separate isolation units, and cows were tied individually. Hygienic rules were strictly followed to prevent transmission of viruses and bacteria between the two groups and from one cow to another. The four cows of the BHV4 group (B1, B2, B3 and B4) were inoculated intramammarily into the right forequarter (RF), and left hindquarter (LH) with $10^{7.3}$ $TCID_{50}$ of the BHV4 strain Tolakker diluted in 20 ml of buffered phosphate salt solution (PBS) per quarter (Table 1). The other two quarters (right hindquarter (RH), and left forequarter (LF)) were not inoculated and served as within-cow control quarters. Prior to each intramammary

inoculation via the teat channel, teats were disinfected with alcohol. After each inoculation, using a syringe with a short blunt ended needle, the udder was massaged to distribute the inoculum. The four cows of the BHV4 group were also intranasally inoculated with one ml containing $10^{7.3}$ $TCID_{50}$ of BHV4 per nostril, using a nozzle attached to a syringe to produce a spray. Cows of the control group (C1, C2, C3, and C4) were mock-inoculated into the same two quarters as described above for the BHV4 group with 20 ml BHV4-free BUE cell culture medium. The cows of the control group were also intranasally inoculated as described for the BHV4 group with BHV4-free BUE cell culture medium.

Fourteen days after the BHV4 inoculation, the RF and LH quarters of cows B1 and B4 from the BHV4 group, and of cows C1 and C2 from the control group, were inoculated intramammarily with 800 colony-forming-units (cfu) of *S. uberis* strain O140J in 20 ml of PBS. Prior to inoculation, teats were disinfected with alcohol and after inoculation the udder was massaged to distribute the bacterium. All inoculations were performed after the morning milking between 10.00 and 12.00 a.m. The cows were milked twice daily using a quarter milking device. During this study, cows were not treated with antibiotics, except cow C4 that was treated with Duoprim (intramuscularly) and Delvomast (LH) daily on days 9 – 17 pi.

Table 1. Inoculation scheme of cows inoculated with BHV4, BUE cell culture, or *Streptococcus uberis*.

	Intranasally		Intramammarily into quarters RF and LH with:		
Cow	BHV4 (day 0)	BUE* (day 0)	BHV4 (day 0)	BUE* (day 0)	*S. uberis* (day 14)
B1	+	-**	+	-	+
B2	+	-	+	-	-
B3	+	-	+	-	-
B4	+	-	+	-	+
C1	-	+	-	+	+
C2	-	+	-	+	+
C3	-	+	-	+	-
C4	-	+	-	+	-

BUE*: BUE cell culture; -**: not inoculated

2.3 Collection of samples

A volume of 50 ml of milk from each quarter of each cow was collected daily just before the a.m. milking of the cows. Milk samples were collected according to the procedure described by the National Mastitis Council (Harmon et al., 1990), and transported immediately to the laboratory, homogenised and divided for storage. Milk samples for virus isolation were directly stored at –70°C. Milk samples used for the determination of somatic cell counts (SCC), bacterial isolation, and antibody detection, were immediately stored at –20°C. Weekly, blood samples were obtained from the vena jugularis, centrifuged at 2000 x g for 10 minutes, and sera were stored at –20°C. Daily, blood samples were collected in tubes containing EDTA starting on day –2 till day 28 pi for white blood cell counts and for blood cell differentiation.

Daily, nasal swabs for BHV4 isolation and titration were collected, processed and stored directly at –70°C, as described by Kaashoek et al. (1994). All collected milk, nasal secretions, and serum samples were stored in duplicate.

2.4 Clinical signs and morphology of the milk

Clinical signs and rectal temperatures were recorded daily in the morning. Udders were palpated for signs of infection (swollen or painful). The morphology of the milk samples was examined daily. Therefore, the first streams of milk secretion were discarded, and the foremilk was checked for colour, clots, and morphology.

2.5 Assays of samples

2.5.1 Somatic cell count (SCC)

Somatic cells in daily quarter milk samples were counted by means of a Fossomatic (Foss Electric, Hillerϕd, Denmark). Subclinical mastitis was defined high quarter milk SCC of ≥250,000 cells/ml (Dohoo and Leslie, 1991; Smith, 1996), without any visible abnormality of the milk or the udder (International Dairy Federation, 1987).

2.5.2 White blood cell counts and differentiation

The white blood cell (WBC) counts were determined using an automated cell counter (Sysmex F-800,

Charles Goffin, The Netherlands). Each day, thin blood films were prepared from venous blood collected in tubes containing EDTA. The slides were stained by using the May-Grunwald-Giemsa staining method (Merck KGaA, Darmstadt, Germany), and the WBC were differentiated by typing 100 cells.

2.5.3 BHV4 isolation

In order to isolate virus, milk samples were thawed and defatted by centrifugation at 1500 x g for 10 minutes. A volume of 0.2 ml of the defatted milk was added to 0.3 ml of DMEM and pipetted on a semi-confluent monolayer of BUE cells in 24-wells cell culture plates. Virus isolation was further performed as described earlier (Wellenberg et al., 2000). Cell cultures were observed daily for cytopathic effect (cpe). After a freeze/thaw cycle, a second passage was performed by inoculating the virus/cell suspensions on semi-confluent monolayers of BUE cells. BUE cell cultures were incubated again for 7 days at 37°C (5% CO_2). In each test run, 5 controls were incorporated. Three controls, containing 10, 100 and 1000 $TCID_{50}$ BHV4/ml milk, served as positive controls. A BHV4-free milk sample and a non-inoculated BUE cell culture control, served as negative controls.

The BHV4 titre was determined in milk samples from two arbitrarily chosen cows B1 (RF) and B3 (LH) on days 1, 3, 5, 7, 9 and 10 or 11 pi, and in BHV4 positive milk samples obtained after *S. uberis* inoculation (Table 2). Virus titration was performed by preparing serial ten-fold dilution steps in DMEM (10^{-1} – 10^{-8}). A volume of 200 µl of each dilution, plus 300 µl of DMEM, was inoculated in 12-fold on BUE cells cultivated in 24-wells cell culture plates. Plates were incubated as described above. After a freeze/thaw cycle, a second passage on BUE cells was performed, and cells were examined for the appearance of cpe after an incubation period of 7 days at 37°C (5% CO_2).
Isolation and titration of BHV4 from nasal secretions was performed on BUE cells using 96-wells cell culture plates. For BHV4 isolation, BUE cells in suspensions of 150 µl were inoculated with 50 µl of thawed nasal swab suspension and cells were examined for cpe after an incubation for 7 days at 37°C (5% CO_2). For virus titration, serial ten-fold dilutions of nasal swab suspensions were prepared in DMEM (10^{0} – 10^{-4}). Each dilution was inoculated in 8-fold on BUE cells as described above and incubated at 37°C (5% CO_2). Plates for virus titration were examined after 7 days for the appearance of cpe. In each virus titration assay, the endpoint titres were expressed as $\log_{10}$ $TCID_{50}$/ml by using the method of Reed and Muench.

2.5.4 BHV4 antibodies

Serum and pooled milk samples from all four quarters, in dilutions of 1:20 and 1:5, respectively, were screened for antibodies against BHV4 by immunoperoxidase monolayer assay (IPMA) (Wellenberg et al., 1999). Samples, containing antibodies against BHV4, were titrated in serial twofold dilutions, starting at a dilution of 1:20 (serum) or 1:5 (milk). The titre of the test sample was taken as the reciprocal of the highest dilution giving a positive reaction.

2.5.5 Bacterial isolation

Bacteriological culturing of milk samples was performed based on standards of the IDF (International Dairy Federation, 1984). Briefly, milk samples (0.025 ml) were inoculated on Oxiod Blood Agar Base No 2, containing 5% blood and 0.1% aesculin. Plates were incubated at 37°C, and bacterial growth was evaluated after 24 and after 48 hours. Bacterial colonies were identified, and bacteria were considered to be pathogenic or non-pathogenic. A quarter was considered to have an intramammary infection when ≥500 cfu/ml of the same udder pathogen was cultured from two out of three consecutive milk samples, or when ≥100 cfu/ml of a bacterial species was cultivated from a quarter with clinical signs of mastitis (Barkema et al., 1999). If three or more bacterial species were cultivated from a sample, the sample was considered to be contaminated.

2.6 Statistical analysis

To approximate the normal distribution, a natural logarithmic transformation of the SCC was used (Shook, 1982). Statistical analysis was used to compare the natural logarithm of SCC between quarters inoculated with BHV4, control quarters of the same cow of the BHV4 group (within-cow controls), quarters of the control cows inoculated with BUE cell culture, and quarters of the control cows that were not inoculated. The following linear mixed model was used:

$$\mathbf{Y_{i,\,j,\,k(i,\,j)} = m + C_i + CQ_{i,\,j} + t_{k(i,\,j)} + e_{i,\,j,\,k(i,\,j)}}$$

where $Y_{i,\,j,\,k(i,\,j)}$ is the average natural logarithm of SCC of quarter j (j=1…4) of cow i (i=1…8) under the assigned treatment k; m is the overall mean; C_i is the random effect of cow i; $CQ_{i,\,j}$ is the random effect of quarter j within cow i; $t_{k(i,\,j)}$ is the fixed effect of the treatment k and finally $e_{i,\,j,\,k(i,\,j)}$ is residual error. The treatment effect has a factorial structure, since it consists of combinations of an

intramammary inoculation ("+" or "-") and inoculum (BHV4 or BUE cell culture). Estimation of the parameters in the model is done by restricted maximum likelihood (REML) and the Wald-test is applied to assess significance of effects. All statistical calculations were performed with Genstat (1993). Statistical significance was declared at $p < 0.05$.

3. Results

3.1 Clinical signs

3.1.1 Days 0-14 pi

Within the period of 0 – 14 days pi, clinical signs of mastitis or rectal temperatures >39.5°C were recorded neither in the four cows of the BHV4 group nor in cows C1 and C2 of the control group. No changes in udder health or morphology of the milk, collected from BHV4-inoculated quarters, BUE cell culture inoculated quarters, and non-inoculated quarters were recorded in these six cows.
Cow C4 of the control group showed an increase in rectal temperatures (> 39.5°C) for 6 days, starting at day 9 pi. Swelling of the udder, and changes in the morphology of the milk from all four quarters from cow C4 were recorded starting at day 9 pi. This was also observed for the LF quarter from cow C3 starting on day 2 pi. No clinical signs or increase of rectal temperature were further recorded for cow C3, and no changes in the morphology of the milk, collected from the three remaining quarters were recorded. Gram negative bacteria (*Klebsiella sp.*) were isolated from the milk of these clinical mastitis cases. Therefore, all data of all four quarters from cow C4, that were obtained after day 8 pi, and all data of the LF quarter from control cow C3 were excluded from statistical analysis.

3.1.2 Days 15-28 pi

After the intramammary inoculation of cows B1 and B4 (BHV4 group) and C1 and C2 (control group) with *S. uberis* on day 14 pi, increased rectal temperatures (> 39.5°C) were recorded for all four cows starting between days 16 and 19 pi. Thereafter, rectal temperatures varied between 38.2 and 41.6°C (Table 2). The morphology of the milk from *S. uberis* inoculated quarters altered and clots were observed initially between days 15 and 20 pi. Changes in the morphology of the milk, in combination with painful and swollen quarters, were recorded for all *S. uberis* inoculated quarters up to day 28 pi. No changes in the morphology were observed in milk from non-*S. uberis* inoculated quarters of these four cows. There were no marked differences in rectal temperatures or the morphology of the milk from quarters inoculated with BHV4 and *S. uberis* (cows B1 and B4) compared to those of the two cows that were inoculated with *S. uberis* only (C1 and C2) (Table 2).

Between days 15 - 28 pi., cows B2 and B3 (cows from the BHV4 group but not inoculated intramammarily with *S. uberis*) and control cow C3 (except for quarter LF) showed no clinical signs, increase of rectal temperatures, painful or swollen udders or any changes in the morphology of the

milk.

3.2 Somatic cell count

3.2.1 Days 0-14 pi

Prior to the inoculation of BHV4, SCC in milk from two quarters (LH from cow B1 and RF from B4) was >250,000 cells/ml, and therefore, SCC of these two quarters was not used for statistical analysis. In 3 of the 6 remaining BHV4 inoculated quarters, the SCC in milk increased above 250,000 cells/ml (cows B3 (RF and LH) and B4 (LH)), and reached levels up to 981,000 cells/ml. No increased SCC was recorded in the quarters of cows B1 (RF) and B2 (RF and LH). An increase in SCC was recorded between days 6 - 14 pi (Figure 1).

