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SHORT REPORT
High-level aminoglycoside resistant enterococci isolated from swine

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SUMMARY

Approximately 42% (187/444) of swine enterococci collected between the years 1999 and 2000 exhibited high-level resistance to gentamicin (MIC $\geqslant 500\,\mu$g/ml), kanamycin (MIC $\geqslant 500\,\mu$g/ml), or streptomycin (MIC $\geqslant 1000\,\mu$g/ml). Eight aminoglycoside resistance genes were detected using PCR, most frequently *ant*(*6*)-*Ia* and *aac*(*6′*)-*Ii* from *Enterococcus faecium*. Twenty-four per cent (45/187) of total high-level aminoglycoside-resistant isolates and 26% (4/15) of isolates resistant to high levels of all three antimicrobials were negative for all genes tested. These data suggest that enterococci isolated from swine contain diverse and possibly unidentified aminoglycoside resistance genes.

The possibility of transfer of antimicrobial-resistant bacteria from animals to humans has caused increased interest in antimicrobials that are used in both veterinary and human medicine. Introduced into clinical application in the mid-1900s, aminoglycosides are one class of antimicrobials that are used in both areas for treatment of bacterial infections [1–3]. Although limited in use in food-animal production due to their toxic nature, aminoglycosides are administered for treatment and control of severe infections caused by both Gram-negative and Gram-positive bacteria. In swine, gentamicin, neomycin, or streptomycin can be used to manage weanling pig scours caused primarily by *Escherichia coli*. Gentamicin is also indicated in the treatment of swine dysentery caused by *Treponema hyodysenteriae*. Until recently, enteritis and swine dysentery were also treated with apramycin, but use of the drug in the United States has been voluntarily withdrawn.

In the United States, resistance to aminoglycosides in human enterococci isolates has been extensively studied, but limited data exists about the prevalence of these genes in enterococci from other sources, particularly of animal origin. In this study, the prevalence as well as distribution of genes encoding aminoglycoside-modifying enzymes (AME) and conferring high-level resistance to gentamicin, kanamycin, and streptomycin in enterococci from swine was investigated.

Enterococci used in this study represented a subset of enterococcal isolates collected for the veterinary surveillance branch of NARMS (National Antimicrobial Resistance Monitoring System) at the USDA-ARS, Athens, GA between the years 1999 and 2000 [4]. Enterococci were isolated from swine faecal samples collected on-farm from three geographically distinct locations within the United States. Each farm was visited a total of eight times with 150 faecal samples collected per visit per farm. One presumptive *Enterococcus* isolate was analysed per animal. All media used in this study were purchased from Becton

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Table 1. *Summary of high-level aminoglycoside resistance among swine enterococci*

	No. of resistant isolates per species (%)*					
Aminoglycoside	*E.c.* ($n=7$)	*E.d.* ($n=48$)	*E.fs.* ($n=61$)	*E.fm.* ($n=59$)	*E.g.* ($n=2$)	*E.h.* ($n=10$)
Gentamicin ($n=28$)	0 (0)	2 (7·1)	16 (57·1)	7 (25·0)	0 (0)	3 (10·7)
Kanamycin ($n=132$)	7 (5·3)	30 (22·7)	32 (24·2)	53 (40·2)	2 (1·5)	8 (6·1)
Streptomycin ($n=140$)	3 (2·1)	44 (31·4)	49 (35·0)	36 (25·7)	1 (0·7)	7 (5·0)

E.c., *E. casseliflavus*; *E.d.*, *E. durans*; *E.fs.*, *E. faecalis*; *E.fm.*, *E. faecium*; *E.g.*, *E. gallinarum*; *E.h.*, *E. hirae*.

* Per cent resistance was determined by dividing the number of resistant isolates (per species) by the total number of resistant isolates per antimicrobial.

Dickinson (Sparks, MD, USA). One gram of faecal sample was diluted 1:10 in phosphate-buffered-saline (PBS; pH 7·4), vortexed and 100 μl of the diluted sample was inoculated onto BBL Enterococcosel agar and incubated for 24 h at 37 °C. Presumptive enterococcal isolates were subcultured onto trypticase soy agar (TSA) containing 5% defribinated sheep blood. Enterococcal species identification was performed as previously described [5].

