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1   **The aetiopathogenesis of equine periodontal disease – a fresh perspective**

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23   <u>Summary</u>

24   Periodontal disease is a painful and highly prevalent disorder of horses and thus causes a significant

25   welfare problem. Despite its importance, few scientific studies on its aetiopathogenesis have been

26   performed. Equine periodontitis differs from the plaque-induced periodontitis found in brachydont

27   species where bacteria accumulating in dental plaque induce a destructive inflammatory response in

28   the periodontium. In contrast, equine periodontitis is usually initiated by entrapment of feed

29   between cheek teeth which causes inflammation of periodontal tissue that likely allows bacterial

30   infection of the periodontal tissues that is later exacerbated by the host's response. Equine oral

31   microbiology is a neglected field of research and identification of the bacteria involved in equine

32   periodontitis by use of molecular bacteriology and examination of the interaction between these

33   bacteria and the equine oral immune response should reveal important information about the

34   pathogenesis of this disease.

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36   <u>1. Introduction</u>

37   Periodontal disease is increasingly recognised as a common and painful equine oral disorder. With

38   progression of this disease, the tissues surrounding and supporting the tooth are destroyed until

39   eventually the tooth itself may be lost. Earliest recorded observations of equine periodontal disease

40   by Aristotle date back to 333BC (Carmalt 2007) and in the early 1900s, several reports described its

41   clinical features and high prevalence, especially in urban horses (Colyer 1906; Little 1913; Harvey

42   1920). Colyer (1906) also acknowledged its substantial welfare impact describing periodontal

43   disease as 'the scourge of the horse.' More recent studies have shown periodontitis to be present in

44   up to 75% of horses (Baker 1970; Ireland *et al*. 2012) with its prevalence increasing with advancing

45   age. Other recent equine studies have confirmed this disorder to be very painful, causing quidding

46   and weight loss (Dixon *et al*. 2008, 2014). Donkeys are also commonly affected with disease noted

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47   in 23.5% of working donkeys (Rodriguez *et al.* 2013) and 100% of diastemata in one donkey study

48   having associated periodontitis (du Toit *et al.* 2009). This disorder is not limited to domesticated

49   equids, with Penzhorn (1984) noting periodontitis in free-ranging Cape Mountain Zebra.

50   Despite the high prevalence of periodontitis in domesticated horses and the significant welfare

51   problem it causes, the disease may initially go unnoticed by owners because associated clinical

52   signs such as quidding and loss of condition can be absent (Dixon *et al.* 2008; 2014). Although

53   there has been increased recognition of the importance of equine periodontal disease in recent years,

54   very few scientific studies on the aetiopathogenesis of this disorder have been performed. This

55   article aims to critically review current knowledge of this disorder and also outline the potential use

56   of novel methods in investigating its aetiopathogenesis.

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58   ## 2. Equine Periodontal Anatomy

59   The periodontium is a complex, dynamic structure compromised of four separate tissues, i.e. the

60   gingiva, peripheral cementum, periodontal ligament and alveolar bone that interact together to

61   protect and support the tooth. Staszyk and Gasse (2005) described the equine periodontium as

62   having three major supportive functions, firstly to secure the tooth in the alveolus, secondly; to

63   accept a variety of masticatory forces and thirdly; to restore the tooth to its original position after

64   temporary displacement during mastication. The equine periodontium must also adapt to allow for

65   the prolonged eruption of hypsodont dentition and cope with masticatory forces of over 1550

66   Newtons exerted on the caudal cheek teeth for up to 18 hours per day (Huthmann *et al.* 2009).

67   Although it is intuitive to think of peripheral cementum as part of the tooth itself, especially in

68   hypsodont teeth, where it compromises a substantial part of the clinical crown (Mitchell *et al.*

69   2003), it can also be considered as a component of the periodontium due to its distinctive

70   odontogenic development (Stasyzk *et al.* 2015). Throughout the life of the tooth peripheral

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71 cementum is continually produced by cementoblasts and deposited at the apex and around the

72 periphery of the reserve crown, a feature unique to hypsodont periodontium (Stasyzk and Gasse

73 2007). Human cementum has an organic content of around 50% primarily collagen, with small

74 collagen fibrils being produced by cementoblasts and nourished by vessels within the periodontal

75 ligament (Hands 2008). Larger fibrils known as Sharpey's fibres originate from the periodontal

76 ligament and are incorporated into both the peripheral cementum and alveolar bone, flexibly

77 anchoring the tooth into the alveolus (Grant and Bernick 1972).

78 In the horse, the periodontal ligament is a highly vascular and cellular structure which largely

79 consists of collagen fibres, fibroblasts and ground substance interspersed with blood and lymphatic

80 vessels (Staszyk and Gasse 2005). The equine periodontal ligament contains unique vasculature

81 which both nourishes and supports the tooth during mastication and prolonged eruption. Periodontal

82 blood vessels are integrated into the equine periodontal ligament in three distinct ways (Staszyk and

83 Gasse 2004). The Type 1 arrangement of periodontal blood vessel groups are protected from

84 masticatory forces by a sheath of 'veil cells' and connective tissue which is thought to protect blood

85 flow during mastication. An elaborate collagen network anchors blood vessels in the Type 2

86 arrangement, resulting in a functional fibro-vascular unit able to resist forces of traction during

87 mastication. In the Type 3 vascular arrangement, dilated ballooned venules running parallel to the

88 tooth surface between collagen fibre bundles have a cushioning effect, absorbing the substantial

89 forces of mastication. Staszyk and Gasse (2004) also noted presence of elastic oxytalan fibres in the

90 equine periodontal ligament, which allow regeneration and remodelling, whilst improving

91 periodontal blood vessel stability.

