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INFECTIOUS DISEASE

Histopathological Characterization of the Lesions of Contagious Ovine Digital Dermatitis and Immunolabelling of *Treponema*-like Organisms

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Summary

Contagious ovine digital dermatitis (CODD) is a cause of severe lameness in sheep and the three *Treponema* phylogroups *Treponema medium*/*Treponema vincentii*-like, *Treponema phagedenis*-like and *Treponema pedis* have been associated with clinical disease. The aims of this study were: (1) to describe the histopathological changes associated with each previously established grade of clinical lesion, and (2) to investigate immunohistochemically the association of the *Treponema*-like organisms with the observed histopathological changes. Early lesions were characterized by lymphoplasmacytic infiltration of the distal digital skin, with suppurative coronitis and intracorneal pustules. In more advanced stages of the disease there was complete separation of the dorsal wall of the hoof with a necrotizing and fibrinosuppurative exudate and dermatitis. The later lesions were mostly resolved, but with milder suppurative changes remaining within the cornified layer and periosteal reaction of the dorsal aspect of the distal phalanx. Large numbers of *Treponema*-like organisms were identified within early grade lesions (as well as later, more advanced grade lesions) and were specifically associated with the observed histopathological changes. The results of this study provide some evidence in support of the hypothesis that the three CODD-associated *Treponema* phylogroups are involved in the aetiopathogenesis of this disease.

Keywords: contagious ovine digital dermatitis; histopathology; sheep; *Treponema*

Introduction

In 1997, a newly emerged disease of the ovine digit was observed in the UK, the lesions of which led to severe clinical signs in affected animals (Harwood *et al.*, 1997). The distinction between this disease and ovine foot rot was made by the differences in clinical appearance, the failure to respond to conventional foot rot therapies, and the isolation of *Treponema* spp. from the lesions, with and without concurrent detection of the causative agent of foot rot, *Dichelobacter nodosus* (Naylor *et al.*, 1998; Davies *et al.*, 1999; Lewis *et al.*, 2001). The disease was named contagious ovine digital dermatitis (CODD) (Davies *et al.*, 1999) and further work has since identified three *Treponema* phylogroups to be associated with clinical disease, namely *Treponema medium*/*Treponema vincentii*-like, *Treponema phagedenis*-like and *Treponema pedis* (Sullivan *et al.*, 2015). These same three *Treponema* phylogroups are reported to be the cause of bovine digital dermatitis (BDD) (Evans *et al.*, 2008), although not all BDD lesions may necessarily contain all three phylogroups (Evans *et al.*, 2009).

Recently, the clinical presentation of CODD was described in detail for the first time, and a five-point lesion grading system was proposed (Angell *et al.*,

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2015). In that study, the range of lesions was organized into a clinical progression of disease such that lesions progressed from grade 1 to 5. Briefly, the disease begins at the coronary band and extends distally, resulting in progressive, and eventually total, separation of the dorsal hoof wall. The lesions may resolve and new horn eventually replaces the horn lost in the fulminant stage of the disease, with some deformity of the regrown hoof. To date, there has been no histopathological characterization of these lesions or immunohistochemical investigation of the presence of *Treponema* spp. associated with the pathological changes.

In cattle, immunohistochemistry (IHC) has been used to identify *Treponema*-like organisms in BDD lesions, and these are associated with hair follicles and sebaceous glands (Evans *et al*., 2009). In the present study, IHC was used to investigate the presence/absence of CODD-associated *Treponema* spp. It was considered that *Treponema* spp. would more likely be identified in earlier grades of lesion, particularly those of grade 1. The organisms may persist throughout all of the stages of the clinical disease, but might not be identifiable due to competition by secondary invaders, tissue necrosis or damage/removal by the immune system.

The aims of this study were: (1) to describe the histopathological changes observed for each grade of clinical lesion and to investigate whether these grades reflected a progression of disease, and (2) to investigate whether the spirochaetes of the three phylogroups, *T. medium*/*T. vincentii*-like, *T. phagedenis*-like and *T. pedis*, were associated with clinical disease.

Materials and Methods

The study protocol was approved by the University of Liverpool Ethics Committee (VREC 13).

Sampling

In a previous study, a novel five-point lesion grading system was proposed to describe clinical cases of CODD (Angell *et al*., 2015). In that study, clinical cases deemed typical of each lesion grade were obtained and the digits were sampled *post mortem* for further radiographic analysis. These digits were removed immediately *post mortem* and placed in 10% neutral buffered formalin. These same digits were used in the current study. In addition, two sheep were purchased from a farm with no clinical history or evidence of CODD and digits from these were used as negative controls, together with interdigital skin from the footpad of a dog with exudative interdigital pyoderma.

Fifteen digits were used, including the two normal digits from the two negative control sheep, a clinically normal digit from a sheep that had CODD in other digits in other feet, five digits with lesions of clinical grade 1, two digits with grade 2 lesions, two digits with grade 3 lesions, one digit with a grade 4 lesion and two digits with grade 5 lesions.

Histopathology

Each digit was sectioned sagittally to obtain a longitudinal slice of approximately 3–5 mm incorporating hoof horn, phalangeal bones and soft tissues up to and including the proximal phalanx. In all cases the following tissue samples were obtained (Fig. 1): (1) distal digital skin including the coronary band, (2) dorsal horn with laminae, (3) solar horn with laminae, and (4) the distal phalanx. These tissues were embedded in paraffin wax. Blocks containing horn underwent a softening pretreatment with hair

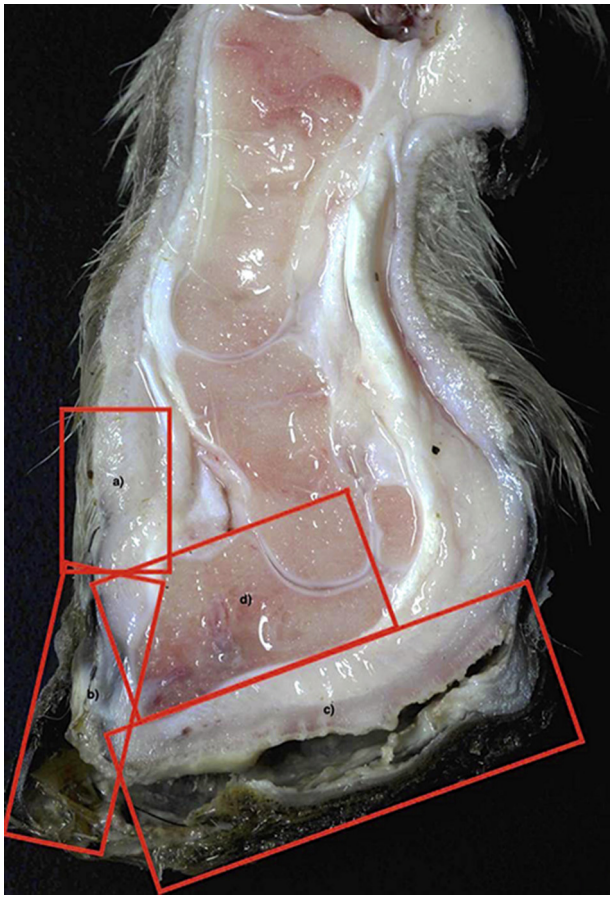


Fig. 1. Sagittal section from an ovine foot with CODD, illustrated to indicate the sites from which tissue samples were obtained: (a) distal digital skin including coronary band; (b) dorsal horn with laminae; (c) solar horn with laminae and (d) the distal phalanx.

