Veterinary Microbiology 239 (2019) 108495

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Monitoring of the shedding and serological dynamics of Bovine gammaherpesvirus type 4 in a dairy cattle herd

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ARTICLE INFO

Keywords:
BoHV-4
PCR
Serology
Cattle
Heifers

ABSTRACT

Bovine gammaherpesvirus type 4 (BoHV-4) is increasingly related with reproductive disease in cattle, but its epidemiology is not fully understood. We monitored the serological response and shedding of BoHV-4 in a positive dairy cattle farm with metritis. First, we performed an ELISA to detect BoHV-4 antibodies in all the animals (n = 104). Afterwards, ten seronegative heifers introduced in the production lot and sera samples were monthly taken for four months and then 6–10 months after introduction to detect BoHV-4 antibodies by ELISA. Moreover, a vaginal swab was taken after calving to detect BoHV-4 by PCR. Concurrently, a weekly collection of vaginal and nasal swabs and milk was performed during the first month post-partum in multiparous cows with metritis (n = 14), heifers with metritis (n = 4), heifers without metritis but positive to BoHV-4 (ELISA or PCR) (n = 2) and multiparous cows without metritis (n = 3).

Seropositivity was higher in older animals and in the production lot. Three heifers which shed BoHV-4 after parturition resulted seronegative at first but eventually seroconverted. In the same vein, most heifers seroconverted after 6–10 months in the production lot (8/10). Multiparous cows shed virus by various routes: 13/14 (93 %) in vaginal secretions, 7/14 (50 %) in nasal exudates and 7/14 (50 %) in milk. However, in the other groups, shedding was only detected in vaginal swabs from the first week post-partum. Our study describes BoHV-4 shedding in field conditions. Seronegative animals may become horizontally infected when moved to a contaminated environment.

1. Introduction

Bovine gammaherpesvirus type 4 (BoHV-4) has been increasingly related to reproductive problems in cattle in the last few years, such as metritis, endometritis, abortion or infertility (Chastant-Maillard, 2015). The role of BoHV-4 in cattle disease is unclear since experimental infections have not always led to the development of clinical signs and the virus is also present in healthy animals (Frazier et al., 2002; Monge et al., 2006; Chastant-Maillard, 2015). The participation of BoHV-4 in metritis is supported by some experimental findings after the inoculation of BoHV-4 in pregnant animals (Wellemans et al., 1986) and by epidemiological observations which reported a significant association of BoHV-4 with clinical metritis (Areda et al., 2018). Uterine disease is currently considered a very likely feature of BoHV-4 infection, with the virus acting at least as a co-factor for the establishment of the disease (Donofrio et al., 2008; Sheldon et al., 2009; Chanrot et al., 2017). Moreover, BoHV-4 has also been isolated in severe cases of metritis which may result in death (Monge et al., 2006; Nikolin et al., 2007). However, the course of BoHV-4 infection is not fully understood and it implies a complicated process in which the virus is thought to be able to cause latent infections (Dubuisson et al., 1989) with macrophages and lymphocytes as the main host cells (Osorio and Reed, 1983; Egyed et al., 1996), among others (Egyed and Bartha, 1998; Campos et al., 2014).

This situation evidences that questions still remain open about why some animals develop the disease and others do not. In this way, several interesting studies have been carried out to unravel the underlying molecular mechanisms involved (Donofrio et al., 2008, 2010; Jacca

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https://doi.org/10.1016/j.vetmic.2019.108495
Received 16 August 2019; Received in revised form 29 October 2019; Accepted 29 October 2019

et al., 2013, 2014). However, it would also be helpful to understand how animals acquire the infection in field conditions as well as the within-herd course of infection. In this regard, sources of infection have not been extensively studied yet. Some authors have reported the existence of vertical transmission (Egyed et al., 2011) and venereal transmission has been suggested as well (Chanrot et al., 2017), but the virus has also been detected in different secretions: vaginal exudates (Monge et al., 2006; Verna et al., 2012), nasal exudates (Castrucci et al., 1987) or milk (Donofrio et al., 2000; Wellenberg et al., 2001). Therefore, it is reasonable that carrier adults may constitute a source of infection for naïve animals through these secretions.

