

# Assessment of *Porphyromonas levii* Antibody Status of Cattle Pre-and Post Partum and the Relationship of Antibody Levels to the Development of Bovine Necrotic Vulvovaginitis

Blum, S.,<sup>1</sup> Zamir, L.,<sup>2</sup> Krifucs, O.,<sup>1</sup> Goshen, T.,<sup>3</sup> Brenner, J.,<sup>1</sup> Leitner, G.<sup>1</sup> and Elad, D.<sup>1\*</sup>

<sup>1</sup>Department of Clinical Bacteriology and Mycology, The Kimron Veterinary Institute, Bet Dagan, Israel,

<sup>2</sup>The Koret School of Veterinary Medicine, Rehovot, Israel,

<sup>3</sup>Hachaklait, Caesarea, Israel

\* **Corresponding author:** Daniel Elad, DVM, PhD, Dept. of Clinical Bacteriology and Mycology, The Kimron Veterinary Institute, P.O.Box 39, Bet Dagan, 50250 Israel. Phone: +972-3-9681688; Fax: +972-3-9688965; Email: danielad@moag.gov.il

### ABSTRACT

The dynamics of anti-*Porphyromonas levii* antibody production was assessed by ELISA on a farm endemic for Bovine Necrotic Vulvovaginitis (BNNV) (farm A), one that underwent an outbreak a year previously (farm B) and one in which sporadic cases were observed in the past (farm C). Blood samples were taken 7, 4 and 1 week prior to the expected date of calving and on the first and fourth week after calving. Antibodies were present, pre-partum, in 44.4% and 34.8% of the animals on farm A and B, respectively. The antibody titre increased after calving in animals that developed BNVV by a factor of 3.3 and 2.8, on farms A and B respectively, but remained largely unchanged in animals that did not develop the disease. The frequency of development of BNVV in pre-partum seronegative and seropositive animals was similar: 42.5% and 38.3%, respectively. Antibody titres were significantly higher in animals that developed BNVV than in those that did not develop BNVV.

**Keywords:** Antibodies; Bovine necrotic vulvovaginitis; *Porphyromonas levii*

### INTRODUCTION

Bovine necrotic vulvovaginitis (BNVV) is a syndrome characterized by necrosis of the vulva and the vagina of heifers during the first two weeks post-partum. It was first described after a number of outbreaks in Israel that started in 2000 (1). *Porphyromonas levii*, a pigmented, gram-negative, anaerobic rod that is part of the ruminal microbiota (2) has been proposed to be the causative agent of this syndrome (3), although it can also be isolated in low numbers from vaginal samples of heifers on farms with no history of the disease (4) and thus, apparently, needs predisposing factors, such as stress, to cause the infection. As BNNV has a significant economic impact (5, 6), the development of a vaccine which stimulates an immune response against *P. levii* is desirable. In

order to develop such a vaccine, understanding the immune response of the host to *P. levii* and the relationship of this response to the development of BNVV is essential. Following the assessment of anti-*Porphyromonas levii* antibodies production in an experimental murine model, (see article in this issue), the objective of this study was to assess the *P. levii* antibody status of cattle pre-and post partum and to relate those changes to development of BNVV.

### MATERIAL AND METHODS

#### Animals and farms

The study was conducted in 2008 in 3 dairy farms. Farms A and B were collective dairy farms (kibbutz farms) located in

central-south Israel. Farm A had approximately 450 cows and 500 heifers. BNVV had become endemic on farm A since its first appearance in 2006, approximately 6 months after the number of cattle in the herd was doubled, following a merger with another farm. Farm B had approximately 490 cows and 170 heifers. An outbreak of BNVV occurred in late spring 2007 and thereafter sporadic cases were observed. Heifers on both farms were raised separately from cows until 21 days before they were due to calve, at which time they were grouped together with older cows until 21 days after calving. They were then separated from older cows until they calved for the second time. One-hundred Israeli-Holstein dairy heifers from these two farms were included in the survey. In addition, seventeen cows in various lactations, eight in the first month post-partum and nine in their last month of pregnancy, located on a third farm (C) were sampled, each on one occasion. Farm C was an experimental dairy farm in central Israel, which had approximately 130 cows and 80 heifers. Heifers on this farm were raised separately from older cows before and after parturition. Only a few sporadic cases of BNVV had been diagnosed in farm C in the past but not during the year of the survey. On all three farms, cattle were kept under a zero-grazing, loose housing management system, in completely covered, open sheds.

