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## **Antibiotic resistance in faecal bacteria isolated from horses receiving virginiamycin for the prevention of pasture-associated laminitis**

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1 **Antibiotic resistance in faecal bacteria isolated from horses receiving**  
2 **virginiamycin for the prevention of pasture-associated laminitis**

3

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24

25

26 Abstract (250 words)

27 *Enterococcus faecium*, a major cause of potentially life-threatening hospital-acquired  
28 human infections, can be resistant to several antimicrobials, such that streptogramin  
29 quinupristin-dalfopristin (Q/D) is one of the few antibiotics still effective.  
30 Consequently use of the streptogramin virginiamycin as an animal growth promoter  
31 was banned in the EU in 1999 as some believed this contributed to the emergence of  
32 Q/D resistant *E. faecium*. Virginiamycin is advocated for preventing equine pasture-  
33 associated laminitis, but its effect on equine faecal bacterial Q/D resistance has not  
34 been determined. Faecal samples were obtained from horses receiving virginiamycin,  
35 horses co-grazing and horses not exposed to virginiamycin. Streptogramin resistant *E.*  
36 *faecium* were cultured from 70% (21/30) of animals treated with virginiamycin, 75%  
37 (18/24) of co-grazing animals and 69% (11/16) of animals not exposed. *ermB* and  
38 *vatD* genes were detected using real time PCR in 63% and 66% of animals treated  
39 with virginiamycin, 75% and 71% of co-grazing animals and 63% and 69% of  
40 animals not exposed. Antimicrobial resistance genes were present only in samples  
41 which had cultured Q/D resistant *E. faecium*. There was no significant difference  
42 between groups with respect to antimicrobial resistance. The gene load of *vatD* was  
43 significantly ( $p=0.04$ ) greater in unexposed animals compared to those treated with  
44 virginiamycin. The use of virginiamycin to prevent pasture-associated laminitis does  
45 not appear to be related to an increased Q/D resistance frequency. However, in view  
46 of the high frequency of resistance within all groups, the horse is a reservoir of Q/D  
47 resistant genes and clones that potentially could be transferred transiently to humans.

48

49 Key words: streptogramin, virginiamycin, *vatD*, *ermB*, enterococci, horse

50

51 **Introduction**

52 Laminitis most commonly occurs when susceptible animals consume large amounts  
53 of grass carbohydrate which are fermented by hindgut bacteria, creating conditions  
54 that favour overgrowth of Gram positive bacteria (Bailey et al., 2003). Virginiamycin  
55 (Founderguard™) therapy is advocated for pasture-associated laminitis prevention to  
56 avoid this Gram positive bacterial overgrowth (Bailey et al., 2003; Rowe et al., 1994).

57

58 *Enterococcus faecalis* and *E. faecium* can cause potentially life-threatening hospital-  
59 acquired infections in man (Giraffa, 2002). These bacteria can be resistant to several  
60 antimicrobials, such that the streptogramin quinupristin-dalfopristin (Q/D) is one of  
61 the few antibiotics still effective (Soltani et al., 2000). The streptogramin  
62 virginiamycin was used as an animal growth promoter, especially in pigs and poultry.  
63 However some (Hammerum et al., 1998; Hayes et al., 2001), but not all (Claycamp  
64 and Hooberman, 2004), believe that the extensive use of virginiamycin may have  
65 contributed to the emergence of Q/D resistance amongst human *E. faecium*.  
66 Consequently, use of virginiamycin as a growth promoter was banned in the European  
67 Union (EU) in 1999 (Soltani et al., 2000). Thus, as the horse is regarded as a food  
68 producing animal by the EU, virginiamycin can only be prescribed for the prevention  
69 of pasture-associated laminitis in the United Kingdom (UK) on a named horse using a  
70 Special Treatment Certificate from the Veterinary Medicines Directorate.

71

72 The aim of this study was to evaluate the effect of virginiamycin use in horses and  
73 ponies for the prevention of pasture-associated laminitis on streptogramin resistance  
74 amongst enterococci from equine faecal samples as this has not previously been  
75 investigated.

