Treponema spp. spirochetes and keratinopathogenic fungi isolated from keratomas in donkeys

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Abstract

Keratoma is an aberrant keratin mass thought to originate from epidermal horn-producing cells interposed between the stratum medium of the hoof wall and the underlying third phalanx. The cause is unknown, although the presence of keratomas is frequently associated with chronic irritation, focal infection, or trauma. A total of 167 donkeys with keratomas were presented in this study. Diagnosis of a keratoma was based on clinical signs, radiography, and histopathologic examination. Surgical excision was attempted on all donkeys with lameness unless euthanasia was advised. Histopathologic examination, including Giemsa, periodic acid Schiff (PAS) and Young's silver special histochemical stains were performed and showed the presence of fungal hyphae and spirochete bacteria within the degenerate keratin. PCR for treponeme bacteria was performed on 10 keratoma lesions and 9 healthy pieces of hoof (controls). All healthy donkey tissues were negative for the three recognised digital dermatitis (DD) treponeme phylogroups whereas 3/10 (30%) of donkey keratoma samples were positive for one of the DD treponeme phylogroups. Routine fungal culture and PCR for fungi was performed on 8 keratoma lesions and 8 healthy pieces of hoof (controls). Keratinopathogenic fungi were detected in 1/8 (12.5%) keratomas while only non-keratinopathogenic, environmental fungi were detected in 8 control healthy hoof samples. This is the first time DD treponemes phylogroup and keratinopathogenic fungi have been detected in keratomas. Further studies are required to assess the significance of this finding.

Keywords: donkey, equine, fungi, hoof, hyphae, keratoma, spirochetes, treponemes

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Keratoma is an aberrant keratin mass thought to originate from epidermal horn-producing cells interposed between the stratum medium of the hoof wall and the underlying third phalanx.[11,20,23,41,47] Keratomas have been described as horn cysts or benign horn tumors; however, there is no evidence that they are neoplastic in nature.[20,47] Typically, keratomas are either spherical or cylindrical and are commonly located at the toe region, the quarter of the hoof, and less frequently at the sole or heel with one case report describing a keratoma located at the frog.[3,11,20,31,32,36,47] Single or multiple keratomas affecting one or more hooves in the same horse have been described.[9,19,42,47,48] The cause is unknown but the presence of keratomas is frequently associated with chronic focal irritation, focal infection such as hoof abscesses, or focal trauma.[40,47] A thorough clinical history, in combination with clinical examination, may raise suspicion of a keratoma. Diagnostic imaging including radiographs, ultrasound of the sole (for solar keratomas), computed tomography, and low field magnetic resonance imaging can be valuable in reaching a provisional clinical diagnosis, but histopathologic examination is required for definitive diagnosis.[17,28,29,47] Keratomas have not been studied extensively histologically.[47] Differential diagnoses should include other rare conditions such as hoof neoplasms (e.g. melanoma, squamous cell carcinoma), proliferative pododermatitis (canker), or intraosseous epidermoid cysts of the third phalanx.[22,37,47] Treatment of choice is complete or partial surgical excision which may lead to complete recovery.[6,8] Keratomas have been recognised as a condition in donkeys.[46] The purpose of this study was to describe the histopathologic changes in the hoof and third phalanx of donkeys' feet affected with keratomas.

Treponema spp. are spiral-shaped bacteria of the phylum Spirochaetes. Specific phylogroups of treponemes that include *Treponema medium*, *Treponema pedis,* and

71 *Treponema phagedenis*-like species are particularly associated with the
72 pathogenesis of bovine digital dermatitis (BDD), but have recently been associated
73 with other hoof diseases of domestic and wild animals such as contagious ovine
74 digital dermatitis (CODD) of sheep, hoof diseases of American elk as well as
75 proliferative pododermatitis (canker) of horses.[2,10,12,14,21,34,35,38,44,45,50] To date,
76 *Treponema* spp. have not been identified in keratoma lesions. The significance of
77 detecting BDD-associated *Treponema* spp. from keratomas is discussed in this
78 study.

79 In humans, invasion of keratinopathogenic molds and keratinophilic dermatophytes
80 is regarded as the most important factor in disease of the nail plate, which is called
81 onychomycosis.[24] There is a paucity of information in the literature regarding the
82 significance of keratinopathogenic molds in equine hoof disorders. The isolation of
83 keratinopathogenic molds from keratomas and the significance of this finding is
84 discussed in this study.

