Macrolide, glycopeptide resistance and virulence genes in *Enterococcus* species isolates from dairy cattle

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The genus *Enterococcus* is known to possess the capacity to acquire and disseminate antimicrobial resistant determinants alongside the ability to produce various virulence genes that enables it to establish infections. We assessed the prevalence and antibiogram profiles of *Enterococcus* spp. in faecal samples of dairy cattle. Faecal swab samples were collected from 400 dairy cattle from two commercial cattle farms in two rural communities in the Eastern Cape, South Africa. Confirmation of enterococci isolates was carried out by PCR targeting of the *tuf* gene. Species delineation was by species-specific primers targeting the superoxide dismutase (*sodA*) gene in a multiplex PCR assay. Isolates were screened for the presence of the following virulence genes (*ace*, *gelE*, *esp*, *efA*, *cylA* and *hyE*) and antimicrobial resistance determinants to erythromycin, vancomycin and streptomycin were evaluated molecularly. A total of 340 isolates were confirmed as belonging to the genus *Enterococcus*. Species distribution among the isolates consisted of *Enterococcus faecium* (52.94 %) and *Enterococcus durans* (23.53 %) in preponderance compared to the three other species, namely *Enterococcus faecalis* (8.8 %), *Enterococcus hirae* (8.6 %) and *Enterococcus casseliflavus* (5.9 %). All were resistant to vancomycin, while 99 % showed resistance to aminoglycoside and 94 % to macrolide. Three virulence genes (*ace*, *gelE* and *esp*) were detected in almost all the confirmed isolates. The resistance determinants *vanB* (19.7 %), *vanC1* (25 %), *vanC2/3* (26.3 %) *ermB* (40.29 %) and *strA* (50.88 %) were detected among the isolates. A high prevalence of multidrug-resistant enterococci isolates was detected in this study and the genetic repertoire to survive in the presence of antimicrobial agents was present in these organisms.

INTRODUCTION

Members of the genus *Enterococcus* are known to be abundantly present in the intestinal tracts of humans and warm-blooded animals, as well as in water and soil environments. These bacteria have become notorious recently because of their ability to cause nosocomial infections, especially among both immunocompromised patients and elderly people. Pathogenic enterococci have been reported to harbour a pathogenicity island that encodes for aggregation substance (AS), gelatinase, extracellular surface proteins (esp), cytolysin, hyaluronidase and other proteins (Giridhara Upadhyaya *et al.*, 2009; Shankar *et al.*, 2002). These virulence factors enable enterococci to cause tissue damage and inflammation.

Besides their possession of several virulence factors, members of the genus *Enterococcus* also have inherent capacity to accumulate and disseminate antimicrobial resistance determinants. Enterococci, like other bacteria are capable of developing strategies to enhance their survival in adverse situations (Garcia-Migura *et al.*, 2005). They have emerged as an important opportunistic pathogen causing life-threatening infections in patients, alongside the fact that they have developed remarkable resistance to a battery of antimicrobial agents belonging to several classes such as the cephalosporins, macrolides and aminoglycosides, as well as the glycopeptide...
antibiotics that are commonly used for the clinical therapy of most Gram-positive nosocomial infections. The development of commercial animal husbandry has resulted in the use of antimicrobials in animal feeds as growth promoters worldwide, though it has been banned within the European Union. Across the globe, a variety of antimicrobial agents are available for therapeutic use in animals. Many studies have supported the claim that with the increased use of antimicrobial agents in animals and humans, an increased prevalence of resistant strains has emerged as a direct consequence of the antimicrobial use (Donabedian et al., 2003; Hershberger et al., 2005). Infections by vancomycin-resistant enterococci (VRE) are directly related to the widespread use of antimicrobials in animal feeds as growth promoters worldwide, though it has been banned within the European Union. Bacteria from animals to humans should not be allowed to zoonotic diseases alone, but attention should be given to those harbouring antimicrobial resistance determinants as they pose great danger to human health. The indiscriminate use of antimicrobials in farm animal managements may have undesirable consequences for human health, because it could result in the selection of a reservoir of opportunistic human pathogens carrying antibiotic resistance determinants.