## 76 **Materials and Methods**

### 77 *Samples*

78 Faecal samples were obtained from 30 animals kept at varying geographic locations  
79 across the UK which had been treated with oral virginiamycin (Founderguard™) for  
80 the prevention of pasture-associated laminitis for at least one month (group 1); from  
81 one control animal not receiving virginiamycin but grazing the same pasture as  
82 virtually every treated animal (group 2, n=24); and 16 animals kept at a single  
83 location which had never been treated with virginiamycin, were grazing pasture which  
84 had never knowingly been exposed to virginiamycin and had no contact with group 1  
85 or 2 animals (group 3). All the faecal samples were collected individually  
86 immediately following defaecation into sterile 50ml universal containers.

87

### 88 *Determination of antimicrobial susceptibility of enterococci*

89 Fresh faecal samples were aerobically cultured on Vancomycin Resistant Enterococci  
90 (VRE) base agar (Oxoid, Cambridge, UK). Enterococci colonies were identified  
91 based on the results of Gram stain, catalase test, Lancefield grouping and API test.  
92 Minimal inhibitory concentrations (MICs) were determined using the agar dilution  
93 method according to the recommendation of the Clinical and Laboratory Standards  
94 Institutes document M31-A3. *E. faecalis* ATCC 51299 and *E. faecium* ATCC 35667  
95 were used as the quality control reference strains. Susceptibility to Q/D (Synercid®,  
96 Aventis, Guilford, UK) using concentrations ranging from 0.5µg/ml to 32µg/ml was  
97 tested. Plates were incubated for 24 hours at 37°C under aerobic conditions and then  
98 read manually. The MIC50, MIC90 and MIC were recorded as the minimum  
99 antibiotic concentration that inhibited bacterial growth by 50%, 90% and completely

100 respectively and a MIC  $\geq 4\mu\text{g/ml}$  was considered resistant. Samples were subsequently  
101 kept frozen at  $-80^{\circ}\text{C}$  until DNA extraction was performed.

102

### 103 *DNA Extraction*

104 Total bacterial DNA was extracted from 200mg of each frozen faecal sample using  
105 QIAamp DNA Stool Mini Kit (Qiagen, Crawley, UK) using the manufacturer's  
106 protocol for isolation of DNA from stool for pathogen detection into 500 $\mu\text{l}$  eluate and  
107 subjected to PCR.

108

### 109 *Polymerase Chain Reaction (PCR)*

110 Oligonucleotide primers were designed to amplify small regions of the streptogramin  
111 resistance genes *vatD* and *ermB* which encode resistance to Streptogramin A and B  
112 respectively and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase  
113 (GAPDH) according to Table 1. Each PCR reaction was performed using 5 $\mu\text{l}$  of  
114 template DNA, 5 $\mu\text{l}$  of GoTaq buffer (Promega UK Ltd, Southampton, UK), 2.5 unit  
115 of *Taq* polymerase (GoTaq, Promega UK Ltd, Southampton, UK), 4.0mM  
116 magnesium chloride ( $\text{MgCl}_2$ ), 0.8mM of deoxynucleotide triphosphates (dNTPs),  
117 1 $\mu\text{M}$  of each primer and 0.1 $\mu\text{g}/\mu\text{l}$  of bovine serum albumin (BSA). Amplification  
118 conditions in GeneAmp<sup>®</sup> PCR System 9700 (Applied Biosystems, Warrington, UK)  
119 were as follows: initial denaturation step of 3 min at  $95^{\circ}\text{C}$ , followed by 40 cycles of  
120 30 sec at  $95^{\circ}\text{C}$ , 30 sec at annealing temperature (table 1), 30 sec at  $72^{\circ}\text{C}$ , and a final  
121 extension step of 10 min at  $72^{\circ}\text{C}$ . Amplicons were visualised after electrophoresis on  
122 1.5% agarose gels using ethidium bromide. Positive control for *vatD* was provided by  
123 Dr Neil Woodford, Health Protection Agency - Centre for Infections, London, UK.