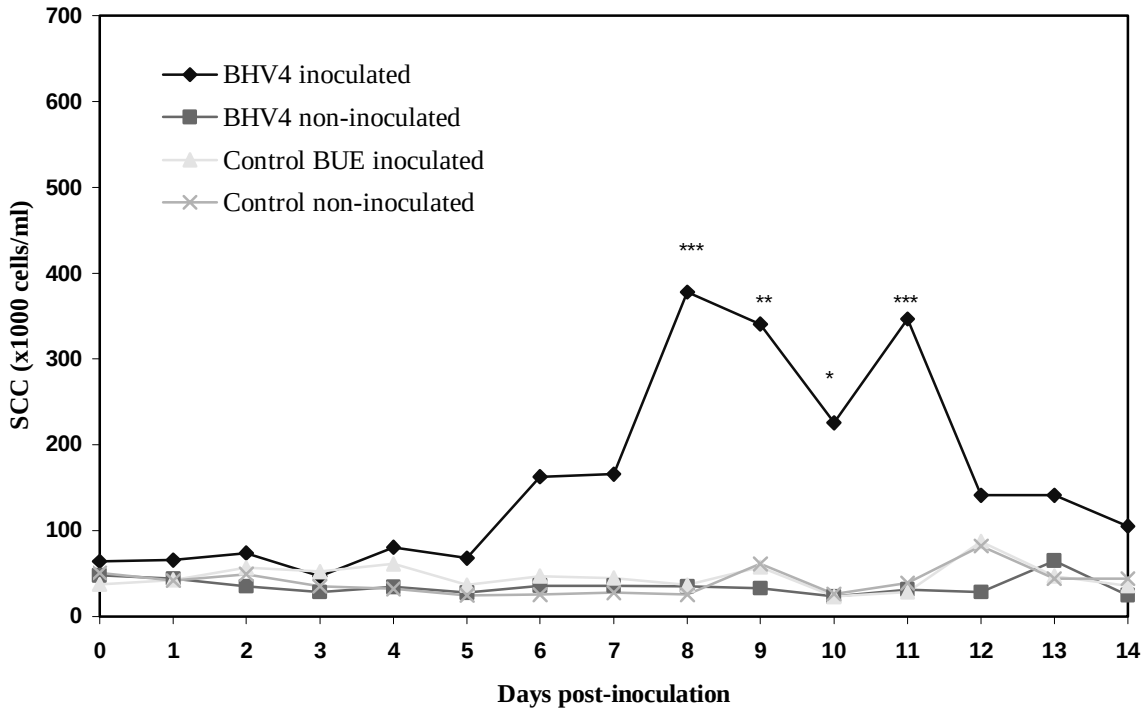

Fig 1. Mean SCC in milk from BHV4 inoculated quarters (n = 6), non-BHV4 inoculated quarters (within cow-controls) (n = 8), BUE cell culture inoculated quarters (n = 6, plus data from cow C4 up to day 9 pi), and non-inoculated quarters (n = 5, plus data from cow C4 up to day 9 pi). SCC in milk from quarters inoculated with BHV4 were significantly higher than the SCC in milk from the other quarters on day 8, 9 and 11 pi (*: $p < 0.1$, **: $p < 0.05$, and ***: $p < 0.01$)

No SCC >250,000 cells/ml was recorded in milk samples collected from the eight non-BHV4 inoculated quarters of four cows of the BHV4 group (within-cow controls), from the two quarters (RF and LH) from cows C1, C2, C3 and C4 (up to day 9 pi) of the control group that were inoculated with BUE cell culture suspension, and from the two non-inoculated quarters of these four cows of the control group (except for the LF quarter of cow C3 as indicated in paragraph *3.1*). In only one occasion, day 12 pi, SCC in milk from quarter LH from cow C1 was >250,000 cells/ml (360,000 cells/ml).

Table 2. Rectal temperatures, morphology of the milk, SCC and peripheral WBC from cows inoculated intramammarily with *S. uberis*, preceded by a BHV4 infection (day 0), or with *S. uberis* only (day 14 pi).

Response of infected cows	(BHV4 +) *S. uberis*		*S. uberis*	
Cow:	B1	B4	C1	C2
Rectal temperature				
First day temperature >39.5°C	5*	5	4	2
No of days >39.5°C (n = …)	4*	4	7	5
Morphology of the milk				
First day clots in the milk	1	5	3	2
No days of abnormal milk (n =…)	13	6	12	12
Somatic cell counts				
First day of >250.000/ml	2	2	2	2
No days >250.000/ml (n = …)	12	13	13	13
Peripheral WBC				
First day of changes in WBC count	4	5	3	5
No days that the amount of band form neutrophils >2 (n = …)	6	1	6	3

*: days after *S. uberis* inoculation (*S. uberis* inoculated on day 14 pi).

On days 8, 9 and 11 pi, SCC in milk from quarters inoculated with BHV4 was significantly higher than: a) SCC in milk from the non-BHV4 inoculated quarters from the same cows (within-cow controls); b) SCC in milk from quarters inoculated with BUE cell culture (control group), and; c) SCC in milk from non-inoculated quarters of the control group (Figure 1). The differences between the SCC in milk from; a) the non-BHV4 inoculated quarters from cows of the BHV4 group; b) the quarters inoculated with BUE cell culture (control group); and c) the non-inoculated quarters of the control group, were not significant.

3.2.2 Days 15-28 pi.

The SCC in milk from the RF and LH quarters from cows C1, C2, B1 (RF), and B4 (LH) that were inoculated with *S. uberis* on day 14 pi (n=6), started to increase above 250,000 cells/ml around day 16 pi (Table 2), and reached levels above 1,000,000 cells/ml. The morphology of the milk changed between days 15 and 20 pi (Table 2). The SCC in milk from the two non-*S. uberis* inoculated quarters LF and RH of the same four cows (n = 8) stayed below 250,000 cells/ml. In only two occasions, the SCC increased above 250,000 cells/ml, namely, for the non-inoculated LF quarter of cow B4 on days 23 and 24 pi (341,000 and 376,000 cells/ml, respectively). However, no BHV4, *S. uberis* or other bacteria were isolated from the milk of this quarter.

3.3 WBC counts and differentiation of WBC

No effects on WBC counts were recorded in blood from the four cows inoculated with BHV4 and the control cows C1 and C2 up to day 14 pi, and no shift was recorded in the peripheral differential WBC of these cows. WBC counts started to decrease between days 17 - 19 pi, 3 – 5 days after the inoculation with *S. uberis*, and WBC counts of 1.3 – 2.4 x 10^9/l were recorded in all four cows between days 19 – 22 pi. In blood smears of all four cows that were inoculated with *S. uberis*, a shift in the peripheral differential WBC was detectable. An increase of band form neutrophils, up to 10% of the WBC, was recorded in blood smears. In all four cows the WBC counts started to increase again to normal values (5 – 10 x 10^9/l) after day 22 pi. No differences in WBC counts, or in the number of band form neutrophils in blood smears were recorded in cows B1 and B4 compared to those in the two cows that were inoculated with *S. uberis* only (C1 and C2) (Table 2). No effects on WBC counts were recorded in blood from cows B2 and B3 of the BHV4 group, which were not inoculated with S. uberis, and no shift was recorded in the peripheral differential WBC of these cows up to day 28 pi.

3.4 BHV4 isolation.

BHV4 was isolated from all eight BHV4 inoculated quarters (RF and LH) from day 1 pi up to days 9 - 14 pi, and the titres in milk samples from cows B1 and B3 varied between $10^{1.3}$ and $10^{2.9}$ $TCID_{50}$/ml (Table 3). BHV4 was isolated neither from milk samples from the two non-inoculated quarters RH and LF of these cows (within-cow controls), nor from all four quarters from the cows of the control group. After the intramammary inoculation with *S. uberis*, BHV4 was isolated from milk collected from the RF quarter from cows B1 and B4 on several days between days 22 and 27 pi; BHV4 titres varied between $10^{1.2}$ and $10^{3.1}$ $TCID_{50}$/ml (Table 3). BHV4 was not isolated from milk samples from cows B2 and B3 that were inoculated intramammarily with BHV4, but not with *S. uberis*.

BHV4 was isolated from nasal swabs from all four cows of the BHV4 group between days 2 and 9 pi. The peak titres varied between $10^{2.9}$ and $10^{3.9}$ $TCID_{50}$/ml (Figure 2). No BHV4 was isolated from nasal swabs after the intramammary *S. uberis* inoculation.

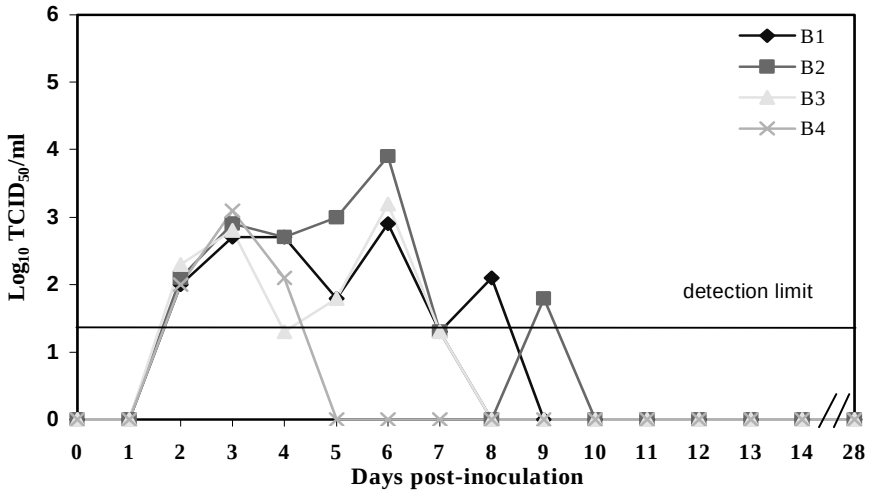

Fig 2. BHV4 titres in nasal secretions from cows intranasally inoculated with BHV4.

Table 3. Isolation of BHV4 from milk and BHV4 titres in milk from cows inoculated with BHV4.

Days pi.	BHV4 isolation (log_{10} $TCID_{50}$/ml)							
Cow:	B1		B2		B3		B4	
	RF	LH	RF	LH	RF	LH	RF	LH
0	-*		-		-		-	
1	+ (2.1)**	+	+	+	+	+ (1.8)	+	+
2	+	+	+	+	+	+	+	+
3	+ (1.4)	+	+	+	+	+ (2.4)	+	+
4	+	+	+	+	+	+	+	+
5	+ (1.4)	+	+	+	+	+ (2.0)	+	+
6	+	+	+	+	+	+	+	+
7	+ (1.8)	+	+	+	+	+ (2.9)	+	+
8	+	+	+	+	+	+	+	+
9	+ (1.6)	+	+	+	+	+ (1.5)	+	+
10	+ (1.3)	-	+	+	+	+	+	-
11	-	-	+	+	+	+ (1.3)	+	-
12	-	-	+	+	+	+	-	-
13	-	-	+	+	+	-	-	-
14	-	-	+	-	-	-	+	-
15	-	-	-	-	-	-	-	-
16 - 19	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-
22	+ (1.2)	-	-	-	-	-	-	-
23	+ (2.3)	-	-	-	-	-	-	-
24	+ (3.1)	-	-	-	-	-	-	-
25	+ (3.0)	-	-	-	-	-	-	-
26	+ (2.4)	-	-	-	-	-	+ (1.3)	-
27	-	-	-	-	-	-	+ (1.9)	-
28	-	-	-	-	-	-	-	-

-* : no BHV4 isolated

( )** : BHV4 titre

3.5 BHV4 antibody responses

In all four cows of the BHV4 group, serum antibodies against BHV4 were first detected on day 14 pi (Table 4), and reached levels between 320 and 2560 on day 28 pi. In milk samples of the four cows of the BHV4 group, antibodies against BHV4 were detectable around day 12 pi, and BHV4 antibody titres increased up to 160. In sera and milk samples of the four cows of the control group no BHV4 antibodies were detected.