Table 1
Summary of lesions in each of the five clinical grades of CODD

Clinical grade	Treponema spp. IHC	*Distal digital skin*	*Coronary band*	*Dorsal hoof wall*	*Solar horn*	*Distal phalanx (P3)*
0		Mild, multifocal perivascular lymphoplasmacytic dermatitis.	NAD*	NAD	NAD	NAD
	TM†	–	–	–	NE	–
	HEL‡	–	–	–		–
1		Mild to severe multifocal, lymphoplasmacytic dermatitis. Moderate to marked epidermal hyperplasia.	Moderate to severe lymphoplasmacytic and mild to marked suppurative coronitis; intracorneal pustules; numerous colonies of coccobacillary and spirochaetal bacteria.	Mild focal suppurative dermatitis to severe suppurative infiltration of the horn and subcorneal epidermis. Spirochaetes within dorsal horn.	Mild lymphoplasmacytic dermatitis.	NAD
	TM	–	+ + +	+ + +	NE§	–
	HEL	–	+ + +	+ + +		–
2		Moderate multifocal, lymphoplasmacytic dermatitis. Marked epidermal hyperplasia.	Complete loss of the superficial cornified layer, fibrinopurulent exudate, necrotic debris. Lymphoplasmacytic dermatitis.	Complete separation of horn, replaced by necrotic debris, marked suppurative infiltrate of epidermal and dermal laminae. Colonies of coccobacillary and spirochaetal bacteria. Lymphoplasmacytic dermatitis.	Mild lymphoplasmacytic dermatitis. One foot shows loss of horn and suppurative dermatitis.	Activation of the cambium layer.
	TM	–	–	+ + +	NE	–
	HEL	–	–	+ + +		–
3		Mild, multifocal perivascular lymphoplasmacytic dermatitis. Marked epidermal hyperplasia.	Mild, multifocal perivascular lymphoplasmacytic coronitis; intracorneal pustules.	Complete separation of horn, replaced by necrotic debris, marked suppurative infiltrate of epidermal and dermal laminae. Colonies of coccobacillary and spirochaetal bacteria. Severe lymphoplasmacytic dermatitis.	NE	Activation of the cambium layer with mild to moderate new bone growth dorsally.

	TM	–	–	+	NE	–
	HEL	–	–	+ + +		–
4		Mild, multifocal perivascular lymphoplasmacytic dermatitis. Marked epidermal hyperplasia.	NAD	Severe necrotizing fibrinosuppurative infiltration of superficial cornified layer. Hyperplastic epidermal laminae. Colonies of coccobacillary bacteria. Granulation tissue formation.	Mild lymphoplasmacytic dermatitis	Multifocal periosteal projections from the dorsal surface.
	TM	–	–	+	NE	–
	HEL	–	–	+ + +		–
5		Mild, multifocal perivascular lymphoplasmacytic dermatitis. Mild epidermal hyperplasia.	NAD	Largely intact, multifocal small intracorneal pustules.	NAD	Dorsal surface of P3 mild to moderately uneven with numerous small bony spicules.
	TM	–	–	–	NE	–
	HEL	–	–	–		–

The *Treponema* spp. immunolabelling patterns are: -, no labelling; +, mild labelling; + +, moderate labelling; + + +, strong labelling. Immunolabelled organisms with distinct spirochaetal morphology were present in the largest numbers at the coronary band and dorsal hoof horn in grade 1 and the dorsal hoof horn in grade 2; fewer organisms were observed in the dorsal hoof wall for grades 3 and 4. Strong homogeneous extracellular immunolabelling was present in the dorsal hoof horn from grades 1 to 4. Immunolabelling was absent from grade 5 and the negative controls (grade 0).

[*]NAD, no abnormalities detected.

[†]TM, *Treponema* spp. morphology clearly observed.

[‡]HEL, homogeneous extracellular immunolabelling.

[§]NE, not examined.

removal lotion (Nair Hair Removal Lotion; Church & Dwight, Folkestone, UK) and blocks containing bone were decalcified using a decalcification product (Calci-Clear; National Diagnostics, Hessle, UK), respectively. These were then sectioned into 5 µm slices and stained with haematoxylin and eosin (HE). A selection of tissues from different digits with necrosuppurative lesions with numerous bacterial colonies were also stained by the Warthin–Starry method.

Immunohistochemistry

IHC was employed to investigate the presence of the three CODD-associated *Treponema* spp. in tissue samples using a polyclonal antibody raised in a rabbit (Evans *et al.*, 2009). Sections were dewaxed using a series of xylene and ethanol baths followed by pretreatment with H_2O_2 in methanol (6 ml of 30% H_2O_2 in 360 ml of methanol) to block endogenous peroxidase activity. Antigen retrieval was performed using bacterial proteinase type XXIV (Sigma; Poole, Dorset, UK) for 5 min before blocking with normal goat serum for 10 min. The primary antibody (1 in 4,000 dilution) was incubated overnight and then slides were washed and incubated with secondary goat anti-rabbit antibody (1 in 100 dilution) for 30 min before being washed again. An avidin–biotin complex method was used to detect binding. The slides were then counterstained with haematoxylin. Following optimization and examination under low power to survey tissue areas, a grading system of no labelling (0), mild (1), moderate (2) or strong (3) labelling was used to give an indication of the varying intensity of the *Treponema* spp. antigen detection. None of

