Pathological and virological features of skin lesions caused by BVDV in cattle

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Abstract

Dermatitis might occur in mucosal disease (MD) caused by bovine viral diarrhea virus (BVDV). This study describes the pathological and virological features of skin lesions associated with BVDV infection in four persistently infected (PI) cattle. Skin samples were reprocessed for histopathology and IHC. BVDV isolates were obtained and were genetically characterized. In addition to upper alimentary system ulcerative lesions, all cattle (one outbreak and three individual cases) presented focal crusty and ulcerative lesions affecting the mucocutaneous and skin-horn junctions, interdigital clefts, pastern, and areas surrounding the dewclaws and diffuse thickened skin within 7–20 days of infection. Microscopic analysis revealed parakeratotic hyperkeratosis and single-cell keratinocyte death, accompanied by ballooning degeneration and spongiosis in the epidermis, as well as intraepithelial and subcorneal pustules. IHC showed BVDV antigen in the cytoplasm of keratinocytes undergoing individual cell death. Phylogenetic analysis revealed that the isolates from cattle #1, #2, and #4 belonged to BVDV-1a, whereas that from cattle #3 belonged to BVDV-1d. Cytopathic BVDV was isolated from cattle #2 and #3 (MD), and non-cytopathic BVDV was isolated from cattle #1 and #4. Thus, BVDV infection might cause acute disease, characterized by skin and upper alimentary system ulcerative lesions, in both MD and PI cattle.

Keywords Dermatitis · Infectious diseases · Mucosal disease · Persistently infected · *Pestivirus*

Introduction

Bovine viral diarrhea virus (BVDV) is a single-stranded RNA virus of the *Pestivirus* genus within the Flaviviridae family [1]. It has been divided into two species, namely, BVDV-1 and BVDV-2 [2], and these can be divided into 21 (1a–1u) [3, 4] and three (2a–2c) subgenotypes, respectively [5]. BVDV is also classified into biotypes according to its effect on cell cultures: cytopathic (cp) or non-cytopathic (ncp) [6].

BVDV infection in naïve pregnant cows (30 and 90 days of gestation) might result in the generation of persistently infected (PI) calves. PI animals are the main viral sources in outbreaks of bovine viral diarrhea (BVD) [7]. Mucosal disease is a lethal disease characterized by lesions in the mucosas of the digestive tract, necrosis of lymphoid tissue, hoof inflammation, and dermatitis and occurs when the PI animal is infected with cp-BVDV [8–10].

BVDV lesions may occur with BVDV infection depending on host factors, such as the immune competence and immune status, and viral factors, such as the genotype/subgenotype, biotype, and virulence of the infecting strain [7]. Dermatitis is frequently present in mucosal disease (MD) [10] and might present as localized lesions in chronically affected MD animals with ulceration of the interdigital cleft and coronary band [11] or as generalized acute or chronic dermatitis [12, 13]. Additionally, generalized dermatitis might be observed in PI animals infected with ncp-BVDV [14].

This study aimed to describe the gross and microscopic features of the BVDV infection-associated skin lesions in four PI cattle through molecular identification of the involved

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Pestivirus and an analysis of the immunohistochemistry staining pattern.

Materials and methods

Clinical, epidemiological, and histopathological features

Necropsy archives of a veterinary pathology laboratory database from 2012 to 2016 were reviewed, and all persistently infected cattle with cutaneous lesions by BVDV were selected for this study, accounting four cattle in total. The animals were classified according to age, breed, sex, and clinical signs. Three out of four cattle were subjected to postmortem examination immediately after euthanasia, whereas the other was subjected to this examination 12 h after death. Multiple samples of the organs and skin were collected and fixed in 10% neutral buffered formalin. Tissue samples were routinely processed for histopathology and stained with hematoxylin and eosin (H&E). Blood samples from these animals were also collected, and the serum was stored at -80°C until analysis.

