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Prevalence of individual and bulk tank milk antibodies of bovine herpesvirus type 1 and its relation to milk quality parameters on dairy farms in Catalonia (north-east Spain)

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ABSTRACT

Bovine herpesvirus type 1 (BoHV-1) is the causative agent for infectious bovine rhinotracheitis and infectious pustular vulvovaginitis in cows or balanoposthitis in bulls. In this study, individual and bulk tank milk (BTM) samples from 5 Catalan dairy farms with different control strategies against BoHV-1 were analysed during the course of a year for milk quality parameters and glycoprotein E (gE) antibodies. Detection of gE antibodies was carried out with ELISA techniques. Prevalence of BoHV-1 varied between farms, and was stable during the study in individual and BTM samples. Comparing the antibody results of samples with milk quality parameters, positive samples with higher levels of antibodies corresponded to lower lactose and to higher percentages of fat and somatic cells.

INTRODUCTION

Bovine herpesvirus type 1 (BoHV-1) is an important pathogen of cattle, mainly in the respiratory and genital tracts, causing infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis, but also causing abortion and infertility. The virus can also cause fatal multisystemic infection and encephalomyelitis before birth or early postnatal BoHV-1 infection in neonatal calves (Muylkens and others 2007), conjunctivitis, mastitis, enteritis and dermatitis (Wyler and others 1989, Straub 2001).

BoHV-1 and other viral infections can play a direct or indirect role in the aetiology of bovine mastitis (Wellenberg and others 2002, Barkema and others 2009), thereby affecting milk quality parameters (Halasa and others 2007). It has been observed that milk parameters of quarters with subclinical mastitis compared with healthy ones had a significantly higher somatic cell count (SCC) but a lower fat content (Tomazi and others 2015).

BoHV-1 can remain latent during the lifetime of the host in the trigeminal ganglion or pharyngeal tonsils following a primary infection of the conjunctiva, oral and/or nasal cavities, or in the sacral ganglia following genitalia infection (Ackermann and Wyler 1984, Winkler and others 2000) or can be reactivated by factors that cause stress or alter the immune status of the animal such as parturition (Thiry and others 1985), transportation (Thiry and others 1987), mixing of animals, animal movements (Jones and Chowdhury 2010), inclement weather (Van DRUNNEN LITTEL-van den HURK 2006), concomitant infection, poor husbandry or diet (Turin and others 1999), overcrowding (Van DRUNNEN LITTEL-van den HURK 2006) or following treatment with corticosteroids (Winkler and others 2000).

BoHV-1 is a widely disseminated pathogen displaying significant differences in regional incidence and prevalence with regard to geographical location and breeding management in the regions under consideration (Van Schaik and others 1998, Ackermann and Engels 2006). IBR/IPV disease is listed by the World Organisation for Animal Health.

The requirement for 'IBR-free' status in EU countries restricts the importation of cattle from endemically infected regions. These rules have motivated several European countries or regions to establish control and/or eradication programmes (Raaperi and others 2014). According to EU directives, Catalonia is under an official IBR eradication programme that combines the use of a strategy for differentiating infected from vaccinated animals and culling seropositive animals. Since January 2013, the purchase of BoHV-1-positive animals has been prohibited

and vaccination of dairy cows is only allowed when a marker (glycoprotein E (gE) deleted) vaccine is used. Elimination of positive animals and the use of any vaccine will be prohibited in 2020 (gencat.cat).

The most common method for the control of IBR infection in Catalan herds has been vaccination of both seropositive and seronegative animals in order to minimise or exclude the possibility of activation of latent IBR infection in the herd by reducing the amount of virus excreted following reactivation (Bosch and others 1997, Mars and others 2001).

The main objective of this study was to examine the relationship between antibody levels in the milk of individual cows and milk quality parameters over the course of a year. At the same time, the authors wanted to determine the percentage of positive cows by measuring BoHV-1 antibodies present in the bulk tank milk (BTM) and in each lactating cow of the five farms over the four samplings. A further aim was to determine whether tank analysis was sufficient to ascertain and predict the health of a farm in terms of BoHV-1 without carrying out individual analyses using concentrated and non-concentrated BTM samples.

MATERIALS AND METHODS

Farms and animals

The study was conducted on five high production commercial closed dairy farms in Lleida, Catalonia (north-east Spain) during the course of a year from September 2013 to August 2014. Farms 1 to 5 had an average of 65, 335, 123, 205 and 441 lactating Holstein-Friesian cows, respectively. The average production of the farms was 9500–11100 kg of milk (3.6% fat and 3.3% protein) in 305 days per cow. Average number of lactation ranged between 2.4 and 2.6. Cows in lactation were housed either in cubicles or free-stall barn straw bedded and they were fed a total mixed ration consisting of corn silage, grass silage and concentrates. Cows were milked two (at 6:00 and 18:00) or three times a day (at 4:00, 12:00 and 20:00).

