Characterization of high level ampicillin- and aminoglycoside-resistant enterococci isolated from non-hospital sources

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Abstract

**Purpose.** High level ampicillin- and aminoglycoside-resistant enterococci are being increasingly reported from non-hospital sources. This study was carried out to characterize these strains from non-hospital sources in Nigeria.

**Methodology.** A collection of Enterococcus faecium isolated from vegetables, soil, farm animals and manure and observed to be resistant to ampicillin (n=63) and gentamicin (n=37) discs, were screened for resistance to high levels of ampicillin and aminoglycoside using E-test strips. Putative high level ampicillin- and aminoglycoside-resistant strains were screened for pbp5 and aminoglycoside modifying enzyme genes, respectively, by PCR. The C-terminal region of the amplified pbp5 gene was also sequenced.

**Results.** Five (5/63) and thirty-five (35/37) of the ampicillin- and aminoglycoside-resistant strains were identified as high level ampicillin- and aminoglycoside-resistant E. faecium strains, respectively, based on the MIC results. The amplified pbp5 gene from the high level ampicillin-resistant isolates displayed 96–99 % nucleotide sequence similarity with the reference strains and three novel insertions (500Glu→Leu, 502Asp→Arg and 614Ile→Phe) in the amino acid sequence. Aminoglycoside modifying enzyme genes aac(6')-Ie-aph(2') (100 %), aac(2')-lc (88.8 %), aac(3')-Ila (90 %) and ant(4')-Ia (40 %) were detected among the high level aminoglycoside-resistant isolates.

**Conclusion.** This is the first report on the characterization of high level ampicillin- and aminoglycoside-resistant Enterococcus faecium among animals and vegetables in Nigeria. The results show that non-hospital sources can constitute a reservoir for potential dissemination of these strains and genes to humans via the food chain or by direct contact.

INTRODUCTION

Antimicrobial agents are widely used in livestock production for growth promotion, prophylaxis and chemotherapy, a practice which promotes the emergence of resistance among bacteria by selective pressure [1]. Enterococcus spp. have been isolated from various sources such as animals, foods of animal origin, clinical samples, food-processing environments and water [2–4]. These species easily respond to the selective pressure of antimicrobial use in addition to their capability to pick and disseminate resistance genes to other bacteria [5]. They are therefore used to effectively monitor the evolution and prevalence of antimicrobial resistance in different ecosystems [6].

There are over 35 species belonging to the genus Enterococcus, and among them, Enterococcus faecium and Enterococcus faecalis are the most important species due to their association with cases of human infection [7]. Worldwide, they are currently ranked third among leading causes of nosocomial infections such as endocarditis, wound and urinary tract infections [8].

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Keywords: Enterococcus faecium; high level ampicillin resistance; high level aminoglycoside resistance; non-human sources; Nigeria; vegetables.

Abbreviations: AME, aminoglycoside modifying enzyme; BHI, brain–heart infusion (broth); HLAR, high level aminoglycoside-resistant; PBP, penicillin-binding protein.

The GenBank/EMBL/DDBJ accession numbers for the pbp5 sequence of Enterococcus faecium strains NEO23, NEO17, NEO45 and NEO22 are KX377704–KX377707, respectively.

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Aminoglycosides, preferably gentamicin, in combination with a cell-wall inhibitor, are the drugs of choice (to achieve a synergistic effect) for the treatment of enterococcal infection [9]. However, the occurrence of enterococci resistant to cell-wall inhibitors (as a result of production of an altered penicillin-binding protein or rarely β-lactamases) [10] and aminoglycosides [mediated by aminoglycoside modifying enzyme genes (AMEs) or mutation in the 30S ribosomal subunit] eliminates this synergistic effect [11]. The occurrence of strains resistant to these classes of drugs is therefore an important problem in clinical settings around the world [12–14]. Recently, these strains have been increasingly detected in animals and their products in different countries [2, 3, 15, 16].

Similarities between the resistant strains from non-hospital sources such as animals and those causing human infections have been reported, raising public health concerns and suggesting livestock environments and products may play an important role in the evolution and dissemination of high level ampicillin- and aminoglycoside-resistant enterococci to humans [17–19]. The detection of these strains among animals may pose a potential public health risk, especially in developing countries where close contact exists between animals and humans. It is a common practice to use animal manure to fertilize farmlands, which leads to contamination of food produce and water bodies [4].