Though much has been learned over the last two decades about the epidemiology of nosocomial enterococci, there is a paucity of information on the prevalence and epidemiology of antimicrobial resistance in enterococcal species from dairy cattle in the Eastern Cape of South Africa. The Eastern Cape Province of South Africa is largely rural and agrarian, with few commercial dairy farms. The use of antibiotics to manage animal productivity is a common practice, and the impacts of bacteria with resistance determinants shed into the environment through faecal samples of animals could pose a huge epidemiological problem. This study was therefore aimed at assessing the prevalence, antimicrobial resistance and virulence profiles of Enterococcus spp. isolated from faecal samples of dairy cattle farms exposed to high levels of tetracycline in the Nkonkobe municipality in the Eastern Cape of South Africa, as part of our larger study on antibiotic resistance determinants in enterococcal isolates from faecal samples of domesticated animals and from environmental samples.

**METHODS**

**Ethical clearance.** Ethical clearance (REC-270710-028-RA Level 01) for the study was obtained from the University of Fort Hare ethics committee prior to sample collection, and approval was granted by farmer owners before commencement of sampling.

**Study population and sampling.** Samples were collected from two commercial dairy cattle farms in the Amathole Districts of the Eastern Cape of South Africa. A total of 400 samples from two dairy farms, A and B, were collected for the study, 270 from farm A and 130 from farm B. Rectal faecal samples were collected from individual cattle using sterile swab sticks during the milking process to avoid duplication in collection. After collection, samples were shipped on ice to the University of Fort Hare Microbiology laboratory for immediate processing. Histories of antibiotic treatments were collected for the purpose of describing the antibiotic usage among the study population. Sampling was done fortnightly between June and August 2014.

**Laboratory detection of Enterococcus spp.** The swab sticks were used to inoculate tryptone soya broth (TSB; Oxoid) and incubated at 37°C for 18 to 20 h. These were then sub-cultured onto Bile Aesculin Azide (BAA) agar and incubated at 37°C for 24 h. Tiny black colonies were assumed presumptive for Enterococcus spp. and an isolated colony per plate was picked into TSB and further incubated for 18 h at 37°C for glyceral stock preparation and preservation at −80°C for future use.

**DNA extraction.** Bacterial DNA was prepared as previously described by Bai et al. (2012). Briefly, a pure bacterial culture was resuscitated from the glyceral stock and grown overnight in TSA at 37°C, with slight agitation. From this culture, 2 ml was centrifuged in a clean Eppendorf tube for 5 min at 14,000 r.p.m. and the pellet was washed using normal saline (0.85% NaCl). After adding 150 µl rapid lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.3, 1 mM EDTA pH 9.0; 1% Triton X-100), the pellet was re-suspended by vortexing and the suspension was boiled for 15 min in a heating block, cooled and centrifuged at 10,000 r.p.m., and the supernatant collected and stored at −20°C. These were then used as templates in all PCRs that were performed in this study.

**Molecular confirmation of the isolates.** PCR identification analysis with Enterococcus genus-specific primers Ent1 and Ent2 targeting the sodA gene, as previously reported by Banbing et al. (1999), was performed. The previously extracted genomic DNA was amplified by PCR performed in a 25 µl mixture of 2× buffer (supplied with Taq polymerase), 2.5 mM MgCl₂, 2.5 U Taq DNA polymerase, 200 µM of each deoxynucleoside triphosphate, and 10 pM of each primer, Ent1F 5’-CACGGTACATGATG-3’ and Ent2R 5’-AACCTCGTACCAACCGGAAAC-3’. E. faecalis ATCC 19433 and E. hirae ATCC 8043 both served as positive controls while nuclease-free water was used as the negative control. The PCR mixture was subjected to a 4 min denaturation step at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s at 53°C and 60 s at 72°C, and a final elongation step of 10 min at 72°C. PCR products were separated by electrophoresis at 110 V for 45 min in a 2% agarose gel with ethidium bromide, and were then viewed under UV light (ALLIANCE 4.7 transilluminator; Merton) and photographed.

