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SHORT PAPER

Epidermolysis Bullosa in Calves in the United Kingdom

**A. P. Foster^{*}, A. M. Skuse[†], R. J. Higgins[‡], D. C. Barrett[§], A. W. Philbey[§],
J. R. Thomson^{||}, H. Thompson[§], M. A. Fraser[¶], P. E. Bowden[#]
and M. J. Day[†]**

^{*} *Veterinary Laboratories Agency – Shrewsbury, Kendal Road, Harlescote, Shrewsbury, Shropshire, SY1 4HD, England,*

[†] *Division of Veterinary Pathology, Infection and Immunity, School of Clinical Veterinary Science, University of Bristol, Langford House, Langford, BS40 5DU, England,* [‡] *Veterinary Laboratories Agency – Lasswade, Pentlands Science Park, Bush Loan, Penicuik EH26 OPZ, Scotland,* [§] *Division of Animal Production and Public Health and Division of Pathological Sciences, Institute of Comparative Medicine, University of Glasgow Veterinary School, Bearsden Road, Glasgow G61 1QH, Scotland,* ^{||} *Scottish Agricultural College, Veterinary Science Division, Allan Watt Building, Bush Estate, Penicuik EH26 0QE, Scotland,* [¶] *School of Nursing, Midwifery and Social Care, Napier University, Canaan Lane Campus, 74 Canaan Lane, Edinburgh EH9 2TB, Scotland and* [#] *Department of Dermatology, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, Wales, UK*

Summary

Epidermolysis bullosa (EB) was diagnosed in eight calves from four farms in the United Kingdom on the basis of clinical, histological and ultrastructural findings. In three affected herds, pedigree Simmental bulls had been mated with Simmental-cross cows. In a fourth herd two Holstein-Friesian calves were affected. Lesions included multifocal erosion and ulceration of the hard and soft palates, tongue, nares and gingiva, with onychomadesis (dysungulation). There was alopecia, erosion and crusting of the coronets, pasterns, fetlocks, carpi, hocks, flanks and axillae. Histopathological findings included segmental separation of full thickness epidermis from the dermis, with formation of large clefts containing eosinophilic fluid, extravasated red blood cells and small numbers of neutrophils. Follicular and interfollicular areas of skin were affected, with clefts extending around hair follicles and sometimes involving whole follicles. Ultrastructurally, there was evidence of vacuolar change within basal keratinocytes, corresponding to areas of histological clefting. Preliminary genetic screening of the candidate keratin genes (*bKRT5* and *bKRT14*) has excluded mutations of these as the cause of this condition.

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Epidermolysis bullosa (EB) is a group of mechanobullous disorders involving defects in the basement membrane zone (BMZ) that are characterized clinically by blistering following minor trauma. In man, EB is classified into three major types: epidermolytic (EB simplex, EBS), lucidolytic (junctional EB, JEB) and dermolytic (dystrophic EB, DEB) (Fine *et al.*, 2008). EBS is characterized by intraepidermal blistering; JEB by blister formation at the level of the lamina lucida and DEB by blister formation below the lamina

densa (McAllister and Marinkovich, 2005). The EBS subtypes have been defined on the basis of genetic mutations in BMZ-associated proteins, including keratins 5 and 14, integrin $\alpha_6\beta_4$, plectin, desmoplakin and plakophilin-1 (Fine *et al.*, 2008).

Herein we report clinical, histological and ultrastructural findings that define a mechanobullous dermatosis resembling EBS. Eight calves with congenital mechanobullous dermatosis were examined from four herds in the UK (Table 1). Postmortem examinations were performed on seven calves, whereas biopsy samples only were examined from calf 8. At the time of presentation, the eight affected calves exhibited skin

Correspondence to: A.P. Foster (e-mail: a.foster@vla.defra.gsi.gov.uk).