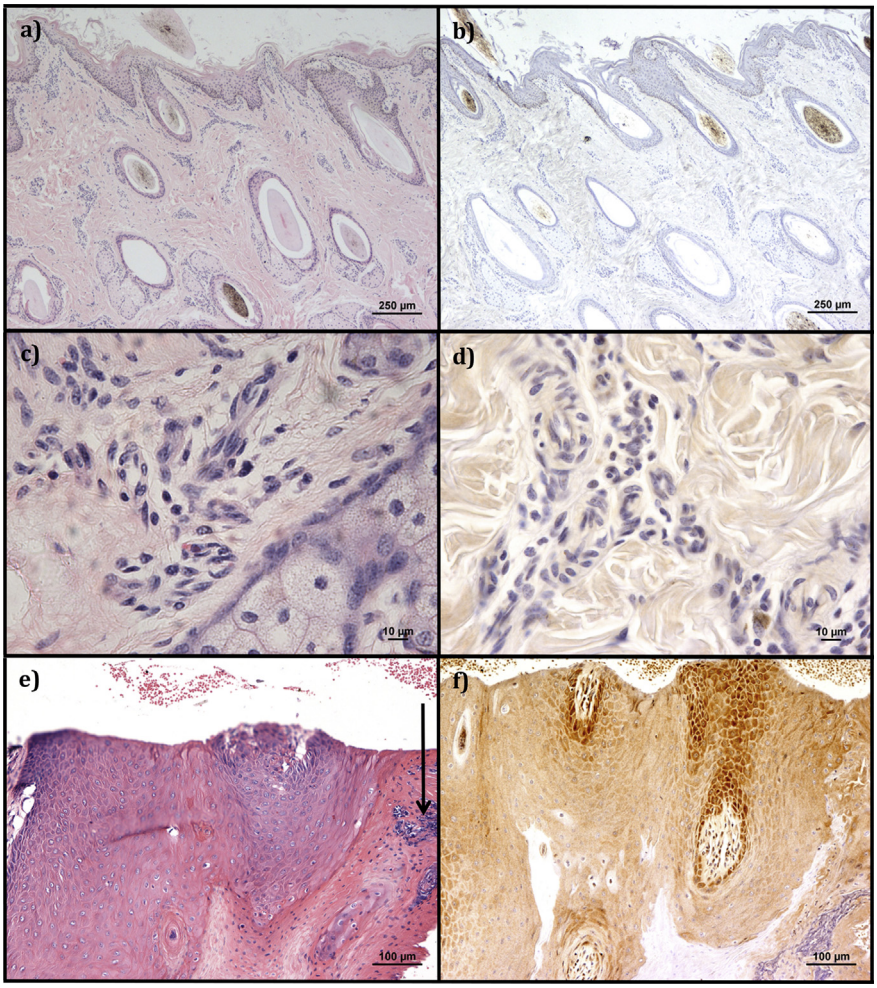


Fig. 2. Skin from a clinically unaffected sheep from a farm without CODD (a–d) and tissue from canine interdigital skin with exudative pyoderma (e and f). HE (a, c and e); IHC (b, d and f). (a) Mild perivascular and periadnexal dermatitis. (b) Mild background immunolabelling. (c) Perivascular lymphoplasmacytic dermatitis. (d) Mild background immunolabelling. (e) Epidermis of canine interdigital skin with bacterial colonies (arrowed). (f) Intracytoplasmic granular background labelling of epidermal keratinocytes together with unlabelled bacteria.

the control tissues exhibited any positive immunolabelling and the bacteria in the canine skin sample were not labelled.

Results

Details of each lesion are summarized in Table 1; key features from each grade of clinical lesion grade are summarized below.

Clinically Normal Digits and Negative Controls

There was mild eosinophilic and lymphoplasmacytic dermatitis of the distal digital skin. No significant change was observed in the hoof. There were no organisms detected by Warthin–Starry stain and there was no specific immunohistochemical labelling. Representative images are shown in Fig. 2.

Clinical Grade 1

The distal digital haired skin and coronary band exhibited mild to severe, multifocal, periadnexal to perifollicular lymphoplasmacytic infiltration within the superficial dermis, in some cases extending into the deep dermis. In some digits there was moderate epidermal hyperplasia and orthokeratotic and/or parakeratotic hyperkeratosis. Bacterial colonies of variable morphology, with haemorrhage and an infiltrate of neutrophils, were sometimes present at the coronary band. At the coronary band and distally

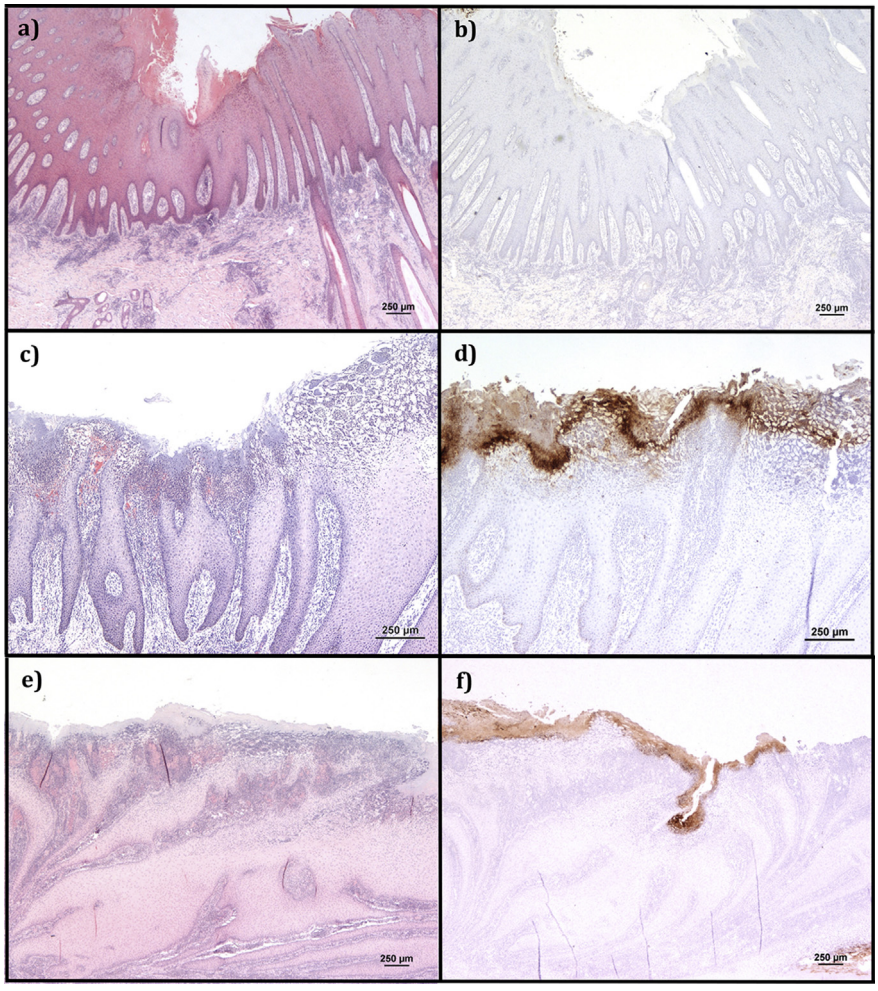
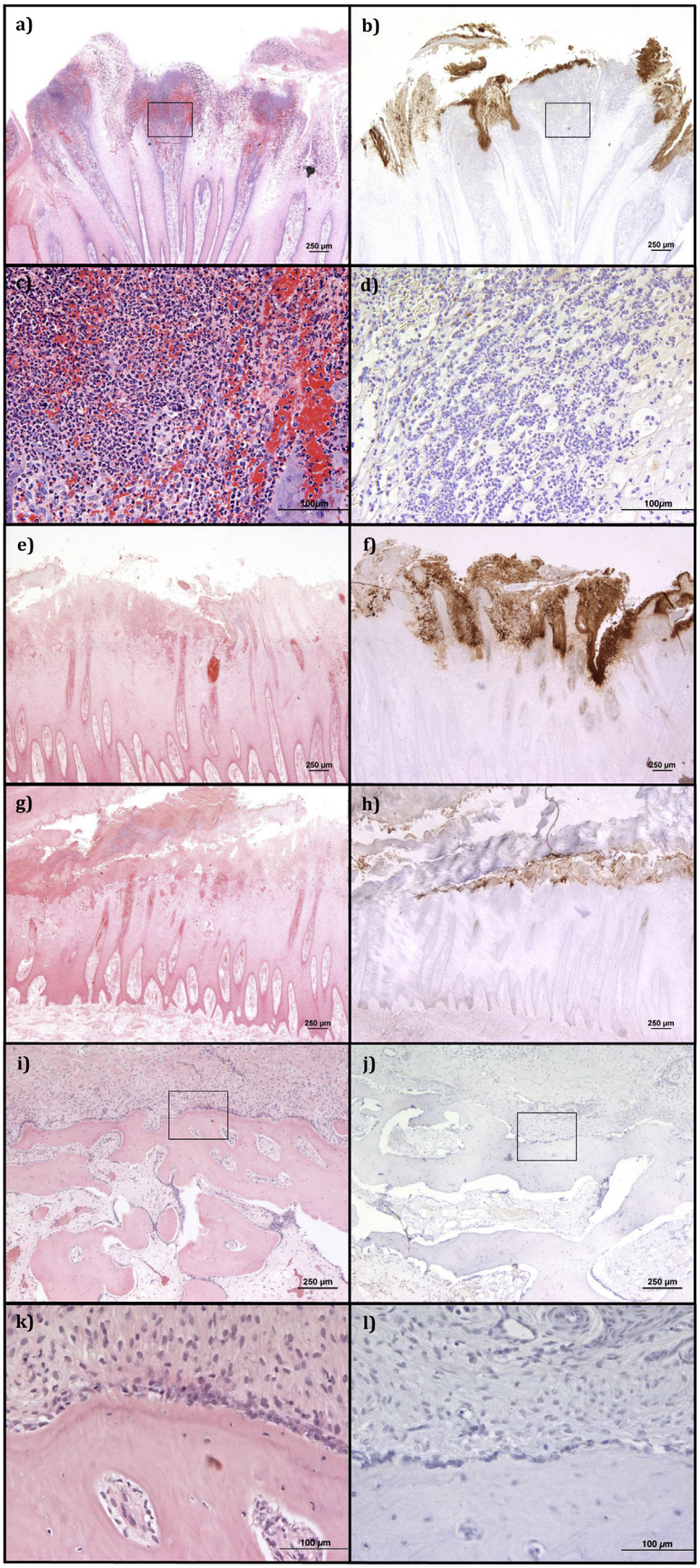