124

125 *Real-Time Polymerase Chain Reaction (QPCR)*

126 The *vatD*, *ermB* and GAPDH PCR products were purified using Wizard SV Gel and  
127 PCR Clean-up System (Promega UK Ltd, Southampton, UK) and cloned following  
128 the manufacturer's recommendations using pGEM-T Easy Vector System with  
129 JM109 High Efficiency Competent Cells (Promega UK Ltd, Southampton, UK).  
130 Plasmid DNA from selected clones was extracted using GenElute HP Plasmid  
131 Miniprep kit (Sigma-Aldrich Company Ltd, Dorset, UK). Plasmid DNAs for each  
132 target gene were diluted 10-fold from  $1 \times 10^8$  copies/ $\mu$ l to  $1 \times 10^1$  copies/ $\mu$ l and used to  
133 generate standard curves in each QPCR assay so that absolute copy number of each  
134 unknown sample could be determined. SYBR<sup>®</sup> Green JumpStart<sup>™</sup> *Taq* ReadyMix<sup>™</sup>  
135 for quantitative PCR (Sigma-Aldrich Company Ltd, Dorset, UK) was used with a  
136 primer concentration of  $1 \mu$ M and concentrations ranging from  $1 \times 10^8$  copies to  $1 \times 10^1$   
137 copies for the standards or 1ng DNA for the samples. Each reaction was carried out in  
138 triplicate. The cycling conditions consisted of an initial denaturation step at  $95^\circ\text{C}$  for  
139 10min followed by 41 cycles of  $95^\circ\text{C}$  for 10 sec, annealing for 20 sec according to  
140 table 1, and extension at  $72^\circ\text{C}$  for 20 sec followed by a fluorescence acquisition. The  
141 reactions were carried out on a DNA Engine Opticon<sup>®</sup> 2 (Bio-Rad Laboratories Ltd,  
142 Hemel Hempstead, Herts, UK) and data analysed using Opticon Monitor Software  
143 Version 3.1.32. Primer efficiency was confirmed for each experiment according to  
144 (Pfaffl, 2001) where a value of 2.0 indicates 100% amplification efficiency.  
145 Efficiency of each primer pair in every experiment was between 1.9 and 2.1 with an  
146  $R^2$  value of 0.99. The efficiencies of each primer pair were linear for DNA  
147 concentrations between 200pg and 10ng. Melting curve analysis of the PCR products  
148 was performed for each experiment to confirm fluorescence was measured from  
149 specific amplicons only.

150

151 *Statistical Analysis*

152 The MIC<sub>50</sub>, MIC<sub>90</sub> and MIC were compared between the 3 groups using a one way  
153 ANOVA with Bonferroni's post hoc test. The proportion of samples which contained  
154 Q/D resistant *E. faecium* and *E. faecalis* was compared between the 3 groups using a  
155 chi-squared test. Expression of *ermB* and *vatD* were normalised to the expression of  
156 GAPDH in the same total bacterial DNA sample, creating a gene expression ratio.  
157 The data was tested for normality using a D'Agostino & Pearson omnibus normality  
158 test and found to be not normally distributed. Therefore data was compared between  
159 the 3 groups of animals using a Kruskal Wallance test. The analysis was performed  
160 using commercially available statistical software (GraphPad Prism version 5.0) and  
161  $p < 0.05$  was considered statistically significant.

162

163 **Results**164 *Antimicrobial susceptibility of enterococci*

165 The MICs for the quality control strains *E. faecalis* ATCC 51299 and *E. faecium*  
166 ATCC 35667 were 16 and 0.5 µg/ml respectively. The MIC<sub>50</sub> (2 µg/ml for all  
167 groups), MIC<sub>90</sub> (4 µg/ml for all groups) and MIC range (0.5 – 32 µg/ml for groups 1  
168 and 3 and 0.25 - 32 µg/ml for group 2) for *E. faecium* was not significantly different  
169 between the 3 groups. Streptogramin resistant *E. faecium* were cultured from 70%  
170 (21/30) of animals treated with virginiamycin, 75% (18/24) of animals grazing the  
171 same pasture and 69% (11/16) of animals grazing pasture where virginiamycin had  
172 never been used. Streptogramin resistant *E. faecalis* were cultured from 7% (2/30) of  
173 animals treated with virginiamycin, 4% (1/24) of animals grazing the same pasture  
174 and none (0/16) of the animals grazing pasture where virginiamycin had never been

175 used. There was no significant difference in the proportion of samples which  
176 contained streptogramin *E. faecium* or *E. faecalis* between the 3 groups of animal.

