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Characterization and identification of microbial communities in bovine necrotic vulvovaginitis

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ABSTRACT

Bovine necrotic vulvovaginitis (BNVV) is a severe and potentially fatal disease of post-partum cows that emerged in Israel after large dairy herds were merged. While post-partum cows are commonly affected by mild vulvovaginitis (BVV), in BNVV these benign mucosal abrasions develop into progressive deep necrotic lesions leading to sepsis and death if untreated. The etiology of BNVV is still unknown and a single pathogenic agent has not been found. We hypothesized that BNVV is a polymicrobial disease where the normally benign vaginal microbiome is remodeled and affects the local immune response. To this end, we compared the histopathological changes and the microbial communities using 16S rDNA metagenetic technique in biopsies taken from vaginal lesions in post-partum cows affected by BVV and BNVV.

The hallmark of BNVV was the formation of complex polymicrobial communities in the submucosal fascia and abrogation of neutrophil recruitment in these lesions. Additionally, there was a marked difference in the composition of bacterial communities in the BNVV lesions in comparison to the benign BVV lesions. This difference was characterized by the abundance of Bacteroidetes and lower total community membership in BNVV. Indicator taxa for BNVV were *Parvimonas*, *Porphyromonas*, unclassified Veillonellaceae, *Mycoplasma* and Bacteroidetes, whereas unclassified Clostridiales was an indicator for BVV. The results support a polymicrobial etiology for BNVV.

Introduction

Fifteen years have elapsed since the emergence of bovine necrotic vulvovaginitis (BNVV) in Israeli dairy herds and the etiology of the disease still remains elusive. Interestingly, the natural history of this disease is linked to the dairy market reform program that was launched by the Israeli government at the end of 1998. Consequently, large numbers of dairy cattle were mobilized and mixed in absorbing herds. Within months, merged farms experienced severe and fatal outbreaks of BNVV in postpartum cows. Although first calving heifers were at higher risk, the disease is known to affect newly introduced stocks and local animals of all age groups after calving (Goshen et al., 2012). To date, large merged herds still experience recurrent outbreaks despite the fact that the original mobilized and absorbing populations have been turned over and replaced years ago. It is now recognized that sporadic and rare cases of the disease occurred for years, and are still occurring, in many herds nationwide. However, the disease was only recognized and named as a consequence of the outbreaks that have occurred since 1998 (Elad et al., 2004).

Immediately after birth, the vagina of the newborn female calf is colonized by microbes originating from their mother's birth canal, oral cavity, skin and feces. The microbial community of the vagina is dynamic and undergoes changes as animals mature and is also affected by diet and other environmental factors. Gut and skin microbiota are important contributors to the vaginal microbial community (Gajer et al., 2012; Hickey et al., 2012; Uchihashi et al., 2015). On sexual maturity, in the case of natural service, the vaginal microbiota is also augmented by the microbial community of the male genitalia (Mandar et al., 2015).

In cows, the vaginal lumen is lined by mucosa of keratinized stratified squamous epithelium with an underlying lamina propria of connective tissue (Appendix: Supplementary Fig. S1). During normal parturition, some damage occurs to the mucosal surface of the vagina, leading to breaching of the epithelial surface, microbial colonization of the exposed tissues and an inflammatory response (bovine vulvovaginitis; BVV). This condition is usually self-limiting and dairy cows recover spontaneously within a few days without further consequences. Unknown factors aggravate these minor

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lesions, resulting in BNVV, a fulminating condition that can be fatal without antimicrobial treatment. The bacterium *Porphyromonas levii* has been suggested as the microbial etiology of BNVV (Elad et al., 2004). The same research group also reported the presence of this organism in vaginal swabs from non-affected animals in BNVV-affected and non-affected farms (Blum et al., 2008a). To date, all attempts to culture a dominant pathogen and to characterize BNVV as a monomicrobial disease have been unsuccessful. We hypothesized that BNVV is a polymicrobial disease which causes remodeling of the normally benign vaginal microbiome and affects the local immune response (Hajishengallis et al., 2012). To this end, we analyzed the histopathological characteristics of the disease and conducted culture-independent, metagenetic analysis of vaginal microbiota in cows with BVV and BNVV.

Materials and methods

Sampling

BNVV and BVV were clinically diagnosed in 17 dairy cows as previously described (Fig. 1; Blum et al., 2007; Blum et al., 2008b; Goshen et al., 2012). As commonly practiced in Israeli dairies, none of the animals were treated with any antimicrobial therapy from 2 months prior to calving and the onset of disease. Using aseptic technique, vaginal biopsy samples were collected under epidural anesthesia after disinfection of the vulva. Eight cows with BNVV, diagnosed 3–13 days after calving, originated from two dairy farms (Farm A and Farm B; Appendix: Table S1) where there was an outbreak of the disease. Nine cows with BVV, diagnosed 2–10 days after calving, originated from a further two dairy farms (Farm C and Farm D; Appendix: Table S1) where BNVV had never been diagnosed.

Vaginal biopsy samples were trisected for histology, transmission electron microscopy (TEM) and DNA extraction. Samples for DNA extraction were snap frozen in liquid nitrogen and stored at –80 ºC until further processing. Samples for histological analysis were fixed in neutral buffer 4% formaldehyde, paraffin-embedded, and sections were cut and stained with hematoxylin and eosin (H and E) and Gram stains. Samples for TEM were fixed in 1% glutaraldehyde/2% formaldehyde in 0.2 M sodium cacodylate buffer, and embedded in Epon; thick sections (1 mm) were stained with toluidine blue for light microscopy and thin sections (70 nm) were stained with uranyl acetate and Reynold's lead citrate for TEM with a Philips CM-12 electron microscope.