In order to gain insight about the transmission of BoHV-4, we analyzed animals from a farm with a severe problem of post-partum metritis in which BoHV-4 had been detected. For that, we monitored the viral elimination and serological status of seronegative heifers around the time of their first calving and analyzed viral excretion in animals with post-partum metritis considering three potential routes of horizontal transmission: vaginal shedding, nasal shedding and milk. Our aims were to determine potential sources of infection with BoHV-4 and to offer information about possible horizontal transmission routes of this virus in a farm.

2. Material and methods

2.1. Characteristics of the farm under study

The study was carried out between January 2015 and January 2016 in a dairy cattle farm in Galicia (NW Spain). The animals in this farm are divided in three production lots housed in separate barns according to age and production status. The youngest animals are housed in lot 1, and subsequently transferred to lot 2, where they are inseminated and kept for about 10 months, until 2–3 months prior to parturition, when they are about 23 months of age. Then, they are moved to lot 3 (production lot), which is consequently composed of multiparous animals, either in milking or in the dry period, and heifers with an upcoming parturition.

In the end of 2014, this farm presented an outbreak of severe metritis that did not respond to antimicrobial treatment (tetracycline and ceftiofur). In the last 21 parturitions, six metritides appeared (28 %) and three of those animals presented a very severe course that eventually resulted in death. In general, affected animals showed a watery red-brown vaginal exudate which started 3–4 days after parturition, as well as systemic signs such as fever ($> 40\,^{\circ}C$), yield reduction, lack of appetite and apathy. The last two animals which presented clinical signs were analyzed and their vaginal exudates tested positive for BoHV-4 by real-time PCR and antibodies against this virus were detected by ELISA. *Escherichia coli* was isolated in the cultures of vaginal exudates by detecting an extended-spectrum β-lactamase mechanism of resistance.

This farm had been performing BVD, IBR and *N. caninum* control programs having detected less than four antibody-positive animals for each pathogen in the previous four years. No vaccination programs had been performed and no other pathologies had been reported, however; the practitioner reported problems in calcium and phosphorus absorption.

2.2. Design of the study

In the beginning of the study, (January 2015) sera samples were taken from all the animals older than six months in the farm (n = 104) to detect the presence of Ig-G antibodies against BoHV-4 by ELISA. Then, from March to October 2015, this study was divided into two parts: monitoring of shedding in the production lot and monitoring of the heifers introduced in the production lot.

Metritis was diagnosed when animals presented clinical signs consisting of pyrexia ($\geq 39.5\,^{\circ}C$) up to 10 days postpartum with a fetid purulent vulvar discharge (Sheldon and Dobson, 2004)

2.2.1. Monitoring of heifers introduced in the production lot

The typical management of the herd was not altered by the performance of this study. Accordingly, heifers were introduced in the production lot three months before their expected due date (n = 10). Prior to their introduction, a serum sample was taken to detect antibodies against BoHV-4. Then, over those three months, a monthly serum sample was also taken until parturition. Additionally, within the first week after parturition, a vaginal swab to detect the virus by PCR was taken and, one month after calving, a serum sample to detect antibodies was also taken. Moreover, the presence of post-partum metritis in these animals was registered. Finally, serum samples of all the monitored animals were taken one year after the beginning of the study (January-2016) to detect the presence of antibodies.

2.2.2. Monitoring of the shedding in the production lot

In this part of the study, animals in the production lot were divided in 4 groups. Group I was composed of all the multiparous animals which presented post-partum metritis in the period of study (n = 14), Group II comprised the introduced heifers that presented post-partum metritis (n = 4), Group III consisted of introduced heifers without metritis which had at least one positive sample (serum or vaginal swab) to BoHV-4 during the monitoring that was carried out in heifers after their introduction in the production lot, (n = 2) and Group IV included multiparous animals with no metritis after parturition in the period of monitoring (n = 3).

For each group, the sample collection was carried out in the same manner. After calving, a serum sample was taken and vaginal swabs, nasal swabs and milk samples were taken weekly during the first month post-parturition for a total of 4 samples per animal and per type of sample.