Minimum inhibitory concentrations (MIC, μg/ml) for enterococci were determined by broth microdilution using the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems Ltd, Westlake, OH, USA) according to the manufacturer's directions. A customized panel of antimicrobials for the NARMS programme was used including gentamicin, kanamycin, and streptomycin. Results were interpreted according to NCCLS guidelines [6]. High-level resistance was defined as MIC $\geqslant 500$ μg/ml for gentamicin and kanamycin and $\geqslant 1000$ μg/ml for streptomycin. *Enterococcus faecalis* ATCC 29212 was the positive control for determination of MIC.

Oligonucleotides were synthesized by Operon Technologies (Alameda, CA, USA). Primers and cycling conditions used for detecting *aph*(2″)-*Ib* and *aph*(2″)-*Id* were as previously published [7]. Multiplexing primers and cycling conditions for *aac*(6′)-*Ii*, *aac*(6′)-*Ie-aph*(2″)-*Ia*, *ant*(4″)-*Ia*, *ant*(6)-*Ia*, *ant*(9)-*Ia*, *aph*(2″)-*Ic*, and *aph*(3″)-*IIIa* were also as previously described [8]. PCR was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) as previously described using an annealing temperature of 50 °C [9]. Positive controls for PCR were as follows: *E. faecium* SF11770 [*aph*(2″)-*Ib*] [7], *E. casseliflavus* SF11300 [*aph*(2″)-*Id*] [10], *E. faecium* G128E-1013 [*aac*(6′)-*Ii*] (this study), *E. faecalis* ATCC 49532 [*aac*(6′)-*Ie-aph*(2″)-*Ia*] [11], *E. gallinarum* 15N12-928 [*ant*(4″)-*Ia*] (this study), *E. faecalis* ATCC 49533 [*ant*(6)-*Ia*] [11], *Escherichia coli* NM554 (pAT28) [*ant*(9)-*Ia*] [12], *E. gallinarum* SF9117 [*aph*(2″)-*Ic*] [13], and *E. faecium* 10N-55-1023 [*aph*(3″)-*IIIa*] (this study). *E. faecium* G128E-1013 [*aac*(6′)-*Ii*], *E. gallinarum* 15N12-928 [*ant*(4″)-*Ia*], and *E. faecium* 10N-55-1023 [*aph*(3″)-*IIIa*] were all obtained from swine faecal samples. To verify PCR products, amplicons were sequenced at the ARS Regional Sequencing Facility, Southeastern Poultry Research Laboratory, Athens, GA, USA and sequences compared to AME genes using NCBI-BLAST analysis [14, 15]. For pulsed-field gel electrophoresis (PFGE), cells from a 5 ml overnight culture were pelleted, embedded in agarose plugs and lysed as previously described [16]. Plugs were digested overnight with 20 U of *Sma*I (Roche Molecular Biochemicals, Indianapolis, IN, USA) and digested DNA separated on a 1·2% SeaKem agarose gel using a CHEF-DRII pulsed-field electrophoresis system (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out at 6 V for 21 h with a ramped pulse time of 5–30 s in 0·5× Tris-borate-EDTA (TBE) buffer (14 °C). Cluster analysis was determined with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair-group method (UPGMA). Optimization settings for dendrograms were 4% with a band tolerance of 2–5%.

From the study, 444 enterococci were isolated and 42% (187/444) of the isolates were resistant to high levels of gentamicin (HLR-G), kanamycin (HLR-K),

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Table 2. *Distribution of aminoglycoside resistance genes among high-level aminoglycoside resistant swine enterococci*