177

#### 178 *Real-Time Polymerase Chain Reaction (QPCR)*

179 Standard curve parameters obtained are shown in table 2. Only one amplification  
180 product was visible on the melting curve. Results are shown in tables 3 and 4. The  
181 antimicrobial resistance genes were present only in those faecal samples which had  
182 cultured streptogramin resistant *E. faecium*. There was no significant difference in the  
183 ratio of the number of copies/ng total bacterial DNA *ermB*:GAPDH between the 3  
184 groups of animals. The ratio of the number of copies/ng total bacterial DNA  
185 *vatD*:GAPDH was significantly ( $p=0.038$ ) greater in animals which had never been  
186 exposed to virginiamycin (group 3) compared to those being treated with  
187 virginiamycin (group 1).

188

#### 189 **Discussion**

190 The effect of oral virginiamycin therapy used for the prevention of equine pasture-  
191 associated laminitis on enterococcal streptogramin resistance has not been previously  
192 evaluated. In the present study, there was widespread streptogramin resistance within  
193 *E. faecium* found in the equine gastrointestinal tract; however treatment with oral  
194 virginiamycin was not associated with a significant increase. Whilst *E. faecalis* is  
195 naturally resistant to Q/D, resistance is acquired in *E. faecium* and it would appear  
196 that this acquired resistance in the horse is at least in part associated with the presence  
197 of *ermB* and *vatD* genes. Virginiamycin therapy was not associated with an increase  
198 in the presence of or gene load of *ermB* or *vatD* genes; rather the gene load of *vatD*  
199 genes was significantly greater in animals not exposed to virginiamycin.

200

201 Antimicrobial resistance in enterococci isolated from faeces of healthy and sick  
202 horses has been evaluated previously in a number of studies and whilst a high  
203 frequency of resistance to a wide range of antimicrobial drugs has been reported (de  
204 Niederhausern et al., 2007; Devriese et al., 1996; Laukova et al., 2008; Rice et al.,  
205 2003; Singh, 2009), resistance to Q/D has not been determined. The high frequency of  
206 Q/D resistance within *E. faecium* found in animals treated with virginiamycin in the  
207 present study is however in accordance with previous studies on pigs and poultry  
208 from farms using virginiamycin as a growth promoter (Hayes et al., 2001; Simjee et  
209 al., 2007; Welton et al., 1998; Werner et al., 2000).

210

211 The resistance to Q/D of enterococci isolated from faecal samples from animals not  
212 receiving antimicrobial therapy has been investigated in a number of species. Low  
213 frequencies of Q/D resistant enterococcal isolates were found in studies evaluating  
214 pigs (Nulsen et al., 2008) and poultry (Kieke et al., 2006) raised without antibiotics  
215 and healthy dogs and cats (Frye et al., 2006). In contrast, a high frequency of isolates  
216 from healthy humans were resistant to Q/D (Novais et al., 2006). Thus it would  
217 appear that the frequency of Q/D resistant *E. faecium* in faecal samples from healthy  
218 horses is greater than in a number of other species and horses are a reservoir of Q/D  
219 resistant genes and clones that potentially could be transiently transferred to humans.

220

221 Whilst the presence of the gene *vanA* which encodes for glycopeptide resistance in *E.*  
222 *faecium* has been previously investigated in the horse (de Niederhausern et al., 2007;  
223 Devriese et al., 1996; Moura et al., 2010), the mechanisms involved in Q/D resistance  
224 in enterococci of equine origin have not been evaluated. In the present study, the

225 frequency of occurrence of the streptogramin resistance genes *vatD* and *ermB* found  
226 in enterococci from other species was investigated. In accordance with the results of  
227 the present study, *ermB* appears to be frequently present in Q/D resistant *E. faecium*  
228 isolates (Lopez et al., 2010; Soltani et al., 2000). However, the presence of the *vatD*  
229 gene is more variable between studies (Donabedian et al., 2006; Soltani et al., 2000).