92 Matrix metalloproteinase-1 which initiates collagen breakdown to allow remodelling has been

93 detected in the equine periodontal ligament (Warhonowicz *et al*. 2007). As eruption progresses,

94 equine teeth reduce in size and shape (narrowing towards the apex), and in turn the surrounding

95 alveolar bone must constantly remodel order to provide sufficient support to the tooth. Sharpey's

96 fibres in the periodontal ligament insert into a thin, compact layer of "cortical" alveolar bone which

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97   lines the alveolus and is radiographically known as the *lamina dura*. The alveolar bone surrounding

98   this superficial compact layer is more porous which may reflect its constant remodelling (Dixon and

99   du Toit 2011).

100   The gingiva is a firm, keratinised epithelium covering the underlying alveolar bone, periodontal

101   ligament and reserve crown and acts as a physical barrier against oral microbial invasion of the

102   periodontal tissues. It is possible to further classify gingival tissue by its location within the oral

103   cavity and with regards to its position relative to the tooth. Stasyzk *et al*. (2015) describe two

104   distinct zones of equine gingiva: the interdental gingiva also known as the interdental papilla and

105   the remaining bulk of the gingiva located on the buccal, labial, palatal and lingual aspects of teeth.

106   The free gingiva is the most occlusal and mobile aspect of the gingiva and acts as an interface with

107   the epithelium of the gingival (crevicular) sulcus which is a shallow pocket between the tooth and

108   adjacent sulcular epithelium. Junctional epithelium at the base of the sulcus adheres tightly to the

109   peripheral cementum on the tooth surface.

110   Gingival sulcus depth may be between 1- 4mm in periodontally healthy horses (Cox *et al*. 2012)

111   and an increase in sulcar depth indicates the presence of periodontal disease. Gingival crevicular

112   fluid containing antibodies, enzymes and other inflammatory mediators and immune components is

113   secreted into the sulcus, and together with gingival tissue plays an important role in responding to

114   immunological challenges posed by oral bacterial communities. In periodontal health, the gingiva

115   provides a tight seal around the erupted crown and protect underlying structures by forming a

116   mechanical barrier. However in periodontal disease, this barrier is damaged leaving underlying

117   sensitives tissues exposed and open to both mechanical damage and bacterial colonisation. A very

118   complete description of equine periodontal anatomy has recently been published by (Stasyzk *et al.*

119   2015).

120   3. Aetiopathogenesis of periodontal disease

121   *Plaque induced periodontal disease in brachydont species*

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122 Periodontal disease is also of major importance in humans and brachydont domestic animals and

123 consequently has been extensively studied in many brachydont species, often as a model for human

124 disease (Giannobile *et al.* 1994). In brachydont dentition, the initiating factor for gingivitis, which is

125 the earliest and often reversible stage of periodontal disease, is the accumulation of dental plaque in

126 the gingival sulcus, which may eventually become calcified (calculus) (Theilade *et al*. 1966;

127 Mariotti 1999). More specifically, the presence of plaque in the gingival sulcus initiates a bacterial–

128 induced inflammatory reaction (gingivitis) that may or may not proceed to involve the deeper

129 periodontal tissues (Page and Kornman 1997). More severe periodontal disease is most frequently

130 due to the host's response to the bacterial invasion (Bartold and Dyke 2013).

131 *Entrapped food induced periodontal disease in hypsodont species*

132 Plaque induced periodontitis does not appear to be a common problem in horses, unlike in

133 brachydont species (Dixon *et al*. 2000), an exception being the canine teeth, where the presence of

134 calculus can cause gingivitis **(Fig 1)** , but rarely, more severe periodontitis.

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142 **Fig. 1 Calculus on a canine tooth with local gingivitis (arrows).**

143 In equidae, food trapping in anatomical defects such as diastemata between adjacent cheek teeth, is

144 the usual instigator of periodontitis. In a study of referred equine cases with cheek teeth disorders,

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145   periodontal disease in the absence of intercurrent dental disorders, and without the presence of

146   plaque was identified in just 3/349 (0.9%) cases (Dixon *et al*. 1999; 2000).

147   Equine periodontitis is particularly associated with diastemata which can be described as abnormal

148   spaces between adjacent teeth - which should normally be in tight occlusal apposition. Food

149   material becomes impacted into this abnormal space during mastication, often becoming tightly

150   entrapped and initiating inflammation of the underlying gingiva initially, that invariably progresses

151   to the deeper periodontal tissues **(Figs 2 & 3).** Colyer (1906) noted the high prevalence and

152   importance of equine periodontal disease and attributed it to a coarse diet, but Dixon *et al.* (2000)

153   noted that the illustrated specimens of cheek teeth periodontal disease from Colyer's study as

154   illustrated by Miles and Grigson (1990) all had diastemata, and that the reported periodontal disease

155   appears to have been initiated by interproximal food trapping. Little (1913) had previously

156   attributed diastemata as a cause of equine periodontal disease, especially periodontal disease in the