**Molecular confirmation of species identity.** In order to delineate isolates into different species of the genus Enterococcus, a PCR was performed as previously described by Jackson et al. (2004) using amplification of the sodA gene, which has sequences specific for the following species: E. faecalis, E. faecium, E. hirae, E. durans and E. casseliflavus in monoplex PCR reactions. The Dream Taq PCR Master Mix (2×) consisting of 4 mM MgCl₂, 0.4 mM deoxynucleoside triphosphate mix and Taq polymerase enzyme (Thermo Scientific), and 1µl of 10 pM of each primer pair was added to constitute the reaction mixture in a PCR tube. PCRs were performed in a final volume of 25 µl consisting of 12.5 µl master mix, 1 µl each of forward and reverse primers, 5.5 µl water of PCR grade and 5 µl DNA template. Following an initial denaturation at 95°C for 4 min, products were amplified in 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C (E. faecalis, E. durans and E. casseliflavus) or 48°C (for E. faecium and E. hirae) for 1 min, and elongation at 72°C for 1 min followed by a final extension at 72°C for 7 min. Five microlitres of product was electrophoresed at 110 V for 45 min on a 2% Tris-borate-EDTA agarose gel containing 2 µg ethidium bromide ml⁻¹ to verify amplification of the targeted genes. DNA molecular weight marker 100bp was used as the standard and gels were photographed under a UV light transilluminator [ALLIANCE 4.7; Merton (a molecular imager gel documentation system)].
Antimicrobial, virulence factors in enterococci isolates from dairy cattle

PCR for the detection of virulence determinants. The isolates were screened for the presence of virulence factors previously described among enterococci. Specific primers that allow for the detection of the following six virulence factors have been reported to aid in the colonization and establishment of infections within the host: ace, efaA, cyIA, geF, esp and hyfE, as previously described in the literature (Shankar et al., 1999; Mannu et al., 2003; Valenzuela et al., 2009) – were used to screen the isolates. The reactions were performed in a total volume of 25 µl using 5 µl DNA, 1 µl of 10 PM of each primer, 12.5 µl PCR Dream Taq Master Mix (Thermo Scientific), and water of PCR grade to make up the volume. PCR conditions for ace and geF genes after the initial heating of the block at 94°C for 4 min were denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min for 35 cycles and final extension at 72°C for 7 min. The PCR cycles for the amplification of the efaA, esp, cyIA and hyfE genes were 35 cycles of denaturation at 94°C for 1 min, annealing at 56.5°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels with 110 V for 45 min, stained in ethidium bromide solution, visualized under a UV transilluminator (ALLIANCE 4.7; Merton) and photographed.

Antibiotic sensitivity testing. The antimicrobial susceptibility of all isolates was assessed according to the Kirby-Bauer disk diffusion method (CLSI, 2014), using the following antibiotic discs (Mast Diagnostics): clindamycin (CD, 2 µg), imipenem (IPI, 10 µg), neomycin (NE, 30 µg), streptomycin (S, 10 µg), vancomycin (V, 30 µg), penicillin G (PG, 10 µg), amoxicillin-clavulanic acid (AUG, 10 µg), ciprofloxacin (CIP, 5 µg), cephalothin (KF, 30 µg), clexacillin (CX, 5 µg), erythromycin (E, 15 µg) and amikacin (AK, 30 µg). The discs were dispensed by an automated disc dispenser (Mast Diagnostics). In the evaluation of the results, strains displaying intermediate resistance were regarded as resistant. The interpretations of zones of inhibition were carried out according to the CLSI (2014) performance standards for antimicrobial susceptibility testing guidelines based on human clinical breakpoints.