230

231 Relatively few studies have reported the frequency of the concurrent presence of both  
232 resistance genes. In the present study both genes were isolated from 56% of animals.  
233 Both genes were found to be present in 30% of isolates from poultry litter (Simjee et  
234 al., 2007) and in 79% of streptogramin resistant *E. faecium* isolates from broilers the  
235 *vatD* and *ermB* genes were physically linked (Jensen et al., 2002). However, other  
236 studies have demonstrated the concurrent presence of *ermB* and *vatD* in very few  
237 isolates (De Graef et al., 2007; Lopez et al., 2010; Thibodeau et al., 2008).

238

### 239 **Conclusion**

240 In conclusion, Q/D resistance is widespread within *E. faecium* found in the equine  
241 gastrointestinal tract, even in animals which have not been previously exposed to the  
242 drug, and it appears to be associated with the presence of the streptogramin resistance  
243 genes *ermB* and *vatD*. The use of virginiamycin for the prevention of pasture-  
244 associated laminitis does not appear to be related to an increase in the presence of  
245 Q/D resistant *E. faecium* or in the presence or copy number of the antimicrobial  
246 resistance genes *vatD* and *ermB* within the faeces. The horse appears to be a reservoir  
247 of Q/D resistant genes and clones that potentially could be transferred to humans. This  
248 enterococcal Q/D resistance would not be expected to alter the ability of  
249 virginiamycin to prevent Gram positive bacterial overgrowth in pasture-associated

250 laminitis as Streptococcal species are the most likely causative agent and there is no  
 251 evidence to date that resistance is transferred between different bacterial genres.

252

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255

### 256 **References**

257

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- 344  
345

346 **Tables**

347 Table 1: Primers used for antimicrobial resistance gene screening

348 Table 1: QPCR standard curve parameters

349 Table 2: QPCR results, expressed as median (interquartile range)

350 Table 3: Frequency of detection of ermB and vatD genes in horses treated with oral

351 virginiamycin (group 1), co-grazing horses (group 2) and horses never exposed to

352 virginiamycin (group 3)

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## Tables

Table 1: Primers used for antimicrobial resistance gene screening

Primer	Sequence (5'-3')	Amplicon length (bp)	Annealing temperature (°C)
vatD	sense: CAAATCATAGAATGGATGGC antisense: TTTCGTTAGCACCATTTCC	251	53
ermB	sense: AAGCCATGCGTCTGACATCT antisense: ATGAAAGCATTCCGCTGGCA	143	55
GAPDH	sense: ACTATCCACGCTTACACAGG antisense: GCAGCACCAAGTTGAGTTAGG	117	55

Table 2: QPCR standard curve parameters

	Slope	Efficiency (%) <sup>a</sup>	Regression coefficient
vatD	-0.296	98%	0.99
ermB	-0.281	91%	0.99
GAPDH	-0.315	106%	0.99

<sup>a</sup> Primer efficiency was calculated according to Pfaffl (2001)

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Table 3: QPCR results, expressed as median (interquartile range)

Group	ermB (copies/ng DNA)	vatD (copies/ng DNA)	ermB:GAPDH ratio	vatD:GAPDH ratio
1	198 (155, 258)	7371 (6423, 18145)	0.022 (0.015, 0.038)	0.083 <sup>a</sup> (0.68, 2.93)
2	197 (146, 243)	8841 (5906, 43640)	0.032 (0.013, 0.045)	0.95 (0.58, 7.38)
3	258 (222, 285)	23730 (14780, 52995)	0.027 (0.019, 0.057)	2.93 <sup>a</sup> (2.25, 8.25)

<sup>a</sup> Values significantly (p=0.038) different compared using Kruskal Wallace test

Table 4: Frequency of detection of ermB and vatD genes in horses treated with oral virginiamycin (group 1), co-grazing horses (group 2) and horses never exposed to virginiamycin (group 3)

Group	ermB	vatD	ermB and vatD	Neither gene
1	19/30 (63%)	20/30 (67%)	16/30 (53%)	7/30 (23%)
2	18/24 (75%)	17/24 (71%)	15/24 (63%)	4/24 (17%)
3	10/16 (63%)	11/16 (69%)	8/16 (50%)	3/16 (19%)
all	47/70 (67%)	48/70 (68%)	39/70 (56%)	14/70 (20%)