### Sampling

Blood from all heifers was collected individually during the seventh, fourth and last week prior to the expected date of calving (270 days post insemination) and during the first and fourth week after calving. Sera were separated and stored at  $-20^{\circ}\text{C}$ . In addition, colostrum was sampled at parturition and stored at  $-20^{\circ}\text{C}$ . Heifers were clinically examined for BNVV during the first week post-partum. BNVV was diagnosed based on extension and severity of lesions according to previously published criteria (3).

Additionally, on Farm A and B, blood was also collected from 21 multiparous cows ( $n = 13$  and  $n=8$ , respectively) once pre-partum (between 40 and 30 days before partum) and once in the fourth week post-partum.

Identification of *P. levis* in the vulva and vagina of heifers was undertaken using anaerobic culture of swabs as previously described (1). The presence of *P. levis* on farm A had been previously evaluated (3) whereas that on farms B and C was tested just prior to this study as described before (3) and found positive in farm B only.

### Serological assay

The level of anti-*P. levis* antibodies was evaluated using an in-house ELISA, as described in the mouse study (7). A strain of *P. levis* isolated from a severe case of BNVV on farm A was used as the antigen. Conjugated antibody in this case was AffiniPure goat anti-bovine IgG (H+L) or IgM (Bethyl Laboratories, USA). Bacteria were grown as described by Blum *et al.*, (3).

Ratio of antibody types (IgM or IgG) was calculated using the formula: IgM titre/total titre. All cows with titre above the threshold determined by the negative controls were considered positive. For negative controls, 10 cows with no previous history of BNVV were tested. Those with the optical density (OD) readings closest to that of uninoculated mice were pooled.

### Total IgG concentration of colostrum

In order to evaluate the overall immunogenic status of each heifer, total IgG concentration in colostrum was determined with a bovine IgG ELISA quantitative kit (Bethyl Laboratories, USA) as previously described (8).

### Statistical analysis

The relative risk of seropositive cows developing BNVV and of animals affected by the syndrome to seroconvert was assessed using an Internet based program (9).

Relative risk =  $(A/A+C)/(B/B+D)$  where

A: Seropositive/BNVV negative

B: Seronegative/BNVV negative

C: Seropositive/BNVV positive

D: Seropositive/BNVV negative

• Standard Error of log Relative risk (SElogR) =  
 $\text{sqrt}((1/A)-(1/(A+C))+(1/B)-(1/(B+D)))$

lower limit =

the exponential of  $(\log(\text{Rel risk})-(1.96*\text{SElogR}))$

upper limit =

the exponential of  $(\log(\text{Rel risk})+(1.96*\text{SElogR}))$

The significance of differences in anti-*P. levis* antibody titres was assessed by one-way analysis of variance using Statistix 7 (Analytical Software, USA).

Statistical significance was considered at  $p < 0.05$

## RESULTS

The numbers of animals tested per farm, the incidence of BNVV during the study period and the proportion of seropositive heifers are summarised in Table 1. The incidence of BNVV was higher on farm A than B whereas farm C remained free of the disease during the study. The proportion of seropositive heifers was consistently lower on farm B than on farm A. On farm A and B antibodies were present, pre-partum, in 44.4% and 34.8% of the heifers and 6/8 (75%) and 9/13 (69%) of the multiparous cows, respectively.

**Table 1:** Number of animals sampled in each farm, incidence of BNVV, and proportion of seropositive animals at each time point

Farm	Number of heifers	BNVV cases	Proportion of seropositive heifers (%) <sup>*</sup>				
			Wk -7	Wk -4	Wk -1	Wk 0	Wk 4
A	54	27 (50%)	51.02	52.17	34.62	40.63	86.67
B	46	13 (28.3%)	24.44	23.81	12.50	17.24	38.64