85

86 **Materials & Methods**

87 History and clinical presentation

88 All 167 donkeys were from a population of equids living at The Donkey Sanctuary in
89 the South West of England, United Kingdom (UK). Pre-mortem diagnosis of
90 keratoma was based on either clinical appearance alone, or a combination of clinical
91 appearance, radiographic findings, and histopathology results. A characteristic
92 clinical appearance consisted of a soft tissue mass of white-cream coloured keratin
93 on the axial surface of the hoof wall, with varying consistency from soft to hard. The
94 abnormal tissue led to either a bulge in the hoof wall or axial deviation of the white

line dependent on the extent of the lesion within the hoof capsule. Lameness was observed in 133/167 (80%) of cases and in 21/167 (12.5%) of cases the keratomas were associated with infection. 57/167 (34%) of donkeys with keratomas were euthanized due to welfare issues associated with uncontrolled lameness despite treatment.

Radiographic examination

Dorso 60^{o} proximal to palmaro-distal oblique views were taken when radiographic evaluation was performed. A positive radiographic finding was considered to be a radiolucent defect in the solar margin of the distal phalanx with a smooth contour and minimal sclerosis, consistent with a space occupying lesion in the hoof capsule (Fig. 1b).[6,41] That is in comparison to an unaffected third phalanx radiograph where no radiolucent defects were observed (fig. 1a).

Surgical procedure

Surgical excision was attempted in all donkeys with lameness associated with keratoma unless confounding factors led the clinician to advise euthanasia instead of treatment. Usually, keratomas that were subject to surgical excision showed no infiltration into the laminae and were commonly able to be ‘peeled’ away.

Euthanasia

Euthanasia was performed on ethical grounds if there was lameness associated with keratoma and additional confounding factors including concurrent health concerns,

lesions in multiple hooves, or extensive lesions that would lead to severe distortion and failure of the hoof capsule following resection.

Post mortem examination

57 euthanized donkeys were subject to full post mortem examination by a board-certified veterinary pathologist at The Donkey Sanctuary. During post mortem examination, all 4 hooves from each donkey were sectioned in sagittal and multiple transverse planes (fig.2a, b).

Histopathologic preparation

Lesions from the affected hooves of all donkeys with keratomas including transverse sections of the affected hooves in 4 donkeys (fig. 2b), as well as transverse sections of normal/control donkey hooves (fig.2a) were collected either at post mortem examination or during surgical excision of keratomas, fixed in 10% buffered formalin, embedded in paraffin-wax, and stained with hematoxylin and eosin for histopathological examination. In addition, Giemsa, periodic acid Schiff (PAS) and Young's silver special histochemical stains were performed.

PCR detection of infectious lameness-associated bacteria and fungal culture/PCR

For isolation of gDNA (genomic DNA), tissues from healthy donkey tissues and foot lesions were thawed DNA extracted using a DNeasy kit (Qiagen, Manchester, United Kingdom) as detailed in manufacturer's instructions, and stored at −20°C. The donkey lesion gDNA samples were investigated using nested PCR assays, both genus specific for *Treponema* and species specific for each of the three aforementioned recognized BDD treponeme phylogroups using Firepol polymerase (Solis, Estonia) as previously described.[18] Assays used reaction conditions and primers as originally detailed and included an initial universal bacterial *16S rRNA* gene step, followed by the nested genus/species specific assays producing 300-500 bp products. gDNA extractions of the three culturable treponemes and double distilled water were used as positive and negative control material, respectively.[12,33] A *Dichelobacter nodosus* specific PCR assay which amplified a 586 bp region of the *D. nodosus 16S rRNA* gene was also used to assess the samples, as previously described.[44] For *Fusobacterium necrophorum* detection, a species-specific PCR assay was also used which targets the lktA gene, as previously described.[4,44] All PCR assays were analyzed in triplicate. All resulting PCR products were subjected to separation by 1% (w/v) agarose (Biorad, Hemel Hempstead, UK) electrophoresis at 110 V, 400 mA for 40 min and visualised by 0.5 mg/ml ethidium bromide staining and subjected to UV illumination and image recording using a standard gel documenting system.

Healthy controls and lesion samples were routinely cultured on Sabouraud dextrose broth (2% [wt/vol] glucose, 1% [wt/vol] peptone) supplemented with chloramphenicol (1 mg liter^{-1}), subcultured onto Sabouraud dextrose agar slants, and kept at 4°C for fungal culture.