Table 4. BHV4 antibody titres in serum and milk from cows inoculated intramammarily and intranasally with BHV4

Days pi.	BHV4 antibody titres							
Cow:	B1		B2		B3		B4	
	serum	milk	serum	milk	serum	milk	serum	milk
0	< 20	< 5	< 20	< 5	< 20	< 5	< 20	< 5
7	< 20	< 5	< 20	< 5	< 20	< 5	< 20	< 5
10	-*	< 5	-	< 5	-	< 5	-	< 5
12	-	< 5	-	20	-	10	-	10
14	40	5	80	40	40	20	20	20
16	-	20	-	80	-	80	-	20
18	-	20	-	80	-	80	-	20
20	-	20	-	80	-	160	-	20
21	80	-	320	-	320	-	80	-
22	-	20	-	80	-	160	-	80
24	-	40	-	80	-	80	-	160
26	-	80	-	80	-	160	-	160
28	320	160	1280	80	2560	160	320	160

-*: not determined

3.6 Isolation of bacteria on days 0-14 pi

No pathogenic bacteria were isolated from milk samples collected from the quarters of the four cows of the BHV4 group, from the control cows C1, C2, from the remaining quarters RF, RH and LH from control cow C3, and from the four quarters of control cow C4 up to day 9 pi.

3.6.1 Isolation of bacteria on days 15-28 pi

Streptococcus uberis was isolated from all inoculated quarters of cows C1, C2, B1, and B4 (LH; intermittent) (Table 2), whereas no *S. uberis* was isolated from the non-*S. uberis* inoculated quarters of these cows. No pathogenic bacteria were isolated from the quarters of cows B2 and B3 that were inoculated with BHV4 only (day 0), or from the remaining quarters RF, RH and LH from control cow C3.

4. Discussion

In this study, a simultaneous intramammary and intranasal inoculation of lactating cows with BHV4 did not lead to clinical mastitis. However, subclinical mastitis was induced, as evidenced by BHV4 replication in all BHV4-inoculated quarters, by the increased SCC of ≥250,000/ml in 2 of 4 cows and in 3 of 6 BHV4-inoculated quarters, and by the absence of such an increase in SCC in controls. General phenomena, such as differences in susceptibility or genetic background of individual cows, might explain why the BHV4 infection induced an increase of SCC in only 50% of the inoculated quarters. The increase in SCC in BHV4 inoculated quarters was not the result of an intramammary infection by bacteria, as evidenced by the absence of udder pathogens in milk during the first 14 days pi.

We have chosen to use two routes of inoculation to enhance the possibility of inducing clinical mastitis. However, a disadvantage of this approach is that a possible interference of the BHV4 infection, that was induced after the intranasal inoculation, on the intramammary BHV4 infection cannot be excluded. But, based on the facts that: 1) BHV4 was only isolated from the inoculated quarters and not from the non-inoculated quarters of the same cows; and 2) an increase in SCC was only detected in milk from BHV4 inoculated quarters, it is likely that the induced subclinical mastitis was primarily the result of the intramammary BHV4 infection.

The SCC is an indicator of subclinical mastitis, and the major factor affecting SCC is infection of the mammary gland (International Dairy Federation, 1987; Dohoo and Leslie, 1991; Harmon, 1994). Inflammation of the mammary gland by udder pathogens often results in an increase of SCC $>10^6$/ml within 1 or 2 days post-infection (Paape et al., 1981), as was the case in all four cows intramammarily inoculated with *S. uberis*. However, the SCC in milk from the BHV4 inoculated quarters did not increase before day 6 pi. The reason for this difference in increase in SCC may be that different defence mechanisms are involved in bacterial and viral infections of the mammary gland. In bacterial udder infections, innate immunity, also known as non-specific responsiveness, is the predominant defense during the early stages of infection (Harmon, 1994; Sordillo et al., 1997). In the early stages of infection, neutrophils, and the migration of neutrophils from the blood into the udder, which occurs by chemotaxis, play a major role in host defence against environmental (coliform) mastitis in cows (Kremer et al., 1990). We may speculate that the increase of SCC in milk from BHV4 inoculated quarters was the result of cytotoxic responses against BHV4 infected cells mediated by other defense mechanisms, i.e. the occurrence of the late cytokine cascade (including the production of interferon-γ), the development of cell-mediated immunity and the more specific antibody dependent cell-mediated cytotoxicity (ADCC), as reported for BHV1 (Rouse et al., 1976; Campos et al., 1994). Interferon-γ

plays an important role in the generation of non-MHC-restricted cytotoxic reponses (Campos et al., 1989), and it can activate macrophages to kill virus infected cells, as reported for BHV1 (Babiuk et al., 1996). The peak activities of these cell-mediated immune responses occurred 7-10 days pi (Campos et al., 1994).

Unlike BHV4, experimental intramammary inoculation of bovine herpesvirus 1 (Greig and Bannister, 1965; Corner et al., 1967; Straub and Kielwein, 1966), foot-and-mouth disease virus (Burrows et al., 1971; Blackwell and Yilma, 1981), and parainfluenza 3 virus (Kawakami et al., 1966) have been found to induce clinical mastitis. Increased temperatures, swollen udders, and changes in the morphology of the milk were reported for all three virus infections. In addition, an increase of SCC was recorded after the BHV1 and PI3 inoculation, and an intramammary inoculation with BHV1 and foot-and-mouth disease virus also resulted in reduced milk yields.

For cases of subclinical mastitis, bovine leukaemia virus has been detected in mammary tissue of affected cows (Yoshikawa et al., 1997). But, there is no clear evidence that this virus plays a role in the aetiology of bovine subclinical mastitis, and no experimental studies have been reported to examine whether this virus was able to induce bovine subclinical or clinical mastitis. Consequently, to our knowledge this is the first report on a virus that induces bovine subclinical mastitis after experimental inoculation.

In the preceding study (Wellenberg et al., 2000), BHV4 was isolated from milk from cows with clinical mastitis in combination with udder pathogens, especially *S. uberis*, a major udder pathogen (Thomas et al., 1994). To study whether BHV4 might play an indirect role in the aetiology of bovine clinical mastitis, we examined whether a preceding BHV4 infection would exacerbate bovine clinical mastitis induced by *S. uberis*. Based on data of clinical signs, morphology of the milk, SCC, and peripheral WBCs, we conclude that a preceding BHV4 infection did not exacerbate bovine clinical mastitis induced by *S. uberis.*

After the *S. uberis* inoculation, BHV4 was isolated from milk from two of four quarters inoculated with BHV4 and *S. uberis*, whereas no BHV4 was isolated from milk from four quarters that were inoculated with BHV4 only. These data indicate that BHV4 remained present in the infected cow, and that the *S. uberis* infection appeared to trigger replication of BHV4. Replication of latent BHV4 from various tissues has been reported after dexamethasone treatment (Castrucci and others, 1987; Dubuisson and others, 1989). Whether BHV4 persisted in latent form in cells of the mammary gland or in peripheral blood leukocytes (Osorio and Reed, 1983; Lopez et al., 1996; Egyed and Bartha, 1998), or as infectious virus in milk cell fractions (Donofrio et al., 2000) was not examined. However, this study demonstrates for the first time that bacteria, i.e. *S. uberis*, appear to trigger the replication of BHV4. Which process triggered BHV4 replication during the *S. uberis* infection is unknown. In an

experimental mastitis model, an increase in serum cortisol level has been detected shortly after *Escherichia coli* infection (Shuster et al., 1993). An increase of serum cortisol levels or comparable processes that activate BHV4 replication could be induced in *S. uberis* mastitis. More research is warranted to define whether bacterial infections may trigger reactivation of latent BHV4 and which processes are involved. After the *S. uberis* inoculation, BHV4 was only detected in milk and not in nasal secretions. This suggests a local trigger on the replication of BHV4 that did not result in systemic spread.

In conclusion, this study shows that a simultaneous intramammary and intranasal inoculation of lactating cows with BHV4 did not induce clinical mastitis, but induced subclinical mastitis. In addition, no exacerbation of a *S. uberis* infection by a preceding BHV4 infection was noted, and *S. uberis* infections may have triggered BHV4 replication. This study indicates that it is unlikely that BHV4 is a major clinical mastitis pathogen, but it may play a role in the aetiology of bovine subclinical mastitis.

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CHAPTER 8

INHIBITION OF BOVINE HERPESVIRUS 4 REPLICATION IN VITRO BY SELECTED ANTIVIRAL AGENTS

G.J. Wellenberg[1], J. Neyts[2], and J.T. Van Oirschot[1]

[1] *Division of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands*

[2] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.*

Abstract

In this study the susceptibility of bovine herpesvirus 4 (BHV4) to various antiviral agents was evaluated. Brivudin inhibited the replication of BHV4 in bovine umbilical cord endothelial cells with a 50% effective concentration (EC_{50}) value of 0.05 µg/ml. Cidofovir and ganciclovir also inhibited the replication of BHV4, but with EC_{50}-values of 0.2 – 0.8 µg/ml and 6.3 – 12.5 µg/ml, respectively. Inhibitory activity on the replication of BHV4 was observed for foscarnet at a concentration of 100 µg/ml, whereas no inhibition of BHV4 replication was noted for acyclovir, adefovir, and penciclovir at concentrations of ≤100 µg/ml. Brivudin and cidofovir, and to a lesser extent ganciclovir, may have potential for the treatment of BHV4 infections.

1. Introduction

Bovine herpesvirus 4 (BHV4), one of the members of the *Gammaherpesvirinae* subfamily, has been isolated from cows with various diseases e.g. mammary pustular dermatitis (Reed et al., 1977). Recently, BHV4 has been isolated from milk samples of cows with clinical mastitis, and not from milk samples of control cows (Wellenberg et al., 2000). Following a simultaneous intramammary and intranasal inoculation of lactating cows with BHV4, replication of BHV4 in the mammary gland and an increase in milk somatic cell counts were noted (Wellenberg et al., 2001). Zadoks et al. (submitted) reported an association between BHV4 seropositivity and the occurrence of bovine mastitis caused by *Staphylococcus aureus*. These reports suggest that BHV4 plays a role in the aetiology of bovine mastitis. A compound that would specifically inhibit the replication of BHV4 may have potential for the treatment of BHV4 infections.

The susceptibility of murine gammaherpesvirus 68 (MHV-68), another member of the subfamily of the *Gammaherpesvirinae*, to antiviral agents has been reported by Sunil-Chandra et al. (1994a; 1994b), and Neyts and De Clercq (1998). There have been no reports on the susceptibility of BHV4 to antiviral agents. Such knowledge may be of interest for the treatment of BHV4 infections, and also provide deeper insight into the mode of replication of this virus. Therefore, we examined the susceptibility of BHV4 to selected compounds with proven anti-herpesvirus activity.

2. Materials and methods

2.1 Virus and cells

The Dutch BHV4 strain Tolakker has been isolated from a cow with signs of clinical mastitis (Wellenberg et al., 2000). Bovine umbilical cord endothelial (BUE) cells were used for BHV4 propagation. BUE cells were grown and maintained in Dulbecco's minimal essential medium (DMEM) (Gibco Laboratories, Life Technologies Inc., USA) supplemented with 2% foetal bovine serum (Gibco Laboratories, Life Technologies Inc., USA). The following antiviral agents were used; penciclovir (PCV), ganciclovir (DHPG), acyclovir (ACV), cidofovir (HPMPC), adefovir (PMEA), foscarnet (PFA), and brivudin (BVDU). Antiviral activity was studied on semi-confluent monolayers of BUE cells grown in 96-well cell culture plates (Costar). Cells were inoculated with BHV4 at a multiplicity of infection (m.o.i.) of 0.1. Following an adsorption period at 37°C for 2 hours, BUE cell cultures were washed with phosphate buffered saline solution (PBS).

2.2 Antiviral agents

Serial two-fold dilutions of each antiviral agent, ranging from 0,05 µg/ml to 100 µg/ml, were prepared in Earle's minimal essential medium (EMEM + 2% normal calf serum) using dummy plates. A volume of 100 µl of each dilution was added in duplicate to the washed BUE cell cultures, and cell cultures were incubated at 37°C for 7 days (5% CO_2). Each serial dilution of the antiviral agents was checked for BUE cell toxicity by adding a volume of 100 µl of each dilution (in duplicate) to semi-confluent monolayers of BUE cells that had not been inoculated with BHV4. For each antiviral agent, also a cell control and a virus-growth control was incorporated into each cell culture plate. Therefore, non-infected semi-confluent monolayers of BUE cells, with the addition of 100 µl EMEM (+ 2% normal calf serum), but without the antiviral agents, were used as cell control. Semi-confluent monolayers of BUE cells that were infected with BHV4 (m.o.i. = 0.1) were used as virus-growth control. The virus-growth control was incubated and washed as described above. After the wash procedure 100 µl EMEM (+ 2% normal calf serum), but without the antiviral agents, was added to the wells. BUE cell toxicity, cell and virus-growth controls, were incubated for 7 days at 37°C (5% CO_2). The appearance and degree of cytopathic effects (cpe) in each well was recorded microscopically at day 7 post-infection. The EC_{50}-value was calculated on the concentration that inhibited virus-induced cpe formation by 50%, and the complete inhibition (CI) of BHV4 replication was recorded in case no cpe was observed.