All animal procedures were performed by licensed veterinarians with consent of the animal owner and approved by the Volkani Center Animal Care Committee for the Hachaklait, Mutual Society for Veterinary Services (Approval no. IL-233/10, 26 January 2010).

Total DNA extraction and sequencing

Total DNA was extracted from biopsy samples using AccuPrep Genomic DNA Extraction Kit (BIONEER, Cat. No. k-3032), in accordance with the manufacturer's instructions.

To identify the microbial constituents of the biopsy samples, 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing was performed using the Roche 454 sequencing system as previously described (Dowd et al., 2008). Amplicons originating from the V1–V3 region (27F– 5′ GAG TTT GAT CNT GGC TCA G 3′ to 519R 5′ GTN TTA CNG CGG CKG CTG 3′, numbered in relation to the *Escherichia coli* 16S rRNA gene) were sequenced.

Metagenetic analysis

The metagenetic analyses of the obtained 16S rRNA gene sequences were performed as previously described (Shpigel et al., 2015). Briefly, reads underwent a cleaning process using MOTHUR v1.24 (Schloss et al., 2009), after which a total of 51,053 sequences, with an average read length of 242 bp, were used further. Pairwise distances were calculated between all DNA reads, which were subsequently clustered into operational taxonomic units (OTUs) at the 0.03 level. Each OTU was phylotyped based on current RDP-II taxonomy (Cole et al., 2009). The resulting OTUs were arranged in a data matrix to represent their abundance relative to the sampling effort.

Multivariate analysis was performed in PC-ORD v5.32 (MjM Software) with Sorensen distances. Ordinations were performed with non-metric multidimensional scaling (NMDS; Mather, 1976) at 500 iterations, and cluster analyses were performed with flexible beta linkages ($\beta = -0.25$). Differences between sample groups were calculated with the multi-response permutation procedure (MRPP; Mielke, 1984).

To determine which OTUs were mainly responsible for differences between groups, we computed indicator values (IVs) which are a combination of the frequency of occurrence and abundance of each OTU in each group. IV ranges between 0 and 100 and is larger if an OTU is more frequent and/or more abundant in a given group compared to another group (Dufrene and Legendre, 1997). The mean relative abundance of these genera was compared between farms using the non-parametric Mann–Whitney two-independent-samples test for comparison of means (GraphPad Prism 6, GraphPad Software). A P value of 0.05 or less was considered significant.

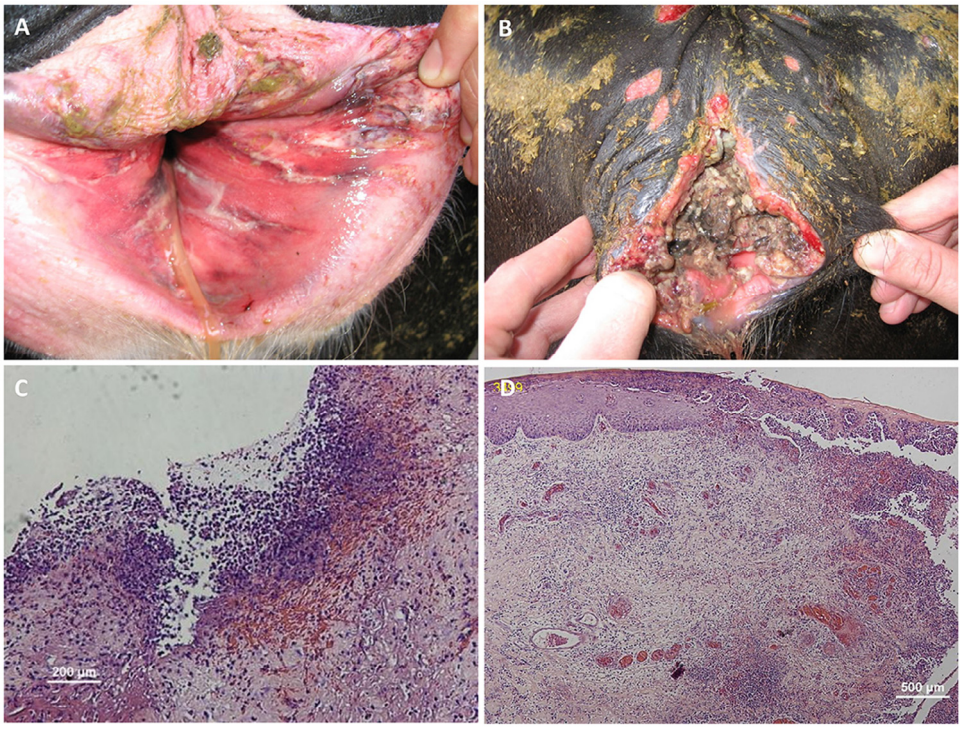

Fig. 1. Clinical and histological images of post-partum dairy cows affected by bovine vulvovaginitis (BVV; A and C) and necrotic vulvovaginitis (BNVV; B and D). Hematoxylin and eosin stain of mucosal lesions from BVV (C) and BNVV (D) cases. Localized areas of mucosal ulcers and purulent discharge (A) characterized by massive neutrophils recruitment (C) are visible in BVV. Severe and extensive necrotic lesions affecting the mucosa and submucosa in BNVV (B and D). All images are representative of the entire sample and the histological morphology and pathology results were very similar for each sample of BVV and BNVV.