However, in spite of these potential risks and the extensive use of aminoglycosides for prophylaxis and chemotherapy, especially in poultry production across Nigeria, there is no such information on the occurrence of high level ampicillin- and aminoglycoside-resistant species of the genus Enterococcus among animals and animal products. Few studies investigating the occurrence and antimicrobial resistance of enterococci have been reported in Nigeria [20–22]. However, none of these studies are targeted towards the occurrence and characterization of strains resistant to high level ampicillin and gentamicin, two important antibiotics used in the treatment of enterococcal infection.

Also much remains to be known in this area on the role animals and related environments may play in the evolution and dissemination of these strains and the potential public health risk they pose in this region. The aim of this study was therefore to investigate and characterize high level ampicillin- and aminoglycoside-resistant E. faecium from livestock and related environments in Nigeria.

**METHODS**

**Bacterial strains**

In this study, strains of E. faecium previously identified as resistant to ampicillin (n=63) and gentamicin (n=37) were studied [23]. The strains were isolated from vegetables (ampicillin n=12), faeces (ampicillin n=33, gentamicin n=27) and litter of poultry (ampicillin n=12, gentamicin n=10) and cattle (ampicillin n=6) [23]. For this study, the isolates were recovered from the stored slants by subculturings on tryptone soya agar (Oxoid) followed by incubation for 18–24 h at 37 °C under aerobic conditions.

**Determination of minimum inhibitory concentration (MIC)**

The ampicillin and gentamicin MICs for the respective isolates were determined using the E-test gradient diffusion strips (bioMérieux) according to the manufacturer’s instructions. Interpretation of resistance breakpoints was based on the Clinical and Laboratory Standards Institute (CLSI) method [24]. Resistance of the isolates to MIC of ampicillin and aminoglycoside (gentamicin) ≥16 µg ml⁻¹ and ≥500 µg ml⁻¹, respectively, were defined as high level resistance [24]. Briefly, Muller–Hinton agar (Oxoid) plates were inoculated with bacterial suspensions adjusted to a turbidity equivalent to a 0.5 McFarland standard. This was followed by placement of the E-test strips and incubation of the plates for 24 h at 37 °C. The MIC of ampicillin-sulbactam was also determined using the agar-dilution method according to the CLSI method [24].

**Detection of β-lactamase activity**

The production of the β-lactamase enzyme was tested using β-lactamase sticks. Briefly, the end of the β-lactamase stick impregnated with nitrocefin, a chromogenic cephalosporin, was moistened with a drop of distilled water and used to touch a colony of the test isolate. The stick was then rotated over the colony to pick up a mass of the cells after which it was kept slanted in a Petri dish for the reaction to occur. The development of a pink/red colour on the impregnated tip within 5–15 min after picking the colony was indicative of a positive reaction (production of β-lactamase).

**Extraction of DNA**

For bacterial genomic DNA extraction, each strain was grown in brain heart infusion broth (BHI) for 24 h. Extraction of genomic DNA was performed as previously described [25] with minor modifications which included an overnight incubation in lysis buffer and ethanol-washing of the DNA pellets. Recovered DNA was kept at 4 °C until use.

**Screening for pbp5 gene and sequencing**

The isolates expressing high level ampicillin MIC were screened by PCR for carriage of the gene pbp5 encoding penicillin-binding protein 5. The C-terminal region of the amplified PCR product was purified and sequenced on both strands to detect mutations in the amino acid sequence coded by the pbp5 gene. The PCR and sequencing were carried out using primers (Table 1) and conditions described by Jureen et al. [26]. Sequencing of the amplicons was performed using an Applied Biosystems 3130xl genetic analyser with the BigDye terminator v3.1 reagent (Applied Biosystems). Alignment and translation were carried out using the BioEdit software, and the nucleotide and amino acid sequences were compared with the pbp5 sequence of the reference strain E. faecium D63r (accession no. X84860) in the GenBank database.
Table 1. Primer sequences used for the amplification of some genes encoding antimicrobial resistance in species of the genus Enterococcus