Calving was either individually or in small groups of dams (maximum 5). Cows were housed in free-stall straw bedded pen, and calves were separated from the mother within three hours of birth in all farms and received colostrum.

Breeding management was carried out by artificial insemination (AI) with Holstein-Friesian (IBR-free) commercial semen performed by highly trained technicians or veterinarians specialised in reproduction.

On four farms (farms 2, 3, 4 and 5), each cow was sampled and analysed for milk, fat, protein, lactose, dry matter extract, and urea concentration and SCC by the Catalan Interprofessional Dairy Association Laboratory (ALLIC, *Associació Interprofessional Lletera de Catalunya*, Cabrils, Barcelona) once a month. Farm 1 was not under individual sampling. On all five farms BTM was analysed daily for milk quality.

Farms were chosen in order to study different IBR statuses and controls, taking account of management, vaccination and biosecurity.

The use of vaccines on the farms was variable and included: double deleted (gE− and thymidine-kinase enzyme (tK)) IBR vaccine (HIPRAbovis IBR Marker live, HIPRA, Girona, Spain) and trivalent vaccine (HIPRAbovis Balance, HIPRA, Girona, Spain) for bovine viral diarrhoea virus, bovine respiratory syncytial viru and parainfluenza virus type 3. The vaccination programme varied: farm 1 never used vaccines; farms 2 and 5 stopped vaccinating animals against the above mentioned diseases in May 2011 and December 2012, respectively. Farm 2 restarted vaccination in May 2013. Farms 3 and 4 followed a standard blanket vaccination protocol of all animals over eight months of age on the holding every six months. No animals were purchased during the last 10 years in any farm except farm 5 which bought 60 IBR-free heifers in 2010.

Samples

For this study, BTM samples and individual milk samples were collected over four consecutive seasons (autumn and winter of 2013, spring and summer of 2014; named as 1, 2, 3 and 4, respectively). Individual (from four farms) and BTM samples were obtained from the routine collections of the ALLIC programme on these farms. This laboratory analysed individual milk samples and BTM samples for fat, protein, lactose, dry matter extract and urea concentration, SCC and bacteriology. Individual milk samples and BTM samples analysis for fat, protein and lactose concentrations was carried out by Fourier transform infrared spectroscopy (FTIR) on a Foss MilkoScan FT+ (Foss A/S, Hillerod, Denmark), SCC was determined using a Foss Fossomatic FC (Foss A/S, Hillerod, Denmark) and bacteriology was determined using a Foss BactoScan FC+ (Foss A/S, Hillerod, Denmark). Once samples had been routinely analysed for milk quality, they were sent in 50 ml containers and refrigerated (+4°C) to the Animal Science Department Laboratory (Universitat de Lleida) where they were processed and analysed using an ELISA technique. On farm 1, which did not follow the ALLIC individual milk sampling protocol, investigators conducted individual milk sampling over all four seasons during the study.

BTM samples contained azidiol (bacteriostatic preservative) and individual milk samples bronopol (bactericidal preservative) to preserve the milk longer. Addition of preservatives in milk and storage time does not influence the results of antibody detection (Wellenberg and others 1998).

ELISA technique

The BoHV-1 antibody levels were determined using a commercial gE-blocking ELISA test kit (CIVTEST BOVIS IBRgE; HIPRA, Girona, Spain) with a sensitivity of 89.5% (95% confidence interval (CI) 84.73% to 92.90%) and specificity of 96.84% (95% CI 95.34% to 97.87%) in sera

(Rebordosa-Trigueros and others 2012). The kit detects antibodies to a specific gE BoHV-1 and is able to distinguish vaccinated from naturally infected animals. The manufacturer's instructions were strictly followed, using the kit reagents and controls provided.

Before the development of the technique, samples were prepared and left in the refrigerator (+4°C) for 20 hours to separate the cream fraction on the top from skimmed milk below. Once the two phases were separated, the top milk cream band was discarded and the skimmed milk obtained. Skimmed milk samples were dispensed into microtubes arranged in special racks with the same layout as the ELISA plates (96 microtubes arranged in 12 columns and 8 rows) and numbered by relating them to the identification number of each cow. These samples were frozen (−20°C) until analysis.