Fig. 3. Tissues from a CODD lesion of clinical grade 1. HE (a, c and e); IHC (b, d and f). (a) Skin/coronary band with moderate, multifocal, periadnexal/perifollicular lymphoplasmacytic dermatitis with hyperplasia and orthokeratotic hyperkeratosis (left of the image). (b) Very mild background immunolabelling in the superficial stratum corneum. (c) Diffuse severe erosion of the dorsal horn with suppurative inflammation, haemorrhage and oedema. (d) Mild extracellular labelling of the exudate with strong labelling of the superficial eroded dorsal horn. (e) Superficial inflammatory exudate and intracorneal pustules in the dorsal horn adjacent to the coronary band. (f) Extracellular labelling of eroded material of skin/dorsal horn at coronary band region, together with labelling within a fissure extending deeper into the epidermis, becoming more intense at the deepest point.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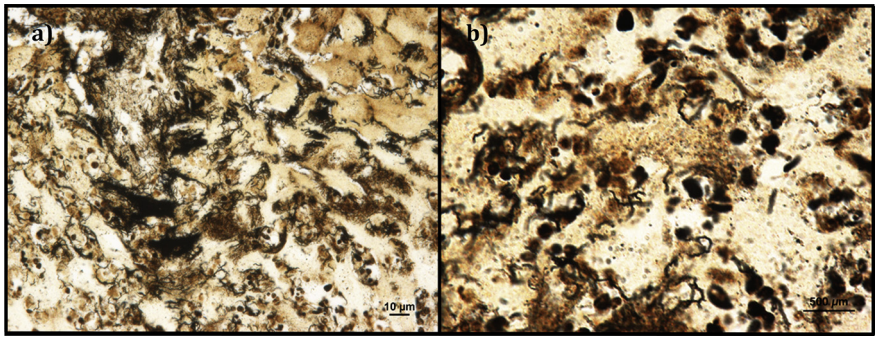


Fig. 5. Tissues from grade 2 and grade 3 lesions stained with Warthin–Starry. (a) Dorsal horn from a grade 2 lesion showing bacteria, many having spirochaetal morphology. (b) Dorsal horn from a grade 3 lesion showing bacteria with clear spirochaetal morphology.

in the superficial dorsal hoof horn there was strong extracellular immunolabelling. Often there was strong and specific immunolabelling of organisms with clear *Treponema* spp. morphology, admixed with the neutrophilic infiltrate, often in the hyperkeratotic regions, at the sites of hoof wall detachment and between the cells of the stratum corneum and stratum spinosum (Fig. 3).

Within the subcorneal epidermal laminae there was mild to severe, multifocal, dermal perivascular lymphoplasmacytic infiltration, sometimes with pustules associated with degenerate keratinocytes. In some digits the proximal dorsal hoof horn (stratum corneum) had become detached from the remainder of the epidermis. Where the horn had separated, the eroded epidermis was lined by a superficial layer of proteinaceous debris, degenerate neutrophils, necrotic keratinocytes and bacterial colonies of variable morphology. The neutrophils extended into the remaining epidermis and were present in the dermal and epidermal laminae.

In the regions of epidermal erosion and suppurative inflammation, superficially there were large, multifocal areas of strong immunolabelling with large numbers of organisms exhibiting clear *Treponema* spp. morphology. These were located extracellularly between the (sometimes sloughed) keratinocytes of the stratum corneum and stratum spinosum. Occasionally, there were small areas of strong extracellular labelling with several organisms exhibiting *Treponema* spp. morphology in the deeper epidermis. Representative images are shown in Fig. 3.