157   interdental (interproximal) spaces adjacent to the mandibular 310 and 410 cheek teeth.
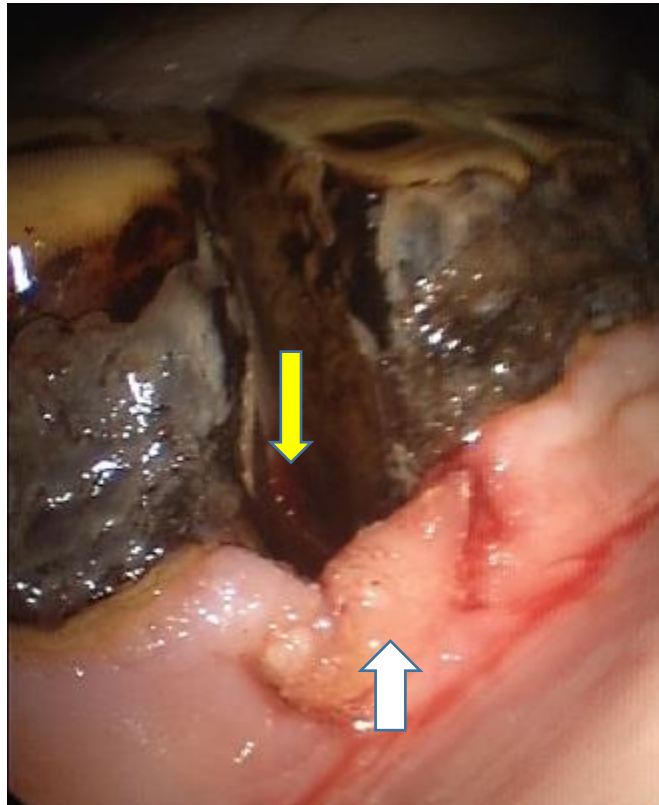
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160   **Fig 2. Post-mortem image of left maxillary cheek teeth showing deep periodontal pockets**

161   **between the mesial 4 cheek teeth with deep periodontal pocketing of feed.**

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Fig 3. Oral endoscopic view of severe equine cheek teeth periodontal disease caused by a diastema. The teeth adjacent to the diastema have caries of the peripheral cementum and are covered in grey-coloured plaque. There is marked loss of periodontal tissues in the interdental space (yellow arrow) but no loss of gingiva which is markedly hyperplastic (white arrow).

Diastemata may be congenital or acquired. Dixon *et al.* (2008; 2014) classified equine diastemata as primary diastemata (where the teeth developed too far apart and/or with insufficient angulation to occlusally compress them) **(Fig 2)** and as secondary diastemata, such as caused by displaced **(Fig 4)** or rotated cheek teeth **(Fig 5)**, dental overgrowths or supernumerary cheek teeth. In addition senile diastemata may form due to the tapering of equine cheek teeth towards their apices resulting in decreased surface area at the occlusal surface, along with age-related loss of angulation of the 06s and 11s **(Fig 6).** Diastemata have been documented in up to 50% of horses in a UK equine practice survey, with feed material becoming trapped in 91.4% of diastemata (Walker *et al*. 2012). In addition, 34% of diastemata had associated gingivitis and 44% were accompanied by periodontal pockets (Walker *et al*. 2012). Impaction of feed material in periodontal pockets has also been

186   recorded in 76% of cheek teeth diastemata in donkeys (du Toit *et al*. 2009), with 71% of these

187   donkeys also having concurrent dental disorders such as displaced cheek teeth, which likely

188   initiated or contributed to diastemata formation. Malerupted (rotated) maxillary cheek teeth have

189   also been described as the primary cause of diastemata formation and associated severe

190   periodontitis (Casey and Tremaine 2010) **(Fig 5).** Voss (1937) suggested that irregular feeding

191   times interrupted salivary flow in horses, which in turn could contribute to the development of

192   periodontal disease.

Fig 4. Secondary diastema. This post-mortem image shows a developmentally displaced (and also curved) cheek tooth with secondary diastemata rostral and caudal distal to it and a deep periodontal pocket on its lingual aspect (arrow). There are also "primary" diastemata between other adjacent teeth.
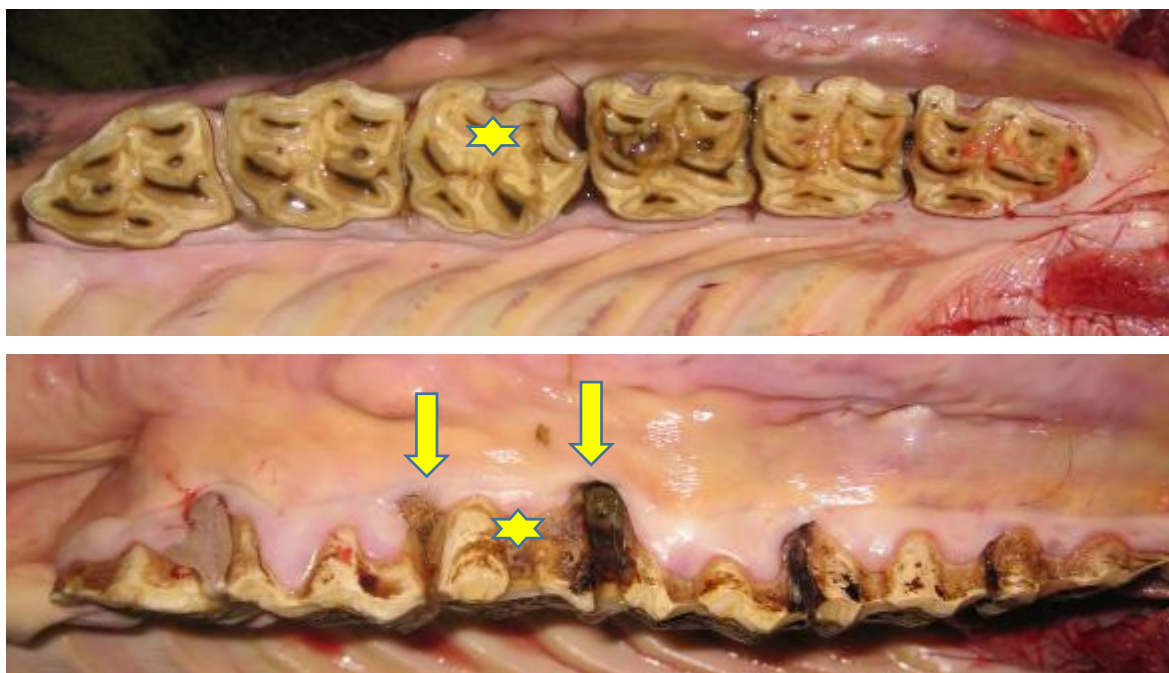
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217 **Fig 5.   These post-mortem images (occlusal aspect –top image: buccal aspect –bottom image)**