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pbp5</em></td>
<td>PBPSF-1: 5'-TACCAATGCAAAACGGG-3'</td>
</tr>
<tr>
<td></td>
<td>PBPSR: 5'-TATCCCTTGGTATCGAGG-3'</td>
</tr>
<tr>
<td><em>aac(6)-Ie</em></td>
<td>F: 5'-CAGACCTTGGGAAAGATGAA-3'</td>
</tr>
<tr>
<td><em>aph(2)-Ia</em></td>
<td>R: 5'-CCTGGTGTAATCTGCTAGGG-3'</td>
</tr>
<tr>
<td><em>aph(2)-Ic</em></td>
<td>F: 5'-CCCAAGTATGCTACGGTCC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCCAACGTGGTTCTGCACAGG-3'</td>
</tr>
<tr>
<td><em>aph(3)-IIa</em></td>
<td>F: 5'-GGCTTAAATGGAATACTACCGGG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTTAAAAATATCATAGCTCCGG-3'</td>
</tr>
<tr>
<td><em>ant(4)-Ia</em></td>
<td>F: 5'-CAATCTGCTAAATCGGTAGAAGCC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGGAATTTGACGACAGATTACGA-3'</td>
</tr>
</tbody>
</table>

Nucleotide sequence accession numbers

The *pbp5* gene sequences of the high level ampicillin-resistant *E. faecium* were deposited in the GenBank database (www.ncbi.nlm.nih.gov/Genbank) with accession numbers KX377704–KX377707.

Detection of aminoglycoside modifying enzyme (AME) genes by PCR

The isolates identified as resistant to high level aminoglycoside were screened by PCR to detect AME genes *aac(6)-Ie-aph(2)-Ia*, *aph(2)-Ic*, *aph(3)-IIa* and *ant(4)-Ia* using the primers and conditions previously described by Vakulenko et al. [27]. The selected primers amplify a 348-, 444-, 523- and 294-bp fragment of the listed AME genes, respectively (Table 1). Briefly, the PCR reactions were performed in a 10 µl reaction mixture containing 3 µl DNA template, 0.5 µl 50 mM MgCl₂, 0.8 µl 2.5 mM dNTPs, 0.8 µl DMSO, 2.9 µl H₂O, 1.0 µl of 10X PCR buffer, 0.1 µl (2.5 U) TaqDNA polymerase (Qiagen) and 0.5 µM each of forward and reverse primers. Amplification was carried out using the following thermal cycling conditions: an initial denaturation for 3 min at 94°C; 35 cycles of 40 s at 94°C (denaturation), 40 s at 55°C (annealing) and 40 s at 72°C (extension); and a final extension step of 2 min at 72°C. *E. faecalis* ATCC 49532 and *E. faecium* SF11770 lb (Chow) were used as positive controls for detection of *aac(6)-Ie-aph(2)-Ia* and *aph (2')-Ib*, respectively. The PCR products were analysed on 1% (w/v) TBE-agarose gels and the bands were visualized by the gel documentation system (ENDURO™ GDS Gel Documentation System, Labnet International, Inc, USA).

RESULTS AND DISCUSSION

Five (7.9%) and 35 (94.6%) of the *E. faecium* isolates tested expressed ampicillin and gentamicin MIC equal to or greater than the 16 µg ml⁻¹ and 500 µg ml⁻¹ recommended resistance breakpoints for each antibiotic, respectively. To our knowledge, this is the first study to characterize high level ampicillin- and aminoglycoside-resistant species of the genus Enterococcus from non-hospital sources in Nigeria, and one of the few reports from Africa. Nonetheless, reports are available from other continents and countries in Africa on the occurrence of these strains in non-hospital sources such as animals and related environments [2, 3], water bodies [4] and food [28].

Antimicrobial resistance is a serious emerging public health concern because of the compromised efficacy of antimicrobial agents used in the treatment of infectious diseases. The emergence and spread of resistance among enterococci isolates is a serious threat to public health [5]. The occurrence of high level ampicillin- and aminoglycoside-resistance among the *E. faecium* isolates in this study, especially the poultry isolates, is not surprising given the indiscriminate use of these agents in livestock production in Nigeria. In Nigeria, like in many developing countries, there is no restriction on the use of antibiotics in animal production [29]. Cell-wall inhibitors and aminoglycosides in various combinations are administered indiscriminately in avian production across Nigeria to prevent or treat infections. Indiscriminate use of antibiotics in livestock production selects for resistance among enteric commensals [1, 6]. We therefore hypothesize that this may have contributed to the occurrence of these strains in addition to the resistance to other antibiotics observed, especially among the poultry isolates in the present study. The finding of this study is in agreement with those of other authors in different countries [3, 18, 19]. The detection of strains resistant to the two agents in this study raises serious public health concerns as it limits the spectrum of antibiotics available for the treatment of enterococcal infection.