3. Results and discussion

The *in vitro* activities of the selected antiviral agents on the replication of BHV4 in BUE cell cultures are presented in Table 1. Complete inhibition of virus-induced cpe formation was observed in BHV4 infected BUE cells treated with BVDU at concentrations of ≥0.05 µg/ml (EC_{50}-value: 0.05 µg/ml), with HPMPC at concentrations of ≥1.6 – 3.2 µg/ml (EC_{50}-values: 0.2 – 0.8 µg/ml), and with DHPG at concentrations of ≥25 µg/ml (EC_{50}-values: 6.3 – 12.5 µg/ml). A limited inhibition of virus-induced cpe formation was observed in BHV4 infected BUE cells treated with PFA at a concentration of 100 µg/ml, and no inhibition was observed with ACV, PMEA or PCV. Complete destruction of the BUE cell monolayers (100% cpe) was observed in all wells used for virus-growth control, and no cpe was observed in all BUE cell control wells.

Table 1. Inhibitory effects of selected anti-herpesvirus agents on the in vitro replication of BHV4 (strain Tolakker).

Antiviral agent	EC_{50}[a] (µg/ml)	CI[b] (µg/ml)	MTC[c] (µg/ml)
BVDU	0.05	≥ 0.05	> 100
HPMPC	0.2 – 0.8	≥ 1.6 – 3.2	> 100
DHPG	6.3 – 12.5	≥ 25	> 100
PFA	100	> 100	> 100
ACV	> 100	> 100	> 100
PCV	> 100	> 100	> 100
PMEA	> 100	> 100	> 100

[a] : concentration of antiviral agent required to reduce BHV4-induced cpe formation by 50%

[b] : concentration of antiviral agent required for complete inhibition of BHV4-induced cpe formation

[c] : minimal toxic concentration (concentration required to alter morphology of BUE cells)

BVDU, ACV, PCV and DHPG all depend on a virus-encoded thymidine kinase (TK) for activation. However, in this study only BVDU and DHPG inhibited BHV4 replication. Compared to MHV-68, BHV4 seems to be more susceptible to BVDU, whereas both viruses are equally susceptible to DHPG. HPMPC, which does not depend on the viral TK for activation, but probably on the DNA polymerisation process, proved to be a potent inhibitor of the replication of BHV4. Two other

gammaherpesviruses, i.e. MHV-68 and Epstein-Barr virus, proved also to be susceptible to HPMPC. Another acyclic nucleoside phosphonate, namely adefovir (PMEA), which has potent anti-retrovirus and anti-herpesvirus activities (Naesens et al., 1997) was devoid of anti-BHV4 activity.

In conclusion, this study provides information on the susceptibility of BHV4 to a selection of anti-herpesvirus agents. This information offers opportunities for designing strategies to inhibit BHV4 replication in e.g. the mammary gland or to treat pustular dermatitis.

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CHAPTER 9

VIRAL INFECTIONS AND BOVINE MASTITIS

A review

G.J. Wellenberg[1], W.H.M. van der Poel[2], and J.T. Van Oirschot[1]

[1] *Division of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB, Lelystad, The Netherlands.*

[2] *Microbiological Laboratory for Health Protection (MGB), National Institute of Public Health and the Environment (RIVM), P. O. Box 1, 3720 BA Bilthoven, The Netherlands.*

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Abstract

This review deals with the role of viruses in the aetiology of bovine mastitis. Bovine herpesvirus 1, bovine herpesvirus 4, foot-and-mouth disease virus, and parainfluenza 3 virus have been isolated from milk from cows with clinical mastitis. Intramammary inoculations of bovine herpesvirus 1 or parainfluenza 3 virus induced clinical mastitis, while an intramammary inoculation of foot-and-mouth disease virus resulted in necrosis of the mammary gland. Subclinical mastitis has been induced after a simultaneous intramammary and intranasal inoculation of lactating cows with bovine herpesvirus 4. Bovine leukaemia virus has been detected in mammary tissue of cows with subclinical mastitis, but whether this virus was able to induce bovine mastitis has not been reported.

Bovine herpesvirus 2, vaccinia, cowpox, pseudocowpox, vesicular stomatitis, foot-and-mouth disease viruses, and bovine papillomaviruses can play an indirect role in the aetiology of bovine mastitis. These viruses can induce teat lesions, for instance in the ductus papillaris, which result in a reduction of the natural defence mechanisms of the udder and indirectly in bovine mastitis due to bacterial pathogens. Bovine herpesvirus 1, bovine viral diarrhoea virus, bovine immunodeficiency virus, and bovine leukaemia virus infections may play an indirect role in bovine mastitis, due to their immunosuppressive properties.

We conclude that viral infections can play a direct or indirect role in the aetiology of bovine mastitis; therefore their importance in the aetiology of bovine mastitis and their economical impact needs further attention.

1. Introduction

Bovine mastitis is a highly prevalent disease in dairy cattle, and one of the most important diseases affecting the world's dairy industry; it places a heavy economic burden on milk producers all over the world (Miller and Dorn, 1990; Schakenraad and Dijkhuizen, 1990; Miller et al., 1993; Bennett et al., 1999). Worldwide, annual losses due to mastitis have been estimated to be approximately 35 billion US dollar. In the US, the annual costs of mastitis have been estimated to be 1.5 – 2.0 billion US dollar, while losses of milk productions, due to subclinical mastitis, and higher cow replacements costs associated with high somatic cell counts were estimated at 960 million US dollar (Wells et al., 1998). Each case of clinical mastitis in the US and California costs approximately 107 and 200 US dollars, respectively (Miller et al., 1993). In Scottish dairy herds, facing high bulk-tank somatic cell count, the average annual costs of subclinical mastitis was 100 Pound Sterling/cow (Yalcin et al., 1999), while in the UK and the Netherlands, the annual average revenue losses were calculated to be 42–84 Pound Sterling/cow (Esslemont and Peeler, 1993) and approximately 130 Dutch Guilders/cow (Schakenraad and Dijkhuizen, 1990).

Mastitis is defined as an inflammatory reaction of the parenchyma of the mammary gland that can be of an infectious, traumatic or toxic nature (International Diary Federation, 1987). Mastitis is characterized by physical, chemical and usually bacteriological changes in the milk and by pathological changes in the glandular udder tissue. The diagnosis of mastitis is based on clinical signs, e.g. swelling of the udder, tender to the touch, fever, and depression. In many cases a reduced milk production can be observed. Because of the large number of subclinical mastitis cases, the diagnosis of mastitis can also depend on indirect tests which in turn depends on e.g. the leukocyte numbers in the milk (Radostits et al., 1994).

Bovine mastitis is generally considered to be of infectious nature leading to inflammation of one or more quarters of the mammary gland and it is often affecting not only the individual animal but the whole herd or at least several animals within the herd. If left untreated, the condition can lead to deterioration of animal welfare resulting in culling of affected cows, or even death.

Mastitis-causing pathogens include bacteria and non-bacterial pathogens, like mycoplasms, fungi, yeasts, and chlamydia (Watts, 1988, Radostits et al., 1994). These pathogens infect the udder generally through the ductus papillaris, which is the only opening of the udder to the outside world.

Despite intensive aetiological research, still around 20-35% of clinical cases of bovine mastitis have an unknown aetiology (Miltenburg et al., 1996; Wedderkopp, 1997). Miltenburg et al. (1996) found a 28% negative rate in 1045 cases of clinical mastitis, and Wedderkopp (1997) did not note pathogens in 35% of 6809 milk quarters in 3783 cows suffering from clinical mastitis. The percentage of culture-

negative samples of both clinical and subclinical mastitis cases in the Netherlands has recently been determined to be approximately 25% (Barkema et al., 1998). An explanation for these high percentages of culture-negative samples might be a low concentration of udder pathogens, e.g. *Escherichia coli*. Other pathogens such as mycoplasma, yeasts and moulds are difficult to cultivate. But these agents cannot be the explanation for all culture negative milk samples from mastitis cows, because these agents are no common udder pathogens (Pfützner, 1994; Wendt, 1994). Due to the high percentages of unknown causes of mastitis, it is obvious to study the role of viruses in the aetiology of bovine mastitis. This in spite of the fact that viruses are generally considered not to play an important role. Watts (1988), for example, identified 137 microbial species as causative agents of bovine mastitis, including agents involved in its pathogenesis. However, viruses were not included.

The reasons for this negligence could be manifold. Historically, mastitis research has concentrated on bacterial pathogens. In case of viral infections, signs of mastitis may not have been recognised because other clinical signs were more prominent. Subclinical mastitis cases are often not diagnosed and consequently their aetiology is not investigated. This may cause an underestimation of virus infections involved in bovine subclinical mastitis. Another reason might be that lactating cows are seldom used in viral pathogenesis studies. In, e.g. most BHV1 pathogenesis studies, young calves are used due to economic aspects. A disadvantages thereof is that it does not yield any indication as to whether BHV1 can be involved in the aetiology of bovine mastitis. In addition, milk samples from mastitis cows are often not properly collected, treated and stored for virological research, as this requires special care. The laboratory diagnosis of viral mastitis is laborious and expensive. Diagnostic tools, e.g. susceptible cells, for the detection of viruses are often not optimally used. These arguments might explain why it is difficult to estimate the importancy of viral infection on the aetiology of bovine mastitis and their economical impact. It also explains the low number of viral mastitis reports, and it may explain why the last brief review on viral infections of the bovine mammary gland has been published 30 years ago (Afshar and Bannister, 1970). This review paper aims to make an inventory of the updated evidence that demonstrate whether viral infections are associated in a direct or indirect way with bovine mastitis (Table 1).

Table 1. Viral infections and their association with bovine mastitis

Virus	Natural cases	Experimental reproduction		Indirect by teat lesions	Epide-miological studies
	Virus isolation	IM route [a]	Natural route		
Bovine herpesvirus 1	+	+			D [b]
Bovine herpesvirus 4	+ [c]	+			+
Foot-and-mouth disease virus	+	+	+	+	
Parainfluenza 3 virus	+	+			
Bovine leukaemia virus	+ [d]				+ / -
Bovine herpesvirus 2				+	
Cowpox virus				+	
Pseudocowpox virus				+	
Vesicular stomatitis virus			+		
Bovine papillomaviruses				+	
Bovine viral diarrhoea virus					+
Bovine immunodeficiency virus					D [b]
Rinderpest virus				D [b]	
Bovine enterovirus		+			

IM [a]: intramammary

D [b]: data are considered to be insufficient for association

+ [c]: virus isolation from cases and not from matched controls

+ [d]: no virus isolated but viral particles detected by electron microscopy

2. Viral infections and bovine clinical mastitis

Bovine herpesvirus (BHV) 1 (Gourlay et al., 1974; Roberts et al., 1974), BHV4 (Wellenberg et al., 2000), foot-and-mouth disease virus (Burrows et al., 1971), and parainfluenza 3 virus (Kawakami et al., 1966a and 1966b) have been detected in milk from cows with clinical mastitis. However, the detection of virus in milk from cows with mastitis obviously does not prove that these agents are the cause of mastitis, or that they are involved in an indirect way.

2.1 Bovine herpesvirus 1

Bovine herpesvirus 1, a member of the *Alphaherpesvirinae* subfamily within the *Herpesviridae* family, causes infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis. In 1974, BHV1 was isolated from a cow with mastitis in the USA. Although, bacterial culture was negative and only BHV1 was isolated from the milk, the evidence that the virus caused the mastitis was, at most, circumstantial as the milk sample was collected three days after vaccination with a live IBR-vaccine (Roberts et al., 1974). In France, BHV1 was isolated from milk samples from cows with mastitis in combination with *Mycoplasma agalactiae* (Espinasse et al., 1974; Gourlay et al., 1974). BHV1 was also isolated from one of the milk samples obtained from one out of 96 cows with mastitis (Bilge, 1998). Besides the isolation of BHV1 in milk, the virus was also isolated from vesicular lesions on the udder and on the teats of a cow. Thus, BHV1 was associated with cutaneous lesions of the bovine udder, however it was difficult to ascertain whether the lesions were primarily caused by the BHV1 infection (Guy et al., 1984).