PCR detection of antibiotic resistance genes. Genetic profiling of resistance genes from isolates exhibiting phenotypic resistance to vancomycin was performed using the previously extracted DNA. PCRs were performed in a BioRad Thermal Cycler. The oligonucleotide primers for PCR amplifications were synthesized by Inqaba Biotech (Pretoria, South Africa). Primer sequences for vanA, vanB, vanC1 and vanC2/3 genes were those described by Nam et al. (2013). The list of specific primers used in this study and the sizes of amplification products are shown in Table 1. The reactions were performed as monoplex in a total volume of 25 µl, using 5 µl cell lysate as DNA template, 1 µl of 10 PM of each of the eight primers, 12.5 µl Dream Taq master PCR mix (Inqaba Biotech) and 5.5 µl PCR-grade water. Amplification conditions were as follows: a first denaturation step of 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 56.5°C for 1 min, extension at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gels at 110 V for 45 min, stained in ethidium bromide solution, visualized under a UV transilluminator (ALLIANCE 4.7; Merton) and photographed.

The presence of erm(B) and strA genes was screened among isolates that exhibited resistance to erythromycin and streptomycin by using the primers pairs erm(B) (ermBN1: 5'-CGAGTGAAGTTACCCAGCA-3', ermBN2: 5'-CGGTGAAATATCCAAAGTACG-3') and strA (strA: 5'-ATCCTGGTGACGGGATTTGG-3', strAR: 5'-CCAGTTCTCCTCGC-3'), respectively. In each PCR tube, a reaction mixture (25 µl) containing 5 µl bacterial DNA, 12.5 µl Dream Taq Master Mix (Thermo Scientific) 10 PM each primer and 5.5 µl water of PCR grade was prepared. The reaction mixture in the tube was subjected to 35 PCR cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C and 56.5°C for erm(B) and strA, respectively, and elongation at 72°C for 1 min in a BioRad thermal cycler with a final extension at 72°C for 7 min. PCR products with specific sizes of the resistance genes were detected by agarose gel electrophoresis at 110 V for 45 min, followed by staining with ethidium bromide. Resulting gels were visualized under UV light in a transilluminator (ALLIANCE 4.7; Merton) and photographed.

RESULTS

A total of 400 faecal samples were collected from two dairy farms with a total herd of 800 and 160 cattle, respectively, that had been repeatedly exposed to antibiotics including tylosin, advacin (danofloxacin), ampicillin and penicillin G. From these samples, 341 isolates were recovered and confirmed to be enterococci by molecular characterization of the tuf gene.

Taxonomic identification of the isolates

The results of speciation of the confirmed 341 isolates by PCR with species-specific primers identified them as being E. faecium, E. durans, E. hirae, E. casseliflavus and E. faecalis. Speciation of the isolates showed that E. faecium (52.94%) and E. durans (23.53%) were predominant compared to the three other species, E. faecalis (8.8%), E. hirae (8.6%) and E. casseliflavus (5.9%). One isolate could not be identified by the species-specific primers used in the screening process.

Antibiotic susceptibility testing

Results of the antibiotic susceptibility testing of the isolates are summarized in Fig. 1. The zones of inhibition data obtained in vitro with 12 antimicrobial agents revealed that all the isolates were resistant to both vancomycin and cloxacillin, while resistance to amikacin, cephalothin, streptomycin, penicillin G, clindamycin, neomycin and erythromycin ranged from

Table 1. List of oligonucleotide primers used in the genetic profiling of resistance genes among the isolates in this study