Immunohistochemistry

Skin sections and samples of the small and large intestine from the four cattle were subjected to immunohistochemistry, according to a previously described protocol [12]. A monoclonal BVDV antibody was employed (BVDV MAb 15c5, Syracuse Bioanalytical, East Syracuse, NY, USA) at a dilution of 1:500 in phosphate-buffered saline (PBS). The reaction was revealed with Permanent Red solution (DakoCytomation, Carpinteria, CA, USA). An ear notch section was used as the positive control, and primary antibody was replaced with PBS in the negative control sections.

RT-PCR and phylogenetic analyses

Cattle #1 (LV/Itabajara/12), #2 (LV/N614/13), and #3 (LV/N62/15) were genetically characterized in previous studies [12, 15]. The RT-PCR and phylogenetic analyses from the serum of cattle #4 (LV/N928/15) were performed according to the previous study, with the primers 324/326 for partial amplification of the 5' untranslated region (5'UTR) of *Pestivirus* [16, 17].

Virus isolation

Serum from the four cattle was subjected to virus isolation using *Pestivirus*-free Madin-Darby bovine kidney cells (MDBK) in 24-well microtiter plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum and antibiotics. The plates were incubated at 37°C with

5% CO_2 and examined daily for up to 4 days to observe the cytopathic effect. Four consecutive passages were performed for each sample. The animals were classified as MD cases if a cp biotype was obtained [18].

Results

Clinical and epidemiological features

All cases occurred in different farms in the state of Rio Grande do Sul, Brazil. The affected cattle presented clinical signs of anorexia, sialorrhea, weight loss, and crusty and ulcerative skin lesions during the clinical course, specifically from 7 to 20 days after infection. Three of the cases occurred individually, whereas cattle #3 formed part of an outbreak of MD.

Cattle #1 did not gain weight, presented retarded growth, and was maintained in the veterinary pathology facilities for 2 months. Cattle #2 was a poorly developed calf that was born weak and presented bloody diarrhea. Cattle #3 was a calf from a property where an extensive outbreak of BVD occurred; this outbreak included 22 persistently infected calves, 11 of which, including cattle #3, died within a short period (MD calves), and the other 11 remained alive for a longer period (PI calves) [2]. Cattle #4 was a recently acquired adult cow with no previous known history that began to lose weight and present skin lesions in the 15 last days.

Data regarding the age, breed, sex, clinical course, and skin lesion locations for the four affected cattle are shown in Table 1.

Gross findings

During physical examination, all cattle presented ulcerative skin lesions of all feet affecting the skin-horn junction (coronary bands), interdigital clefts, pastern, and areas surrounding the dewclaws, and these lesions extended to the fetlock joint in cattle #2, #3, and #4. The skin close to these areas was also thickened and dry and showed marked crust formation (eczema) and alopecia (Fig. 1a), and these signs were particularly prevalent at mucocutaneous junctions (ocular area, nostrils, preputial opening, and vulva), which showed moderate multifocal areas of erosion and/or associated ulceration. The ventral body skin (thoracic, abdominal, submandibular, inguinal, and axillar regions) of cattle #3 was diffusely thickened and presented a moist and yellowish appearance (Fig. 1b). Cattle #4 presented a diffusely thickened, fissured and dry skin along the neck and head (Fig. 1c) as well as the ventral body skin (Fig. 1d).

The oral mucosa, hard palate, and tongue of all cattle presented mild to severe multifocal to coalescing areas of ulceration. The abomasum mucosa of cattle #2 and #3 showed multifocal to coalescing rounded ulcers. The esophagus of

Table 1 Age, breed, sex, skin lesion locations, and clinical course of the four cattle affected by BVDV