The first BTM samples were also processed as concentrated (n=9), in order to increase sensitivity of the test without affecting specificity and thus validate the result of non-concentrated samples. The IgGs concentration procedure was carried out in strict accordance with the manufacturer's instructions, using the kit reagents and controls provided. (HIPRA-CIVTEST BOVIS IBRgE MILK [Ab] leaflet, 709031–00 version)

Before analysis, samples were thawed at room temperature. Samples were placed in the ELISA microplate wells according to the protocol. All tests were performed according to the manufacturer's instructions. The absorbance was measured as optical density (OD) values at 450 nm using a microplate reader (Multiskan FC Thermo Fisher Scientific). The results were expressed as inhibition percentages (%IN) calculated as follows

$$\%IN = \frac{\text{Average OD450 Negative Control} - \text{OD450 Sample}}{\textit{Average OD450 Negative Control}} x100$$

According to manufacturer's instructions (Table 1) samples of non-concentrated BTM and individual milk were considered negative if the %IN value was up to 25 and positive if the value was more than 25 (in BTM samples this cut-off value corresponds to prevalence ≥25%). Samples of concentrated BTM were considered negative if the %IN value was less than 5 and positive if the value was at least 5 (which corresponds to prevalence ≥4%). Combination of %IN values in BTM samples considered positive when concentrated (%IN≥5) and negative when non-concentrated (%IN≤25) was useful to determine some estimated 'low-positive' prevalence (4%–25%).

Data analysis

Prevalence of animals positive to BoHV-1 was calculated every time that individual milk sample collection was performed. This analysis was conducted using the frequency (FREQ) procedure of SAS V.9.2 (Statistical Analysis Software, Cary, North Carolina, USA). Lower and higher confidence limits for the proportion of positive animals was calculated at 95%. For individual milk samples, a repeated measurements analysis of variance (PROC MIXED of SAS) was performed to determine whether the quality parameters of milk, such as protein, lactose, fat, dry matter extract, urea and somatic cells varied between positive and negative animals. The statistical model included cow as a random effect (to account for repeated data) and the fixed effects of farm, date of collection and status of BoHV-1 positivity. Data imbalance between farms was taken into account, as some contributed with many positive animals and others with few positives, and least square means and standard errors were calculated.

TABLE 1: Relation between inhibition percentage (IN%) values of the ELISA, prevalence value of the bulk tank milk (BTM) sample and positivity to bovine herpesvirus type 1 (BoHV-1) of individual samples. Source: HIPRA-Civtest bovis IBRgE MILK [Ab] leaflet, 709031–00 version

IN% value	BTM sample	Individual sample
Non-concentrated milk samples:		
Higher than 25.0	Prevalence ≥25%	Positive sample
Lower or equal to 25.0	Prevalence <25%	Negative sample
Concentrated milk samples		
Higher than or equal to 5.0	Prevalence ≥4%	Positive sample
Lower than 5.0	Prevalence <4%	Negative sample

RESULTS

During the study, individual milk (n=4250), concentrated BTM (n=9) and non-concentrated BTM (n=20) samples were analysed. The total positive individual samples against BoHV-1 on all farms during the study period were 1403 (33%) and 2847 (67%) samples were negative. As regards concentrated BTM samples, one sample was negative (prevalence <4%) and eight samples were positive (prevalence ≥4%). When BTM samples were not concentrated, 5 were positive (prevalence ≥25%) and 15 negative (<25%). Six samples were negative when non-concentrated and became positive when concentrated, where interpretation is: prevalence 4%–25% (Table 2).

Relation between individual milk and BTM samples

Farm 1 was always BoHV-1-negative in all individual or bulk tank analyses. The four remaining farms (2–5) always had BoHV-1-positive cows: farm 2 always showed a very high positivity for both individual (>92% of positive animals) and BTM, whether concentrated or not (%IN >81%). Farms 3, 4 and 5 maintained a proportion of positive animals below 10.0%, except for sampling 1 on farm 5, with a prevalence of 10.1% although this decreased in subsequent samplings (Table 2).