DNA extraction, preparation of the PCR mixture, and post-PCR analysis were carried out in separate rooms using equipment designated for each area to minimize the possibility of specimen contamination.[16]

The fungal strains were inoculated in 1.5-mL Eppendorf tubes containing 0.5 mL of Sabouraud dextrose broth supplemented with chloramphenicol and incubated overnight in an orbital shaker at 150 rpm and 30°C. Thereafter, fungal cultures were adjusted photometrically (absorbance at 530 nm; McFarland 0.5 standard) to a concentration of 1×10^6 to 5×10^6 cells/mL. In the case of filamentous fungi, conidia were separated from the rest of the mycelium by filtration through sterile glass wool.[26] The fungal suspensions with predetermined concentrations were centrifuged at 5,000 × g, and then the pellet was frozen at −20°C for 1 h and incubated at 65°C for 1 h in 0.5 mL of extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). The lysate was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). Then, 65 µL of 3 M sodium acetate and 75 µl of 1 M NaCl were added to 350 µl of the supernatant and the resulting volume was incubated at 4°C for 30 min. DNA was recovered by isopropanol precipitation and washed with 70% (vol/vol) ethanol. The concentration was measured by monitoring the UV absorbance at 260 nm (Gene Quant System; Pharmacia, LKB Biochrom).[16]

Extracted DNA was amplified using a RoboCycler 96 temperature cycles (Stratagene, La Jolla, Calif). The primers used are specified below. PCR amplification was carried out in two steps.[16] The universal primers used for fungal amplification were ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′), which hybridizes at the end of 18S rRNA gene, and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3), which hybridizes at the beginning of 28S rDNA (Life Technologies, Barcelona, Spain).[49]

For the second amplification, the primers used were ITS86 (5′GTG AAT CAT CGA ATC TTT GAA C 3), which hybridizes with the 5.8S rDNA region, and ITS4 (Life Technologies, Barcelona, Spain).[27] PCR products were Sanger sequenced on both strands with the amplifying primers and identification done using www.boldsystems.org.

Results

Radiographic findings

Dorso 60^{o} proximal to palmaro-distal oblique views of the affected hooves were taken in 140/167 (83%) donkeys. Lesions strongly suspected of a keratoma, namely radiolucent defect with smooth contour and minimal sclerosis affecting the solar margin and/or the wings of the third phalanx were noted in 115/140 (82%) donkeys (Fig 1b).

Macroscopic findings

All four hooves of the affected donkeys subjected to post mortem examination were examined and a sagittal section as well as multiple transverse sections of the hooves were made to better reveal the extent of the lesions (Fig. 2a, b). Focally, the hoof wall of one or more feet of the affected donkeys was expanded and replaced by a spherical, cylindrical, or irregularly shaped, pale white or grey to dark grey, and varying in consistency from soft and friable to hard and solid mass that effaced and/or compressed the stratum lamellatum and the laminar corium. Often, compression bone resorption of the third phalanx was present. The mass either

213 affected the whole hoof wall thickness or just the inner part. Similar masses in the
214 solar part of the hoof were less often observed.

215

216

217 Histopathologic findings

218

219 Samples of keratomas from all 167 donkeys were examined histologically. The
220 histopathologic findings in the affected hooves were compared to the normal
221 histologic anatomy of non-affected hooves (Fig.2c,3a, c). Focally, the normal
222 architecture of the stratum medium and often stratum externum of the hoof was
223 effaced and replaced by degenerate laminar keratin, often admixed with nucleated
224 keratinocytes (orthokeratotic and parakeratotic hyperkeratosis) (Fig. 2d). This
225 aberrant keratin mass, which was rarely admixed with moderate to high numbers of
226 neutrophils (suggesting secondary bacterial involvement), compressed the
227 underlying structures, namely the stratum lamellatum, the laminar corium, and the
228 third phalanx (Fig. 2d). Focally there was either loss of differential staining (necrosis)
229 (Fig. 3d) or atrophy, stunting, and fusion of the primary and secondary epidermal and
230 dermal lamellae (fig. 3b). Focally, there was regular epidermal hyperplasia (Fig. 3b).
231 Multifocally, the primary dermal lamellae were infiltrated by low numbers of
232 lymphocytes and plasma cells. Focally, the third phalanx trabecular bone was lined
233 by osteoblasts and fewer osteoclasts (remodelling). There was focal bone lysis of the
234 third phalanx (fig. 2b, d). There was focal, sclerosis affecting the laminar corium
235 (Fig.2d).