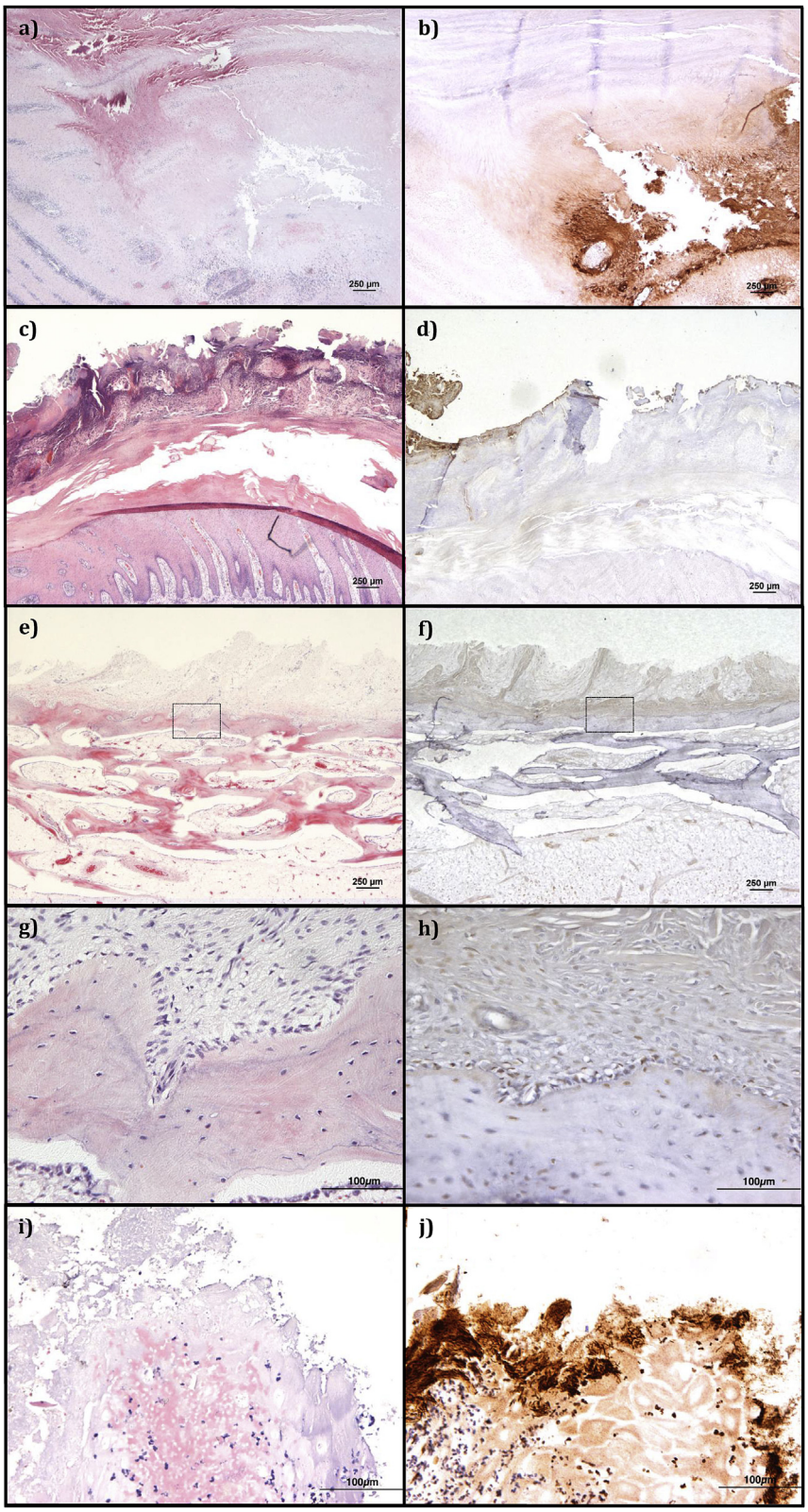
Clinical Grade 2

Within the distal digital haired skin there was moderate to severe epidermal hyperplasia with rete ridge formation and orthokeratotic hyperkeratosis. There was moderate, multifocal to diffuse, lymphoplasmacytic infiltration of the superficial and mid dermis, becoming progressively more severe towards the coronary band. No immunolabelling was observed in the distal digital skin.

There was complete loss of the superficial cornified layer (horn) of the proximal dorsal hoof wall, with replacement by a band of degenerate neutrophils, fibrin, necrotic debris, haemorrhage and numerous colonies of coccobacilli. The epidermal laminae were markedly infiltrated by the suppurative inflammatory process, which extended into the dermal laminae becoming mixed with a moderate to severe lymphoplasmacytic infiltrate. In the superficial stratum corneum there were numerous groups of bacteria with coccoid, rod and spirochaetal morphology.

There were large multifocal to coalescing areas of strong extracellular immunolabelling in the eroded dorsal horn, which extended to the dorsal laminae and was admixed with large numbers of positively-labelled organisms with clear *Treponema* spp. morphology. This was mostly observed superficially

Fig. 4. Tissues from a CODD lesion of clinical grade 2. HE (a, c, e, g, i and k); IHC (b, d, f, h, j and l). (a) Erosion of the dorsal horn with multifocal to coalescing suppurative infiltration and haemorrhage. (b) Extracellular labelling in the exudate and the eroded dorsal horn; the labelling is of greater intensity deeper in the fissures formed in the lesions extending deeper into the epidermis. (c) Higher magnification of the boxed area of (a) showing marked infiltration of neutrophils. (d) Higher magnification of the boxed area of (b) showing very mild background immunolabelling. (e) Erosion of the dorsal horn together with an exudate. (f) Extracellular labelling in the exudate of the eroded dorsal horn, together with labelling within a fissure extending deeper into the epidermis; the labelling is more intense at the deepest part of the fissure. (g) Partial separation of the superficial dorsal horn together with a superficial exudate. (h) Higher magnification of the partial horn separation with immunolabelling between the separated layers where the dorsal hoof wall detaches from the remainder of the epidermis. (i) Periosteal reaction of the distal phalanx. (j) Very mild background immunolabelling. (k) Higher magnification of the boxed area in (i) demonstrating activated osteocytes. (l) Absence of immunolabelling in the area shown in (k).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in areas of suppurative inflammation, but strong extracellular immunolabelling was also found deep within the horn.

In the caudal solar horn and laminae there was background mild lymphoplasmacytic infiltration throughout the superficial dermis. In the more distal sections, the horn was separated from the remainder of the epidermis. Superficially, there was marked infiltration of neutrophils with fibrin, necrotic debris and haemorrhage. The dermal laminae were similarly infiltrated by neutrophils, mixed with lymphocytes and plasma cells. Subjacent to the epidermis was a lymphoplasmacytic band with fewer neutrophils, throughout the superficial dermis and almost obscuring the dermo epidermal junction. There was a distinct boundary (distal to caudal) between the severely affected tissue and relatively mildly affected tissue, which exhibited an intact cornified epidermis and mild lymphoplasmacytic and very mild suppurative dermatitis of the dermal laminae. No immunolabelling was observed in the caudal and solar horn/laminae.

In the distal phalanx, in one digit, the cambium layer of the periosteum subjacent to the dermal inflammation consisted of a continuous row of plump (activated) osteoblast-like cells. There were no organisms detected in the distal phalanx by Warthin–Starry staining and no immunolabelling was observed. Representative images are shown in Figs. 4 and 5.

Clinical Grade 3

The histological appearance was similar to that of the grade 2 lesions, although there was moderate, homogeneous non-specific immunolabelling at the level of the coronary band. The stratum corneum of the dorsal hoof horn was markedly and irregularly thickened where present, but in some areas was eroded or partially detached exposing the subcorneal layers of the epidermis, which was, in some areas, expanded by intracorneal pustules. The eroded tissues and subjacent dorsal laminae exhibited histological changes similar to those of the grade 2 samples, also demonstrating strong extracellular immunolabelling, but there were fewer organisms exhibiting clear *Treponema* spp. morphology.

The solar horn was thickened by moderate diffuse orthokeratotic hyperkeratosis. Within the solar laminae and subjacent dermis there was moderate, multifocal to coalescing lymphoplasmacytic infiltration. There was no evidence of suppuration within the solar horn or associated laminae or dermis.

The periosteum on the dorsal aspect of the distal phalanx was activated with mild to moderate new bone growth dorsally. Within the adjacent dermis there were mild lymphoplasmacytic infiltrates, together with variably sized collections of activated fibroblasts. No immunolabelling was present. Representative images are shown in Fig. 6.