218 **show a rotated 208 tooth (yellow star) with a narrow diastema rostrally and wide diastema**

219 **caudally (yellow arrows).**

220

221 **Fig 6. Caudal cheek teeth of a horse with senile diastemata. Note the 209 (yellow star) worn**

222 **down to roots with compensatory cementum deposition and loss of some infundibulae and**

223 **senile excavation in the other two teeth (red stars).**

224

### *Feed Stasis and Bacterial Proliferation*

226   The above described trapping, stasis and subsequent decomposition and fermentation of food

227   material in equine interdental spaces can as noted, abrade the sensitive gingiva, causing mechanical

228   damage and gingival inflammation (Little 1913; Baker 1979; Cox *et al.* 2012). Over time, impacted

229   feed, (a porous foreign body) acts as a bacterial nidus, further supporting the proliferation of

230   bacteria which ferment the trapped feed. The Lactobacillales order of bacteria which include

231   Lactobacilli, Streptococci and Enterococci ferment plant material by anaerobically metabolising

232   carbohydrates to produce lactic acid (Gänzle 2015).

233

Inflammation of the periodontium

235   The initial insult provokes a substantial inflammatory response within gingival tissue due to both

236   mechanical abrasion of sensitive gingival epithelium and bacterial proliferation, as noted earlier.

237   This is apparent clinically as gingivitis with hyperaemia and bleeding upon gingival probing. In

238   man, a number of different pathogenic bacteria are implicated in the induction of a marked host

239   inflammatory response which in turn leads to destruction of the periodontal ligament and resorption

240   of alveolar bone and cementum which can leads to end stage disease, i.e. loss of the tooth. This

241   exaggerated inflammatory response shown in human periodontitis cases is the result of prolonged

242   cytokine production in gingival tissue leading to increased production of proteases, which although

243   can break down invading microbes, can also damage the host's periodontal tissues (Teng 2003).

244   This response may show individual variation, including a genetic component in humans (Yoshie *et*

245   *al*. 2007).

246   Apart from the study of Cox *et al*. (2012) there appears to be no published work on equine

247   periodontal disease histology. These authors showed the mucosal surface of equine periodontal

248   pockets to be hyperplastic, with epithelial disruption and presence of large numbers of

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249 inflammatory cells such as neutrophils in the lamina propria **(Fig 7)** and adjacent connective tissues

250 **(Fig 8)** and destruction of the periodontal tissue including peripheral cementum in which the

251 periodontal membranes were once attached **(Fig 8).**
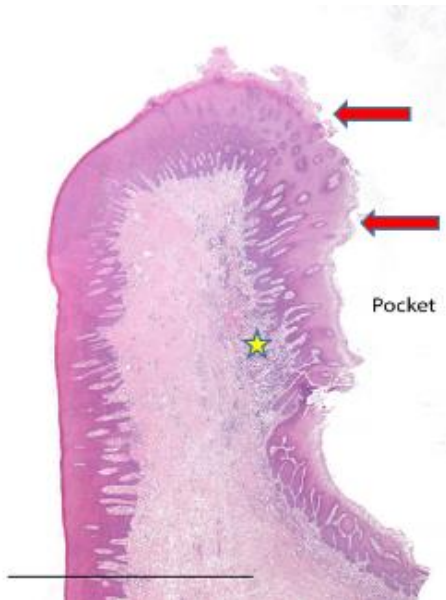
Pocket

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253 **Fig 7.   Histological section of periodontium from a horse with periodontitis. This shows a**

254 **periodontal pocket circa 5mm deep. The calcified dental tissue (i.e. tooth) on the right side of**

255 **periodontal pocket has been lost during decalcification. Moderate gingival hyperplasia (red**

256 **arrows) is present on the gingiva facing the periodontal pocket and at the free gingival margin**

257 **–(as is grossly seen in another horse with periodontitis in Fig 3). There is also modest**

258 **infiltration of inflammatory cells into the lamina propria (yellow star). (bar = 2mm). Image**