Giemsa, Gram, PAS, and Young's silver special histochemical stains revealed numerous filamentous, up to 200 µm long, frequently branching fungal hyphae (fig. 3f) and fewer wavy Spirochete-like bacteria (fig. 3e) measuring 80-100 µm in length. All microorganisms detected were strongly Giemsa and PAS positive and weakly positive with Young's Silver histochemical stain (Fig. 3e, f). Interestingly, no fungal hyphae or Spirochete-like bacteria were detected within the healthy hoof wall.

PCR detection for potential bacterial pathogens and fungal culture

PCRs for bacterial pathogens were performed in 10 keratomas and 9 healthy controls. All donkey samples were negative for both of the ovine scald/ footrot associated pathogens *D. nodosus*- and *F. necrophorum*-specific PCR assays (Tables 1 and 2).

All healthy donkey foot tissues (n=9) were negative for the three recognised DD treponeme phylogroups whereas 3/10 (30%) of donkey keratoma samples were positive for one of the DD treponeme phylogroups (Tables 1 and 2). No lesions contained multiple of the recognised DD treponeme phylogroups. The phylogroup-specific PCR for *T. medium*, *T. phagedenis*, and *T. pedis* DD spirochetes showed they were present in 0/10 (0%), 2/10 (20%), and 1/10 (10%) of donkey keratomas, respectively.

Of the healthy donkey tissues, 4/9 samples (44.4%) were positive with the general *Treponema* PCR whereas 7/10 (70%) of the keratomas were positive for the presence of general treponemes (*Treponema* genus-specific PCR). Culture for treponemes was not attempted.

Fungal cultures were performed in 8 keratomas. In 1/8 (12.5%) keratomas, the keratinopathogenic fungus *Scopulariopsis brevicaulis* was detected (Table 3).[24] In 3/8 (37.5%) keratoma lesions, four fungi of unknown keratin pathogenicity (*Lichtheimia corymbifera, Lichtheimia ramosa, Alternaria sp.,* and *Geotrichum sp.*) were detected.[24] However, *Lichtheimia corymbifera* produces keratinases and *Lichthemia ramosa* has been involved in cutaneous infections in humans.[1,5] In 3/8 (37.5%) keratomas, there was no growth of fungi. In 1/8 (12.5%) keratomas, a non-keratinopathogenic fungus (*Cryptococcus albidus*) was detected. In all healthy hoof samples only environmental, non-keratinopathogenic fungi were detected.

Discussion

Keratomas are rare lesions of horse's hooves but should be included in the differential diagnosis in foot-oriented lameness cases.[17,23,29,36,42,47,48] Although keratomas have been recognized as a condition in donkeys, the associated literature is sparse.[46] To the authors' knowledge, this study comprises the largest number of donkeys with keratomas. A potential reason for this is the fact that donkey hooves are evolved to absorb a vast amount of moisture in order to stay hydrated and flexible in an arid environment.[7] When exposed to long periods of high environmental humidity, as in the UK, donkey hooves absorb excessive moisture and as a result they are prone to recurrent abscess formation, one of the possible causes of keratomas.

Histopathologic examination is the gold standard for diagnosing keratomas; however, they have not been studied extensively. In this study, we aimed for a

concise and thorough histopathologic description of keratomas including pathologic changes of the stratum medium, stratum lamellatum, the laminar corium, and the third phalanx, which can be used as a guide by pathologists presented with hoof masses.

Partial or complete surgical excision, which is the treatment of choice, will typically result in complete recovery. However, in one report there was evidence that post-operative complications such as excess granulation tissue formation, hoof crack formation and recurrence occurred more often in keratomas subject to complete resection than the ones subject to partial resection (71% versus 25%).[6]

The cause of keratomas is unknown, but it is thought that chronic focal irritation, focal infection, such as hoof abscesses, or focal trauma are commonly associated with keratoma pathogenesis.[40,47] In this study, 61 out of 167 (37%) donkeys with keratoma lesions had a history of recurrent hoof abscess or other trauma on the affected foot. Interestingly, in this study, both keratoma and healthy samples were negative for both of the ovine scald/footrot associated pathogens *D. nodosus*- and *F. necrophorum*-specific PCR assays. Similar PCR assays in one paper investigating the same anaerobic bacteria in horses with equine hoof thrush revealed the presence of *Fusobacterium necrophorum* in 1/14 control healthy hooves and 5/14 hooves with thrush, whilst *Dihelobacter nodosus* was not isolated in any of the control or affected hooves.[37] The literature lacks in similar studies in donkeys. Therefore, to date it is unknown if those bacteria are part of the normal hoof flora in donkeys. The involvement of *Treponema* spp. and keratinopathogenic molds as a primary or secondary cause of keratoma has not been previously investigated. BDD-