2.3. Laboratory analysis

Serum samples were analyzed to detect the presence of antibodies against the virus using a commercial ELISA kit: ELISA: BOHV4 ELISA Kit (Bio-X Diagnostics, Jemelle, Belgium). Seroconversion was considered when an increase in the manufacturer's classification derived from the ratio S/P was observed in consecutive samples (with a separation of 30 days).

Vaginal and nasal swabs were eluted in 1 ml of PBS. 200 µl of vaginal and nasal elutions and 200 µl of milk were taken to perform the DNA extraction using the commercial kit NucleoSpin® Tissue (Macherey-Nagel, Düren, Germany). PCR analyses were performed with the commercial kit TaqVet™ Bovine Herpesvirus Type 4 (LSI, Lisseu, France) which targets the glycoprotein B (gB). These procedures were carried out following the manufacturers' recommendations.

2.4. Statistical analysis

Differences in seroprevalence regarding lot (1: calf lot; 2: heifer lot; 3: production lot) and group of age (< 23 months, 23–36 months, 36–48 months and > 48 months) were analyzed by univariate logistic regression. Each category was contrasted with the previous category. The cut-off of the first category in the age variable (23 months) was the average age of entrance in the production lot in this farm.

General estimation equations were used to analyze the presence of differences in shedding according to the type of route (vaginal, nasal or milk) and the week of sampling in multiparous animals with metritis (Group I), adding "week of sampling" as a within-subjects factor. The rest of the groups were not statistically analyzed due to their low number of animals. The statistical analyses were performed using the software SPSS v.20 (IBM, NY, USA).

Table 1
Seropositivity of BoHV-4 by lot and age group.

Seropositivity by lot				Seropositivity by age			
Group	Positives/total (%)	p	OR (CI95%)	Group	Positives/total (%)	p	OR (CI95%)
1: calves	10/28 (35.7)			1: < 23 months	9/32 (28.1)		
2: heifers	3/9 (33.0)	0.896	0.9 (0.2-4.4)	2: 23-36 months	11/19 (58.0)	0.039	3.5 (1.2-6.1)
3: production	50/67 (74.6)	0.002	4.6 (1.8-11.8)	3: 36-48 months	14/17 (82.3)	0.121	3.4 (0.7-16)
				4: > 48 months	30/35 (85.7)	0.929	1.1 (0.2-4.9)

Each category is contrasted with the previous category.

3. Results

Sixty-three out of 104 animals tested seropositive to BoHV-4 (61 %) at the beginning of the study. Seroprevalences were significantly higher in lot 3 (75 %; 50/67) (p = 0.002; OR: 4.6; CI95 %: 4.8–11.8) and lower in lot 2 (3/9; 33 %) and in lot 1 (10/28; 36 %) with no differences among these two latter lots (p = 0.896; OR: 0.9; 0.2–4.4) (Table 1). An increase in seropositivity with age was also found, detecting a significant rise in the percentage of seropositive animals when comparing < 23 month-old animals and 23–36 month-old ones (p = 0.039; OR: 3.5; CI95 %: 1.2–6.1) (Table 1).

3.1. Monitoring of heifers introduced in the production lot

The ten animals introduced in the production lot during the period of study resulted seronegative prior to the entry and none of them seroconverted one month after calving. However, 2/10 animals seroconverted after the three months of follow up and 8/10 animals tested seropositive in their final sample (Fig. 1) (i.e. 6–10 months after their introduction in the production lot).

Table 2 compares PCR and serological results and post-partum metritis detection. Shedding of BoHV-4 was detected in 3/10 animals and they tested seronegative during the three months of follow-up after calving, but all the shedders eventually tested seropositive at the end of the study. Post-partum metritis was detected in 2/3 PCR-positive animals. Among the 7/10 PCR-negative animals, two seroconverted during the follow-up without signs of metritis and five resulted seropositive at the end of the study. Post-partum metritis was detected in 2/7 PCR-negative animals.

3.2. Monitoring of the shedding in the production lot

All multiparous animals with metritis (Group I) were seropositive after calving. BoHV-4 DNA was detected in 13/14 (93 %) animals: 12/14 (86 %) animals presented vaginal shedding, 7/14 (50 %) presented nasal shedding and 9/14 (64 %) presented milk shedding. 7/14 animals presented simultaneous viral shedding by various routes (50 %) at some point of the four month follow-up. The fourth sample could not be taken in 4/14 animals since they died during the study.