A possible role of BHV1 in the aetiology of bovine mastitis, without or in combination with bovine viral diarrhoea virus (BVDV), was also suggested by Siegler et al. (1984), who described a high incidence of mastitis cases in a number of herds with BHV1 and BVDV infected cows. Bacteriological examination of bovine milk samples in a number of herds suffering from mastitis revealed no udder pathogens, while others suffered from mastitis induced by e.g. staphylococci and streptococci. Immunisation of cows in the affected herds with IBR/IPV vaccine, without or in combination with mucosal disease/BVD vaccine, resulted in an effective control of their mastitis problems, including the herds suffering from mastitis induced by e.g. staphylococci and streptococci. Any clear evidence that BHV1 or BVDV were involved in these mastitis cases was not presented, as no attempts were made to isolate BHV1 or BVDV from milk of affected cows, and no data were presented about unvaccinated control cows within the same herds.

Bovine herpesvirus 1 has been shown to replicate in the bovine mammary gland and to induce signs of clinical mastitis after an intramammary inoculation (Greig and Bannister, 1965; Straub and Kielwein, 1966; Corner et al., 1967). An intramammary inoculation of one young heifer with a BHV1-IBR or a BHV1-IPV strain induced swollen quarters, hard and tender to the touch (Greig and Bannister, 1965). A strong reduction in milk yield was recorded, and milk samples showed abnormal morphology, with clots and blood, after the intramammary inoculation of cows with the BHV1-IBR strain. Virus was first isolated from infected quarters on day 2 post-inoculation (pi) which continued up to day 10-15 pi. The virus reached titres up to $10^6 – 10^7$ $TCID_{50}$/ml. No virus was detected in the milk from the two uninoculated control quarters. Clinically, the experimental mastitis produced by the BHV1-IBR strain was similar to that induced by the BHV1-IPV strain. Dilution series further demonstrated that about 10^3 $TCID_{50}$ BHV1/ml was required to produce infection of the mammary gland by the intramammary route (Greig and Bannister, 1965). In another study, an intramammary inoculation with BHV1-IPV resulted in clinical mastitis as evidenced by an increase in body temperature, decreased appetite, painful and swollen udders, and a strong reduction of the milk yield. It was possible to isolate the virus from the milk of inoculated quarters until day 11 pi (Straub and Kielwein, 1966).

Experimental BHV1 infections of the mammary gland resulted in necrosis of the alveolar epithelial layer, infiltration and accumulation of polymorphic and mononuclear cells, and inclusion bodies in the nuclei of epithelial cells (Corner et al., 1967).

The above mentioned studies demonstrate that BHV1 has been isolated from natural cases of mastitis and that the bovine udder is susceptible to BHV1. However, its impact on bovine mastitis cases in general is unclear. In view of the ubiquitous character of this virus, the number of reported mastitis cases in which BHV1 played a role is probably low. Bovine herpesvirus 1 was not isolated from milk of any of the 58 natural clinical mastitis cases from 10 herds examined virologically by Wellenberg et al. (2000). BHV1 is probably not a major primary udder pathogen.

2.2 Bovine herpesvirus 4

Bovine herpesvirus 4, a rhadinovirus and member of the *Gammaherpesvirinae* subfamily within the *Herpesviridae* family, has been isolated from cows with various clinical signs, including mammary pustular dermatitis (Reed et al., 1977), and chronic ulcerative mammary dermatitis (Cavirani et al., 1990). Recently, BHV4 has been isolated from 3 milk samples of 3 (5%) out of 58 cows with clinical mastitis, and not from the 58 matched control cows. Two of the three cows from which BHV4 was

isolated developed antibodies against BHV4, while no increase in antibodies against BHV4 were detectable in the third cow within 21 days. A possible role of BHV4 in bovine mastitis was further supported by the fact that in 4 of the 10 herds examined there was an ongoing BHV4 infection at the same time as mastitis occurred (Wellenberg et al., 2000). In a second case-control study, a part of the gene coding for BHV4-glycoprotein B was detected by PCR (Wellenberg et al, 2001) in milk samples from 2 (4%) out of 54 mastitis cows. From the same milk samples, BHV4 was isolated on bovine umbilical cord endothelial cells, a cell type highly susceptible to bovine herpesviruses (Wellenberg et al., submitted). A significant increase in BHV4 antibody titres was detected in one of these two mastitis cows at the same time as mastitis occurred. No BHV4 was detected in milk from their matched control cows by gB-PCR or virus isolation. In both case-control studies, the presence of BHV4 was in most cases accompanied by bacterial udder pathogens, e.g. *Staphylococcus aureus* and *Streptococcus uberis.* An experimental study, performed to further investigate the role of BHV4 in bovine mastitis, showed that a simultaneous intramammary and intranasal inoculation of lactating cows with BHV4 did not result in clinical mastitis. However, subclinical mastitis was induced in 2 out of 4 inoculated lactating cows (Wellenberg et al., in press). A significant increase of SCC was recorded in milk from 50% of the BHV4 inoculated quarters on days 8, 9 and 11 pi, compared to the non-inoculated quarters of the same cows (within-cow controls) and the quarters of the mock-inoculated cows. Virus was isolated from milk samples of inoculated quarters only; from day 1 pi to days 9 - 14 pi. A *Streptococcus uberis* infection appeared to trigger BHV4 replication in cows infected 2 weeks before with BHV4. Bovine herpesvirus 4 was isolated from the milk from 2 out of 4 quarters after an intramammary *Streptococcus uberis* inoculation.

During an epidemiological study, a positive association between the presence of BHV4 antibodies in cows and the incidence of bovine mastitis caused by *Staphylococcus aureus* was recorded (Zadoks et al., submitted). This finding suggests that a previous BHV4 infection promotes the development of mastitis especially caused by *Staphylococcus aureus*. BHV4 has also been isolated from the cellular fraction of milk samples from cows with antibodies against BHV4. Unfortunately, no clinical data were reported on mastitis in these cows (Donofrio et al., 2000).

All above mentioned studies strongly suggest a role for BHV4 in bovine mastitis. Although, BHV4 probably does not appear to play an important role as primary udder pathogen in the aetiology of clinical mastitis, it may play a role in subclinical mastitis cases, or in an indirect way. More research is warranted to establish a possible indirect role of BHV4 infections in bovine mastitis, e.g. as a result of immunosuppression. The virus can infect cells involved in the immune system, e.g. mononuclear blood cells (macrophages), and recently, a possible role of BHV4 has been postulated by playing a role in damaging vascular tissues (Lin et al., 2000). In addition, bovine endothelial cell cultures are highly

susceptible to BHV4.

2.3 Foot-and-mouth disease virus

Foot-and-mouth disease (FMD) virus, a member of the *Aphthovirus* genus within the family of the *Picornaviridae*, in general causes an infection whereby the virus is widespread through various tissues and organs of the host. Although, a primary infection of the mammary gland is unlikely to be a common occurrence in the pathogenesis of FMD, the virus can also replicate in the secretory epithelial cells of the mammary gland. Many researchers have isolated FMD virus from milk of FMD affected cows (Burrows, 1968; Ray et al. 1989; Fuchs, 1994), and also teat and udder lesions have been reported in FMD-affected cattle during an outbreak with an Asia-1 serotype (Firoozi et al., 1974).

The results of experimental inoculations of the udder show that it is a highly susceptible organ that is capable of producing large amounts of virus. Evidence for the replication of FMD virus in the mammary glands, as a result of a systemic infection, was found in cattle that were infected by (simulated field-type) contact exposure to FMD virus infected animals (Blackwell et al., 1983). Infection of FMD virus by the oronasal route also resulted in virus replication in secretory epithelial cells of the alveoli of the udder (Blackwell and Yilma, 1981), and in progressive temporal necrosis in the alveoli. Clumps of necrotic secretory epithelial cells and detached membrane-limited structures (cellular debris) were observed within the alveolar lumen and in the milk (Blackwell et al., 1983). During experimental infection, an increase of leukocytes was not recorded up to day 17 pi. This means that FMD virus infections of the bovine udder result in necrosis of the alveolar epithelial cells, but this occurs without a strong increase in leukocytes as observed for most bacterial udder infections. So, FMD virus is not the cause of a viral clinical mastitis as such, but secondary bacterial infections may result in clinical mastitis. The necrosis process is probably responsible for the observed decrease in milk yield (Blackwell and Wool, 1986).

Replication of FMD virus in the mammary gland has also been reported after cows were exposed to the virus either by aerosol, by a combination of intramammary-intravenous inoculation (Blackwell and Yilma, 1981), or after an intramammary inoculation via the ductus papillaris (Burrows et al., 1971). After intramammary inoculation, affected quarters became swollen and tender to the touch. The milk showed abnormal morphology (with clots), and a drop in the milk yield of approximately 60% was recorded. The FMD virus multiplied rapidly and virus titers of $>10^7$ plaque forming units/ml were recorded within 8-32 hours pi. Dissemination of the virus from the mammary gland was recorded by virus isolation from milk within 4-24 hours pi. The ability of FMD virus to persist in the mammary tissue was confirmed by the intermittent recovery of the virus from cows up to day 51 pi, which indicates virus multiplication in the udders of immune cows (Burrows et al., 1971).

Based on reports of natural mastitis cases and experimental infections, we may conclude that the udder is a highly susceptible organ for FMD. Infection of the secretory epithelial cells of the mammary gland will usually be the result of a systemic infection, because a primary infection of the mammary gland by this virus is unlikely to be a common occurrence. Mastitis associated with FMD virus is assumed to be due to secondary bacterial infections.

2.4 Parainfluenza 3 virus

In 1966, parainfluenza 3 (PI3) virus, a member within the *Paramyxoviridae* family, was recovered from Japanese cattle with acute respiratory illness from nasal secretions, and also from milk (Kawakami et al., 1966a). On one of the examined farms, the virus was recovered from milk in 14 of 58 cows (24%). The cows from which PI3 virus was recovered from the milk did not show signs of clinical mastitis, but an increased milk SCC was recorded in many milk samples.

Parainfluenza 3 virus was also isolated from quarter milk from one cow of the same farm with typical aseptic mastitis. An intramammary inoculation of PI3 virus resulted in respiratory signs and other signs e.g. fever, malaise, and losing condition, as observed in calves infected with the same PI3 virus by intravenous or intranasal inoculation. The affected udders developed swelling and induration. The milk showed a color change, an increased pH and increased numbers of glandular epithelial cells, neutrophils, lymphocytes and monocytes. Virus was excreted in high titers (up to 10^7 $TCID_{50}$/ 0.1 ml) in milk from inoculated quarters up to day 10 pi. The histological examination revealed that the major change was an interstitial inflammation, consistent of large lymphoid cells (Kawakami et al., 1966b). Both studies indicate that the mammary gland is highly susceptible to PI3 virus, and that in naturally PI3 virus infected cows udder infections may also occur. In some cases the infection may result in overt clinical mastitis. These findings await confirmation.

3. Viral infections and bovine subclinical mastitis

A possible role of viruses in bovine subclinical mastitis has been suggested before (Fuchs, 1994). Subclinical mastitis occurs frequently, and may lead to high economical losses due to reduced milk yields, and to penalties because of too high bulk-tank somatic-cell counts. Losses resulting from both clinical and subclinical mastitis may amount to 20% of the potential production (Beck et al., 1992). In practice, subclinical mastitis cases are often not detected rapidly, or may even not be recognized by the farmer.

3.1 Bovine leukaemia virus

Bovine leukaemia virus (BLV), belonging to the *Deltaretrovirus* genus and member of the family of *Retroviridae* (Pringle 1999), causes enzootic bovine leukosis. It preferentially infects lymphocytes of the B-lineage in cattle. Recently, BLV particles have been detected by electron microscopy around lymphocytes in the mammary tissue of BLV antibody positive cows affected by subclinical mastitis (Yoshikawa et al., 1997). No macroscopical lesions were detected in the mammary glands of the 6 cows examined, but histological lesions were found in some lobules of the mammary gland, i.e. an infiltration of lymphocytes, plasma cells, and neutrophils into alveoli and interlobular connective tissue. The alveoli also contained numerous macrophages and desquamated alveolar lining cells. No information was recorded about the milk SCC, the presence of bacterial udder pathogens or milk yields. Consequently, whether this virus was the causative agent in this "subclinical" mastitis case is unknown.