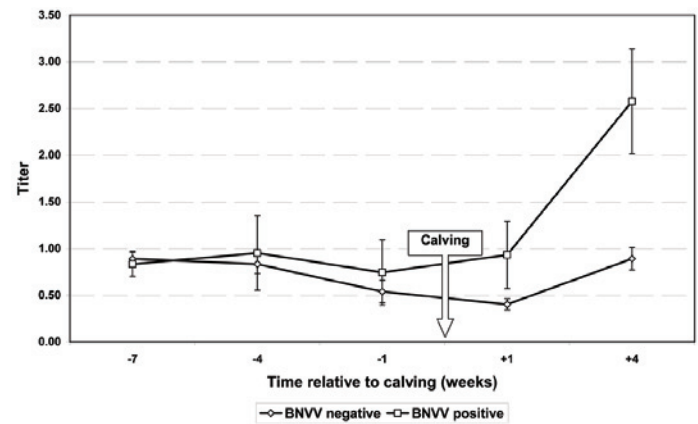
\* Wk: Weeks in relation to calving

On farm C, 4/8 preparturient and 5/9 post-parturient cows were seropositive. In multiparous cows, the percentage of animals with a positive titre pre-partum was higher (~70%). Animals, which were identified as seronegative at any stage pre-partum, did not seroconvert before calving.

**Table 2:** Association between pre-partum antibody status, BNVV incidence, post-partum seroconversion and mean antibody titre 4 weeks after calving.

Pre-partum serology	Diagnosis of BNVV	Post-partum seroconversion <sup>*</sup>	Mean antibody titre
Negative: A: 30/54 (55.6%) B: 30/46 (65.2%)	Negative: A: 17/30 (56.7%) B: 20/30 (66.7%)	Negative: A: 6/15 (40%) B: 14/20 (70%)	0
		Positive: A: 9/15 (60%) B: 6/20 (30%)	0.54
	Positive: A: 13/30 (43.3%) B: 10/30 (33.3%)	Negative: A: 1/12 (8.33%) B: 6/10 (60%)	0
		Positive: A: 11/12 (91.7%) B: 4/10 (40%)	0.8
Positive: A: 24/54 (44.4%) B: 16/46 (34.8%)	Negative: A: 10/24 (41.7%) B: 13/16 (81.3%)		0.89
	Positive: A: 14/24 (58.3%) B: 3/16 (18.7%)		2.58

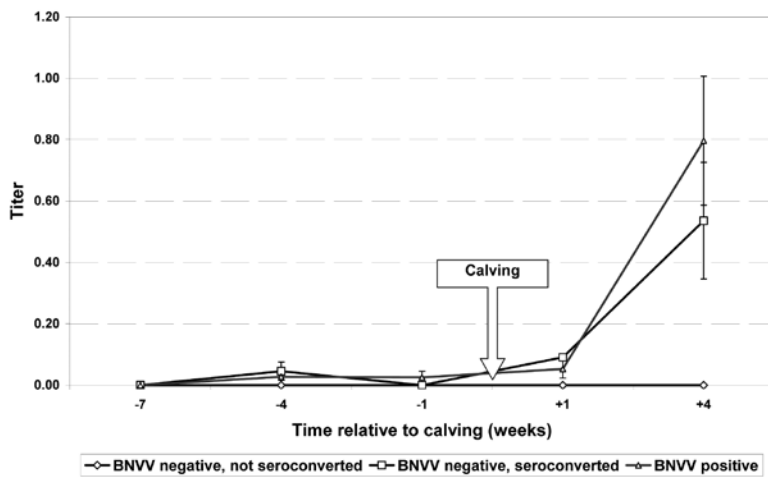
\* Two animals not sampled in negative BNVV group and one in positive group  
A, B: Farm designations



**Figure 1:** Heifers with anti-*P. levisi* antibodies pre-partum (results from farms A and B combined). Mean antibody titers in heifers recovering from bovine necrotic vulvovaginitis and those that did not develop the disease. Bars: Standard error of the means.

Seropositivity, seroconversion and BNVV prevalence data of the heifers on farms A and B are shown in Table 2. The risk of developing BNVV was found to be RR=0.73, 95% CI=0.42-1.30 and RR=1.22, 95% CI= 0.86-1.72 on farm A and B respectively.

Heifers that were seropositive before parturition showed an increase in titres after calving, and this increase was more marked in animals that developed BNVV (Fig. 1).



