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

3

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20

21

22 **Abstract**

23 Keratoma is an aberrant keratin mass thought to originate from epidermal horn-
24 producing cells interposed between the stratum medium of the hoof wall and the
25 underlying third phalanx. The cause is unknown, although the presence of
26 keratomas is frequently associated with chronic irritation, focal infection, or trauma. A
27 total of 167 donkeys with keratomas were presented in this study. Diagnosis of a
28 keratoma was based on clinical signs, radiography, and histopathologic examination.
29 Surgical excision was attempted on all donkeys with lameness unless euthanasia
30 was advised. Histopathologic examination, including Giemsa, periodic acid Schiff
31 (PAS) and Young's silver special histochemical stains were performed and showed
32 the presence of fungal hyphae and spirochete bacteria within the degenerate keratin.
33 PCR for treponeme bacteria was performed on 10 keratoma lesions and 9 healthy
34 pieces of hoof (controls). All healthy donkey tissues were negative for the three
35 recognised digital dermatitis (DD) treponeme phylogroups whereas 3/10 (30%) of
36 donkey keratoma samples were positive for one of the DD treponeme phylogroups.
37 Routine fungal culture and PCR for fungi was performed on 8 keratoma lesions and
38 8 healthy pieces of hoof (controls). Keratinopathogenic fungi were detected in 1/8
39 (12.5%) keratomas while only non-keratinopathogenic, environmental fungi were
40 detected in 8 control healthy hoof samples. This is the first time DD treponemes
41 phylogroup and keratinopathogenic fungi have been detected in keratomas. Further
42 studies are required to assess the significance of this finding.

43

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

45

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

69 *Treponema* spp. are spiral-shaped bacteria of the phylum Spirochaetes. Specific
70 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

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

100

101 Radiographic examination

102 Dorso 60° proximal to palmaro-distal oblique views were taken when radiographic
103 evaluation was performed. A positive radiographic finding was considered to be a
104 radiolucent defect in the solar margin of the distal phalanx with a smooth contour and
105 minimal sclerosis, consistent with a space occupying lesion in the hoof capsule (Fig.
106 1b).^{6,41} That is in comparison to an unaffected third phalanx radiograph where no
107 radiolucent defects were observed (fig. 1a).

108

109 Surgical procedure

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

114

115 Euthanasia

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

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

120

121 Post mortem examination

122

123 57 euthanized donkeys were subject to full post mortem examination by a board-
124 certified veterinary pathologist at The Donkey Sanctuary. During post mortem
125 examination, all 4 hooves from each donkey were sectioned in sagittal and multiple
126 transverse planes (fig.2a, b).

127

128 Histopathologic preparation

129

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

137

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

139

140 For isolation of gDNA (genomic DNA), tissues from healthy donkey tissues and foot
141 lesions were thawed DNA extracted using a DNeasy kit (Qiagen, Manchester, United
142 Kingdom) as detailed in manufacturer's instructions, and stored at -20°C . The
143 donkey lesion gDNA samples were investigated using nested PCR assays, both
144 genus specific for *Treponema* and species specific for each of the three
145 aforementioned recognized BDD treponeme phylogroups using Firepol polymerase
146 (Solis, Estonia) as previously described.¹⁸ Assays used reaction conditions and
147 primers as originally detailed and included an initial universal bacterial *16S rRNA*
148 gene step, followed by the nested genus/species specific assays producing 300-
149 500 bp products. gDNA extractions of the three culturable treponemes and double
150 distilled water were used as positive and negative control material, respectively.^{12,33}
151 A *Dichelobacter nodosus* specific PCR assay which amplified a 586 bp region of the
152 *D. nodosus 16S rRNA* gene was also used to assess the samples, as previously
153 described.⁴⁴ For *Fusobacterium necrophorum* detection, a species-specific PCR
154 assay was also used which targets the *lktA* gene, as previously described.^{4,44} All
155 PCR assays were analyzed in triplicate. All resulting PCR products were subjected
156 to separation by 1% (w/v) agarose (Biorad, Hemel Hempstead, UK) electrophoresis
157 at 110 V, 400 mA for 40 min and visualised by 0.5 mg/ml ethidium bromide staining
158 and subjected to UV illumination and image recording using a standard gel
159 documenting system.

160

161 Healthy controls and lesion samples were routinely cultured on Sabouraud dextrose
162 broth (2% [wt/vol] glucose, 1% [wt/vol] peptone) supplemented with chloramphenicol
163 (1 mg liter^{-1}), subcultured onto Sabouraud dextrose agar slants, and kept at 4°C for
164 fungal culture.