A possible association of BLV infections and mastitis in dairy cows has been investigated on individual and on herd level, however with contradictory results. A positive association has been reported by Milojevic et al. (1991) and Rusov et al. (1994), who reported that the occurrence of mastitis and increased cell counts are more often recorded in cows with enzootic leukosis than in healthy cows. A significant association between BLV seropositivity and higher milk SCC has also been recorded for older cows (Jacobs et al., 1995). According to Emanuelson et al. (1992), the risk for infectious diseases seemed to be greater among BLV-infected herds than among non-BLV infected herds. In this study a positive association between BLV antibody positive bulk milk and bovine mastitis, and also for bulk SCC, was recorded. However, no data were presented on the differences in management on the herd level, which is an important factor in bovine mastitis prevalence.

In the above mentioned studies a possible association of BLV infections and mastitis in dairy cows

is noted, but this is not the case in all studies performed. In one of these studies, 226 adult dairy cows were examined for BLV infection and mastitis, but no statistically significant association was found between the BLV infection and mastitis (Fetrow and Ferrer, 1982). Of the cows positive for BLV-infection, 22.7% had bacteriological cultures positive for mastitis, whereas in the group of BLV-infection negative cows 23.2% suffered from bacteriological mastitis pathogens. Scott et al. (1991) reported that the number of subclinical mastitis cases in BLV positive cows was even lower than that in BLV negative cows. In this study, the number of *Staphylococcus sp.* and *Micrococcus sp.*, isolated from milk from 50 BLV-positive cows, was significantly lower than that in 35 BLV-seronegative cows. In a matched case-control study, to assess the risk of clinical mastitis in BLV-infected cows, the BLV-infected cows did not produce less milk, or did not develop mastitis more often than did noninfected cows ($P > 0.05$) (Huber et al., 1981).

Also studies on milk yields, which also might be an indication for the presence of subclinical mastitis cases within herds, on BLV positive herds showed contradictory results. Heald et al. (1992) could not find a significant association between milk yield, somatic cell counts and BLV-seropositivity. One of the reasons why results on studies on herd level are not in agreement is that many studies did not use a proper study design. None of the studies performed on individual or herd level clarify the role of BLV in the aetiology of bovine subclinical mastitis. No experimental studies have been reported to investigate whether BLV is able to induce subclinical or clinical bovine mastitis. Such experiments are necessary to gain more insight into the role of BLV in the aetiology of bovine mastitis.

4. Viral infections and their indirect role in bovine mastitis

Can viral infections play an indirect role in the pathogenesis of bovine mastitis? Damage of teat and ductus papillaris (as natural barrier) and immunosuppression may lead to a higher susceptibility for bacterial mastitis cases, and bacterial infections may run a more severe course.

4.1 Teat lesions

Bovine herpes mammillitis virus (BHV2), vaccinia, cowpox, pseudocowpox, FMD viruses, and to a lesser extent vesicular stomatitis virus can cause a local dermatitis, often with ulcerations in the ductus papillaris, leading to secondary bacterial infections in the sinus lactiferus and the corresponding mammary gland (Turner et al, 1976; Francis, 1984; Scott and Holliman, 1984).

4.1.1 Bovine herpesvirus 2

Bovine mammillitis is an acute viral disease of cattle caused by BHV2 (Martin et al., 1966), a virus of the genus *Simplexvirus* and member of the *Alphaherpesvirinae* subfamily within the *Herpesviridae* family. Bovine herpesvirus 2 often infects young heifers and young cows at first parity or in the first lactation period. The infection may be subclinical or relatively mild (Turner et al., 1976; Letchworth and LaDue, 1982; Scott and Holliman, 1984), but it can also be very severe causing extensive painful ulcerations on one or more teats and udders (Scott and Holliman, 1984). Lesions can range from vesicles and ulcerations of large (up to 10 cm wide) areas of teat skin to single small (2-3 cm wide) plaques of oedema. Severe BHV2 infections may also result in damage of the ductus papillaris. The functions of the keratin in the ductus papillaris, with its fatty acids and proteins, and the macrophages, lymphocytes and plasma cells in the ductus papillaris and the sinus lactiferus may be impaired due to this BHV2 infection (Paape et al., 1985; Senft and Neudecker, 1991). This may enhance the susceptibility of the mammary gland for bacterial mastitis (Martin et al., 1969; Letchworth and LaDue, 1982; Scott and Holliman, 1984; Gourreau et al., 1989).

BHV2 has also been isolated from milk from cows with ulcera on teats (Martin et al., 1969), but leakage from these lesions was probably the cause of the presence of BHV2 in the examined milk samples. Turner et al. (1976) recorded mastitis cases in cows with BHV2 infection. These cows had ulcera in the ductus papillaris, and therefore its function was impaired. Chronic mastitis was observed in cows with udder ulcera up to the ductus papillaris. These reports suggest that BHV2 may induce mastitis due to damage of the mechanical defence of the udder. Mastitis indirectly due to BHV2

infections (Letchworth and LaDue,1982: Scott and Holliman, 1984) mostly affect a few cows within a herd, but also percentages of 22% have been recorded for BHV2 affected cows that developed mastitis (Martin et al., 1969). Under experimental conditions, an intradermal and intravenous inoculation of a 30-month-old heifer with BHV2 resulted in several clinical signs e.g. mammilitis. However, in this study mastitis has not been recorded and the mammary gland has not been examined for histopathological lesions (Tabbaa et al., 1987).

In conclusion, BHV2 infections can result in damage of the natural defence mechanisms of the udder, which results in a higher susceptibility to bacterial mastitis.

4.1.2 Vaccinia virus and cowpox virus

Infections with vaccinia virus and cowpox virus, both belonging to the genus *Orthopoxvirus* within the subfamily *Chordopoxvirinae* of the *Poxviridae* family, do not occur anymore or are very rare, respectively (Mayr and Czerny, 1990). Clinical signs are comparable to those described for BHV2 infections. As a result of teat lesions, mastitis may occur on the same way as it occurs after a BHV2 infection. After an intramammary inoculation with vaccinia virus, the virus that has been used for smallpox vaccination, an inflammatory reaction in the bovine mammary gland was produced (Easterday et al., 1959). The intramammary inoculation of the mammary glands via the ductus papillaris of 6 cows with vaccinia virus (strain IHD) induced systemic signs e.g. elevated body temperatures, and udder swelling in 5 of the 6 cows inoculated. Lesions appeared on the ends of all vaccinia virus inoculated teats, and progressed from a papule to a vesicle to a scab. The SCC increased up to >500.000/ml, and vaccinia virus was isolated from the milk of 4 out of 4 lactating cows up to 9 days pi (Easterday et al., 1959). Natural cases of bovine mastitis, in which vaccinia virus was involved, are unknown.

Outbreaks of cowpox virus are extremely rare. The virus enters through teat skin injuries and several stages of lesion development can be observed. Erythematous areas appear on the teat and can change into raised papule and ruptures with pitted centers. Lesions spread rapidly throughout the herd. Healing occurs within two to three weeks although secondary bacterial infections may delay resolution (Francis, 1984). During a cowpox virus infection in India of cows and buffalo's some animals suffered from mastitis (Sambyal et al., 1983). Most of the affected cows showed teat lesions, and udder pathogens like *Staphylococcus aureus* and *Klebsiella spp.* were isolated from the milk of affected cows. The role of cowpox virus in this case of mastitis was not clear, but the teat lesions, induced by cowpox virus, might have resulted in secondary bacterial mastitis. The above mentioned studies indicate that cowpox virus may play a role in bovine mastitis, but the incidence is probably very low.

4.1.3 Pseudocowpox virus

The pseudocowpox virus belongs to the genus *Parapoxvirus* within the subfamily *Chordopoxvirinae* of the *Poxviridae* family. Only one report was found concerning the isolation of a poxvirus from milk (Dawson et al., 1968). The virus was isolated from a pooled milk sample and typed as a virus from the paravaccinia subgroup, but no lesions suggestive of pseudocowpox were recorded, neither was clinical or subclinical mastitis. An intramammary inoculation of one lactating cow with this strain did not result in systemic disturbance, swelling or induration of the udder. Only a few small clots were recorded in the milk on days 4 and 5 pi. No lesions developed on teats and on the udder. In milk from one out of two inoculated quarters, the virus was isolated on only 24 hours pi, but clinical mastitis was not noted (Dawson et al., 1968). No further reports on pseudocowpox virus and bovine mastitis were found, despite the fact that this virus is ubiquitous and the infection induces comparable clinical signs as reported for BHV2 infections (Gibbs, 1984). This suggests that, in addition to BHV2, pseudocowpox virus may also induce mastitis due to damage of the mechanical defence mechanism of the udder. The role of pseudocowpox virus in the aetiology of bovine mastitis is still an interesting area for research; this virus was detected in 5 out of 14 cases of bovine teat lesions in Dutch cattle (Wellenberg, 2001, unpublished data).

4.1.4 Foot-and-mouth disease virus

FMD virus can play a secondary role in bovine mastitis in that FMD virus infection may result in ductus papillaris lesions and therefore enhances bacterial infections as reported for an experimental *Arcanobacter pyogenes (A. pyogenes)* udder infection (Saini et al., 1992). After an infection of lactating cows with FMD virus, *Arcanobacter pyogenes* had been isolated from 15 quarters showing purulent mastitis (Saini et al., 1992), while an intramammary inoculation of quarters with *A. pyogenes* alone did produce only mild inflammatory reactions (Vecht et al., 1987). This suggests that the teat epithelium of the quarters had already been damaged by FMD virus that supported the involvement of *A. pyogenes* as the causative agent of purulent mastitis. The injury to teat epithelium was essential for the establishment of infection (Seinhorst et al., 1991). Field studies also support a secondary role of FMD in bovine mastitis. An increased incidence of bovine mastitis cases with secondary bacterial pathogens has been reported after an infection with FMD virus (Ray et al., 1989; Seinhorst et al., 1991).

4.1.5 Vesicular stomatitis virus

Mastitis has been associated with some other virus diseases, but it has not been demonstrated that these viruses were the primary invaders of the mammary gland. Strozzi and Ramos-Saco (1953) reported teat lesions and associated mastitis in cases of vesicular stomatitis virus infections; a virus belonging to the genus *Vesiculovirus* within the *Rhabdoviridae* family. An intramammary inoculation of 8 cows with vesicular stomatitis virus (New Jersey) did not induce udder swelling, but it resulted in increased milk SCC of >500.000/ml in all 5 inoculated lactating cows. The virus was isolated from milk from 4 out of 5 lactating cows. In 5 out of 8 cows elevated body temperatures were recorded. No changes were recorded in the bacterial flora of any quarter inoculated with vesicular stomatitis virus during this study (Easterday et al., 1959). Although vesicular stomatitis virus may play a role in bovine mastitis, the incidence is probably very low as the number of reported mastitis cases in which vesicular stomatitis virus has been involved is nil.

4.1.6 Bovine papillomaviruses

The bovine papillomaviruses belong to the genus *Papillomavirus* within the family *Papillomaviridae*. At least six types of bovine papillomavirus (BPV) have been recognised, and certain types can cause fibropapillomas on teats (Olson, 1990). Fibropapillomas in the ductus papillaris due to bovine papillomavirus may result in damage of the natural defence mechanisms of the udder and therefore in a predisposition for mastitis (Francis, 1984). An ascending bacterial infection may result in mastitis (William et al., 1992).

4.2 Immunosuppression

In addition to viruses that cause teat lesions, other viral infections may induce or enhance bovine mastitis due to their immunosuppressive effects. Although, so far there is not any clear evidence for this.

4.2.1 Bovine herpesvirus 1

Bovine herpesvirus 1 infections can impair the bovine immune system (Bielefeldt-Ohmann and Babiuk, 1985; Straub, 1991; Nataraj et al., 1997; Saini et al., 1999; Koppers-Lalic et al., 2001). Based on the immunosuppressive properties of BHV1, it has been proposed that the virus may play a

secondary role in the aetiology of diseases caused by bacteria (Filion et al., 1983; Bielefeldt-Ohmann and Babiuk, 1985; Hutchings et al., 1990), but whether and which secondary role BHV1 plays in the aetiology of bovine mastitis is not clear. Epidemiological studies, to examine whether BHV1 seropositive animals are more prone to bovine mastitis than BHV1 seronegative animals, are unknown. Hage et al. (1998) reported a significant drop in milk production, which might be an indication for subclinical mastitis, during a subclinical BHV1 infection on a dairy herd. However, no association was found between the BHV1 infection and mastitis, since the milk SCC was unaltered and clinical mastitis was not observed.