259 **courtesy of Dr. A. Cox.**

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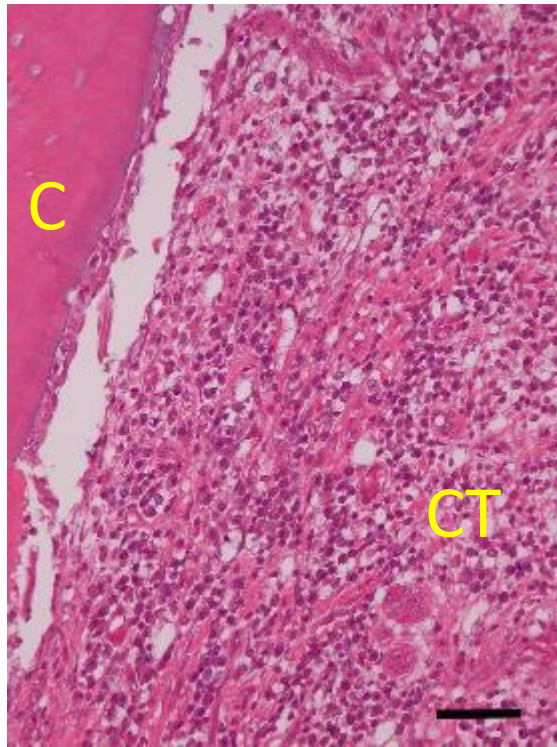
Fig 8.   Massive infiltration of inflammatory cells into interdental subgingival connective tissue (CT) of a horse with periodontitis. The separation of this connective tissue from cementum (C) is artefactual during histological preparation. (bar = 50μm . Image courtesy of Dr. A. Cox.

Accumulations of food material which may be obvious clinically, has also been confirmed on histopathology, alongside large numbers of bacteria and micro abscesses in the submucosa of equine periodontal pockets (Cox *et al*. 2012). In absence of clinical intervention, the disease progresses and inflammation spreads to the periodontal ligament with infiltration of mononuclear cells **(Figs 7 and 8)**. The ligament is gradually destroyed over time, as is the surrounding alveolar bone and cementum **(Fig 9),** decreasing tooth support and further deepening periodontal pockets. Teeth may become mobile at this stage. The increasing depth of the equine periodontal pocket provides the ideal environment for further invasion and proliferation of anaerobic bacteria and the cycle of inflammation and tissue degradation continues until tooth loss occurs.
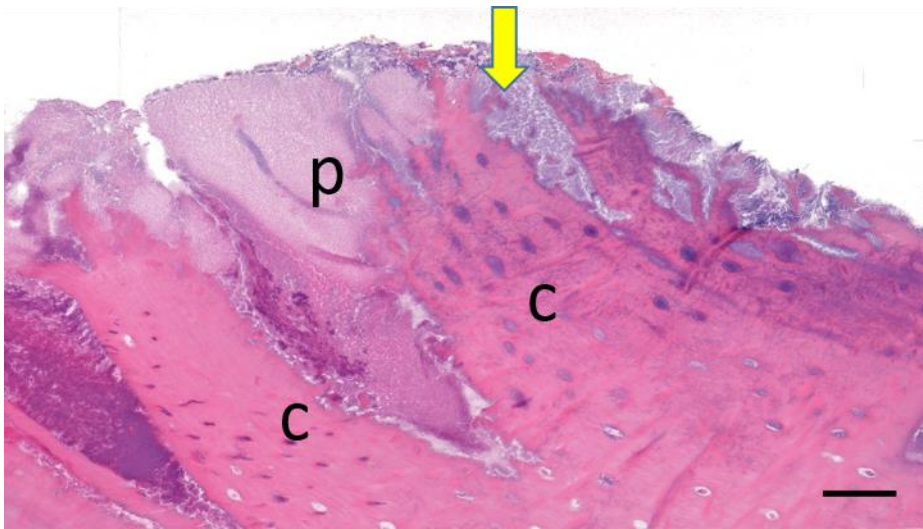
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Fig 9. Disintegration of peripheral cementum (C) of the periodontium due to advanced periodontal disease includes the development of cemental clefts (yellow arrow) that become infilled with plaque (P) –as also grossly seen in another horse with periodontitis in Fig. 3. Image courtesy of Dr A Cox.