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Product size (bp)</th>
<th>Primer name and sequence (5' to 3')</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA</td>
<td>314</td>
<td>AF- GCCGCGGTCACCTTGTAGATA</td>
<td>105–124</td>
</tr>
<tr>
<td>vanB</td>
<td>220</td>
<td>AR- TGAGGAAACCCCCCAAACTGA</td>
<td>399–418</td>
</tr>
<tr>
<td>vanC1</td>
<td>402</td>
<td>BF- AGACATTCCGGTGAGGAAC</td>
<td>844–863</td>
</tr>
<tr>
<td>vanC2/3</td>
<td></td>
<td>BR- GCTGTCAATTAGTGCGGGAA</td>
<td>1044–1063</td>
</tr>
<tr>
<td>C1F</td>
<td></td>
<td>C1F- ATCCAAAGCTATGACCCCGCT</td>
<td>290–309</td>
</tr>
<tr>
<td>C1R</td>
<td></td>
<td>C1R- TGGTCAGGATCGTTTCTAT</td>
<td>672–691</td>
</tr>
</tbody>
</table>

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74% to 99%. Resistance to imipenem, amoxicillin/Clavulanate and ciprofloxacin was comparatively lower at 0.6%, 8% and 12%, respectively. Multiple resistances to various antibiotics were observed among the isolates as shown in Table 2, while the percentage resistance to glycopeptide, macrolides and aminoglycosides is shown in Table 3.

Genetic profiling of resistance genes
The presence of resistance genes to vancomycin, erythromycin and streptomycin was investigated and results showed that the isolates harboured strA, erm(B), vanC2/3, vanC1 and vanB in their order of prevalence. The prevalence of all resistance genes screened was homogeneously distributed among the different enterococcal species identified in this study, while the vanA gene was not detected from any of the isolates investigated. The strA gene had the highest prevalence among the other resistance genes (Table 4).

Table 2. Observed phenotypic multiple resistance patterns among the isolates

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Multiple antimicrobial resistance pattern (phenotypic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>310</td>
<td>V/CD/S/E/ /CX/PG/NE</td>
</tr>
<tr>
<td>7</td>
<td>V/CD/S/E/AK/CX/PG/NE/KF/CIP/AUG</td>
</tr>
<tr>
<td>2</td>
<td>V/IMI/CD/S/E/ /CX/PG/NE/KF</td>
</tr>
<tr>
<td>12</td>
<td>V/CX/E/S/P/CD/CIP/KF/NE</td>
</tr>
<tr>
<td>2</td>
<td>V/CX/IMI/AUG/CIP/CD/S/E/NE/AK/KF/PG</td>
</tr>
<tr>
<td>250</td>
<td>V/CD/S/E/AK/CX/PG/NE/KF</td>
</tr>
</tbody>
</table>

V, vancomycin; CX, cloxacillin; S, streptomycin; E, erythromycin; NE, neomycin; CD, clindamycin; AUG, amoxicillin and clavulanate; IMI, imipenem; CIP, ciprofloxacin; KF, cephalothin; AK, amikacin; PG, penicillin G.

Table 3. Susceptibility to macrolide, aminoglycosides, lincosamide and vancomycin

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Class</th>
<th>Susceptibility</th>
<th>No. of strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>Macrolide</td>
<td>R</td>
<td>338 (99 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>3 (1 %)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Lincosamide</td>
<td>R</td>
<td>330 (97 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>11 (3 %)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Glycopeptide</td>
<td>R</td>
<td>341 (100 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Aminoglycoside</td>
<td>R</td>
<td>309 (91 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>32 (9 %)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Aminoglycoside</td>
<td>R</td>
<td>320 (94 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>21 (6 %)</td>
</tr>
</tbody>
</table>

S, susceptible; R, resistant.

Prevalence of virulence genes among the isolates
Among the virulence genes profiled, only ace, gelA and esp were detected in virtually all the isolates that were genetically investigated, species notwithstanding (Table 5), while the other virulence genes were not detected.