4.2.2 Bovine viral diarrhoea virus (BVDV)

Another virus that causes immunosuppression is BVDV, a member of the *Pestivirus* genus, within the family of the *Flaviviridae* (Roth et al., 1981; Bolin et al., 1985; Markham and Ramnaraine, 1985; Welsh et al., 1995). Persistently infected animals show chronically impaired immunoresponses (Roth and Bolin, 1986; Brownlie, 1989), and a delay in the onset of BRSV-specific IgG response and reduced antibody titres has been noted in cattle infected concurrently with BVDV (Elvander, 1996). These data indicate that BVDV may play an (indirect) role in the susceptibility of the animal to secondary infections, or may enhance the possibility of secondary infections to run a more severe course (Potgieter et al., 1984).

Studies on the immunosuppressive role of BVDV in relation to bovine mastitis are very scarce. Siegler et al. (1984) reported an increased amount of mastitis cases in BVDV and BHV1 seropositive herds, however which role BVDV played in these mastitis cases in unclear. Furthermore, a positive association between BVDV and bovine mastitis, based on the BVDV antibody titres in bulk milk of 237 herds, has been reported. The number of mastitis cases increased in herds with an increased BVDV antibody milk titre (Niskanen et al., 1995). In a retrospective longitudinal study, which was conducted to examine whether the exposure of dairy herds to BVDV affected udder health, a 7% increase was noted in the incidence rate of clinical mastitis in herds exposed to BVDV as compared with non-BVDV exposed herds (Waage, 2000). A reduction in the milk yield was shown in cows that seroconverted for BVDV antibodies, although no information was presented on mastitis (Moerman et al., 1994). Further studies are warranted to clarify the role of BVDV in bovine mastitis. No intramammary inoculation of cows with BVDV has been reported, and in addition there are no reports on the isolation of BVDV from milk of cows with mastitis. However, BVDV genomic sequences can be detected by PCR in milk and bulk milk samples (Radwan et al., 1995; Drew et al., 1999), but this is likely to be caused by the presence of persistently infected cows in the herd, and consequently does not

mean that the virus is involved in a direct or indirect way in bovine mastitis cases.

4.2.3 Bovine leukaemia virus

An association of a virus infection with a higher susceptibility for bovine mastitis has also been suggested for BLV. The primary target cells for BLV are cells of the B-lymphocyte lineage in cattle. Infection of B-lymphocytes may influence the humoral immune responses, e.g. a reduction in plasma IgM levels, and the cellular responses are very probably as well impaired in BLV-infected cattle (Yamamoto et al., 1984; Meiron et al., 1985). These data suggest that the immune system of BLV infected calves can be affected, although its effect on the incidence and the severity of bovine mastitis cases is unclear.

4.2.4 Bovine immunodeficiency virus

Bovine immunodeficiency virus (BIV), a lentivirus within the *Retroviridae* family, was detected for the first time in 1972. Although most infections run a subclinical course, BIV infections may also result in clinical signs such as lymphadenopathy, lymphocytosis, lesions of the central nervous system, wasting and several secondary bacterial infections (Snider et al., 1996). Lymphoid depletion with a reduction of the follicular development and depletion of B and T-cell compartments in lymph nodes are observed in BIV infected animals. Secondary infections were often multiple such as, e.g. metritis and mastitis (Snider et al., 1996). In this study, 24 (40%) out of 59 cows with a BIV infection showed chronic mastitis. Necrotising udder tissue were recorded in combination with a few udder pathogens like *Escherichia coli*.

The effect of co-infection with BLV and the influence of immunosuppression on the severity of chronic bovine mastitis cases remains to be of interest for future investigations.

5. Other viral infections, including non-bovine related virus infections, of the bovine mammary gland

A few other viral infections have been associated with bovine mastitis. For example, mastitis, which may be secondary, has been attributed to a systemic virus disease such as malignant catarrhal fever (Beckman, et al., 1960). This report suggests that severe lesions in the mammary gland may account for a decline in milk production and cracking of the epithelium of the teats. However, this is the only report on any possible relation between malignant catarrhal fever and mastitis.

5.1 Rinderpest virus

The role of rinderpest virus, a Morbillivirus and one of the members of the *Paramyxoviridae* family, in bovine mastitis has not been examined thoroughly. Infection of two swamp buffaloes with a rinderpest virus, isolated from the spleen of an infected buffalo, resulted in clinical signs as fever, depression and conjunctivitis, and vesicles appeared on lips and mammary gland (Tesprateep et al., 1987). Natural primary or secondary cases of mastitis due to rinderpest virus infections have not been reported.

5.2 Bovine enterovirus

Bovine enteroviruses are members of the genus *Enterovirus* in the *Picornaviridae* family. This virus has been isolated from healthy cattle and cattle with enteric, respiratory and reproductive disease problems (Knowles and Mann, 1990), but its role in mastitis is unknown. After an intramammary inoculation of two cows with bovine enterovirus, an acute catarrhal mastitis with marked increased milk SCC, and only mild clinical symptoms were recorded in both cows. The virus was isolated from milk of inoculated quarters (Straub and Kielwein, 1965).

5.3 Non-bovine related viruses

Also non-bovine related viruses have been shown to replicate in the bovine mammary gland after intramammary inoculation (reviewed by Afshar and Bannister, 1970). An inflammatory reaction in the bovine mammary gland was produced by infusion of the Newcastle disease virus (strain Roakin). The intramammary inoculation of 3 cows, one of which did not lactate, with Newcastle disease virus resulted in udder swelling and an increase in body temperature in one of the inoculated cows. An

increase in milk SCC was recorded in the two inoculated lactating cows, and the milk also contained virus on day 6 after inoculation, but not on day 9 (Easterday et al., 1959).

Mitchell et al. (1956) have demonstrated that influenza and NCD viruses will multiply when inoculated into the mammary gland (Mitchell et al., 1956). No mention was made of any inflammatory processes. These studies show that non-bovine viruses are able to replicate in the bovine mammary gland.

6. Concluding remarks

This review shows that viruses can be involved, in a direct or indirect way in the aetiology of bovine mastitis. In natural cases of mastitis, BHV1, BHV4, FMD and PI3 viruses have been isolated from milk. In addition, experimental infections via the ductus papillaris clearly demonstrated that these viruses replicated in the mammary gland tissue, followed by clinical mastitis in the cases of BHV1, FMD virus and PI3 virus infections, and subclinical mastitis after a BHV4 infection. However, no investigations have been performed to examine whether this route of infection is of importance in the field. Because bacterial pathogens usually infect the udder through the ductus papillaris, it may be expected that also viruses can infect the mammary gland tissue via this route after transmission by, e.g. milking devices. Especially in cases when hygienic measures are not well taken. However, we assume that BHV1, BHV4, FMD and PI3 viruses are mostly transmitted by direct contact and by aerosols. That an experimental infection through the natural route leads to mastitis, has been shown only for FMD (Table 1). However, in the Western world FMD virus induced bovine mastitis is not of practical relevance because cattle infected with FMD virus will be destroyed immediately after the diagnosis has been made. With regard to BHV1, BHV4, PI3 virus and BLV infections, the data in the literature do not convincingly demonstrate that these viruses can play a primary role in causing mastitis in the field.

It is likely that viruses that cause teat lesions (BHV2, cowpox, pseudocowpox, FMD, vesicular stomatitis virus, and papillomaviruses), and thereby damaging the integrity of the bovine udder, indirectly contribute to mastitis. The impact of these virus infections may be great within individual herds but less within regions or districts of countries.

Although it is plausible that virus-induced immunosuppression underlies mastitis, there are no data that underpin this assumption. In addition, only very few well-designed epidemiological studies have been performed to support a causal relationship between virus infections and mastitis.

Further research should be performed to firmly establish the importance of viral infections on bovine mastitis in the field. Such research should certainly take into account that mastitis is a multifactorial disease, consequently such studies are difficult to design. Investigations should deal with well-designed case-control studies, more experimental viral infections whether or not in conjunction with bacterial infections, and various epidemiological studies. Application of new more powerful diagnostic tools, based on the detection of viral genomic sequences e.g. by (multiplex) PCR or micro-array devices, offer new opportunities for a rapid simultaneous detection of most viruses involved in bovine mastitis. In the future, these new screening methods may also provide a better insight in the prevalence of viruses in milk from cows with mastitis.

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SUMMARY

SAMENVATTING

LIST OF PUBLICATIONS

DANKWOORD

CURRICULUM VITAE

Summary

Mastitis is an often occurring disease in dairy cattle with an enormous economic impact for milk producers worldwide. Despite intensive research, which is historically based on the detection of bacterial udder pathogens, still around 20-35% of clinical cases of bovine mastitis have an unknown aetiology. Due to the high number of unknown causes of clinical mastitis, studies were undertaken to gain more insight into the role of viruses in this important disease. A case-control study was set up, in which non-conventional cell types were used for virus isolation. We found that BHV4 may be involved in the aetiology of bovine mastitis, because it was isolated from milk of 3 (5%) out of 58 cows with clinical mastitis and not from the 58 matched control cows. Concomitant with the isolation of BHV4, an increase in antibody titre against BHV4 was noted in two of these three mastitis cows from which BHV4 was isolated. Bovine herpesvirus 4 (BHV4), a member of the *Gammaherpesvirinae* subfamily, has a worldwide distribution. It has been isolated from cows with various clinical signs, but never from milk of mastitis cows before. Restriction enzyme analyses studies showed that the first isolated BHV4 strain from milk of cows with clinical mastitis belonged to the Movar 33/63 group. Because there was a lack of well-validated tests for the detection of BHV4, BHV4 antibodies and BHV4 DNA when we began our investigations, new tests for the diagnosis of BHV4 infections were developed and validated.

The replication of BHV4 is absent or slow in many routinely used cell types. Therefore non-conventional cell types such as bovine udder epithelial cells, bovine umbilical cord endothelial cells and bovine alveolar lung macrophages were additionally used for virus isolation. The use of the bovine umbilical cord endothelial cells, a cell type seldom used in bovine virology, proved to be a good choice. In the first case-control study, BHV4 was isolated only on this cell type and not on three other cell types used. Susceptibility studies showed that BHV4 grew to much higher titres in bovine umbilical cord endothelial cells than in the routinely used Madin Darby bovine kidney cells. The bovine umbilical cord endothelial cell type was not only highly susceptible to BHV4, but it also proved to be susceptible to other bovine herpesviruses, such as BHV2 and BHV5 (Chapter 2).

For the detection of BHV4 antibodies only one commercial indirect-ELISA was available. We developed and evaluated an immunoperoxidase monolayer assay (IPMA) for the detection of antibodies directed against BHV4. The newly developed BHV4 IPMAs were based on the detection of antibodies against the American BHV4 reference strain DN-599 and on LVR 140, a reference strain belonging to the European Movar 33/63-like group. After validation, the IPMA proved to be a reliable test, and it was found more sensitive for the early detection of BHV4 antibodies than the indirect-ELISA. In the IPMA, BHV4 antibodies were first detectable 16-18 days post infection and in the

indirect-ELISA only after 30 days post-infection. In addition, a serological survey showed that the estimated seroprevalence of BHV4 in Dutch cattle was 16-18% and that the percentage of BHV4 seropositive cattle varied by age category between 6 and 43% (Chapter 3).

A polymerase chain reaction for the detection of BHV4-glycoprotein-B (gB) DNA was developed and validated, and a nested-PCR was modified to detect BHV4-thymidine kinase (TK) DNA in bovine milk samples. Both methods proved to be rapid and reliable tests for the screening of BHV4 DNA in milk (Chapter 4). In a second case-control study, using these newly developed diagnostic tools, BHV4 was detected again from mastitis cows only and not from matched controls (Chapter 6). BHV4 and BHV4-gB DNA was detected in milk from 2 (4%) out of 54 cows with clinical mastitis, whereas no BHV4 was detected by virus isolation and PCR in 54 matched controls. Also other results from the second case-control study largely confirmed those from the first case-control study. An experimental study was performed to examine whether a simultaneous intramammary and intranasal inoculation of lactating cows with BHV4 induced clinical mastitis (Chapter 7). No clinical mastitis was noted in the four inoculated lactating cows, but the somatic cell counts increased significantly in milk of 50% of the BHV4 inoculated quarters, compared to the non-inoculated quarters of the same cows and quarters of mock-inoculated cows (control group) on days 8, 9, and 11 post-inoculation. Another interesting finding was that a bacterial infection, *Streptococcus uberis* infection, triggered BHV4 replication.

Replication of latent BHV4 from tissues has been reported after dexamethasone treatments, but it has never been described that its replication may be triggered by a bacterial infection. This study also showed that a preceding BHV4 infection did not exacerbate clinical mastitis induced by an intramammary *Streptococcus uberis* inoculation.