Clinical Grade 4

There was moderate epidermal hyperplasia and orthokeratotic hyperkeratosis of the distal digital haired skin. There was mild multifocal lymphoplasmacytic infiltration of the dermis, but the epidermis of the coronary band appeared unaltered. In the skin and coronary band there were numerous groups of bacteria with mostly coccoid and rod morphology superficially, but no immunolabelling was observed.

The superficial cornified layer of the dorsal hoof horn exhibited extensive areas of marked infiltration by neutrophils admixed with fibrin and necrotic debris, together with proliferation of well vascularized fibrous tissue (granulation tissue) with plump fibroblast and endothelial cell nuclei. Superficially within the suppurative and necrotic areas there were numerous groups of bacteria with mostly coccoid, rod and spirochaetal morphology, and there was strong extracellular immunolabelling and labelling of small numbers of organisms exhibiting clear treponemal morphology. These areas were not as extensive and there appeared to be fewer organisms than in grade 1 and 2 lesions, and there was no involvement of the solar horn and laminae. The dorsal epidermal laminae were hyperplastic and the dermal laminae and sub epidermal dermis exhibited background mild multifocal lymphoplasmacytic infiltration.

Within the connective tissue between the dorsal hoof wall and the distal phalanx, there was moderate, multifocal lymphoplasmacytic infiltration within moderately well vascularized, dense, fibrous connective tissue with plump fibroblast nuclei (mature granulation tissue). The dorsal surface of the distal phalanx was highly irregular with multifocal

Fig. 6. Tissues from a CODD lesion of clinical grade 3. HE (a, c, e, g and i); IHC (b, d, f, h and j). (a) Intracorneal pustule in the dorsal horn. (b) Positive labelling of an intracorneal pustule within the dorsal horn. (c) Erosion of stratum corneum and separation of the dorsal horn containing intracorneal pustules. (d) Very mild labelling of the eroded horn. (e) Periosteal reaction of the third phalanx, with evidence of new bone growth. (f) Mild background labelling of the distal phalanx and surrounding collagen. (g) Higher magnification of the boxed area of (e) showing periosteal reaction of the distal phalanx. (h) Higher magnification of the boxed area of (f) showing mild background labelling. (i) Superficial proteinaceous exudate admixed with neutrophils and bacteria. (j) Extracellular labelling of superficial exudate, with individual organisms and groups of labelled organisms with *Treponema*-like morphology.

projections extending from the bone into the overlying connective tissue. The projections were well mineralized closer to the bone, but more distally were predominantly fibrous, and distally they were poorly demarcated from the surrounding connective tissue and had no periosteal lining. There were islands of osseous tissue separate from the distal phalanx within the fibrous connective tissue. The distal tip of the distal phalanx was partially lost in one of the digits and replaced by fibrous connective tissue. Where present, the periosteal cambium exhibited hyperplastic osteoblast-like cells. No immunolabelling was present in the distal phalanx. Representative images are shown in Fig. 7.

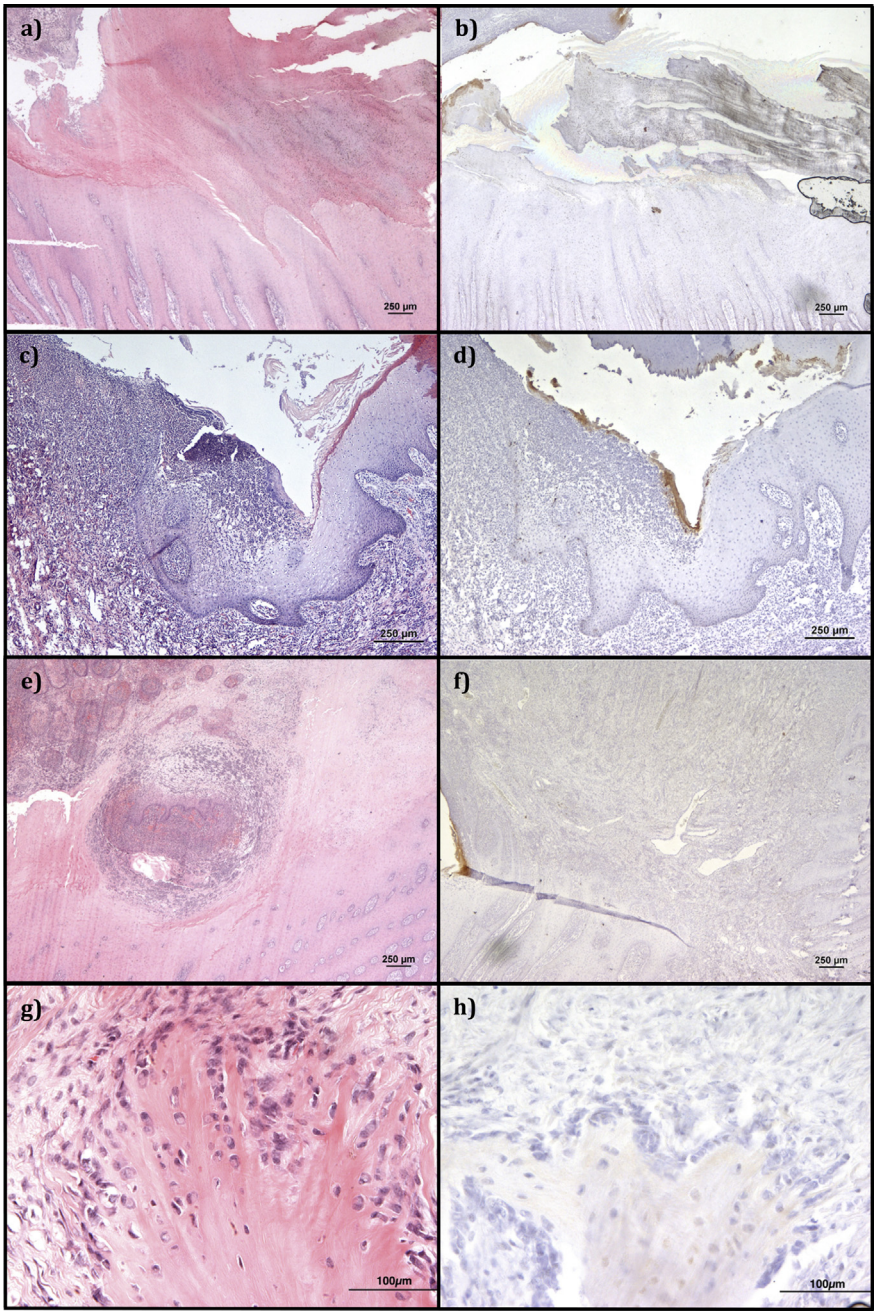

Fig. 7. Tissues from a CODD lesion of clinical grade 4. HE (a, c, e and g); IHC (b, d, f and h). (a) Hyperplastic dermal laminae and granulation tissue of the dorsal horn. (b) Very scant, superficial background immunolabelling of the granulation tissue. (c) Marked suppurative inflammation surrounding a fissure deep within the dorsal horn. (d) Very scant, superficial immunolabelling deep in the fissure in the dorsal horn. (e) Intracorneal pustule in the dorsal horn. (f) Scant background immunolabelling of the tissue. (g) Distal phalanx showing osteoblast-like cells and periosteal reaction. (h) Very scant background immunolabelling of the periosteal reaction.