DISCUSSION
Enterococci are Gram-positive bacteria ubiquitously found in the gastrointestinal tracts of animals, birds and humans, as well as in soil and water (Byappanahalli et al., 2012; Hancock & Gilmore., 2006). Commensal bacteria, including species of the genus Enterococcus in commercial livestock and poultry, can contaminate the food chain during processing or find their way into the environment (Diarra et al., 2007; Diarrassouba et al., 2007). Diarrassouba et al. (2007), in a survey of retail raw meat, revealed that E. faecium was the predominant species of Enterococcus recovered from ground turkey (60 %), ground beef (65 %) and chicken breast (79 %), while E. faecalis was the predominant species (54 %) recovered from pork chops. In our study, the prevalence of E. faecium was 52.9 % while the other species (E. durans, E. hirae, E. casseliflavus and E. faecalis) were lower in their occurrence. Enterococci from faecal samples of cattle can spread to humans through direct contact with animals,
Table 5. Prevalence of virulent genes amplified from the study isolates

<table>
<thead>
<tr>
<th>Virulent genes</th>
<th>No. of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ace</td>
<td>320 (94.11 %)</td>
</tr>
<tr>
<td>getE</td>
<td>330 (97.1 %)</td>
</tr>
<tr>
<td>efaA</td>
<td>0</td>
</tr>
<tr>
<td>hylE</td>
<td>0</td>
</tr>
<tr>
<td>cyl</td>
<td>0</td>
</tr>
<tr>
<td>esp</td>
<td>270 (79.4 %)</td>
</tr>
</tbody>
</table>

through manure, through the use of manure for crop production, through milk or dairy products and also through faecal contamination of water via agricultural runoff into water bodies.

Antibiotic-resistant Enterococcus isolates from animal production have been reported (Aslam et al., 2012; Iwerebor et al., 2015a, b; Iwu et al., 2015; Jackson et al., 2007; Simjee et al., 2006). The resistance levels observed among our enterococcal isolates were higher than those reported in a Slovakian study on 82 isolates (Brtkova & Bujjakova, 2009). A very high percentage of neomycin, streptomycin, erythromycin, vancomycin and clindamycin resistance was found in the present study. The resistance patterns in all the isolates was very similar. Resistance among the isolates to ciprofloxacin, amoxicillin/clavulanate and imipenem were relatively low. This study to the best of our knowledge provides, for the first time, detailed antibiotic resistance genotypes of a variety of enterococci isolated from the faeces of commercial dairy cattle in the Nkonkonbe Municipality of the Eastern Cape of South Africa.

Tylosin is one of the antimicrobial agents used in both farms under study and it is known to select for erythromycin resistance due to similarity of target structure in the bacterial cell. The prevalence of phenotypic resistance among the isolates across all identified species was 99 % to erythromycin. The vanB, vanC1 and vanC2/3 genes were found across the species examined, with 19.7 %, 25 % and 40.29 %, respectively. Non-susceptibility to glycopeptide antibiotics like vancomycin and teicoplanin is a key resistance characteristic in enterococci (Werner et al., 2011). Acquired resistance to vancomycin is mediated by various genetic mechanisms (types vanA/B/D/E/G/L/M/N); the vanA and vanB resistance genotypes are by far the most prevalent worldwide (Sujatha & Praharaj, 2012). Isolates of E. casseliflavus are by nature intrinsically resistant to vancomycin at low levels, and the resistance is mediated by the vanC-1/2 type encoding gene (Sujatha & Praharaj, 2012). Jung et al. (2007) previously reported high vancomycin resistance among enterococci isolated from meat, faeces and raw milk samples collected in Korea, which is similar to our findings.

