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

N.J. Menzies-Gow, N.J. Young

To cite this version:

N.J. Menzies-Gow, N.J. Young. Antibiotic resistance in faecal bacteria isolated from horses receiving virginiamycin for the prevention of pasture-associated laminitis. Veterinary Microbiology, Elsevier, 2011, 10.1016/j.vetmic.2011.05.026. hal-00724201
Antibiotic resistance in faecal bacteria isolated from horses receiving virginiamycin for the prevention of pasture-associated laminitis

N J Menzies-Gow, MA VetMB PhD DipECEIM CertEM(Int.med) MRCVS
N J Young, BSc MSc PhD

Department of Veterinary Clinical Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire AL9 7TA, UK

Corresponding author:
N Menzies-Gow
Department of Veterinary Clinical Sciences
Royal Veterinary College
Hawkshead Lane
North Mymms
Herts. AL9 7TA
UK
Tel: 01707 666297
Fax: 01707 666304
Email: nmenziesgow@rvc.ac.uk

Dr Young’s current address is Biotherapeutics, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts, EN6 3QG, UK.
Abstract (250 words)

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

Key words: streptogramin, virginiamycin, vatD, ermB, enterococci, horse
Introduction

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

Enterococcus faecalis and E. faecium can cause potentially life-threatening hospital-acquired infections in man (Giraffa, 2002). These bacteria can be resistant to several antimicrobials, such that the streptogramin quinupristin-dalfopristin (Q/D) is one of the few antibiotics still effective (Soltani et al., 2000). The streptogramin virginiamycin was used as an animal growth promoter, especially in pigs and poultry. However some (Hammerum et al., 1998; Hayes et al., 2001), but not all (Claycamp and Hooberman, 2004), believe that the extensive use of virginiamycin may have contributed to the emergence of Q/D resistance amongst human E. faecium.

Consequently, use of virginiamycin as a growth promoter was banned in the European Union (EU) in 1999 (Soltani et al., 2000). Thus, as the horse is regarded as a food producing animal by the EU, virginiamycin can only be prescribed for the prevention of pasture-associated laminitis in the United Kingdom (UK) on a named horse using a Special Treatment Certificate from the Veterinary Medicines Directorate.

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

Samples

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

Determination of antimicrobial susceptibility of enterococci

Fresh faecal samples were aerobically cultured on Vancomycin Resistant Enterococci (VRE) base agar (Oxoid, Cambridge, UK). Enterococci colonies were identified based on the results of Gram stain, catalase test, Lancefield grouping and API test. Minimal inhibitory concentrations (MICs) were determined using the agar dilution method according to the recommendation of the Clinical and Laboratory Standards Institutes document M31-A3. E. faecalis ATCC 51299 and E. faecium ATCC 35667 were used as the quality control reference strains. Susceptibility to Q/D (Synercid®, Aventis, Guilford, UK) using concentrations ranging from 0.5µg/ml to 32µg/ml was tested. Plates were incubated for 24 hours at 37°C under aerobic conditions and then read manually. The MIC50, MIC90 and MIC were recorded as the minimum antibiotic concentration that inhibited bacterial growth by 50%, 90% and completely
respectively and a MIC >4μg/ml was considered resistant. Samples were subsequently kept frozen at -80°C until DNA extraction was performed.

**DNA Extraction**

Total bacterial DNA was extracted from 200mg of each frozen faecal sample using QIAamp DNA Stool Mini Kit (Qiagen, Crawley, UK) using the manufacturer’s protocol for isolation of DNA from stool for pathogen detection into 500μl eluate and subjected to PCR.

**Polymerase Chain Reaction (PCR)**

Oligonucleotide primers were designed to amplify small regions of the streptogramin resistance genes vatD and ermB which encode resistance to Streptogramin A and B respectively and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to Table 1. Each PCR reaction was performed using 5μl of template DNA, 5μl of GoTaq buffer (Promega UK Ltd, Southampton, UK), 2.5 unit of Taq polymerase (GoTaq, Promega UK Ltd, Southampton, UK), 4.0mM magnesium chloride (MgCl₂), 0.8mM of deoxynucleotide triphosphates (dNTPs), 1μM of each primer and 0.1μg/μl of bovine serum albumin (BSA). Amplification conditions in GeneAmp® PCR System 9700 (Applied Biosystems, Warrington, UK) were as follows: initial denaturation step of 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at annealing temperature (table 1), 30 sec at 72 °C, and a final extension step of 10 min at 72 °C. Amplicons were visualised after electrophoresis on 1.5% agarose gels using ethidium bromide. Positive control for vatD was provided by Dr Neil Woodford, Health Protection Agency - Centre for Infections, London, UK.
Real-Time Polymerase Chain Reaction (QPCR)