Clinical Grade 5

In the distal digital skin/coronary band there was a mild diffuse hyperplasia, with mild diffuse orthokeratotic hyperkeratosis together with mild to moderate multifocal lymphoplasmacytic infiltration of the superficial and mid dermis. In the necrotic debris sloughed from the distal skin there were small bacterial colonies mostly with indistinguishable morphology. No immunolabelling was observed in the distal digital skin/coronary band or the sloughed necrotic debris.

The cornified layer of the dorsal horn was largely intact, but exhibited numerous areas of nucleation of corneal keratinocytes. There were multifocal intracorneal aggregates of degenerate neutrophils and necrotic cells admixed with haemorrhage and multifocal small bacterial colonies mostly with coccoid, rod or indistinguishable morphology. The epidermal laminae were shortened, thickened and often fused into wide bands. There was mild to moderate multifocal lymphoplasmacytic infiltration, together with active fibroblasts. No immunolabelling was observed in the dorsal horn.

The dorsal margin of the distal phalanx was mild to moderately uneven with numerous small spicules. The cambium layer of the periosteum contained numerous plump osteoblast-like cells. No immunolabelling was observed. Representative images are shown in Fig. 8.

Overall Summary

There was a background diffuse, mild, perivascular, dermal inflammatory infiltrate of eosinophils, lymphocytes and plasma cells in the majority of sections of distal digital haired skin, which was also present in control animals. In the grade 1 lesions, this was more severe and was mildly suppurative and there were intracorneal pustules within the stratum corneum of the coronary band and proximal hoof wall. In grade 2 lesions, severe distal digital lymphoplasmacytic dermatitis was associated with rete ridge formation and epidermal hyperplasia and hyperkeratosis. The superficial cornified layer of the proximal dorsal horn (i.e. hoof wall) was partially or totally absent and replaced by inflammatory cells, bacteria, protein and associated necrotic debris. In grade 3 lesions, the pathological changes were similar to those of grade 2, but were more severe and extensive (reflecting the clinical grading determined by the extent of horn loss). In grade 4 lesions, the lesions of the distal digital skin and coronary band were chronic and less severe, although the superficial cornified layer of the dorsal hoof wall was still multifocally infiltrated by neutrophils and necrotic debris and there was granulation tissue formation. Similarly in the connective tissue between the hoof wall and the distal phalanx there was lymphoplasmacytic infiltration within the granulation tissue, and on the surface of the distal phalanx there were small mineralized projections. In grade 5 lesions, the skin remained hyperplastic, the laminae were shortened, thickened and fused into wide bands, the dorsal hoof wall was intact, although with multifocal small intracorneal pustules still present, and on the surface of the distal phalanx there were mineralized projections of new bone growth. These findings are summarized in Table 1.

Summary of Immunohistochemical Observations

Immunolabelling was observed, predominantly extracellularly, in lesions graded 1–4. The labelling was strongest in the superficial tissues and associated with the fibrinosuppurative exudates present in areas of separated horn with numerous bacterial colonies, including intracorneal pustules. *Treponema* spp. organisms were labelled and observed, in particular, at the leading edge of the separation of the horn from the underlying epidermis, and in grades 1 and 2 lesions, organisms also tracked between keratinocytes and were occasionally deep within the epidermis. In many sections, immunolabelled bacteria exhibiting distinct, spirochaetal morphology were observed within areas of more strongly immunopositive homogeneous material. The numbers of organisms with *Treponema* spp. morphology was greater in earlier lesions (i.e. grades 1 and 2), while numbers declined in later lesions (i.e. grades 3 and 4) and positive immunolabelling was completely absent in grade 5 lesions. Much of the immunolabelling appeared homogeneous and was concentrated in the areas of necrosis and exudation. Spirochaetal organisms were observed within this material, but in many areas the immunolabelling appeared amorphous in addition to being strongly and specifically positive. This reaction was markedly stronger than the very faint background labelling observed in clinically normal tissues.

Discussion

The sampling strategy used in this study ensured that fresh specimens of clinical cases were obtained and placed in fixative within minutes of death. However, due to on-farm practical constraints and in order to focus resources, this meant that the number of lesions in each grade was small and was not uniform. However, this bias is not considered to invalidate the histopathological description due to the consistent changes noted across the different lesion grades, nor invalidate the immunolabelling due to the consistent