In the present study, BTM samples were positive when individual prevalence was very high (>92%) as observed on farm 2. Non-concentrated BTM samples were negative (cut-off ≤25) when individual

TABLE 2: Proportion of individual positive animals and milk tank (concentrated and not concentrated) results to antibodies to BoHV-1

		Individual milk samples	Non-concentrated BTM samples	Concentrated BTM samples	Status
Farm	Sampling*	% positive animals† (LCL–HCL 95%)	%IN‡ BTM (cut-off≤25)	%IN BTM concentrated (cut-off≤5)	Calculated prevalence
1	1	0.0	0.0 (neg)	0.0 (neg)	<4%
	2	0.0	0.0	–	–
	3	0.0	0.0	–	–
	4	0.0	0.0	–	–
2	1	97.6 (96.1–99.2)	92.5 (pos)	96.0 (pos)	≥25%
	2	93.9 (91.3–96.4)	89.7 (pos)	93.5 (pos)	≥25%
	3	98.2 (96,7–99.6)	81.9	–	–
	4	92.12 (89.2–95.0)	89.3	–	–
3	1	4.5 (0.6–8.4)	6.4 (neg)	35.5 (pos)	4%–25%
	2	1.7 (0.00–4.0)	10.6 (neg)	12.1 (pos)	4%–25%
	3	1.5 (0.0–3.5)	0.00	–	–
	4	0.8 (0.0–2.4)	0.9	–	–
4	1	5.0 (1.8–8.2)	11.2 (neg)	40.5 (pos)	4%–25%
	2	4.00 (1.1–6.9)	18.1 (neg)	30.3 (pos)	4%–25%
	3	2.2 (0.1–4.3)	0.00	–	–
	4	2.8 (0,4–5.3)	19.5	–	–
5	1	10.1 (6.9–13.2)	20.8 (neg)	48.6 (pos)	4%–25%
	2	6.5 (4.0–9.0)	19.9 (neg)	37.6 (pos)	4%–25%
	3	5.3 (3.1–7.5)	0.00	–	–
	4	6.3 (3.8–8.8)	19.5	–	–

*Number of sampling. 1=autumn, 2= winter, 3= spring and 4= summer.
†% Positive animals referred for individual analysis.
‡%IN: inhibition percentages.
Estimated prevalence according to the results of the BTM, whether concentrated or not.
BoHV-1, bovine herpesvirus type 1; BTM, bulk tank milk; HCL, higher concentration limit; LCL, lower concentration limit.

prevalence was below 11.00%. Concentration of BTM samples was useful to determine some estimated 'low-positive' prevalence (4%–25%) that resulted as negative in non-concentrated BTM samples. Farms 3, 4 and 5 always showed low individual prevalence data (<10.11%) and a good correlation between individual and bulk tank results, whether concentrated or not.

Trends in individual results over the course of a year

As cows were analysed several times, depending on the lactation period of each one, in order to validate consistency of the results of the different sampling periods, the cows were classified as follows: positive (all samples were positive) (n=468), negative (all samples were negative) (n=1021), converted to positive (negative samples followed always by positive samples) (n=23) and inconsistencies (positive animals becoming negative, or after a first positive assessment alternating between negative and positive results) (n=46).

Comparison with milk quality parameters

A total of 4150 individual milk samples from farms 2, 3, 4 and 5 were analysed for milk quality parameters. Only consistent cows, always positive or always negative, were used to find the relationship between positivity to BoHV-1 and a change in any of the milk quality parameters (Table 3).

Fat, logarithm of somatic cells and lactose differed significantly (P=0.045, P<0.0001 and P<0.0001, respectively) between BoHV-1-positive and BoHV-1-negative samples. Milk from animals positive for BoHV-1 had a higher percentage of fat (3.90%), a higher SCC (2.26×1000/ml) and lower lactose (4.68%) compared with milk from animals negative for BoHV-1 (3.71%, 1.99×1000/ml and 4.82%). Linear regression detected no linear relationship between the level of BoHV-1 antibodies and any of the milk quality variables. The statistically significant higher levels of SCC and fat, and lower levels of lactose, for positive cows in the overall data set was consistent within farms.

TABLE 3: Regression results (GLM) between the milk quality parameters of 3874 samples compared with BoHV-1-positive and BoHV-1-negative antibodies status. Values are means±se (n=1331 samples from 462 cows always positive and n=2819 samples from 916 cows always negative)

Milk quality parameters	Positive samples	Negative samples	P value
Fat *	3.90±0.073	3.71±0.030	0.045
Protein*	3.39±0.025	3.32±0.014	0.2
Lactose*	4.68±0.025	4.82±0.010	<0.0001
ESM*	8.81±0.045	8.87±0.019	0.3
Log(som cells)†	2.26±0.059	1.95±0.025	<0.0001
Urea‡	98.18±3.86	90.37±1.58	0.1

*%.
†Somatic cell count (x1000/ml).
‡ mg/L.
ESM, dry non-fat-milk-material content; GLM, generalized linear model.