Cell-wall inhibitors and aminoglycosides are the mainstay drugs in the treatment of enterococcal infection [9, 11]. Therefore, resistance to these drugs is of major concern from a clinical point of view. The results observed for the MIC of ampicillin-sulbactam among the isolates was the same as that observed for ampicillin. Also none of the isolates was positive to the β-lactamase test, further confirming the resistance observed against ampicillin was not as a result of the production of β-lactamase. Enterococcal resistance to the β-lactams is mainly mediated by the production of altered penicillin-binding proteins (PBPs) with decreased affinity for the β-lactams, and rarely by the production of β-lactamases [30].

The decreased affinity of the PBP is usually due to point mutations around three specific conserved motifs; 425STF, 482SDN and 619KTG in the C-terminal region of the *pbp5* gene identified as the main targets of β-lactams [31]. These amino acid substitutions selectively influence the binding of β-lactam antibiotics to one or both active sites of the PBP5 which are close to the site of mutation [31–33]. Sequence analysis of the C-terminal region of their amplified products revealed a total of nine different amino acid changes: His470→Gln, 496Asn→Lys, 497Phe→Ile, 499Aa→Thr, 500Glu→Leu, 502Asp→Arg, 525Glu→Asp, 612Ile→X and 614Le→Phe. Three variants (A, B and C) based on the pattern of amino acid substitutions in the C-terminal region were detected (Table 2). The sequence
Table 2. Amino acid polymorphisms detected in the C-terminal region of the pbp5 gene of five high level ampicillin-resistant isolates of Enterococcus faecium recovered from non-human sources

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Source of isolate</th>
<th>Amino acid changes at different positions of the C-terminal region</th>
<th>Alleles</th>
<th>Similarity to X84860 (%)</th>
<th>MIC (µg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>X84860</td>
<td>Unknown</td>
<td>H N F A G D E I I</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>(51) 9730357-1</td>
<td>Unknown</td>
<td>Q N F A G D E I I</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>41</td>
<td>Cloacal swab</td>
<td>Q N I Y L R D I I A</td>
<td>96</td>
<td></td>
<td>≥265</td>
</tr>
<tr>
<td>23</td>
<td>Lettuce</td>
<td>Q K F T G D D X F B</td>
<td>96</td>
<td></td>
<td>≥265</td>
</tr>
<tr>
<td>45</td>
<td>Cloacal swab</td>
<td>Q N F A G D E I I C</td>
<td>99</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>17</td>
<td>Cattle manure</td>
<td>Q N F A G D E I I C</td>
<td>99</td>
<td></td>
<td>≥265</td>
</tr>
<tr>
<td>22</td>
<td>Cattle manure</td>
<td>Q N F A G D E I I C</td>
<td>99</td>
<td></td>
<td>≥265</td>
</tr>
</tbody>
</table>

H, histidine; Q, glutamine; N, asparagine; K, lysine; A, alanine; T, threonine; E, glutamic acid; D, aspartic acid; I, isoleucine; F, phenylalanine; Y, tyrosine; X, undetermined or non-standard amino acid; ND, not determined; MIC, minimum inhibitory concentration. The bold amino acids are the novel substitutions detected.

data of the pbp5 gene in the present study revealed that amino acid substitution (470His→Gln) located around two active-site-defining motifs (STFK and SDN) was significantly common in all the high level ampicillin-resistant isolates. In addition to substitutions 496Asn→Lys, 497Phe→Ile, 499Ala→Thr and 525Glu→Asp which have been previously described in some high level ampicillin-resistant E. faecium strains [32, 33, 35], novel substitutions at positions 500Glu→Leu, 502Asp→Arg and 614Ile→Phe, which to the best of our knowledge have not been reported, were detected in this study.

In the present study, a difference in the ampicillin MIC was observed in three strains harbouring similar mutations in the C-terminal region of the pbp5 gene. This finding agrees with earlier reports that pbp5 mutation alone cannot explain the difference in ampicillin MICs among resistant E. faecium [31, 33–35]. Factors such as regulation, expression, translational modifications, or other genes have been suggested to account for the difference in ampicillin MICs of resistant E. faecium [10]. Mutations in the N-terminal sequence of pbp5 [35], overexpression/production of PBP5 [36, 37], and the nature of the peptidoglycan precursors [38] have earlier been reported to play a significant role. However, recently the contribution of genes (fbsW and psr) located at the upstream region of pbp5 [38, 39] and the role of host/source background of the isolates have been increasingly reported [39, 40].