	No. of isolates (%)*					
AME gene	*E.c.* (*n*=7)	*E.d.* (*n*=48)	*E.fs.* (*n*=61)	*E.fm.* (*n*=59)	*E.g.* (*n*=2)	*E.h.* (*n*=10)
ant(6)-la (*n*=96)	2 (28·6)	30 (62·5)	27 (44·3)	31 (52·5)	1 (50·0)	5 (50·0)
ant(9)-la (*n*=14)	0 (0)	2 (4·2)	11 (18·0)	1 (1·7)	0 (0)	0 (0)
ant(4′)-la (*n*=17)	3 (42·9)	4 (8·3)	5 (8·2)	4 (6·8)	1 (50·0)	0 (0)
ant(3′)-llla (*n*=71)	2 (28·6)	23 (47·9)	21 (34·4)	23 (39·0)	0 (0)	2 (20·0)
aac(6′)-le-aph(2″)-la (*n*=21)	0 (0)	2 (4·2)	14 (23·0)	5 (8·5)	0 (0)	0 (0)
aph(2″)-ld (*n*=2)	0 (0)	2 (4·2)	0 (0)	0 (0)	0 (0)	0 (0)
aac(6′)-li (*n*=82)	2 (28·6)	5 (10·4)	12 (19·7)	59 (100)	1 (50·0)	3 (30·0)
Negative† (*n*=45)	2 (28·6)	15 (31·3)	24 (39·3)	0 (0)	0 (0)	4 (40·0)

E.c., *E. casseliflavus*; *E.d.*, *E. durans*; *E.fs.*, *E. faecalis*; *E.fm.*, *E. faecium*; *E.g.*, *E. gallinarum*; *E.h.*, *E. hirae*.
* Per cent positive was determined by dividing the number of isolates containing the AME gene by the total number resistant isolates (per species).
† No PCR products were detected for any of the AME genes tested.

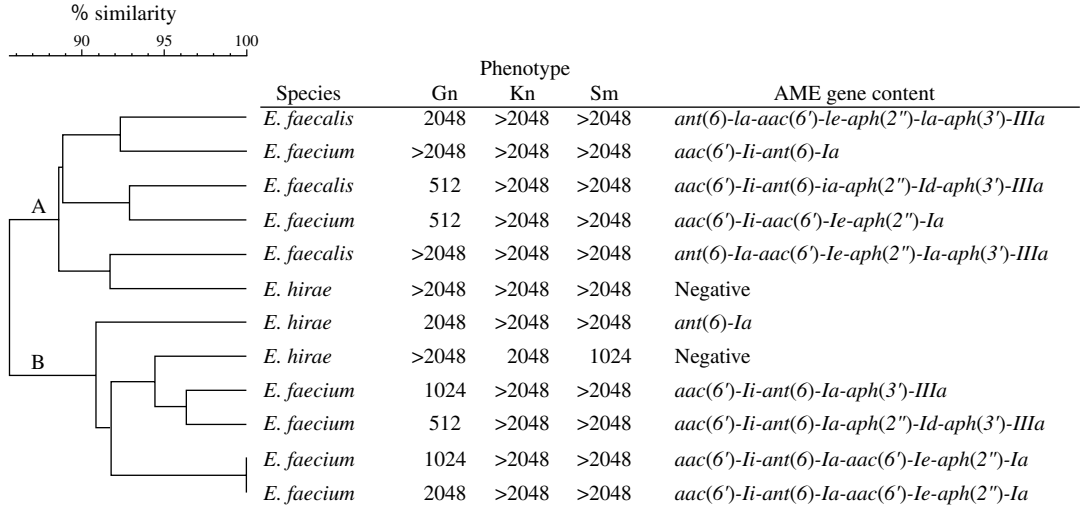

Fig. Dendrogram of high-level aminoglycoside-resistant enterococci from a single farm. Species, aminoglycoside resistance levels and gene content are shown. Two clusters (A and B) were formed with 86% overall similarity. Levels of similarity were determined using Dice coefficient.

or streptomycin (HLR-S). Table 1 shows the distribution of high-level aminoglycoside resistance in enterococci isolated from swine faecal samples. Only 6% (*n*=28) of isolates were HLR-G, whereas 30% and 32% were HLR-K and HLR-S respectively. These percentages are lower than ones previously reported for kanamycin and streptomycin resistance in pigs [17]. The predominant HLR species was *Enterococcus faecalis* (*n*=61) followed by *E. faecium* (*n*=59) and *E. durans* (*n*=48). *E. faecalis* isolates were more frequently resistant to gentamicin and streptomycin, but *E. faecium* isolates were more frequently highly resistant to kanamycin. Interestingly, HLR-G was not found in *E. casseliflavus* or *E. gallinarum*, but HLR-G was found in *E. durans* and *E. hirae*, two species rarely associated with HLG-R (Table 1).