Table 1
Case details and farm history of affected calves

<i>Farm</i>	<i>Case details and farm history</i>
1	<p>Calf 1: 2-week-old Simmental-cross heifer calf</p> <p>Calf 2: 2-week-old Simmental-cross heifer calf</p> <p>Herd comprised 15 cows and heifers that were all mated to the same pedigree Simmental bull. Nine were Holstein-Friesian cross Simmental heifers purchased from one herd and sired by the one Simmental bull. Four of the nine calves born to these heifers had skin lesions (one bull and three heifers). The other six Simmental cows produced unaffected calves. The Simmental bull that had served the whole herd that year had previously sired approximately 300 normal calves on that farm and a previous farm.</p>
2	<p>Calf 3: 1-day-old Simmental-cross bull calf</p> <p>Calf 4: 10-day-old Simmental-cross bull calf</p> <p>Pedigree Simmental bull mated to 20 Simmental-cross cows. Two affected calves were born. A separate group of 20 cows mated with the same bull produced no affected progeny.</p>
3	<p>Calf 5: 3-day-old Simmental-cross bull calf</p> <p>Calf 6: 7-day-old Simmental-cross bull calf</p> <p>Simmental bull mated with Simmental-cross cows. When calf 6 was presented for examination, the farmer had reported 3/25 calves affected. A further 15 cows subsequently calved with no further cases.</p>
4	<p>Calf 7: 4-week-old Holstein-Friesian heifer calf</p> <p>Calf 8: 16-day-old Holstein-Friesian heifer calf</p> <p>Originally an Ayrshire herd on the farm, with anecdotal evidence of a condition resembling EB when homebred bulls were used. Breeding policy changed and many generations were bred using Holstein-Friesian bulls providing semen for artificial insemination, including the grandsires of the affected calves, before the farmer again used a homebred bull. Grand dams were homebred from long-standing cow families that could be traced back to the original Ayrshire breed. The immediate sire and dams were homebred. Only two clinical cases were observed out of 100 calves sired by the one bull.</p>

and oronasal lesions (Fig. 1). The six Simmental calves from farms 1–3 developed dysungulation within hours of birth, but this was not a feature in the two Holstein-Friesian heifer calves from farm 4. The two calves from farm 1 initially had claw lesions only, but multiple cutaneous lesions developed immediately after transportation to the post-mortem facility. All eight calves were unable or reluctant to stand, but could suckle their dams with assistance. Once standing, they were reluctant to walk and drooled saliva. All eight calves were euthanased on humane grounds due to the severity, extent and progression of the skin and oral lesions. At post-mortem examination, all calves exhibited various degrees of multifocal erosion and ulceration of the hard and soft palates, the dorsal and ventral aspects of the tongue and the gingiva. The coronets, pasterns, fetlocks, carpi, hocks, flanks and axillae exhibited alopecia and various degrees of erosion and crusting. Calf 6 had macroscopic cutaneous vesicles and bullae, together with vesicles on the nares and ventral aspect of the tongue. Calf 8 also had bullae on the ventral aspect of the tongue.

Samples of skin, oral mucosa, nares and tongue were collected from affected calves, fixed in 10% neutral buffered formalin, dehydrated in graded ethanol and embedded in paraffin wax. Tissue sections 3–6 µm thick were stained with haematoxylin and eosin and serial sections from some tissues were stained by the periodic acid-Schiff (PAS) method.

Areas of skin, oral mucosa, nares and tongue corresponding to the location of suggestive histological lesions were processed for transmission electron microscopy (TEM). Tissue excised from paraffin wax blocks was dewaxed, then rehydrated, post-fixed in osmium tetroxide and embedded in Agar 100 epoxy resin. Resin sections were stained with toluidine blue and representative areas were selected by light microscopy for thin sectioning (100 nm). Ultrathin sections were mounted on 300 mesh copper grids, stained with uranyl acetate and Reynold's lead citrate and examined using a Philips EM201 transmission electron microscope.

Histological findings in the skin and oral mucosa were similar in all eight calves, but they varied in degree depending on the stage of presentation, site and the presence of secondary changes, such as bacterial infection. The earliest abnormality detected in haired skin was the presence of narrow supra-dermal clefts filled with clear or pale eosinophilic fluid. In slightly more advanced lesions, variable lengths of intact full thickness epidermis were separated from the dermis by larger clefts containing eosinophilic fluid, extravasated red blood cells and occasional neutrophils. There were no degenerative changes in the stratum basale or other layers in these detached segments of epidermis. Follicular and interfollicular areas of skin were extensively affected. Cleft formation extended around hair follicles to various depths, sometimes involving entire follicles, which occasionally were

Fig. 1. Ulceration of distal limbs and hooves in calf 5.

displaced into subepidermal bullae, leaving an oedematous space in the dermis surrounded by sebaceous and apocrine glands. In ulcerated lesions the surface was lined by a serocellular exudate composed of eosinophilic proteinaceous material, haemorrhage and neutrophils. There were various degrees of bacterial colonization of the ulcerated surface. Granulation tissue with fibrovascular hyperplasia, congestion, oedema and exudation of neutrophils had formed in the superficial dermis in older ulcers. Similar fluid-filled clefts had formed between the epithelium and an intact basement membrane in the mucosae of the oral cavity, nares and tongue. Recently ulcerated lesions were infiltrated by neutrophils and exhibited necrosis, haemorrhage and formation of microthrombi. Chronically ulcerated areas showed active formation of granulation tissue, with a mixed cellular infiltrate including plasma cells. PAS staining of histological sections from calves from farms 1 and 3 demonstrated cleft formation involving the epidermis, with the basement membrane attached to the floor of the cleft (Fig. 2).