No.	Age	Breed	Sex	Clinical Course (days)	Skin lesion locations
1	1.5 years	Hereford	M	20	Feet (coronary bands, interdigital, pastern, and dewclaws), nostrils, ocular and preputial regions
2	1 month	Angus	M	20	Feet (coronary bands, interdigital clefts, pastern, dewclaws, and fetlock joint), nostrils, submandibular region, ventral abdomen and thorax
3	4 months	Angus	F	7	Feet (coronary bands, interdigital clefts, pastern, dewclaws, and fetlock joint), inguinal and axillar regions, ventral abdomen and thorax
4	3 years	Mixed breed	F	15	Feet (coronary bands, interdigital clefts, pastern, dewclaws and fetlock joint), vulva, nostrils, ocular, limbs, inguinal and axillar regions, ventral abdomen and thorax, neck and head

F, female; M, male;

cattle #4 and the rumen mucosa of cattle #2 exhibited multifocal linear ulcers. Intestinal lesions were grossly absent in all of the investigated animals.

Microscopic findings

A microscopic analysis of the skin lesions revealed that these consisted of diffuse and severe parakeratotic hyperkeratosis, which thickened the stratum corneum (Fig. 2a). These lesions included multiple keratinocytes of the epidermis and follicular epithelium displaying a hypereosinophilic and retracted cytoplasm with pyknotic nuclei (single-cell death), associated to multifocal ballooning degeneration and severe multifocal spongiosis of keratinocytes in the stratum spinosum and basale, respectively (Fig. 2b). Multifocally, these necrotic and degenerate cells tended to coalesce in the epidermis to form numerous intraepithelial and subcorneal pustules, which contained large numbers of degenerate neutrophils and occasional necrotic keratinocytes. The interface between the epidermis and the superficial dermis showed a moderate to severe diffuse inflammatory infiltrate of lymphocytes, plasma cells,

and macrophages (Fig. 2c, d). The hyperkeratotic areas were intermixed with areas of necrosis in the epidermis in which the lamina propria was covered by myriad bacterial coccobacilli and fibrin. The moist appearance of the skin of cattle #3 corresponded microscopically to multifocal areas of severe necrosis of the epidermis, which were covered by myriad bacterial coccobacilli and fibrin.

The ulcerative lesions of the oral mucosa, hard palate, tongue, esophagus, rumen, and abomasum were similar and composed of multifocal areas of epithelial necrosis associated with numerous epithelial cells displaying single-cell death and a moderate inflammatory infiltrate of lymphocytes and macrophages at the mucosa-submucosa junction. Small and large intestines did not present any microscopic lesions.

Immunohistochemistry, RT-PCR, phylogenetic analysis, and virus isolation

On immunohistochemistry of skin samples, the keratinocytes displaying individual cell death in both the epidermis and the follicular epithelium (Fig. 2e), and the necrotic keratinocytes

Fig. 1 Gross findings of dermatitis caused by BVDV in cattle. **a** The skin of the limbs at the coronary band, interdigital cleft, pastern and areas surrounding the dewclaws was thickened and dry. **b** Inguinal and ventral abdomen skin of cattle #3 was diffusely thickened and showed a moist and yellowish appearance. **c, d** The skin of the neck, head, inguinal and ventral abdomen of cattle #4 was diffusely thickened, fissured and dry. Detail: cut section of severely thickened skin showing marked crust formation

Fig. 2 Photomicrographs of skin sections with dermatitis caused by BVDV. **a** Diffuse and severe parakeratotic hyperkeratosis that thickened the stratum corneum. Hematoxylin and eosin (H&E), 10x. **b** The epidermis and follicular epithelium showed multiple keratinocytes displaying a hypereosinophilic and retracted cytoplasm with pyknotic nuclei (single-cell death) associated with spongiosis in the stratum basale. H&E, 40x. **c** Moderate inflammatory infiltrate of lymphocytes, plasma cells and macrophages in the interface between the epidermis and the superficial dermis. H&E, 20x. **d** Intraepithelial pustule containing large

numbers of degenerate neutrophils and occasional necrotic keratinocytes. H&E, 40x. **e** Marked cytoplasmic immunostaining of keratinocytes displaying individual cell death at the epidermis and mild immunostaining of macrophages at the superficial dermis. Streptavidin-biotin-alkaline phosphatase complex method, chromogen permanent red, hematoxylin counterstain, 20x. **f** Marked cytoplasmic immunostaining of epithelial cells at intraepidermal pustules. Streptavidin-biotin-alkaline phosphatase complex method, chromogen permanent red, hematoxylin counterstain, 40x.