associated *Treponema* phylogroup spp. have been isolated in 3/10 keratomas, but not from 10 healthy control hoof walls. Keratinopathogenic molds were isolated in 1/8 keratomas whilst only non-keratinopathogenic, environmental fungi were detected in 8 healthy control hoof walls. It is unknown how those microorganisms penetrate to the keratoma. It is believed that BDD-*Treponema spp.* and keratinopathic molds are found on the soil.[14,24,25]Although donkeys at The Donkey Sanctuary farms do not co-graze with any ruminants, sheep graze in some fields when there are no donkeys. Shedding of *Treponema* spp. by those sheep may be a source of soil, and subsequently donkey hoof, contamination. It has been demonstrated that the bovine gut is an important reservoir of microbes involved in bovine digital dermatitis pathogenesis.[15,51] This assumption remains to be proven in equines. In terms of the treponemal species identified here, both are considered serum dependent and to cluster closely on phylogenetic analysis to the agent of human syphilis *Treponema pallidum,* differentiating them from treponemes typically considered as commensal.[13,43] Both species have been implicated in the digital dermatitis of cattle, sheep, and American elk with *T. pedis* recently reported as the most highly associated with equine canker samples.[10.12.30] Of note, recent data suggests that digital dermatitis *T. phagedenis* strains exhibit genetic evidence of pathogenicity islands including a type IV secretion system that differentiate it from human non-pathogenic strains.[43] While further studies are needed to establish the contribution of *Treponema* spp. and keratinopathogenic molds to the pathogenesis of keratomas, the findings of this study are strongly indicative that there is at least secondary involvement in the pathogenesis and prognosis.

Conclusion

To the author's knowledge this is the most detailed and conclusive histopathologic description of keratomas in equines and could be used as a guide for the diagnostic work up of equine hoof masses where keratoma is included in the differential diagnosis. This is the first time BDD-associated *Treponema* phylogroup and keratinopathogenic fungi have been detected in keratomas. Further studies are required to assess the significance of this finding.

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Figure Legends

Figure 1. (a) Donkey hoof, normal radiograph. Dorso 60^{o} proximal to palmaro-distal oblique view. (b) Donkey hoof, keratoma, radiograph. Dorso 60^{o} proximal to palmaro-distal oblique view. Two radiolucent defects in the solar margin of the distal phalanx with a smooth contour and minimal sclerosis, consistent with a space occupying lesions in the hoof capsule.

Figure 2. (a) Donkey hoof, normal, transverse section. (b) Donkey hoof, keratoma, transverse section.(c) Donkey hoof, normal. Subgross, area within dashed square in (a). P3, third phalanx; LC, laminar corium; SL, stratum lamellatum; SM, stratum medium; SE, stratum externum. Hematoxylin and eosin (HE) stain.(d) Donkey hoof, keratoma. Subgross, area within dashed square in (b) showing an irregular mass composed of abundant laminar degenerate keratin (stars) compressing and effacing the stratum lamellatum (SL), and the stratum medium (SM). The third phalanx (P3) is compressed and subject to bone remodelling. There is sclerosis of the laminar corium (LC). HE.

Figure 3. (a) Donkey hoof. Normal histomorphology of stratum lamellatum and stratum medium. PEL, primary epidermal lamellae; PDL, primary dermal lamellae; Star, secondary epidermal and dermal lamellae; T, tubular horn; I, intertubular horn. HE. (b) Donkey hoof, keratoma. Abundant degenerate laminar keratin (star). Stunting, fusion, and distortion of the primary lamellae (closed arrow). Regular epidermal hyperplasia (open arrow). HE. (c) Donkey hoof. Normal histomorphology of stratum lamellatum. PEL, primary epidermal lamellae; PDL, primary dermal lamellae; Star, secondary epidermal and dermal lamellae. HE. (d) Donkey hoof. Loss

of differential staining (necrosis) of the stratum lamellatum. HE. (e) Donkey, hoof, keratoma. Multiple, strongly Giemsa positive, wavy spirochete-like bacteria (arrow) within degenerate keratin, Giemsa stain. Inset; similar bacteria, Young's silver stain. (f) Donkey, hoof, keratoma. Multiple, strongly Giemsa positive, filamentous and branching fungal hyphae (arrows) within laminar degenerate keratin admixed with nucleated keratinocytes, Giemsa stain. Inset; similar fungal hyphae, Young's silver stain.