4. Oral Microbiology

It is well recognised that bacteria play a major role in the aetiopathogenesis of human (Socransky *et al*. 1998), canine (Hennet and Harvey 1991a; 1991b) and feline (Harris *et al*. 2015) periodontal disease and it is easy to appreciate the potential importance of bacteria in equine periodontitis. This role was recently supported by the histopathological finding of spirochetes in the sulcar epithelium **(Fig 10)** of diseased equine periodontal pockets, which also had cocci on the epithelial surface **(Fig 11)** (Cox *et al*. 2012)**.** The ecological community of bacteria both commensal and pathogenic inhabiting the oral cavity is known as the oral microbiome. There are approximately 700 different species identified to date in human healthy and diseased oral cavities,(Dewhirst *et al*. 2010), even though the human oral microbiome has not yet been fully characterised. This bacterial community is incredibly complex and dynamic, with species interacting with each other and also with the host immune system. In order to survive in an environment being constantly washed with host saliva, in addition to being challenged with mechanical abrasion from masticatory movements, bacteria have found a method of adhering to the surface of oral tissue and so exist in a biofilm.
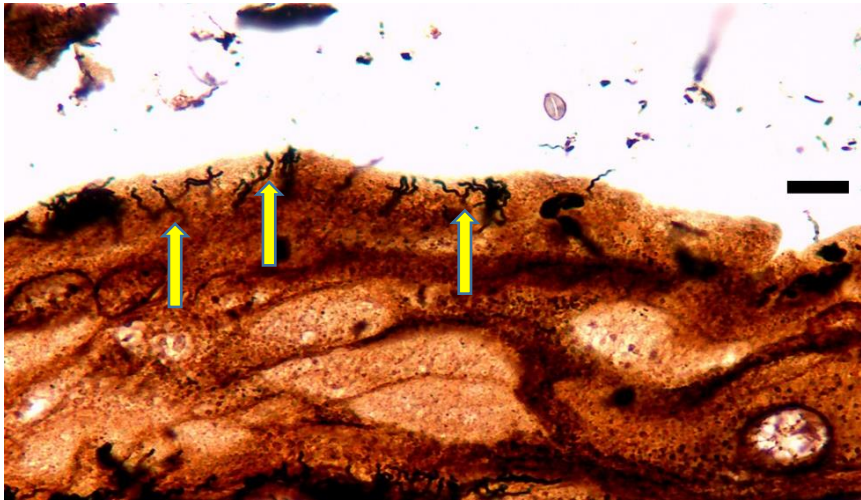
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Fig 10: Spirochaetal bacteria in gingival epithelium of a diseased equine periodontal pocket.

Modified Young's silver stain (bar= 10μm). Image courtesy of Dr A Cox.
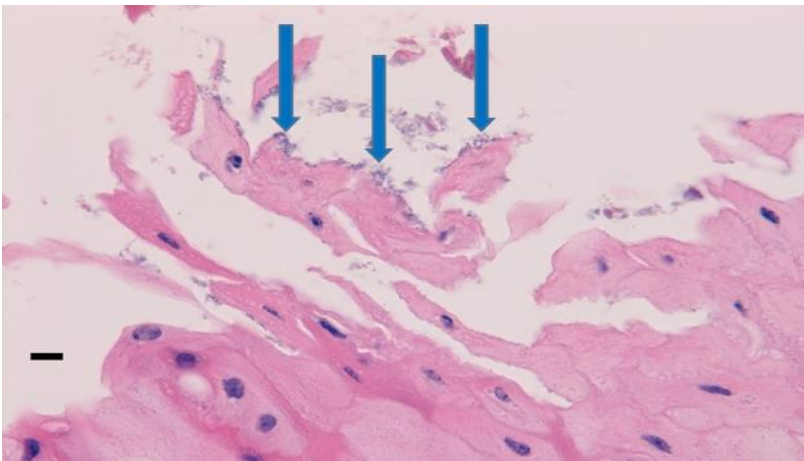
Fig 11: Cocci on the gingival epithelial surface of a periodontitis affected horse. Bar= 10mcm.

Image courtesy of Dr A Cox.

The Oral Biofilm

A biofilm is defined as 'a biopolymer matrix-enclosed bacterial population, adherent to each other

and surfaces' (Costerton *et al*. 1999). Multispecies bacterial communities existing within the oral

biofilm are supported and protected by the surrounding matrix. The composition of an oral biofilm

is very much dependent upon its location within the oral cavity, and thus tooth-associated biofilms

have been divided into two categories in the human mouth. Supragingival biofilms adhere to the

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surface of the clinical crown, and subgingival biofilms adhere below the gum line, either within the

(normal) gingival sulcus or (abnormal) periodontal pocket (Kolenbrander *et al.* 2010). Early

bacterial colonisers which are well adapted to community formation and multispecies growth

(Kolenbrander *et al.* 2010) initially adhere to the salivary pellicle, a layer of proteins and

glycoproteins which permanently coats all normal oral surfaces as recently reviewed by Borkent

and Dixon (2015). Adhesion and subsequent proliferation of early colonisers is followed by co-

adhesion of genetically distinct bacteria to the existing attached population. The salivary pellicle

functions as a defensive layer, lubricating and protecting the surface of the tooth itself (the enamel

surface in brachydont dentition) and surrounding soft tissue (Gibbins *et al.* 2014) and is distinct

from the oral biofilm which attaches to its surface and the subsequent plaque which develops

(Marsh and Bradshaw 1995).

In addition to co-adhesion, distinct bacterial species also interact via cell surface components when

both are suspended in fluid, a process that is termed co-aggregation. For example, strains of

Fusobacterium nucleatum are able to co-aggregate with early and late oral biofilm and may play a

bridging role in the development of human dental plaque (Kolenbrander and London 1993;

Kolenbrander *et al.* 2010). Under certain conditions, the biofilm can become increasingly complex

and mature into dental plaque. The oral biofilm is highly intricate with dynamic microbial

interactions, including complex cell signalling between bacteria of differing genera as well as

transfer of DNA between bacteria. Conjugative transposons which facilitate DNA transfer between

bacteria have been detected in many genera of human oral bacteria such as *Fusobacterium,*

Streptococcus and *Veillonella* (Rice 1998). This is a cause for concern as there is potential for

transference of antibiotic resistance genes between different species of bacteria in the dental

biofilm.