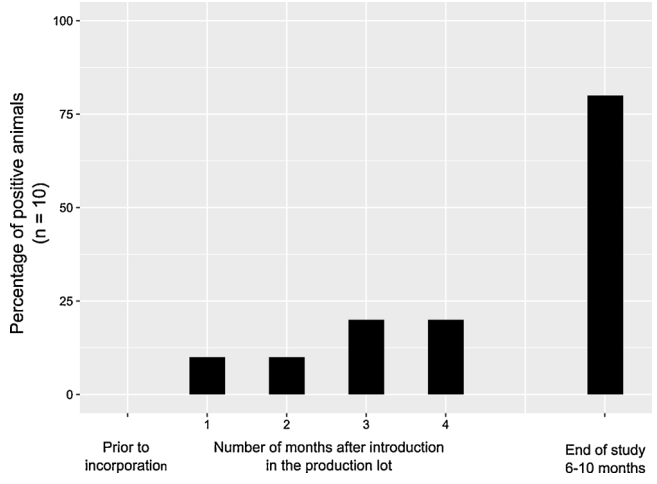

Fig. 1. Serological results to BoHV-4 in heifers (n = 10) prior to the follow up, during the 4-monthly follow-up and at the end of the study.

The pattern of shedding was rather variable between animals and groups (Fig. 2). In Group I, a positive result was found 34 times (65 %) out of the 52 times in which the set of different samples (vaginal, nasal and milk) was taken. Twenty-five out of 52 (48 %) vaginal swabs and 11 nasal swabs and milk samples (21 %) tested positive. BoHV-4 was simultaneously detected in the three types of samples in 10/52 times (19 %). The percentage of positive samples in Group I was significantly higher in vaginal swabs (40/52; 77 %), than in nasal swabs and milk (11/52; 21 %, in both cases) (p = 0.049 and 0.025, respectively; OR = 3.6; CI95 %: 1.1–10, in both cases). In vaginal samples, the percentage of positive samples was significantly higher in the first week (p = 0.043; OR: 5.8; CI95 %: 1.1–9.5).

In the rest of the groups (Group II, Group III and Group IV), positive samples to PCR were only detected in vaginal samples and in the first week of sampling (Fig. 2). In group II, BoHV-4 was detected in 2/4 animals and all animals were seronegative. In group III, 1/2 animal was seropositive without shedding and the other animal was positive to PCR but seronegative. In group IV, all animals were seropositive and BoHV-4 was detected in 1/3 animals.

4. Discussion

BoHV-4 has been related with uterine disease but the ways in which animals naturally acquire the infection and develop disease are still to be determined. This study offers information about the possible serological dynamic of BoHV-4 in cattle herds and potential sources of infection. The significant increase in seropositivity between animals < 23 months and those between 23–36 months suggests that the production lot is the main source of exposure to BoHV-4, since it coincides with the moment when heifers are moved to this lot. In addition, the higher percentages of seropositive animals in the production lot and in older animals (which have lived longer in the production lot) are also consistent with this scenario. Our results are consistent with Wellenberg et al. (1999) who also suggested a progressive acquisition of the infection as animals are introduced in production lots.

Most of the animals did not seroconvert in the first four months after introduction. However, they eventually did after they had been housed for some months in the production lot, which is consistent with the age pattern detected in the herd. Nevertheless, the detection of shedders that tested seronegative to the IgG-based ELISA reveals that serology is not always reliable to determine BoHV-4 exposure, at least when this immunoglobulin is used as a target, and prevents from affirming that seronegative animals are not really infected. In this way, an early viral neutralization by IgM has been suggested (Dubuisson et al., 1989) and a better performance of an immunoperoxidase monolayer assay able to detect IgG and IgM compared to an IgG-based ELISA has been reported (Wellenberg et al., 1999). Therefore, an early control of the infection before the expression of IgG might be an explanation to this result. The

Table 2
Comparison of the results obtained by serology and PCR of BoHV-4 in heifers (n = 10). The presence/absence of post-partum metritis is indicated.

