

SHORT COMMUNICATIONS

Bovine herpesvirus 4 in bovine clinical mastitis

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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 viral 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 varied from one to 22 per herd (Table 1). Milk samples were examined for the presence of viruses, using four different types of cell cultures: embryonic bovine trachea cells (EBT_r; 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 specific 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 were pipetted into the wells of a 96-well cell culture plate, containing 3 to 5 $\times 10^4$ 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, the cell cultures were observed every day for a cytopathogenic effect (cpe). Four controls were included in each cell culture run. Two controls, containing 10^1 and 10^3 median tissue culture infective dose (TCID₅₀) 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 EBT_r cells with 0.2 per cent guinea pig erythrocytes, and incubated at 37°C for one hour. An EBT_r cell culture inoculated with parainfluenza virus 3 was used as a 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 4 (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), 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 the 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 (Harmon and others 1990). 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.

No cpe was observed during the first and second passages in EBT_r cell cultures, 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 EBT_r cell cultures after the second passage, was negative for all these samples. The BUE cell cultures inoculated with milk samples from 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 day 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 mono-

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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	BHV-4 isolation (milk) (M/C)	BHV-4 antibody at day 0 (M/C)	Pathogenic bacteria in milk (day 0)	
				M	C
1	1	-/-	1/-	<i>Escherichia coli</i>	-
2	7	-/-	1/3	<i>E coli</i> (x2), <i>Streptococcus uberis</i> (x2)	-
3	3	-/-	3/1	-	-
4	22	-/-	2/1	CNS (x2), <i>E coli</i> (x2), <i>Staphylococcus aureus</i> (x2), <i>S uberis</i> (x3)	-
5	1	-/-	-/-	CNS	-
6	1	-/-	1/1	-	-
7	2	-/-	1/-	<i>S aureus</i> , <i>S aureus</i> + <i>Arcanobacterium pyogenes</i>	<i>S aureus</i>
8	3	1/-	1/1	<i>S uberis</i> , <i>Streptococcus dysgalactiae</i>	-
9	10	1/-	-/1	<i>E coli</i> , <i>S aureus</i> (x2), <i>E coli</i> + <i>S uberis</i> , <i>S aureus</i> (x2)	-
10	8	1/-	2/4	CNS (x2), <i>S dysgalactiae</i> , <i>S uberis</i> , <i>A pyogenes</i> , <i>Corynebacterium bovis</i> (x2), <i>S uberis</i>	<i>C bovis</i> (x3)
Total	58	3/-	12/12	31	6

M Mastitis cows, C Control cows, CNS Coagulase-negative staphylococci

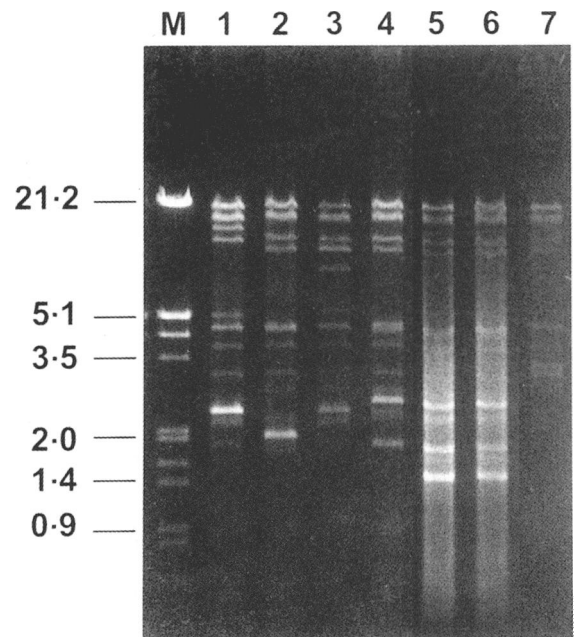
specific antisera against BHV-1 and BHV-2. In an immunoperoxidase monolayer assay, the three virus isolates and the BHV-4 reference strains DN-599 and LVR 140 reacted with monoclonal antibody 123 which is directed against glycoprotein 1 of BHV-4 (Dubuisson and others 1992), while BHV-1, BHV-2 and bovine herpesvirus 5 (BHV-5) did not react with monoclonal antibody 123. Restriction enzyme analyses of DNA from 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 ($P=0.125$).

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

Herd	Cow	Case/control	Virus isolation from milk	BHV-4 Antibody development (serum)*		Pathogenic bacteria in milk (day 0)
				Acute phase	Convalescent phase	
1	24	Case	-	-	-	<i>Escherichia coli</i> (sporadic)
		Control	-	-	800	<i>Corynebacterium bovis</i> (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	<i>Streptococcus uberis</i> (sporadic)
		Control	-	-	-	-
8	49	Case	+	100	400	<i>S uberis</i>
		Control	-	-	-	-
	52	Case	-	-	-	<i>Streptococcus dysgalactiae</i>
		Control	-	200	800	-
9	28	Case	-	-	800	<i>S uberis</i> and <i>E coli</i>
		Control	-	-	-	-
	22	Case	-	-	-	<i>Staphylococcus aureus</i>
		Control	-	-	200	<i>S aureus</i> (day 21)
	12	Case	+	-	-	<i>E coli</i>
		Control	-	-	-	-
10	275	Case	-	-	100	<i>Arcanobacterium pyogenes</i>
		Control	-	200	800	-
	400	Case	+	-	200	<i>S uberis</i>
		Control	-	-	-	-