Of the 10 AME genes examined, eight AME genes [*ant(6)-Ia*, *ant(9)-Ia*, *ant(4′)-Ia*, *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Id*, and *aac(6′)-Ii*] were identified from the swine samples (Table 2). Two AME genes [*aph(2″)-Ib* and *aph(2″)-Ic*] were not detected in any of the isolates and 45 isolates did not contain any known AME genes. These results were not unexpected because *aph(2″)-Ib*, and *aph(2″)-Ic* have only recently been detected in *Enterococcus* suggesting that they may not be as widely disseminated among enterococci as other AME genes [7, 13].

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HLR-S isolates were more frequently found without a corresponding AME resistance gene compared to HLR-G and HLR-K isolates. In the absence of a detected AME gene, HLR-S is most likely due to mutation in the 30S ribosomal subunit [18]. The *ant*(*6*)-*Ia* gene was most often identified in HLR-S isolates and *aac*(*6′*)-*Ii* for HLR-K isolates. Although the most frequently detected AME genes were *ant*(*6*)-*Ia* and *aac*(*6′*)-*Ii* both in *E. faecium, E. durans* strains (62·5%) contained a higher percentage of *ant*(*6*)-*Ia* than *E. faecium* strains (52·5%) (Table 2). *E. durans* strains (47·9%) also contained a higher percentage of *aph*(*3′*)-*IIIa* than *E. faecium* strains (39%). In addition, *E. durans* was the only species in which *aph*(*2″*)-*Id* was detected. The majority of gentamicin resistance was conferred by the bi-functional gene *aac*(*6′*)-*Ie-aph*(*2″*)-*Ia*. The low number of isolates identified containing *aac*(*6′*)-*Ie-aph*(*2″*)-*Ia* was surprising (Table 2). This gene has been found on plasmids in *Enterococcus* and *Staphylococcus aureus* and mediates resistance to all clinically important aminoglycosides [19, 20]. The scarcity of this gene in these isolates suggests that it may not be as common in these animal enterococci as in enterococci from other sources.

Enterococcal isolates resistant to gentamicin, kanamycin, and streptomycin were examined for their AME gene content to determine the relationship between the resistance profile and AME gene content. Fifteen isolates were highly resistant to all three aminoglycosides, 12 from the same farm. Four of the 15 (~27%) were negative for all AME genes in this study. Two of the four negative isolates were *E. durans* from two different farms, and the remaining two were *E. hirae* isolated from the same farm. The Figure shows cluster analysis of the 12 isolates originating from the same farm. Two clusters were evident from the analysis with an overall similarity of 86%. Isolates in cluster A were 89% similar while isolates in cluster B were 91% similar. Isolates were examined based upon isolation date, age of animal, species identification, aminoglycoside resistance profile and AME gene content, but no obvious relatedness could be discerned from the analysis. Only two *E. faecium* isolates were 100% identical based upon PFGE results and these two also contained the same AME genes (Fig.). These data suggest that a single source was not responsible for dissemination of aminoglycoside resistance in these isolates from this farm. In addition, two HLR-G isolates, one HLR-G, HLR-K and one HLR-S isolate did not contain genes conferring resistance to those antimicrobials suggesting either the presence of previously unidentified AME genes or, for streptomycin resistance, ribosomal mutations [18, 21].

Surveys of AME genes have revealed that strains of enterococci can harbour multiple genes [8, 22, 23]. Infections caused by *Enterococcus* spp. are increasing and these bacteria may serve as reservoirs of resistance [24]. In addition, food-animals have been implicated as primary sources of antimicrobial-resistant bacteria, and in order to fully understand the role that food-animals have in the dissemination and perpetuation of antimicrobial resistance, bacteria from veterinary sources must continue to be studied. In this study, prevalence of aminoglycoside resistance and AME resistance genes in enterococci from swine was investigated. High-level aminoglycoside resistance was low in the isolates ranging from 6% to 32%. Future studies will involve both investigation of the genetic source of aminoglycoside resistance in isolates in which an AME gene was not detected and mechanisms of dissemination of AME genes among enterococci from swine.

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