in the intraepidermal and subcorneal pustules presented marked immunostaining in the cytoplasm (Fig. 2f). Immunostaining was also observed in the cytoplasm of inflammatory cells at both the epidermis-dermis interface and the pustules. Immunostaining was absent in the sections of the small and large intestines.

According to the phylogenetic analysis, the virus isolates from cattle #1, #2, and #4 belonged to the BVDV-1a subgenotype, whereas that of cattle 3 belonged to the BVDV-1d subgenotype. Cp and ncp-BVDV were isolated from cattle #2 and #3 (MD cases), whereas only ncp-BVDV was isolated from cattle #1 and #4 (PI).

Discussion

The diagnosis of BVDV persistent infection in the four cattle included in the present study was based on pathological,

immunohistochemical, and virological findings. All of the cattle included in this study were diagnosed as PI cattle based on the pattern of IHC staining on the skin sections. Pronounced immunostaining in the keratinocytes and follicular epithelium has been described as an effective method for identifying PI animals [19]. A definitive diagnosis of MD might require demonstration of a cp biotype in tissues [18, 20], and thus, cp-BVDV was obtained from two of the four cases, confirming that MD occurred in these animals. Additionally, the staining intensity of MD and non-MD PI animals has been found to not differ [18]. This finding was also obtained in our study, which showed that all of the cattle, including those infected with ncp-BVDV and cp-BVDV, presented a similar immunostaining pattern on the skin sections, and the observed immunostaining was located primarily in the cytoplasm of keratinocytes displaying individual cell death in both the epidermis and the follicular epithelium. Specific BVDV antigen was detected in all skin samples from two

previously described outbreaks in which ncp-BVDV caused generalized dermatitis [14]. In our cases, however, immunostaining was mainly observed in keratinocytes displaying individual cell death and in necrotic cells at intraepidermal and subcorneal pustules.

Gross skin lesions associated with BVDV are described as localized lesions in chronically affected MD animals with ulceration of the interdigital cleft and coronary band [11] but might also present as generalized dermatitis in cattle with a clinical course of 30 to 45 days [13, 14]. However, all of the cattle included in the present study presented an acute clinical course, with a maximum of 20 days, similar to the findings observed in a similar MD animal infected with “HoBi”-like *Pestivirus*, which showed a clinical course of 15 to 20 days [20]. Ulcerative lesions at the interdigital skin involving the pastern and coronary junctions were observed in all of our animals, in agreement with previous reports [11], and these lesions also involved the skin at mucocutaneous junctions, as has been observed in PI cattle showing erosions of the nose, muzzle, interdigital cleft, and crustaceous dermatitis [21]. These findings demonstrate that BVDV has a certain tropism for these areas. However, cattle #2, #3, and #4 presented diffuse involvement of the skin, which was thickened, as has been observed in two Nelore cattle presenting with MD [13]. The lesions in cattle #3, however, had a moist appearance and mainly affected the inguinal and axillar regions. This appearance might be related to microscopic findings of multifocal necrosis of the epidermis covered by large amounts of myriad bacterial coccobacilli and thus an exudative aspect. However, the lesions in cattle #4 had a dry appearance and mainly affected the ventral parts, neck, and head. These findings have been previously observed in the head, coronary band, and interdigital space of two cattle with MD, whereas an exudative or moist appearance was previously observed mainly in the cervical, axillar, inguinal, and perianal regions [13], differing from the case included in the present study because cattle #4 was a PI animal infected with ncp-BVDV-1a. Similar crust thickening of the skin surrounding the nose and eyes and the skin of the neck, and inguinal and lumbar regions was previously described in two calves [20].