Skin from six of eight calves examined by TEM had distinct cleft formation between the epidermis and

Fig. 2. Photomicrograph of PAS-stained nasal skin from calf 1, showing cleft formation involving the epidermis, with the basement membrane attached to the floor of the cleft. Bar, 50 μ m.

dermis that was confined to the region above the basement membrane and through the basal epithelial cells. There were small individual or aggregated vacuoles in the basal cytoplasm of these cells, associated with a degree of dissolution of the adjacent cytoplasm (Fig. 3). No ultrastructural abnormalities were observed in hemidesmosomes or tonofilaments of basal epithelial cells. Cells in the stratum spinosum were unremarkable. No morphological changes were detected in the basement membrane or dermal collagen. No clefting was observed in calves 6 and 8, but samples of skin from these animals had extensive secondary inflammation that may have obscured other changes in the epidermis.

Blood samples were obtained from calves 1 and 2 and total genomic bovine DNA was isolated from both samples by standard methods (Liovic *et al.*, 2001). Human keratin *K5* and *K14* sequences were compared with a bovine DNA database and two equivalent bovine *K5* gene sequences (*bKRT5*) were obtained (AY740402 and Z32746) and combined. The resulting sequence was 88.2% identical to human *K5* at the mRNA level and 94.7% homologous at the

Fig. 3. TEM image of skin from Calf 1, showing basal keratinocyte cytolysis and cleft formation, with splitting of keratinocytes from the BMZ $\times 7,000$. Bar, 1.4 μm .

protein level. However, human *K14* sequences only identified a large (900 Kb) bovine chromosome contig (NW931763) and further bioinformatic analysis was used to identify bKRT14 within this region. The bovine K14 sequences showed 85.8% homology at the mRNA level and 93.8% homology at the protein level to the human *K14* sequence. Bovine oligonucleotide primers were designed for each gene (*bKRT5* and *bKRT14*). These primers were used to amplify PCR products for each exon of both genes from both calves and these were directly sequenced using standard automated technology (Han *et al.*, 2006) and the results compared with the database reference sequence. With the exception of a few polymorphisms, the two calves under investigation did not show any variance from the normal sequence in exons 1–8 of bKRT5 or 1–8 of bKRT14.

In previous reports of bovine EB-like conditions Bassett (1987) described histopathological findings of dermo-epidermal separation in follicular and inter-follicular areas, with the basement membrane remaining attached to the dermal aspect of the zone of separation and areas of cytolysis in the stratum basale. Since no lesions with intact bullae were observed, the condition was designated bovine epidermolysis, but no ultrastructural studies were undertaken to define the site of primary cleavage. EBS was diagnosed on the basis of histological findings in a Simmental-cross Red Holstein calf in Switzerland (Stocker *et al.*, 1995) and a Charolais cross Limousin calf in Denmark (Agerholm, 1994), but no ultrastructural studies were performed in these cases. New Zealand Kiwi-cross calves with EBS had variable cleft formation in the skin at the BMZ (Ford *et al.*, 2005). Clefting above the BMZ was subsequently observed by

EM, along with some clumping of tonofilaments in basal cells, similar to cases reported by Jolly *et al.* (1973). In previous case reports, ultrastructural observations have only been reported in cattle from New Zealand (Jolly *et al.*, 1973; Ford *et al.*, 2005). In our cases the stratum spinosum was not affected and there were no eosinophilic inclusion bodies or clumping of tonofilaments, which were seen in cattle with EB in New Zealand (Jolly *et al.*, 1973; Ford *et al.*, 2005).

Given the lack of mutations in bKRT5 and bKRT14, the genetic defect may be present in other proteins; indeed, mutations in two proteins (integrin $\alpha 6\beta 4$ and plectin) have been reported as causal in humans (Jonkman *et al.*, 2002; Koss-Harnes *et al.*, 2002), so the cause of EBS in these calves may lie in the genes encoding these other proteins.

The clinical, histological and ultrastructural findings in eight calves examined in this study were consistent with EBS. Further studies are warranted to clarify the genetic mutation involved in this condition.

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