Serology results	PCR-Positive animals (after calving)		PCR-Negative animals (after calving)	
	Nº of positive animals (n = 3)	Nº of animals with post-partum metritis (n = 2)	Nº of negative animals (n = 7)	Nº of animals with post-partum metritis (n = 2)
After 3 months in production lot				
Positive	0	0	2	0
Negative	3	2	5	2
After calving (4 months)				
Positive	0	0	2	0
Negative	3	2	5	2
End of study (6-10 months)				
Positive	3	2	5	1
Negative	0	0	2	1

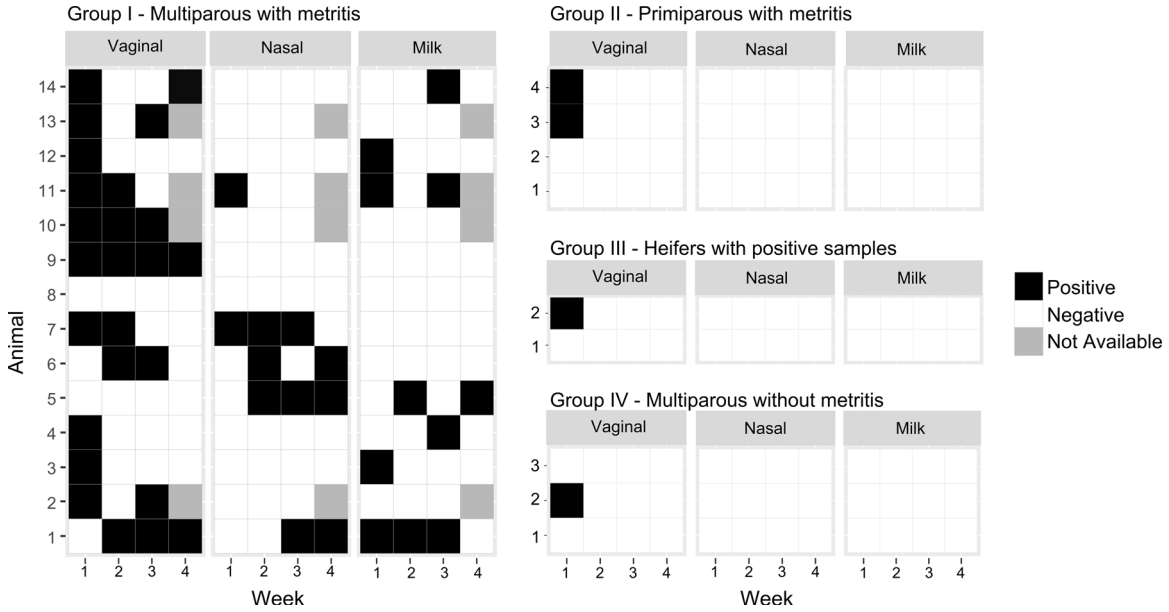

Fig. 2. PCR results of BoHV-4 shedding in vaginal exudates, nasal exudates and milk in cows by week after parturition.

members of gammaherpesvirus have a slow replication cycle and the predominant pathway after infection leads to the establishment of a latent phase instead of a lytic phase (Ackermann, 2006). However, it is questioned whether gammaherpesviruses go deep into their latency and if constant virus excretion at a very low level is possible (Ackermann, 2006). It is uncertain if seronegative shedders reflect a situation like this, but it would be a hypothesis to consider in further studies.

The eventual seropositivity of seronegative shedders at the end of the study may indicate that sometimes a long time is needed before seroconversion takes place. Parturition has been proposed as a factor related to seroconversion (Graham et al., 2005). Nevertheless, we did not find such trend (Fig. 1). False seronegative animals may have an important consequence for serological analysis. Since BoHV-4 might be related to severe metritis, accurate diagnostic techniques would be valuable to determine animals at risk, even when the virus is only a co-factor for disease. Thus, a serological diagnosis in pregnant animals may be used for an early diagnosis in order to implement preventive measures prior to calving. However, if the IgG-based serological techniques fail to detect infection in the first stages of it, they would not be useful or would have limited efficacy for this purpose.