**Figure 2:** Heifers without anti-*P. leyii* antibodies pre-partum (results from farms A and B combined). Mean antibody titers in BNVV positive animals, BNVV negative animals that seroconverted and BNVV negative animals that did not seroconvert. Bars: Standard error of the means.

Differences between animals that developed and those that did not develop BNVV were not significant before and by the first week after calving and became significant by the fourth week thereafter ( $P=0.0028$ ).

In heifers that were seronegative before calving, three types of reactions were observed (Fig. 2): BNVV positive, BNVV negative that seroconverted and BNVV negative that remained seronegative. No change in the titers was observed until the fourth weeks after calving, when antibody titers increased in both BNVV positive and BNVV negative heifers which seroconverted to statistically significant levels ( $P=0.0040$  and  $P=0.0015$ , respectively). By four weeks after calving, antibody titers in the heifers which developed BNVV were higher than those in heifers that seroconverted without clinical signs but the difference was not statistically significant ( $P=0.3891$ ).

IgM titers increased by a factor of between 2.5 and 3.5 post-partum, exclusively in heifers that seroconverted after calving. The range of colostrum IgG levels in all heifers was  $80 \pm 4$  mg/mL. No differences were found in total IgG levels in the colostrum of BNVV positive and negative heifers (data not shown).

## DISCUSSION

The development of BNVV has been associated with infection by *P. leyii* (1, 3). No other potential pathogen has been identified; studies investigating the role of alternative patho-

gens, such as bovine herpes viruses, have not found any link between these pathogens and BNVV (1, 3). However, the presence of *P. leyii* in both cows and herds without BNVV suggests that infection by *P. leyii* alone may not be a sufficient cause for the development of BNVV. Other pathogens and/or specific risk factors seem to be necessary for *P. leyii* to colonise the vulvovagina and to trigger the development of BNVV. These suggestions need to be confirmed by further studies on the pathogenesis of BNVV. The present study focused on the dynamics of the humoral response to *P. leyii* pre- and post partum and its relationship to the development of BNVV.

In the present study, we found that on farms with BNVV infections, between 44.4% and 34.8% of the examined heifers had anti-*P. leyii* antibodies titres pre-partum, values slightly lower than the ones observed farm C (50%-55.5%) in spite of there being no BNVV cases on that farm during the survey. It seems likely that the source of these antibodies was previous contact with the microorganism, probably in the digestive tract.

The relative risk of developing BNVV according to the presence of antibodies differed on the farms A and B. This may be the result of the relatively low number of BNVV positive cows and other factors such as husbandry. Following calving, about two-thirds of seronegative heifers developed antibody titres, resulting in a total of about 70% of the population being positive. This proportion seems to be stable since it is similar to those observed in multiparous cows (75% and 69%). Since BNVV cases in adult cows are extremely rare, their resistance is probably not solely based on antibodies. Other factors, such as lower stress levels following parturition, are likely to be important. Interestingly, the proportion of seropositive heifers remained consistently lower on farm B than on farm A. This may be the result of the later farm being endemic for the syndrome and thus having been exposed for a longer period to infection with *P. leyii*, whereas the former only experienced one outbreak. This may indicate the possibility of an immune memory at the herd level.

For heifers that were seropositive before calving, mean antibody titres increased in those animals which developed BNVV but not in those which did not show clinical signs,



indicating the possibility that the intense exposure to the microorganism associated with the development of BNVV induced a booster effect on the humoral response against the microorganism.

In pre-partum seronegative animals an increase in IgM anti-*P. levii* antibodies ratio was observed after parturition confirming that the contact between animal and microorganism was recent. While the prepartum antibody levels, when present, probably resulted from the exposure in other sites such as the gastrointestinal tract, were unable to avert the syndrome, higher antibody levels, similar to those observed following recovery from the infection, or possibly inducible by a vaccine, may contribute to its prevention. One possible mechanism that may contribute to this humoral activity is the increased macrophage phagocytic activity against *P. levii*, observed *in vitro*, that may be achieved by opsonisation of bacteria with high-titre anti-*P. levii* serum (10).

## CONCLUSIONS

Anti-*P. levii* antibodies may be present in heifers pre-partum, but they do not prevent the development of BNVV. Recovery from BNVV leads to seroconversion in most pre-partum seronegative heifers and to a significant increase in anti-*P. levii* antibody titres in pre-partum seropositive animals.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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