The vatD, ermB and GAPDH PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega UK Ltd, Southampton, UK) and cloned following the manufacturer’s recommendations using pGEM-T Easy Vector System with JM109 High Efficiency Competent Cells (Promega UK Ltd, Southampton, UK). Plasmid DNA from selected clones was extracted using GenElute HP Plasmid Miniprep kit (Sigma-Aldrich Company Ltd, Dorset, UK). Plasmid DNAs for each target gene were diluted 10-fold from 1x10^8 copies/μl to 1x10^1 copies/μl and used to generate standard curves in each QPCR assay so that absolute copy number of each unknown sample could be determined. SYBR® Green JumpStart™ Taq ReadyMix™ for quantitative PCR (Sigma-Aldrich Company Ltd, Dorset, UK) was used with a primer concentration of 1μM and concentrations ranging from 1x10^8 copies to 1x10^1 copies for the standards or 1ng DNA for the samples. Each reaction was carried out in triplicate. The cycling conditions consisted of an initial denaturation step at 95°C for 10min followed by 41 cycles of 95°C for 10 sec, annealing for 20 sec according to table 1, and extension at 72°C for 20 sec followed by a fluorescence acquisition. The reactions were carried out on a DNA Engine Opticon® 2 (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK) and data analysed using Opticon Monitor Software Version 3.1.32. Primer efficiency was confirmed for each experiment according to (Pfaffl, 2001) where a value of 2.0 indicates 100% amplification efficiency. Efficiency of each primer pair in every experiment was between 1.9 and 2.1 with an R^2 value of 0.99. The efficiencies of each primer pair were linear for DNA concentrations between 200pg and 10ng. Melting curve analysis of the PCR products was performed for each experiment to confirm fluorescence was measured from specific amplicons only.
Statistical Analysis

The MIC50, MIC90 and MIC were compared between the 3 groups using a one way ANOVA with Bonferroni’s post hoc test. The proportion of samples which contained Q/D resistant E. faecium and E. faecalis was compared between the 3 groups using a chi-squared test. Expression of ermB and vatD were normalised to the expression of GAPDH in the same total bacterial DNA sample, creating a gene expression ratio. The data was tested for normality using a D’Agostino & Pearson omnibus normality test and found to be not normally distributed. Therefore data was compared between the 3 groups of animals using a Kruskall Wallace test. The analysis was performed using commercially available statistical software (GraphPad Prism version 5.0) and p<0.05 was considered statistically significant.

Results

Antimicrobial susceptibility of enterococci

The MICs for the quality control strains E faecalis ATCC 51299 and E. faecium ATCC 35667 were 16 and 0.5µg/ml respectively. The MIC50 (2 µg/ml for all groups), MIC90 (4 µg/ml for all groups) and MIC range (0.5 – 32 µg/ml for groups 1 and 3 and 0.25 - 32 µg/ml for group 2) for E. faecium was not significantly different between the 3 groups. Streptogramin resistant E. faecium were cultured from 70% (21/30) of animals treated with virginiamycin, 75% (18/24) of animals grazing the same pasture and 69% (11/16) of animals grazing pasture where virginiamycin had never been used. Streptogramin resistant E. faecalis were cultured from 7% (2/30) of animals treated with virginiamycin, 4% (1/24) of animals grazing the same pasture and none (0/16) of the animals grazing pasture where virginiamycin had never been
used. There was no significant difference in the proportion of samples which contained streptogramin E. faecium or E. faecalis between the 3 groups of animal.