We have detected BoHV-4 shedding in all the studied secretions: vaginal and nasal excretions and milk, so they may constitute a source of infection. PCR detection does not imply viral viability, but BoHV-4 has been cultured from these secretions in literature (Castrucci et al., 1987; van Opdenbosch et al., 1988; Donofrio et al., 2000) so viral particles that are shed in these secretions may be infective as it occurs in other bovine herpesviruses (Muylkens et al., 2007). The predominance of vaginal shedding is not unexpected since BoHV-4 has shown endometrial cell tropism (Donofrio et al., 2007; Yang et al., 2017) where the virus replicates actively (Jacca et al., 2014). In experimental infections, the virus could be detected in vaginal secretions up to 21 days after calving (Wellemans et al., 1986). These results are not completely consistent with ours, because, although in some animals in group I the virus was detected for a comparable period, in others, shedding was intermittent. A simultaneous excretion by various routes was also found and vaginal excretion was not detected in all the cases in which viral shedding by other routes occurred. The presence of BoHV-4 in several tissues during persistent/latent infection has been previously demonstrated (Egyed and Bartha, 1998; Boerner et al., 1999). The mobilization of macrophages or other leukocytes would also explain why different secretions from infected animals may be positive (Egyed et al., 1996; Egyed and Bartha, 1998; Donofrio and van Santen, 2001).

The clinical implications of BoHV-4 detection cannot be elucidated from our study, but we have also found healthy animals with viral shedding. BoHV-4 could replicate in tissues with no apparent clinical consequences, but it could also be carried to damaged tissues where its replication may enhance or favor a clinical condition (Chastant-Maillard, 2015). However, viral presence could just be the consequence of macrophage recruitment. Independently from the implications that BoHV-4 may have, shedding may constitute a route of infection.

An apparent difference was observed between multiparous animals with metritis and the rest of the groups of cows, because animals of the

latter groups only showed shedding in vaginal secretions during the first week. The nature of this difference is hard to unravel. Given the age pattern of the farm most of the animals in the multiparous group were unlikely to be in a recent state of infection, so these animals might have presented latently infected macrophages which may have favored viral diffusion after a clinical event, but that cannot be verified.

Overall, our results seem to point out that horizontal transmission may play a determinant role in the transmission of BoHV-4 and that the contamination inside the lots is enough to produce a high population of seropositive animals. Egyed et al. (2011) reported an intrauterine transmission of this virus and this route could hugely contribute to the maintenance of the virus in a farm. Nevertheless, our results do not seem to adjust to a major predominance of vertical transmission. However, since seronegative infected animals have been detected in this study, it is not strictly possible to determine how many seronegative animals are actually infected. Furthermore, some seropositive animals were detected in lots other than the production one. Therefore, the vertical route of transmission might have some implication. In addition, husbandry practices may play some role in the propagation of the infection between lots, for example, we have previously described how personnel clothes can become contaminated with different viruses (Díaz Cao et al., 2018) illustrating how inadequate biosecurity measures may easily favor the diffusion of diseases within a herd. Finally, it must also be considered that our results reflect the characteristics of a single farm, so global trends cannot be extrapolated from it. However, in our research group we have found other farms with high within-herd seroprevalences and similar age patterns in the same region. On the basis of our results, it would be valuable to evaluate the relationship between the appearance of clinical outcomes and the amount of time that the animals are exposed to a contaminated environment prior parturition

In conclusion, our study provides information about possible dynamics of infection with BoHV-4 in field conditions. A presumptive predominance of horizontal transmission can be suspected and heifers introduced in production lots with BoHV-4 shedding are expected to show IgG seroconversion after a few months. Further studies are mandatory to better characterize BoHV-4 epidemiology, but our findings may be helpful and serve as a starting point for such work.

Funding statement

This work was supported by the Program for consolidating and structuring competitive research groups (GRC2015/003, Xunta de Galicia, Spain), by the Research Project 'RUMIGAL: Rede de estudo multidisciplinar dos ruminantes en Galicia' (R2014/005, Xunta de Galicia, Spain) and by a predoctoral grant to J.M. Díaz (Plan I2C, Xunta de Galicia, Spain).

Declaration of Competing Interest

The authors do not have any competing interest to declare

Acknowledgements

The authors want to acknowledge Xunta de Galicia for supporting this study and practitioners that collaborated in the sample taking.

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