Until recently ampicillin-resistant Enterococcus faecium were rarely isolated from animals and humans in the community [30]. They were thought to occur only in the hospital environment and are therefore used as markers for the identification of hospital-acquired resistant E. faecium clones [18]. However, in agreement with the findings of other studies [3, 18, 41], the results of this study show that other non-hospital sources are emerging as reservoirs for the dissemination of these ampicillin-resistant enterococci. The detection of these isolates on lettuce suggests they can contaminate vegetables through manure and be transmitted to human consumers via the food chain.

The 35 high level aminoglycoside-resistant (HLAR) isolates originated from cloacal swabs (n=25), rectal swabs (n=8) and manure (n=2). The bifunctional aminoglycoside modifying enzyme gene aac(6\(^{-}\))-Ie-aph(2\(^{\prime}\)) was the most prevalent (100 %) AME gene detected among the HLAR isolates. Other AME genes detected include aph(2\(^{-}\))-Ic (88.8 %), aph(3\(^{-}\))nia (90 %) and ant(4\(^{-}\)) (40 %). Three of the HLAR isolates harboured all four AME genes (Table 3). Resistance of enterococci to gentamicin is a good predictor of its resistance to other aminoglycosides with the exception of streptomycin [42]. The most common mechanism of resistance to high level aminoglycosides among enterococci is the production of aminoglycoside modifying enzymes (AMEs) [43]. The AME genes aac(6\(^{-}\))-Ie-aph(2\(^{\prime}\))-Ia, aph(2\(^{-}\))-lc, ant(4\(^{-}\)) and aph(3\(^{-}\))nia were detected in this study. Similar genes have been detected among HLAR Enterococcus isolated from hospital environments in some countries [3, 9, 44]. The aac(6\(^{-}\))-Ie-aph(2\(^{\prime}\))-Ia gene that was frequently harboured by the isolates in this study encodes the bifunctional aminoglycoside modifying enzyme that causes Enterococcus to be resistant (gentamicin MIC ≥500µg ml\(^{-1}\)) to all the clinically important aminoglycosides and eliminates the synergism between aminoglycosides and cell-wall-active agents. Other authors have

Table 3. High level aminoglycoside-resistant (HLAR) Enterococcus faecium and aminoglycoside modifying enzyme (AME) genes detected from non-human samples

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>No. of HLAR positive isolates</th>
<th>AME genes detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloacal swabs</td>
<td>25</td>
<td>aac(6(^{-}))-Ie-aph(2(^{\prime}))-Ia, aph(2(^{-}))-Ic, aph(3(^{-}))nia, ant(4(^{-}))-Ia</td>
</tr>
<tr>
<td>Manure</td>
<td>8</td>
<td>aac(6(^{-}))-Ie-aph(2(^{\prime}))-Ia, aph(2(^{-}))-Ic, aph(3(^{-}))nia, ant(4(^{-}))-Ia</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>2</td>
<td>aac(6(^{-}))-Ie-aph(2(^{\prime}))-Ia</td>
</tr>
</tbody>
</table>
reported this gene to be the most predominant among aminoglycoside-resistant enterococci [3, 45]. This enzyme possesses the ability to phosphorylate and acetylate the aminoglycoside using $6'$-acyltransf erase and $2'$-phosphotransferase activity [43]. The $\text{aac(6')-ie-aph(2')-Ia}$ gene is commonly harboured on the composite transposon \textit{Tn}5281 (IS256-related) indicating it can easily be transferred horizontally to other bacteria and suggests the potential for dissemination into the environment [42, 46].

**Conclusion**

The findings of this study showed that non-hospital sources, specifically poultry, cattle and vegetables, harbour \textit{E. faecium} resistant to high level ampicillin and aminoglycoside, suggesting these sources may contribute to the evolution and likely spread of the strains and genes. Detailed molecular comparison of genomes of isolates from hospital and non-hospital sources in Nigeria will help to better define the transmission dynamics of ampicillin- and aminoglycoside-resistant enterococci from non-hospital sources to humans. Abuse of antimicrobials in animal production contributes significantly to increasing antimicrobial resistance among bacteria. Based on the findings of this study, legislative regulation, and judicious and prudent use of antimicrobials in animal production in Nigeria is recommended.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


