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HIGH LEVEL RESISTANCE TO GENTAMICIN AND VANCOMYCIN IN ENTEROCOCCI ISOLATED FROM POULTRY IN ARGENTINA

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ABSTRACT

In chicken husbandry, antimicrobial resistance among enterococci has been observed. Glycopeptides and gentamicin Minimum Inhibitory Concentrations in fecal poultry enterococci were determined. Glycopeptide and high-level gentamicin resistance (HLGR) genes were investigated. *E. faecalis* (*n* = 105) and *E. faecium* (*n* = 70) were the predominant species. In *E. faecalis*, HLGR (19%) was observed. In high-level gentamicin resistant *E. faecalis*, presence of *aac (6´) -Ie-aph (2´´)-Ia* gene was confirmed. VanA *E. faecium* (7.1%) were found, with HLGR (20%) and high-level streptomycin resistance (40%). *vanA* genotype was detected in glycopeptide resistant *E. faecium* (100%). These results highlight the presence of horizontally transferable antimicrobial resistance determinants in *E. faecalis* and *E. faecium* recovered from poultry. Existence of enterococcal reservoirs in poultry should be taken into account as a potential source of antimicrobial resistance genes, strengthening their potential spread to humans through the food chain of horizontal transferable resistance determinants.

INTRODUCTION

The emergence of antimicrobial resistance is a priority for human health. In this sense, detection of reservoirs of potentially pathogenic bacteria with transferable resistance mechanisms that may spread from the environment to man is an important challenge[1-3].

In animal husbandry, antimicrobials are heavily used for prevention and treatment of bacterial diseases; but also as growth promoters in animal feed[4-6]. Among Gram-positive bacteria, the genus *Enterococcus* shows a wide distribution in the environment. Enterococci are recognized as part of the indigenous microbiota of animal and human gastrointestinal tract. In fecal samples from farm animals, such as poultry, resistance to antimicrobials used in humans (*e.g.* aminoglycosides, glycopeptides) has been observed[7,8].

Enterococci can acquire high-level gentamicin resistance (HLGR) determinants by horizontal gene transfer[2,9]. Their expression result in the loss of synergistic bactericidal effect achieved with cell wall-active agents, posing a serious therapeutic problem for invasive infections such as enterococcal endocarditis[10,11].

Among enterococcci with HLGR, *aac (6´)-Ie-aph (2´´)-Ia* is the most prevalent gene, which encodes a bifunctional enzyme, AAC(6')-APH(2"), that confers resistance to aminoglycosides, except for streptomycin. Other monofunctional chromosomal [e.g. *aph(2´´)-Ib*, *aph(2´´)-Id*] and plasmidic [e.g. *aph(2´´)-Ic*] genes also encode gentamicin resistance[12-14].

Glycopeptide resistance in enterococci is mediated by *van* genotypes. Expression of *vanA* gene leads to inducible high-level vancomycin (Minimum Inhibitory Concentration, MIC ≥ 64 µg/ml) and teicoplanin (MIC ≥ 16 µg/ml) resistance[15].

In farm animals, such as poultry, *E. faecalis* are antimicrobial resistance determinants` reservoirs (HLGR, glycopeptide)[16-17]. In addition, there is evidence supporting the feasibility of genetic determinants transfer between poultry and human *E. faecalis*[18]. In Argentina, however, there is no available information in this regard.

The aim of this study was to investigate HLGR and vancomycin resistance determinants in fecal poultry enterococci isolated from farms located at a region in Argentina.

Material and methods

Fecal samples (N = 120) were randomly collected from two poultry farms (GPT-A; GPT-B) in Tandil district, Buenos Aires Pr o v i n c e (Argentina), from March to September 2015. GPT-A: a 9,000 broilers/year average production. GPT-B: a 7,000 broilers/year average production.

Samples (0.5 g) were added to 5 mL of Azide-Glucose broth. Cultures at 35 şC for 18 h in Bile-Esculin Azide agar (BEA) were carried out. Black pigmented colonies were selected for characterization at species level[19]. Genotypic confirmation by PCR for *tuf* and *ddl* genes (Table 1) was performed [1,20].

Resistance to vancomycin, teicoplanin, gentamicin and streptomycin was investigated. MIC was determined by the agar dilution method. Quality control strains: *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299[21].

Detection of glycopeptide resistance (*vanA*, *vanB*) and HLGR [*aac (6´)-Ie-aph (2´´)-Ia*, *aph (2´´)-Ib*, *aph (2´´)-Ic*, *aph (2´´)-Id*] genes was carried out by PCR (Table 1)[20,22].

Table 1. Primers employed in PCR.

Gene	Primers (sequence 5`to 3´)	Reference
Tuf	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	20
$ddl_{E.\ faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	20
$ddl_{E.\ faecalis}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	20
vanA	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	20
vanB	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	20

aac (6´) -Ie-aph	GAGCAATAAGGGCATACCAAAAATC CCGTGCATT TGTCTTAAAAAACTGG	[22]
aph (2´´)-Ib	TATGGATTCATGGTTAACTTGGACGCTGAG ATTAAGCTTCCTGCTAAAATATAAACATCTC	[22]
aph (2´´)-Ic	GAAGTGATGGAAATCCCTTCGTG GCTCTAACCCTTCAGAAATCCAGTC	[22]
aph (2´´)-Id	GGTG GTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	[22]

RESULTS

Enterococci were isolated from 80% (96/120) of the poultry fecal samples. Phenotypic and genotypic characterization showed that *E. faecalis* (*n* = 105) was the predominant enterococcal species found (52.5%), followed by *E. faecium* (*n* = 70).