Bacteria within the biofilm matrix can be protected from exposure to host innate and adaptive

immune mechanisms as well as to administered antimicrobial compounds. Front-line immune

responses such as phagocytosis are ineffective in the biofilm matrix as bacterial cells cannot be

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348 readily engulfed at this site (Kharazmi 1991) and infiltration of neutrophils into the plaque may

349 even provide an additional matrix for bacterial attachment in man (Walker *et al*. 2005). Although

350 ineffective in removing the bacterial biofilm, the immune response has a significant side effect on

351 surrounding tissue, stimulating inflammation and often destruction of the human periodontium

352 (Teng 2003). Due to the limitations of the host immune system and of antimicrobial therapy in

353 combating potentially periodonto-pathogenic bacteria within the dental biofilm, mechanical

354 removal of dental plaque is necessary to treat brachydont periodontal disease.

355 *Bacteria in oral health and disease*

356 Current understanding of the role of bacteria in disease is changing and traditional principles such

357 as Koch's postulates are becoming increasingly irrelevant in modern microbiology. Due to the

358 difficulties in culturing some bacteria, it cannot be said that an organism is not involved in disease

359 purely because it cannot be grown in culture. Likewise, to suggest that an organism is not involved

360 in a disease because it may be found in healthy individuals or is unable to replicate disease when

361 inoculated into a healthy individual would be to ignore variations in host-pathogen interactions. The

362 human oral microbiome in health is markedly different to that found in periodontitis lesions (Wang

363 *et al*. 2013). Analysis of the equine oral microbiome, has shown similar findings, with samples

364 from orally healthy horses showing major dissimilarities to samples taken from diseased periodontal

365 pockets (Kennedy *et al*. 2015). This substantial shift in oral microbiota with periodontitis can be

366 interpreted in several ways. The traditional hypothesis of pathogenic (or certain) bacteria occurring

367 in high numbers or solely detected in periodontitis lesions being the cause of the disease appears to

368 be overly simplistic and it is more likely that many factors with complex interactions are involved.

369 Significant local environmental changes occur during the development of periodontal pockets

370 (Loesche *et al 1983)*, which may be especially deep around equine cheek teeth (Cox *et al*. 2012)

371 The anaerobic or partially aerobic environment of deep human periodontal pockets encourages

372 invasion and proliferation of micro-aerophilic organisms, anaerobes and spirochetes, while the

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373   environment of the general oral cavity supports a significantly different microbiota (Loesche *et al*.

374   1983). Another hypothesis which can be applied to the diseased oral microbiome is the *keystone*

375   *pathogen hypothesis* which maintains that certain pathogens existing at low abundance in the oral

376   cavity may modulate their environment, disturbing the normally symbiotic relationship between the

377   oral bacteria , creating a state of *dysbiosis* (dysregulation of commensal oral bacteria) thus

378   contributing to the development of inflammatory disease. *Porphyromonas gingivalis,* is a well-

379   known keystone pathogen in human periodontal disease due to its ability to modulate the host

380   immune system, and thus alter host immune responses to the entire oral biofilm (Hajishengallis *et*

381   *al*. 2012).

382   ***The oral microbiome in health and periodontitis***

383   It has been acknowledged that the equine oral microbiome has been a neglected field of research

384   until recently (Dacre *et al.* 2008; Sykora *et al.* 2014) and the microbiome in equine oral health

385   received little attention. Baker (1979) performed traditional bacterial culture and biochemical

386   identification of orally healthy and periodontitis affected horses and recorded a significant bacterial

387   population shift between oral health and periodontitis. High counts of *Streptococci* and *Micrococci*

388   were detected in orally healthy samples with intermediate counts of *Veillonella* and low counts of

389   *Lactobacillus spp., Fusobacteria* spp. and coliforms. In periodontitis, the predominant genera

390   present were *Streptococci, Fusobacteria* and coliforms. In addition, *Campylobacter* and spirochetes

391   were detected upon direct smears of diseased samples (Baker 1979). In addition, Sykora *et al*.

392   (2014), found *Porphyromonas gingivalis,* Tannerella and Treponema species were more commonly

393   isolated from horses with periodontitis secondary to Equine Odontoclastic Tooth Resorption and

394   Hypercementosis (EOTRH) than in orally healthy horses by using PCR assays of gingival

395   crevicular fluid samples.

396   When analysing any microbiological community, the number and diversity of bacterial species

397   detected is dependent on the method of analysis used (Lozupone and Knight2008). Studies in other

398   species have estimated that around 50% of oral bacteria cannot be cultured by conventional means

399   (Socranksy *et al*. 1963). It is therefore most likely that previous culture studies have vastly

400   underestimated the number and variety of bacterial species present in the equine oral cavity because

401   novel species and uncultivable bacteria would have gone undetected. It is now possible to

402   characterise the oral microbiome using methods which do not rely on culture and can detect novel

403   and uncultivable bacteria. One such method involves high-throughput sequencing of the gene

404   encoding the 16s sub-unit of the bacterial ribosome (16s rRNA) that is useful in assessing the

405   composition of complex microbial communities directly from clinical samples (Song *et al*. 2013).

406   The 16s rRNA gene is universal in bacteria but not found in mammalian cells. It is around 1550

407   base pairs long and consists of nine hypervariable regions (V1-V9) which are between constant

408   regions (Song *et al*. 2013). These constant regions are highly conserved between phyla, thought to

409   be due to the critical importance of the ribosome to basic cell function (Clarridge 2004). Sequencing

410   of hypervariable regions may allow for differentiation up to species level (Chakravoty *et al*. 2007).