Erythromycin resistance in species of the genus Enterococcus is mediated by methylation of the 23S rRNA by methylase enzymes encoded by erm and other genes, and they are equally involved in the resistance of these bacteria to MLSB (macrolides, lincosamides and streptogramins B) antibiotics. There was a very high phenotypic resistance among the isolates to erythromycin and clindamycin, both of which belong to the macrolide and lincosamide class of antibiotic with frequencies of 99 % and 91 %, respectively. In the current study, erm(B) was detected in 137 (40.29 %) of the isolates. Sixty (43.8 %) of E. faecium isolates harboured erm (B), while the gene was also detected in 15 (10.9 %) E. faecalis, 37 (27 %) E. hirae and 25 (18.2 %) E. durans isolates. It is noteworthy to know that both farms sampled in this study used tylosin as a therapeutic agent and this could have resulted in the observed high resistances among the isolates to erythromycin, which has a similar structure to tylosin. Resistance to MLS antibiotics is encoded by the widespread erm(B) gene and only occasionally via erm(A) or erm(C) (Roberts et al., 1999). Erm (erythromycin resistance methylases) confer resistance by modifying nucleotide A2058 of the bacterial 23S rRNA (methylation), resulting in resistance to MLSB antibiotics. In enterococci, the erm(B) gene is constitutively expressed (Roberts et al., 1999). The erm (B) genetic determinant is widespread among enterococci, especially E. faecium and E. faecalis, and is part of many multi-resistance plasmids that are often linked to Tn1546-like vanA elements (Lavarde-Gomez et al., 2010; Werner et al., 2006). The findings in this study on erythromycin resistance are very similar to those reported by Klibi et al. (2015), who studied the prevalence of antibiotic resistance among enterococcal isolates from farm animals in Tunisia. They also reported that the erm(B) gene was responsible for the observed resistance among their isolates to erythromycin, and this is in agreement with our findings.

There was also a very high level of resistance to streptomycin and neomycin among the isolates: 309 isolates exhibited phenotypic resistance to both streptomycin and neomycin. Genetic profiling of the strA gene revealed that 50.88 % of the isolates harboured the resistance gene. The findings in this study are very similar to those of our previous study on virulence and antimicrobial resistance profiles of enterococcal isolates from pig farms within the same study area (Iwerebor et al., 2015b). Diarra et al. (2010) also reported the prevalence of high antimicrobial resistance among enterococcal isolates from broiler chickens in Canada.

The presence of the surface protein gene esp, the accessory colonization factor ace, the E. faecalis endocarditis antigen efaA and the gelatinase gelE, the hyaluronidase hylA, and cytolysinA cylA genes, which are involved in the virulence of enterococci, was also investigated by PCR. Apart from an unusually high antimicrobial resistance among the enterococci, the virulence of this organism could still be enhanced by the presence of additional virulence characteristics. In E. faecalis strains, several virulence molecules have been described that are associated with infection (Aslam et al., 2012). Many of these may interact with host cells and colonize the mucosal surfaces. Such interactions and colonizaion are considered as the early steps in the events that trigger pathogenesis in many infectious agents.

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The *ace* (adhesion of collagen) gene, which encodes a protein that mediates the association of bacteria to matrix protein of the host cells and the *esp* (enterococcal surface protein) gene encoding another surface protein, have been suggested to be involved in pathogenesis through adhesion of enterococcal cells to biotic and abiotic surfaces and in biofilm formation (Mannu et al., 2003). Another property of enterococci that has been considered a possible virulence factor is gelatinase gelE, a metalloprotease. It is mainly found in endocarditis isolates and can contribute to virulence. A higher prevalence of genotypic virulence markers (*gelE, esp* and *ace*) was detected in the majority of the isolates across species lines that we profiled, and this is in partial agreement with results from earlier studies (Aslam et al., 2012; Eaton & Gasson, 2001; Hamelin et al., 2007).

Enterococci readily acquire antibiotic resistance genes and lately they have become a serious cause of hospital infections, while only occasionally causing disease in animals. However, humans may be exposed to resistant enteric bacteria from animals via the food chain. Considering the ecological aspects of the use of antimicrobials, the use of antimicrobials as feed additives and in veterinary medicine should be properly regulated such that antimicrobial agents used in veterinary therapy are not employed in humans.