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

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

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

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

195

196 **Results**

197 Radiographic findings

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

203

204 Macroscopic findings

205 All four hooves of the affected donkeys subjected to post mortem examination were
206 examined and a sagittal section as well as multiple transverse sections of the hooves
207 were made to better reveal the extent of the lesions (Fig. 2a, b). Focally, the hoof
208 wall of one or more feet of the affected donkeys was expanded and replaced by a
209 spherical, cylindrical, or irregularly shaped, pale white or grey to dark grey, and
210 varying in consistency from soft and friable to hard and solid mass that effaced
211 and/or compressed the stratum lamellatum and the laminar corium. Often,
212 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).

236 Giemsa, Gram, PAS, and Young's silver special histochemical stains revealed
237 numerous filamentous, up to 200 µm long, frequently branching fungal hyphae (fig.
238 3f) and fewer wavy Spirochete-like bacteria (fig. 3e) measuring 80-100 µm in length.
239 All microorganisms detected were strongly Giemsa and PAS positive and weakly
240 positive with Young's Silver histochemical stain (Fig. 3e, f). Interestingly, no fungal
241 hyphae or Spirochete-like bacteria were detected within the healthy hoof wall.

242

243 PCR detection for potential bacterial pathogens and fungal culture

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

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

255

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

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260 Fungal cultures were performed in 8 keratomas. In 1/8 (12.5%) keratomas, the
261 keratinopathogenic fungus *Scopulariopsis brevicaulis* was detected (Table 3).²⁴ In
262 3/8 (37.5%) keratoma lesions, four fungi of unknown keratin pathogenicity
263 (*Lichtheimia corymbifera*, *Lichtheimia ramosa*, *Alternaria sp.*, and *Geotrichum sp.*)
264 were detected.²⁴ However, *Lichtheimia corymbifera* produces keratinases and
265 *Lichtheimia ramosa* has been involved in cutaneous infections in humans.^{1,5} In 3/8
266 (37.5%) keratomas, there was no growth of fungi. In 1/8 (12.5%) keratomas, a non-
267 keratinopathogenic fungus (*Cryptococcus albidus*) was detected. In all healthy hoof
268 samples only environmental, non-keratinopathogenic fungi were detected.

269

270

271 **Discussion**

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

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

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

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

293

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

308 associated *Treponema* phylogroup spp. have been isolated in 3/10 keratomas, but
309 not from 10 healthy control hoof walls. Keratinopathogenic molds were isolated in 1/8
310 keratomas whilst only non-keratinopathogenic, environmental fungi were detected in
311 8 healthy control hoof walls. It is unknown how those microorganisms penetrate to
312 the keratoma. It is believed that BDD-*Treponema* spp. and keratinopathic molds are
313 found on the soil.^{14,24,25} Although donkeys at The Donkey Sanctuary farms do not co-
314 graze with any ruminants, sheep graze in some fields when there are no donkeys.
315 Shedding of *Treponema* spp. by those sheep may be a source of soil, and
316 subsequently donkey hoof, contamination. It has been demonstrated that the bovine
317 gut is an important reservoir of microbes involved in bovine digital dermatitis
318 pathogenesis.^{15,51} This assumption remains to be proven in equines. In terms of the
319 treponemal species identified here, both are considered serum dependent and to
320 cluster closely on phylogenetic analysis to the agent of human syphilis *Treponema*
321 *pallidum*, differentiating them from treponemes typically considered as
322 commensal.^{13,43} Both species have been implicated in the digital dermatitis of cattle,
323 sheep, and American elk with *T. pedis* recently reported as the most highly
324 associated with equine canker samples.^{10,12,30} Of note, recent data suggests that
325 digital dermatitis *T. phagedenis* strains exhibit genetic evidence of pathogenicity
326 islands including a type IV secretion system that differentiate it from human non-
327 pathogenic strains.⁴³ While further studies are needed to establish the contribution
328 of *Treponema* spp. and keratinopathogenic molds to the pathogenesis of keratomas,
329 the findings of this study are strongly indicative that there is at least secondary
330 involvement in the pathogenesis and prognosis.

331 **Conclusion**

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

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345

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496

497 **Figure Legends**

498

499 **Figure 1.** (a) Donkey hoof, normal radiograph. Dorso 60° proximal to palmaro-distal
500 oblique view. (b) Donkey hoof, keratoma, radiograph. Dorso 60° proximal to
501 palmaro-distal oblique view. Two radiolucent defects in the solar margin of the distal
502 phalanx with a smooth contour and minimal sclerosis, consistent with a space
503 occupying lesions in the hoof capsule.

504

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

514

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

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

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