411   Most hypervariable regions occur within the first 500 bases (Keller *et al.* 2010) and so sequencing

412   of the whole gene is not required, with read lengths of 500-700 base pairs being sufficient for

413   identification at species level (Clarridge 2004; Paster *et al*. 2006; Song 2013). Whole 16s rRNA

414   sequencing is desirable if a previously unknown species is identified (Clarridge 2004) and it is

415   highly likely that the equine oral microbiome in both health and disease contains many novel and

416   previously uncharacterised species as no prior studies have been published using this technique.

417   A recent high throughput 16s rRNA sequencing study performed by the authors (Kennedy *et al*.

418   2015) has revealed a population shift towards gram negative organisms as well as increasing

419   numbers of spirochetes in horses with periodontal disease, as would be expected. Bacteria

420   belonging to the Prevotella, Veillonella, Treponema and Tannerella genera were found to

421   significantly increase in equine periodontitis lesions (Kennedy *et al*.2015). with some species

422   belonging to these genera being well known human periodontal pathogens (Sykora *et al*. 2014).

423 In addition to high throughput 16s rRNA sequencing to uncover the oral microbiome, the 16s rRNA

424 gene can also be used as a target in PCR reactions, to screen samples for specific bacteria as used by

425 Sykora *et al*. (2014).

426 ## 5. Conclusion

427 Periodontal disease is a common and painful equine disease that is usually induced by the

428 mechanical impaction of food between and around teeth. Further progression of the disease is very

429 likely dependent on invasion of the periodontal tissues by the extremely complex oral bacteria along

430 with the host's immune response to these microorganisms, with a severe host inflammatory

431 response resulting in increased tissue breakdown and progression of the disease. In addition

432 keystone oral bacterial pathogens may alter the host's immune response to other components of the

433 biofilm. Whilst it is clear that feed stasis and subsequent bacterial proliferation play an important

434 role in the initiation and progression of periodontitis in the horse, there is a great need for further

435 studies into the aetiopathogenesis of this disorder. Recent work has given an insight into which

436 bacterial species are present in the periodontal pockets of horses with periodontitis however it is

437 crucial to distinguish which species are important in disease pathogenesis and which simply flourish

438 due to the change in oral environment. In particular, the interaction between bacteria of the

439 diseased equine oral microbiome and the local immune system requires further investigation in

440 order to provide additional insights into the aetiopathogenesis of equine periodontal disease.

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602

603   Legends of Figures

604 Fig. 1 Calculus on a canine tooth with local gingivitis (arrows).

605 Fig 2. Post-mortem image of a left maxillary cheek teeth row showing deep periodontal pockets

606 between the rostral 4 cheek teeth with deep periodontal pocketing of feed.

607 Fig 3. Oral endoscopic view of severe equine cheek teeth periodontal disease caused by a diastema.

608 The teeth adjacent to the diastema have caries of the peripheral cementum and are covered in

609   plaque. There is marked loss of periodontal tissues in the interdental space (yellow arrow) but no

610   loss of gingiva which is markedly hyperplastic (white arrow).

611   Fig 4. Secondary diastema. This post-mortem image shows a developmentally displaced (and also

612   curved) cheek tooth with secondary diastemata distal to it and a deep periodontal pocket on its

613   lingual aspect (arrow). There are also "primary" diastemata between other adjacent teeth.

614   Fig 5.   These post-mortem images (occlusal aspect –top image: buccal aspect –bottom image) show

615   a rotated 208 tooth (yellow star) with a narrow diastema rostrally and wide diastema caudally

616   (yellow arrows).

617   Fig 6. Caudal cheek teeth of a horse with senile diastemata. Note the 209 (yellow star) worn down

618   to roots with compensatory cementum deposition and loss of some infundibulae and senile

619   excavation in the other two teeth (red stars).

620   Fig 7.   Histological section of periodontium from a horse with periodontitis. This shows a

621   periodontal pocket circa 5mm deep. The calcified dental tissue (i.e tooth) on right side of

622   periodontal pocket has been lost during decalcification. Moderate gingival hyperplasia (red arrows)

623   is present on the gingiva facing the periodontal pocket and at the free gingival margin – as grossly

624   seen in another horse with periodontitis in Fig 3. There is also modest infiltration of inflammatory

625   cells into the lamina propria (yellow star). (bar = 2mm). Image courtesy of Dr. A. Cox.

626   Fig 8.   Massive infiltration of inflammatory cells into interdental subgingival connective tissue

627   (CT). The separation of this connective tissue from cementum (C) is artefactual during histological

628   preparation. (bar =50µm). Image courtesy of Dr. A. Cox.

629   Fig 9. Disintegration of peripheral cementum (C) of the periodontium due to advanced periodontal

630   disease includes the development of cemental clefts (yellow arrow) that become infilled with plaque

631   (P) –as also grossly seen in another horse with periodontitis in Fig 3 (bar ?mm). Image courtesy of

632   Dr. A. Cox.

633   Fig 10: Spirochaetal bacteria in gingival epithelium of a diseased equine periodontal pocket.

634   Modified Young's silver stain (bar= 10mcm)Image courtesy of Dr. A. Cox.

635   Fig 11: Cocci on the gingival epithelial surface of a periodontitis affected horse. Bar= 10mcm.

636   Image courtesy of Dr. A. Cox.

637   <u>Authors' declaration of interest</u>

638   No conflicts of interest have been declared

639   <u>Ethical animal research</u>

640   Ethical review not applicable for this review article.

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644