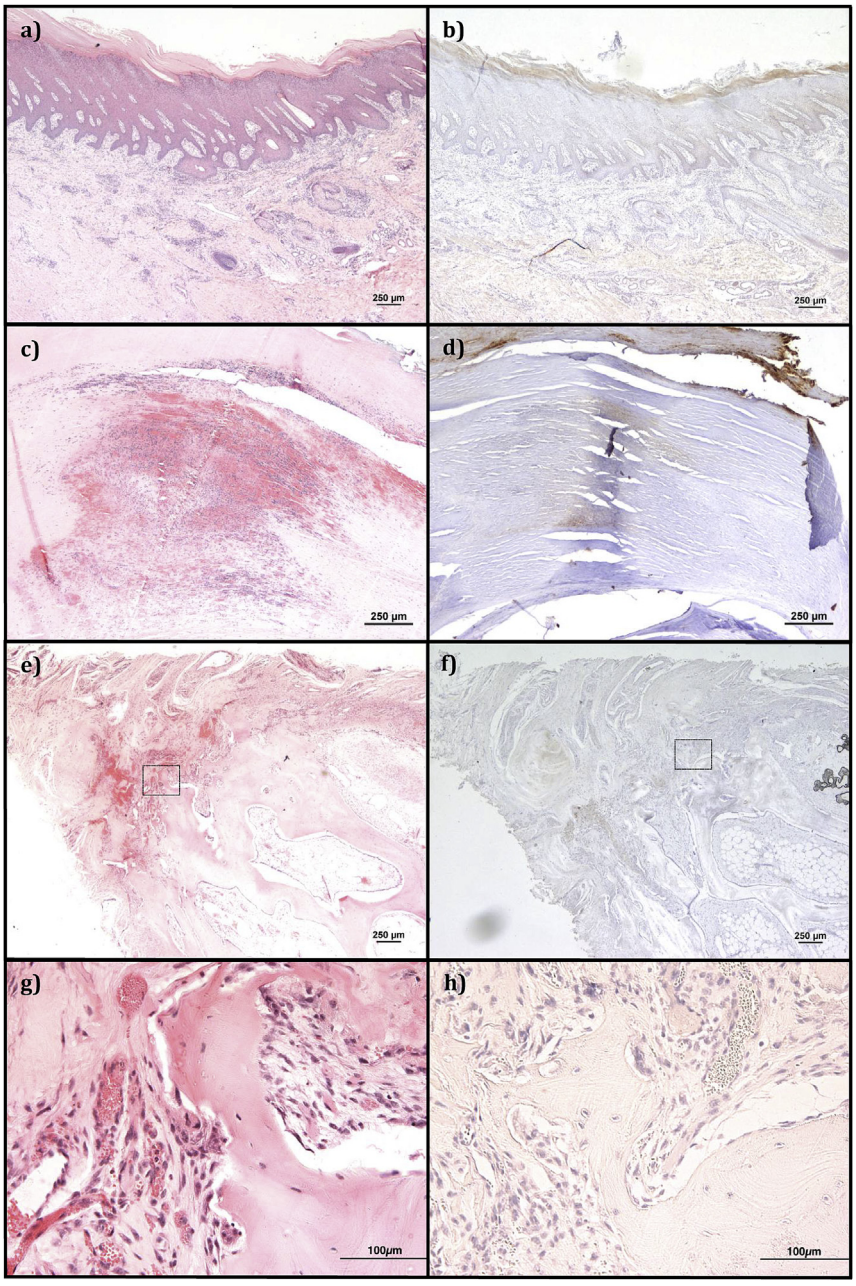

Fig. 8. Tissues from a CODD lesion of clinical grade 5. HE (a, c, e and g); IHC (b, d, f and h). (a) Moderate multifocal chronic lymphoplasmacytic dermatitis of the distal haired skin/coronary band region. (b) Mild background immunolabelling of the superficial stratum corneum. (c) Intracorneal pustule in the dorsal horn. (d) Scant background immunolabelling of the pustule. (e) Periosteal reaction of the distal tip of the distal phalanx. (f) Mild background immunolabelling of the distal tip of the distal phalanx. (g) Higher magnification of the boxed area of (e) showing osteoblasts and osteoclast periosteal reaction. (h) Higher magnification of the boxed area of (f) showing mild background immunolabelling of the distal phalanx.

and predictive pattern of reaction. Attention was focused on the early lesions (i.e. grade 1) as clinically these lesions appear to progress to grades 2–5 (Angell *et al.*, 2015) and it was considered that information from these early lesions would add more in understanding the aetiopathogenesis of the disease. Furthermore, in considering the associations between *Treponema* spp. and the pathological changes present, and their putative role as causal agents, it was considered important to identify whether the bacteria could be detected in early lesions and thus help to determine their role in the aetiopathogenesis of CODD.

In the tissues obtained from the clinically normal negative control animals, there was a mild, perivascular, dermal, mainly lymphoplasmacytic infiltrate. To our knowledge, this 'background infiltrate' has not been reported in sheep before and we are not aware of any similar reports in other species. Indeed, there has been limited published histological investigation of the ovine digit and as such this finding should be interpreted cautiously. It is not necessarily surprising that this part of the skin, being exposed to wet conditions and persistent low-level trauma, should have a low-grade chronic inflammatory reaction present in clinically normal animals. However, in viewing and interpreting the pathological changes described in this paper, this background infiltrate should be borne in mind.

This is the first time to the knowledge of the authors that CODD lesions have been documented histologically and the first time immunohistochemical techniques have been used to determine the presence and location of *Treponema* spp. in ovine feet.

In an earlier paper (Angell *et al.*, 2015), it was suggested that the clinical lesion grades may form a progression of disease such that CODD may initially present as a grade 1 lesion and then progress through the other grades. The histological observations made in this current study appear to support this theory, in that acute changes were first observed at the coronary band, followed by very severe necrotizing and fibrinosuppurative lesions with separation of the horn in tissues from grade 2 and 3. Tissues from grade 4 lesions showed signs of chronicity (e.g. granulation tissue), but with suppurative lesions still present, and grade 5 lesions showed evidence of healing, albeit with mild intracorneal suppurative changes still present in some cases. The histological changes in the low-grade lesions clinically appear to commence at the coronary band and then the lesion appears to progress distally down the dorsal horn while the initial site (coronary band) heals. As such, the clinical and histopathological descriptions from both this current study and the previous clinical description appear well correlated and this may support the use and reliability of the CODD lesion grading system.

In cattle, BDD-associated *Treponema* spp. have been observed in and around hair follicles and sebaceous glands, and these structures have been suggested as a potential route of entry/exit of the bacteria into the tissues (Evans *et al.*, 2009). In the current study, the distal digital skin above the coronary band exhibited progressive lymphoplasmacytic dermatitis with epidermal hyperplasia, but in no cases was the very destructive suppurative lesion present in this location. Furthermore, no *Treponema*-like organisms were observed in hair follicles or sebaceous glands. This may suggest that in sheep the pathological processes target the keratin of the hoof horn more specifically.

In this study it was considered that CODD-associated *Treponema* spp. would be observed in clinically active lesions, but possibly be absent in healed (grade 5) lesions, which appears to be the case in these samples. The immunolabelling showed microorganisms with morphology consistent with *Treponema* spp. predominantly present extracellularly between the keratinocytes. Semiquantitatively, there appeared to be stronger immunolabelling in the earlier lesions (i.e. grades 1 and 2) compared with lesions of grades 3 and 4, and certainly greater numbers of organisms with *Treponema*-like morphology were readily identified in the earlier lesions. When present, these organisms appeared to penetrate for a short distance between the keratinocytes. These findings demonstrate that *Treponema*-like organisms are associated with active lesions, and it may be that they are involved in the initiation and/or propagation of the disease. This was further supported by the absence of *Treponema*-like organisms in healed (grade 5) lesions.

It was beyond the scope of this study to determine the causal nature of the *Treponema*-like organisms identified; however, the distribution within the tissues strongly suggests that they are causally involved to some extent. For example, in Fig. 4 the immunolabelling is stronger at the leading edge of the fissuring in (b) and (f), and in image (e) the immunolabelling is strongly associated with the pathological separation of the superficial dorsal horn from the remaining epidermis. Furthermore, intracorneal pustules were observed in many sections and the examples presented here demonstrate a possible association of *Treponema* spp. immunolabelling with these intracorneal pustules.

In the sections with strong, specific immunolabelling, there were extensive areas of amorphous positively-labelled material, centred on the exudates in the areas of sloughed horn. The *Treponema* spp. organisms were often embedded within this exudate, but the exact nature of the homogeneous labelling is unclear. It could either represent excreted *Treponema* spp. antigen, either free or bound by local tissues, or antigen released as a result of organism degeneration. It is possible that *Treponema* spp. organisms are lysed as a result of enzymes secreted during necrosis and/or inflammation or as a result of the strong host immune response (Dhawi *et al.*, 2005). This detection of *Treponema* spp. antigen in tissues, and the relative absence of intact *Treponema* spp. in later-stage CODD lesions, again supports the hypothesis of a potential key role for the organisms in the initiation of

CODD lesions. The exudate in these lesions appeared to label strongly and specifically with the anti-*Treponema* spp. antibody, and negative control tissues (i.e. unaffected sheep feet and exudative pyoderma from a dog) were negative with only very faint background labelling, which in the case of the dog was also predominantly intracellular.

A further possibility is that in more chronic lesions with avulsion of the hoof horn capsule, the *Treponema*-like organisms are fewer in number due to them being shed with the keratin. Further testing is required in order to investigate these observations more fully.

In conclusion, CODD remains a challenge to the sheep industry. This study documents for the first time the histopathological changes associated with clinical lesions of each grade and provides clear evidence of how the lesions develop as the disease progresses. Furthermore, the presence of *Treponema*-like organisms associated with active lesions adds weight to the hypothesis that these bacteria are involved in the aetiopathogenesis of the disease.

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