* Antibody titres expressed as the reciprocal of the highest serum dilution giving a positive reaction

- Not detected, + Detected

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

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, 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 from 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 day 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 experi-

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mental 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 developed 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), suggests 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 leucocytes 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 may also 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 EBT 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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Preputial diverticulum stone in a boar

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AN intact male three-year-old Vietnamese potbellied pig was presented with a preputial swelling of several month's duration. The owner reported that the pig had no apparent discomfort associated with either urination or breeding. Physical examination revealed a firm, circumscribed 4 cm swelling in the subcutaneous tissues approximately 3 cm lateral to the preputial orifice in the area of the right horn of the preputial diverticulum. No other abnormalities were observed on physical examination. The owner requested that the mass be removed.

The boar was fasted overnight and then premedicated with 0.007 mg/kg medetomidine (Domitor; Pfizer) intramuscularly, 0.2 mg/kg butorphanol (Torbugesic; Fort Dodge) intramuscularly, and 0.02 mg/kg atropine (Atropine sulfate; Phoenix). Anaesthesia was induced by intravenous administration of 10 mg/kg ketamine (Ketaset; Fort Dodge) and the pig was intubated. Anaesthesia was maintained by inhalation of isoflurane, the pig was positioned in dorsal recumbency, and the ventral abdomen was prepared for the aseptic surgical removal of the mass.

Allis tissue forceps were inserted through the orifice of the preputial diverticulum and both horns of the diverticulum were explored. The left horn was partially everted, examined, and no abnormalities were observed. A firm mass was identified within the lumen of the right horn and this horn could not be everted.

A paramedian skin incision was made directly over the right horn of the preputial diverticulum, and the diverticulum was dissected free from the surrounding subcutaneous tissues. The base of the right horn was ablated using a circumferential ligature of 0 polyglactin (Vicryl; Ethicon) and the right horn was transected distal to the ligature. The removed diverticulum horn was incised and a 4 cm stone was identified (Fig 1). Subcutaneous tissues were closed with 2-0 polyglactin (Vicryl; Ethicon) in a simple continuous pattern and the skin was closed using a braided, non-absorbable suture (2-0 polyamid; Schering-Plough Animal Health) in a continuous lock stitch. Recovery from anaesthesia was uneventful. Postoperative antibiotics (22,000 iu/kg procaine penicillin G) were administered and no postoperative complications were observed. The skin sutures were removed 12 days postoperatively, and the pig was reported to be normal two months after surgery. The preputial stone, or diverticulith, was submitted for diagnostic analysis, and was found to be composed of magnesium ammonium phosphate (struvite).

The preputial diverticulum of male swine is a bilobed structure with an orifice located in the dorsal aspect of the

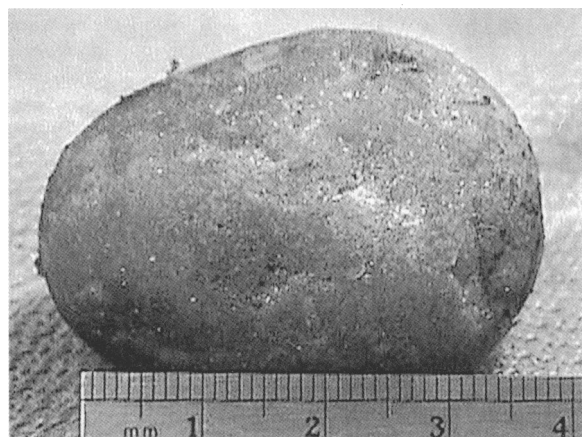


FIG 1: Stone removed from the right horn of the preputial diverticulum in a male Vietnamese potbellied pig

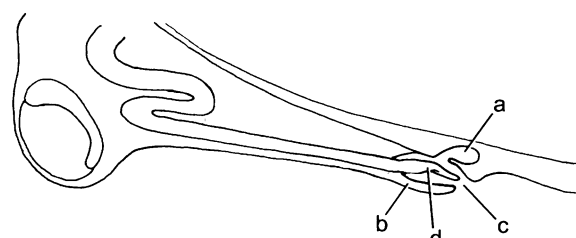


FIG 2: Diagram of the preputial diverticulum of a boar (a). The prepuce (b), preputial orifice (c) and the free portion of the penis (d) can also be seen

cranial aspect of the prepuce (Fig 2). The paired lobes of the diverticulum are located in the subcutaneous tissues cranial and lateral to the preputial orifice (Roberts 1971, Engel and St Clair 1981). Techniques for removal of the diverticulum have been described as treatments for localised infection or, alternatively, for aesthetic considerations (Aamdal and others 1958, Lawhorn and others 1994, Powe 1998). Occasionally, diverticuli are removed when boars develop the vice of repetitive diverticular masturbation, which limits a boar's breeding capacity (Cutler and others 1981).

Although the potential for concretions and preputial calculi to form in the preputial diverticulum has been alluded to by Roberts (1971), the present case is, to the authors' knowledge, the first report of a preputial diverticulith in a boar. Conditions observed in Vietnamese potbellied swine may or may not be representative of domestic swine raised for agricultural purposes.

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Bovine herpesvirus 4 in bovine clinical mastitis

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