Real-Time Polymerase Chain Reaction (QPCR)

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

Discussion

The effect of oral virginiamycin therapy used for the prevention of equine pasture-associated laminitis on enterococcal streptogramin resistance has not been previously evaluated. In the present study, there was widespread streptogramin resistance within E. faecium found in the equine gastrointestinal tract; however treatment with oral virginiamycin was not associated with a significant increase. Whilst E. faecalis is naturally resistant to Q/D, resistance is acquired in E. faecium and it would appear that this acquired resistance in the horse is at least in part associated with the presence of ermB and vatD genes. Virginiamycin therapy was not associated with an increase in the presence of or gene load of ermB or vatD genes; rather the gene load of vatD genes was significantly greater in animals not exposed to virginiamycin.
Antimicrobial resistance in enterococci isolated from faeces of healthy and sick horses has been evaluated previously in a number of studies and whilst a high frequency of resistance to a wide range of antimicrobial drugs has been reported (de Niederhausern et al., 2007; Devriese et al., 1996; Laukova et al., 2008; Rice et al., 2003; Singh, 2009), resistance to Q/D has not been determined. The high frequency of Q/D resistance within E. faecium found in animals treated with virginiamycin in the present study is however in accordance with previous studies on pigs and poultry from farms using virginiamycin as a growth promoter (Hayes et al., 2001; Simjee et al., 2007; Welton et al., 1998; Werner et al., 2000).

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

Whilst the presence of the gene vanA which encodes for glycopeptide resistance in E. faecium has been previously investigated in the horse (de Niederhausern et al., 2007; Devriese et al., 1996; Moura et al., 2010), the mechanisms involved in Q/D resistance in enterococci of equine origin have not been evaluated. In the present study, the
frequency of occurrence of the streptogramin resistance genes vatD and ermB found in enterococci from other species was investigated. In accordance with the results of the present study, ermB appears to be frequently present in Q/D resistant E. faecium isolates (Lopez et al., 2010; Soltani et al., 2000). However, the presence of the vatD gene is more variable between studies (Donabedian et al., 2006; Soltani et al., 2000).

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

Conclusion

In conclusion, Q/D resistance is widespread within E. faecium found in the equine gastrointestinal tract, even in animals which have not been previously exposed to the drug, and it appears to be associated with the presence of the streptogramin resistance genes ermB and vatD. The use of virginiamycin for the prevention of pasture-associated laminitis does not appear to be related to an increase in the presence of Q/D resistant E. faecium or in the presence or copy number of the antimicrobial resistance genes vatD and ermB within the faeces. The horse appears to be a reservoir of Q/D resistant genes and clones that potentially could be transferred to humans. This enterococcal Q/D resistance would not be expected to alter the ability of virginiamycin to prevent Gram positive bacterial overgrowth in pasture-associated
laminitis as Streptococcal species are the most likely causative agent and there is no evidence to date that resistance is transferred between different bacterial genuses.

Acknowledgements

This study was generously funded by the PetPlan Charitable Trust.

References


Werner, G., Klare, I., Heier, H., Hinz, K.H., Bohme, G., Wendt, M., Witte, W., 2000, Quinupristin/dalfopristin-resistant enterococci of the satA (vatD) and satG (vatE) genotypes from different ecological origins in Germany. Microb Drug Resist 6, 37-47.
Tables
Table 1: Primers used for antimicrobial resistance gene screening

Table 1: QPCR standard curve parameters

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

Table 3: 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)
### Tables

Table 1: Primers used for antimicrobial resistance gene screening

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Amplicon length (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
</table>
| vatD   | sense: CAAATCATAGAATGGATGGC  
         | antisense: TTTCGTTAGCACCATTTC | 251 | 53 |
| ermB   | sense: AAGCCATGCCTCGACATCT  
         | antisense: ATGAAAGCATTCCGCTGGCA | 143 | 55 |
| GAPDH  | sense: ACTATCCACGCTTACACAGG  
         | antisense: GCAGCACCAGTTGAGTTAGG | 117 | 55 |
Table 2: QPCR standard curve parameters

<table>
<thead>
<tr>
<th></th>
<th>Slope</th>
<th>Efficiency (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Regression coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>vatD</td>
<td>-0.296</td>
<td>98%</td>
<td>0.99</td>
</tr>
<tr>
<td>ermB</td>
<td>-0.281</td>
<td>91%</td>
<td>0.99</td>
</tr>
<tr>
<td>GAPDH</td>
<td>-0.315</td>
<td>106%</td>
<td>0.99</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primer efficiency was calculated according to Pfaffl (2001)
Table 3: QPCR results, expressed as median (interquartile range)

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

<sup>a</sup>Values significantly (p=0.038) different compared using Kruskall 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)

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