There is a general agreement that the indiscriminate use of antibiotics in farm animal production has created drug-resistant bacteria and that these resistant bacteria could be transmitted to people through food and then spread in the community by person-to-person transmission. In addition, antimicrobial resistant determinants are known to be transferable to other bacteria of the same or a different strain or species. Some severe infections by foodborne bacteria include life-threatening urinary infections and blood poisoning. In farm animals, the use of antibiotics that are critically important in human medicine could result in the emergence of new forms of multi-resistant bacteria that infect people. Some new strains of multi-resistant foodborne bacteria, such as *Campylobacter*, *Salmonella* and *Escherichia coli* that produce the ESBL and/or AmpC enzymes that inactivate nearly all beta-lactam antibiotics and the critically important 3rd and 4th generation cephalosporins, have been isolated from meat and faecal samples of farm animals (Dierikx et al., 2012; Iweriebor et al., 2015b). The over-use of antibiotics in intensive pig farming has been implicated in the emergence of a new ‘pig’ strain of the superbug methicillin-resistant *Staphylococcus aureus* (MRSA), first identified in 2004–2005 in the Netherlands (Geenen et al., 2013). This has spread rapidly among pigs in many European countries, to people who are in contact with the animals, and to the community and hospitals (Huijsdens et al., 2006). The livestock-associated MRSA strain has also colonized chickens, dairy cattle and veal calves and the people who handle them, and may also be emerging as a food safety risk (Casey et al., 2013; Geenen et al., 2013).

Bonten et al. (2001) reported on the occurrence of VRE carriage among healthy individuals in the community and in farm livestock in Europe. The possibility of colonization of humans by VRE predominantly through the food chain is very high, and infection with multidrug and/or virulent enterococcal strains from cheese to humans has been reported (Jamet et al., 2012; Macovei et al., 2007; Templer & Baumgartner, 2007). Moreover, similar strains of VRE have been isolated from farm animals and humans (Freitas et al., 2011). Even though the colonization of humans by animal isolates may be brief according to Sorensen et al. (2001), the risk of transfer of resistance genes during the colonization period is quite possible.

Since resistance development arises due to exposure of microorganisms to antimicrobial agents, bacterial populations isolated from the gut of animals exposed to antibiotics are more likely to be resistant to any given antibiotic agent. This can be further aggravated in animal manure through excretion and through the sharing of extra-chromosomal antibiotic resistance plasmids (R-plasmids) and determinants with non-resistant microbes. According to Ding et al. (2014), the widespread use of antibiotics and the agricultural application of manure can negatively affect the population of environmental microorganisms, and as well as stimulate the development of antimicrobial resistance among them. The possibility of antimicrobial resistant determinants accumulating in soil after application of manure from animal waste onto arable land leading to a build-up and extended bacteria, survival seems high (Gaze et al., 2013; Heuer et al., 2011a, b).

In conclusion, we report here the high prevalence of enterococcal isolates from faecal samples of dairy cattle exposed to tylosin that are harbouring multi-resistance antimicrobial determinants and virulence genes. The use of antimicrobials as growth promoters at sub-therapeutic levels in the management of domesticated animals carries the potential for the evolution and spread of resistance bacteria in both zoo-notic and non-pathogenic environmental isolates. The high levels of antibiotic resistance observed among the isolates suggest a huge threat to both human and veterinary medicine, and calls for urgent action on the need for prudent usage of antimicrobials. The limitation of the study is our inability to perform multi-locus sequence typing to enable delineation of the isolates into clonal types which could have provided valuable epidemiological information.

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**REFERENCES**


Antimicrobial, virulence factors in enterococci isolates from dairy cattle


Enterococcus faecium minants and antibiotic resistance between strains of dairy, animal and clinical origin.