The study on the susceptibility of BHV4 to various anti-viral agents showed that brivudin effectively inhibited the *in vitro* replication of BHV4. These anti-viral agents offer opportunities for designing strategies to inhibit BHV4 replication in cattle with e.g. mastitis. Cidofovir and ganciclovir inhibited the replication to a lesser degree (Chapter 8).

Based on the studies performed, we conclude that BHV4 may play a role in the aetiology of bovine mastitis, albeit likely a minor one in clinical mastitis. In both case-control studies, the presence of BHV4 in milk was mostly accompanied by the presence of known udder pathogens, e.g. *Staphylococcus aureus* and *Streptococcus uberis,* which makes it even more difficult to understand the direct role of BHV4 in causing bovine clinical mastitis. It suggests rather a role in subclinical mastitis and an indirect role for BHV4 in the aetiology of bovine mastitis. More research is warranted to investigate its indirect role as a result of e.g. immunosuppression. BHV4 has a close relationship with cells of the immune system, and it grows to high titres in endothelial cell cultures *in vitro*, but

whether this virus has immunosuppressive properties is not completely clear.

Finally, based on the studies performed during this thesis and the review study on viral infections and bovine mastitis (Chapter 9), we may conclude that viruses can play a direct or indirect role in the aetiology of bovine mastitis.

Samenvatting

Mastitis is één van de belangrijkste gezondheidsproblemen bij melkkoeien met enorme economische gevolgen binnen de melkveehouderij. Ondanks intensieve onderzoeks- en bestrijdingsprogramma's, die voornamelijk gebaseerd zijn op de detectie en behandeling van bacteriële infectieziekten, is bij 20-35% van het aantal klinische gevallen van mastitis de oorzaak nog steeds niet vast te stellen. Omdat de rol van het virus bij klinische gevallen van mastitis niet geheel duidelijk was en van ondergeschikt belang werd geacht, werd er ook niet veel onderzoek naar gedaan. Toch zou een deel van het aantal onbekende mastitis gevallen een virale oorzaak kunnen hebben. Om de mogelijke rol van virussen bij het ontstaan van bovine mastitis nader te onderzoeken werd een case-control studie uitgevoerd. In deze eerste case-control studie werd gebruik gemaakt van non-conventionele cellen voor de isolatie van virussen, gecombineerd met verschillende gevalideerde serologische testen voor het aantonen van een antilichaamrespons.

Op basis van de resultaten van de case-control studie werd voor het eerst een relatie gelegd tussen bovine herpesvirus 4 (BHV4) infecties en bovine mastitis. BHV4 werd bij 3 (5%) van de 58 onderzochte lacterende mastitis runderen aangetoond in de melk, en bij geen van de 58 controle runderen. Gelijktijdig met de isolatie van BHV4 werd bij 2 van de drie mastitis runderen een significante stijging van BHV4 antilichamen in serum aangetoond. Het aantal dat BHV4 vaker werd geïsoleerd uit melk van mastitis runderen dan bij controle runderen was bijna statistisch significant ($P = 0.125$).

Bovine herpesvirus 4 behoort tot de *Gammaherpesvirinae*, een subfamilie van de *Herpesviridae* familie. Het virus is wijd verspreid, en kan geïsoleerd worden uit runderen met verschillende klinische symptomen, maar een BHV4 infectie was nog nooit eerder geassocieerd met klinische mastitis.
Omdat het virus langzaam groeide op veel routinematig gebruikte cellijnen, er geen commerciële gevalideerde test was om BHV4 antilichamen aan te tonen, en er geen geavanceerde moleculair-biologische technieken bestonden voor de detectie van BHV4-DNA in melk, zijn we begonnen met het opzetten en valideren van een aantal diagnostische methoden voor de detectie van BHV4 infecties.

In de eerste case-control studie werden verschillende cellen gebruikt voor de isolatie van virussen, namelijk; bovine uier epitheelcellen, bovine navelstreng endotheelcellen, bovine alveolaire long macrofagen en de routinematig gebruikte embryonale bovine tracheacellen. De non-conventionele bovine navelstreng endotheelcel (BUEc) bleek uitermate geschikt te zijn voor de isolatie van BHV4. In de eerste case-control studie werd BHV4 namelijk alleen geïsoleerd op BUEc en niet op de drie andere celtypen. Op de BUEc werd een duidelijke verlaging van de detectielimiet en werden hogere BHV4 virustiters verkregen t.o.v. de routinematig gebruikte Madin Darby Bovine Kidney (MDBK) cellijn.

De BUEc bleek niet alleen geschikt te zijn voor de isolatie van BHV4 maar deze cellen bleken ook erg gevoelig te zijn voor andere bovine herpesvirussen, m.n. BHV2 en BHV5 (Hoofdstuk 2).

Voor het specifiek aantonen van BHV4 antilichamen in serum werd een immunoperoxidase test (IPMA) ontwikkeld en gevalideerd. Deze IPMA was gebaseerd op de detectie van antilichamen gericht tegen de Amerikaanse BHV4 referentiestam DN-599 en tegen LVR-140, een referentiestam behorende tot de Europese Movar 33/63-groep. Na validatie bleek dat de IPMA een specifieke en betrouwbare test was voor het aantonen van BHV4 antilichamen in serum. Het is een gevoeligere test dan de indirecte-ELISA voor de vroege detectie van BHV4 antilichamen. De IPMA toonde BHV4 antilichamen aan op dag 16-18 na experimentele infectie en de indirecte-ELISA pas op dag 30. Een pilot-studie werd uitgevoerd, omdat er geen data waren over de BHV4 seroprevalentie in het Nederlandse rund. Op basis van deze pilot-studie werd de BHV4 seroprevalentie in runderen in Nederland geschat op 16-18%. Ook bleek dat de BHV4 seroprevalentie varieerde per leeftijdscategorie en wel van 6 – 43% (Hoofdstuk 3).

Om sneller en gevoeliger BHV4 aan te kunnen tonen in melk werd een polymerase ketting reactie (PCR) opgezet en gevalideerd voor de detectie van BHV4-glycoprotein B (gB) DNA. Daarnaast werd een bestaande nested-PCR voor de detectie van BHV4-thymidine kinase (TK) DNA gemodificeerd. Beide PCR's bleken snelle en betrouwbare testen te zijn voor het screenen van melk op BHV4; de gB-PCR is een snellere. Deze test verdiende daarom de voorkeur boven de BHV4-TK PCR in ons verdere onderzoek (Hoofdstuk 4).

In een tweede case-control studie, waarbij gebruik werd gemaakt van de nieuw ontwikkelde testen voor het detecteren van BHV4 infecties, werd selectiever gekeken naar de rol van BHV4 in de etiologie van bovine klinische mastitis. Wederom werd BHV4 alleen gedetecteerd in melk van klinische mastitis runderen en niet in controle runderen. Bovine herpesvirus 4 en BHV4-gB DNA werd gedetecteerd in melk van 2 (4%) van de 54 onderzochte mastitis runderen en niet in melk van de 54 controles (Hoofdstuk 6).

In een experimentele infectie, waarbij lacterende koeien gelijktijdig intramammair en intranasaal geïnoculeerd werden met BHV4, werden geen klinische mastitis gevallen waargenomen, maar wel werd in 50% van de geïnoculeerde kwartieren een verhoogd celgetal in de melk waargenomen, wat een indicatie is voor subklinische mastitis. Op de dagen 8, 9, en 11 na infectie waren de celgetallen in melk van BHV4-geïnoculeerde kwartieren significant hoger dan in melk van niet-geïnoculeerde controlekwartieren van dezelfde koeien (within-cow controles), en de celgetallen in melk afkomstig van mock-geïnoculeerde kwartieren van controle koeien (controle groep) (Hoofdstuk 7).
Vier van de 8 BHV4 geïnoculeerde kwartieren werden op dag 14 na de BHV4 inoculatie geïnfecteerd met *Streptococcus uberis* om de invloed van een voorafgaande BHV4 infectie op de ernst van een

klinische mastitis geïnduceerd door een *S. uberis* infectie te bestuderen. Hieruit bleek dat een voorafgaande BHV4 infectie niet van invloed was op de ernst van een klinische mastitis geïnduceerd door een *S. uberis* infectie. Wel kon op diverse dagen na de *S. uberis* inoculatie wederom BHV4 geïsoleerd worden uit de melk van twee van de vier kwartieren, terwijl vanaf dag 14 na de BHV4 infectie geen BHV4 meer kon worden geïsoleerd uit melk van kwartieren die alleen met BHV4 waren geïnoculeerd. Dit duidt op een activatie van de BHV4 replicatie door *S. uberis*. Activatie van de replicatie van BHV4 door bepaalde bacterien is nog nooit eerder beschreven, terwijl reactivatie van latent BHV4 door een dexamethason-behandeling een bekend fenomeen is.

In hoofdstuk 8 werd onderzocht of enkele geselecteerde virale agentia de replicatie van BHV4 *in vitro* kunnen remmen. Hieruit bleek dat brivudin (BVDU) de replicatie van BHV4 in BUE cellen efficiënt remt tot concentraties van minimaal 0.05 µg/ml. Ook cidofovir en ganciclovir (DHPG) remmen de replicatie van BHV4, maar dan bij hogere concentraties. Deze studie kan als uitgangspunt dienen voor het ontwikkelen van geneesmiddelen om BHV4 infecties te behandelen.

Op basis van de twee case-control studies, de experimentele infectiestudie en de epidemiologische studie (Zadoks et al., submitted) kunnen we concluderen dat BHV4 waarschijnlijk een rol speelt in de etiologie van bovine mastitis. Het speelt geen (grote) rol als primair agens in klinische mastitis gevallen, maar op basis van gegevens van de experimentele infectieproef kunnen we concluderen dat het virus mogelijk wel een rol kan spelen als subklinische mastitis verwekker. Op basis van de epidemiologie studie zijn er aanwijzingen dat BHV4 ook op een indirecte wijze betrokken kan zijn bij bovine mastitis. Het virus kan o.a. macrofagen latent infecteren, en daarnaast groeit het virus goed in endotheelcelcultures. Of dit de aanwijzingen zijn waarom BHV4 van indirecte invloed kan zijn op bovine mastitis, en of BHV4 infecties kunnen leiden tot immuunsuppressie, zal nader onderzocht moeten worden.

Het review artikel “Viral infections and bovine mastitis” geeft aan dat virussen een rol kunnen spelen in de etiologie van bovine mastitis. Hoe groot deze invloed is zal nader onderzocht moeten worden. In de praktijk zal wat vaker gedacht moeten worden aan een mogelijke virale oorzaak bij mastitis gevallen met onbekende etiologie. De opkomst van micro-array technieken biedt hierbij mogelijkheden tot verbetering.

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Curriculum vitae

Gerard Wellenberg werd geboren op 20 juli 1958 te Heino. Na het behalen van het HAVO diploma aan de Thomas á Kempis Scholengemeenschap te Zwolle in 1975 begon hij een HBO-opleiding voor medisch analisten te Deventer. Deze opleiding werd in 1977 afgerond waarna hij van 1977 tot 1980 werkzaam was als HBO-medisch analist bij het Streeklaboratorium voor de Volksgezondheid te Zwolle. In de periode 1980 tot 1992 was hij werkzaam bij de Gezondheidsdienst voor Dieren te Overijssel. In deze periode werden verschillende opleidingen gevolgd waaronder de HLO-immunologie opleiding te Groningen. Met het behalen van deze HLO-opleiding werd het mogelijk een doorstroom-programma te volgen aan de Universiteit. In 1992 werd de baan bij de Gezondheidsdienst opgezegd en startte hij met de studie Biologie (medische afstudeerrichting) te Haren. In september 1994 werd het doctoraal diploma gehaald. Onderzoekservaring heeft hij opgedaan bij de vakgroepen Medische Genetica en Immunologie bij het Academisch Ziekenhuis te Groningen. In augustus 1994 trad hij in dienst van Pharma-Bio Research. Een vooraanstaand contract research organisatie waarbij fase I en –II studies werden uitgevoerd ten behoeve van de registratie van geneesmiddelen bij de FDA. In maart 1996 kwam hij in dienst als onderzoeker / laboratoriumhoofd binnen de afdeling Zoogdier Virologie van ID-DLO (ID-Lelystad). Naast het uitvoeren van de daarbij behorende taken werd in 1998 gestart met het BHV4/mastitis onderzoek. In 2001 werd het onderzoek afgerond met als resultaat dit proefschrift. Vanaf begin 2001 is hij als projectleider betrokken bij het PMWS / PDNS onderzoek.