DISCUSSION

Relationship between individual and bulk tank results

Using milk tank samples is of great interest due to the ease of processing and low cost (Nylin and others 1999). Nevertheless, the results of these samples should be interpreted only as indicative due to the low sensitivity that blocking IBR gE-ELISAs have on tank samples (Kramps and others 2004). BTM samples should be accompanied regularly by an individual test and concentrated tank samples in order to increase the sensitivity of the ELISA technique without affecting specificity (Schroeder and others 2012, Rebordosa-Trigueros and others 2012). Unfortunately, concentration of milk samples is not automated yet and requires specialised personnel and time. For this reason, the study only used the technique of concentration for the first two sampling seasons to compare with the non-concentrated BTM technique which is less expensive at the laboratory level. Low sensitivity of the IBR gE-ELISA in non-concentrated BTM samples can be a major problem in IBR control or eradication programmes, above all on farms with low individual prevalence (Raaperi and others 2010, Rola and others 2015).

In this study, a prevalence below 11% proved negative in non-concentrated BTM testing and positive in concentrated BTM testing. An individual prevalence higher than 80% proved positive (estimated prevalence ≥25%) in BTM testing, whether concentrated or not. Similar results have also been observed in other studies (Wellenberg and others 2002) where in individual testing (milk or serum) less than 10% was considered 'BTM negative' and when individual prevalence was more than 50%, this was considered 'BTM positive' (Hartman and others 1997, Raaperi and others 2010, Porquet garanto 2012). Unfortunately, the farms under study had both a very high or very low individual prevalence, and the relationship between individual performance and milk tank farms with an average prevalence of disease could not be studied.

When BTM sampling, the recommendation for repeated testing in order to increase sensitivity is justified (Eliot and Franken (1997)). Importantly, a total correlation between individual and bulk tank results in the case of a totally 'IBR-free' farm (farm 1) was maintained.

Individual results

The total number of individual milk samples varied during the study because lactating cows were not always the same with regard to the dynamics of the dairy production system (dry-off period, first parturition and culled cows). In order to avoid false-negative animals, repeated testing was carried out wherever possible on all farms included in the study (Raaperi and others 2014).

Conversion of animals to positive was low on all the farms studied (average 1.6%; from 0.0% to 2.7%), this could be due to the use of a 'double deleted' vaccine on all the farms (except in farm number 1), reducing the risk of spread and vaccine virus recombination (Raaperi and others 2014). Inconsistent results were also low (average 3.4%; from 0.0% to 5.3%), these cases can be attributed to false-positives, especially on farms with low prevalence (Geraghty and others 2012).

Comparison with milk quality parameters

Although many infectious and non-infectious factors can affect individual SCC and milk quality of cows, this study suggests that BoHV-1 infection may influence these parameters. The role that BoHV can play in the immunosuppression status of animals could explain these results. Individual fat and SCCs were significantly higher in BoHV-1-positive samples (3.90% and 2.19×1000/ml) compared with BoHV-1-negative samples (3.69% and 1.99×1000 ml). On the other hand, individual lactose was significantly lower in BoHV-1-positive samples (4.68%) compared with negative samples (4.82%). A Polish study found similar results evaluating BoHV-1 status and milk quality parameters in BTM during the course of a whole year, observing that farms with a higher BoHV-1 infection status had significantly higher BTM SCCs and fat content, but differences in protein and total bacteria were not observed (Rola and others 2015). Milk quality parameters and milk production were studied during a subclinical infection of BoHV-1 on a Dutch dairy farm, where no influence of the infection on SCC and fat were observed, although a significant drop in milk production in initially seronegative cows was reported (Hage and others 1998). Another study found a statistical difference in the mean SCC between cows positive for BoHV but without concurrent bacterial infection and cows without BoHV and bacterial intramammary infection (IMI). In this study, the authors concluded that the major influence on milk SCC was bacterial IMI rather than viral influence (Herlekar and others 2013). To the authors' knowledge, no studies have reported differences in lactose percentage in individual milk samples considering positivity to BoHV-1 infection.

The higher SCC and fat values of BoHV-1-positive samples detected within all the farms supports the results obtained overall, although unfortunately, the reduction in sample size when comparing within farms fails to confirm statistically the effect on all the farms. More studies should be conducted in the future in order to increase the sample size within the farm and the number of farms to compare and to confirm whether BoHV-1 affects the milk quality parameters in closed and quite stable farms, under control and vaccination programs for BoHV-1 and without any recent or ongoing outbreaks caused by BoHV-1.

CONCLUSIONS

The results of the present study showed that repeated BTM ELISA tests for BoHV-1 appear to be a very useful tool to evaluate the development of seropositivity for BoHV-1 within a herd. BoHV-1 infection can affect milk quality parameters, increasing SCC and fat in individuals.

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