BVDV has tropism for epithelia of both the alimentary and integumentary systems, and an analysis of skin lesions caused by this virus in two outbreaks affecting calves revealed that the most striking microscopic lesions are characterized by severe hyperparakeratosis associated with necrosis of isolated or grouped cells at the epidermis [14]. Similar histopathological lesions characterized by ballooning degeneration and individual cell necrosis affecting keratinocytes of the upper alimentary system have been described previously in association with BVDV infection [14, 22], suggesting that cp-BVDV replication causes direct cellular damage [23]. MD pathogenesis is related to an intrinsic apoptotic pathway of epithelial cells, associated with major expression of active caspase-3 and

caspase-9, but this expression has not been observed in non-MD PI cases [18]. Despite this finding, individual cell death was observed in all MD and non-MD PI cases included in the present study, similarly to previous findings in calves experimentally infected with ncp biotypes of BVDV [23, 24] and in seven ncp-BVDV-infected calves that presented with generalized dermatitis and were most likely born PI [14]. Additionally, a recent study described similar lesions in an MD calf and a PI calf [20], demonstrating that both ncp- and cp-BVDV might cause similar lesions.

Intraepidermal vesicles and pustules, similar to those described in the present study, can be observed in acute or subacute dermatosis, viral infections, hydropic degeneration of keratinocytes and acantholysis [25]. Viral infection with BVDV appears to be the cause of these lesions in this study because immunostaining was observed in association with the microscopic lesions. This finding is supported by previous descriptions of intraepidermal vesicles and pustules in the skin of a calf and in the forestomachs of experimentally infected calves, which were characterized by several vacuolated cells in the basal layer that coalesced to form microvesicles [20, 23].

MD is characterized mainly by erosions and ulcers in the epithelium of oral and nasal cavities, esophagus, forestomachs, and small intestine mucosa overlying Peyer’s patches [8]. The gross, microscopic, and immunohistochemical findings obtained in this study revealed a limited lesion distribution, mainly restricted to the upper alimentary system, mucosa, and skin, whereas other studies showed no intestinal lesions [12, 13, 22, 26], which might be related to the short clinical course of the cases described. Additionally, clinical and pathological lesions associated with BVDV might vary according to the herd immunity, vaccination, regional location, and direct contact with other herds, whereas the genotypes and subgenotypes are not related to distinct clinical courses [27]. However, the present study revealed that the majority of the cases with cutaneous lesions (3/4) had BVDV-1a characterized as both cp and ncp (1/3; cattle #2) and ncp (2/3; cattle #1 and #4) virus biotypes. Still, this is a questionable finding since BVDV-1a is the most common subgenotype of BVDV in Brazil and the second most predominant worldwide [15, 28], whereas BVDV-1d is the fourth most common BVDV subgenotype in Brazil [15]. This study found that BVDV-1d may cause lesions similar to those obtained with BVDV-1a, which might be related to the particular virulence of these subgenotypes, and similar hypothesis have been proposed for two outbreaks of generalized dermatitis with absence of digestive pathology caused by BVDV-1b [14] and in the skin of a PI alpaca with disseminated BVDV [29].

BVDV infection in both MD (cattle #2 and #3) and non-MD PI (cattle #1 and #4) animals might cause an acute disease with anorexia, sialorrhea, weight loss, upper alimentary system ulcerative lesions, and skin lesions. These lesions are characterized by thickened and dry skin, mainly at the

interdigital and coronary bands, and mucocutaneous junctions and are occasionally associated with a diffuse involvement of the skin. Microscopic analysis revealed that the lesions mainly consisted of keratinocytes undergoing single-cell death, intraepithelial to subcorneal pustules and parakeratotic hyperkeratosis. Immunohistochemistry showed that BVDV antigens were located mainly in the cytoplasm of keratinocytes undergoing individual cell death.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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