In 19% (20/105) of poultry *E. faecalis* (FLS-29, FLS-38, FLS-59, FLS-77, FLS-81, FLS-84, FLS-90, FLS-91, FLS-100, FLS-103, FLS-110, FLS-115, FLS-122, FLS-128, FLS-138, FLS-146, FLS-150, FLS-153, FLS-164, FLS-171), HLGR was observed ($MIC_{gentamicin}$= 512-1,024 µg/mL). In high-level gentamicin resistant *E. faecalis* (100%), presence of *aac (6´) -Ie-aph (2´´)-Ia* gene was confirmed by PCR (Fig. 1a). High-level streptomycin resistance (HLSR) was not detected in *E. faecalis* isolates ($MIC_{streptomycin}$= < 2,000 µg/mL). Glycopeptide resistant *E. faecalis* were not recovered from any of the fecal samples analyzed in this study ($MIC_{vancomycin}$= < 4 µg/mL; $MIC_{teicoplanin}$= < 8µg/mL).

Vancomycin and teicoplanin resistant *E. faecium* (7.1%, 5/70) were found (Table 2). In all cases, VanA phenotype was observed ($MIC_{vancomycin}$= 64-1,024 µg/mL; $MIC_{teicoplanin}$= 64-512 µg/mL). Molecular analysis confirmed *vanA* genotype in all glycopeptide resistant *E. faecium* (Fig. 1b). Glycopeptide resistant *E. faecium* (FCM-70) showed HLGR (20%) and HLSR (40%; FCM-08, FCM-62). In all HLGR *E. faecium* isolates, *aac (6´) -Ie-aph (2´´)-Ia* gene was detected.

Table 2. Glycopeptide and hlgh-level aminoglycoside resistance in poultry *E. faecium*.

Isolate	Genotype	MIC_{van}	MIC_{tei}	HLGR genotype	MICgen	MICstr
FCM-14	*vanA*	64	64	ND[g]	128	128
FCM-22	***vanA***	128	128	ND	256	2,048
FCM-70	***vanA***	64	128	*aac (6´) -Ie-aph (2´´)-Ia*	2,048	256
FCM-93	***vanA***	512	128	ND	256	256
FCM-97	***vanA***	1,024	512	ND	256	2,048

Genotype: glycopeptide resistance gene. MICs in µg/mL. van: vancomycin; tei: teicoplanin. HLGR: High-level gentamicin resistance. gen: gentamicin; streptomycin. ND: not detected.

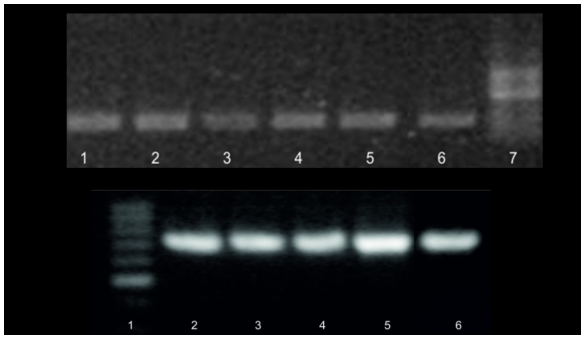

Fig. 1. a) PCR for *aac (6´) -Ie-aph (2´´)-Ia* gene (369 bp) detection in cattle enterococcal isolates. Ladder 1: *Enterococcus faecalis* FLS-29. Lane 2: *E. faecalis* FLS-38. Lane 3: *E. faecalis* FLS-59. Lane 4: *E. faecalis* FLS-77. Lane 5: *E. faecalis* FLS-81. Lane 6: *E. faecium* FCM-70. Lane 7: MW marker. b) PCR for *vanA* gene (732 bp) detection in cattle enterococi. Lane 1: MW marker. Lane 2: *Enterococcus faecium* FCM-14. Lane 3: *E. faecium* FCM-22. Lane 4: *E. faecium* FCM-70. Lane 5: *E. faecium* FCM-93. Lane 6: *E. faecium* FCM-97.

DISCUSSION

Over the last years, *E. faecalis* showed a dualistic behavior. Biotechnological and immunomodulatory properties have been proven in several strains[23-25]. However, nowadays, is considered as one of the most prevalent health-care associated infections pathogens[26].

In our investigation, *E. faecalis* was the most frequent isolated enterococci. In previous reports, it was the most (46.1%) or the least (11.5%) prevalent recovered species from poultry[16,27].

In this study, high-level gentamicin resistant *E. faecalis* (19%) were recovered and *aac (6´) -Ie-aph (2´´)-Ia* gene was detected. A low prevalence of enterococci harboring *aac (6´)-Ie-aph (2´´)-Ia* gene (2.7%) was observed for Australian poultry[27]. Likewise, *aac (6´)-Ie-aph (2´´)-Ia* gene was detected as the only HLGR determinant in *E. faecalis* isolated from poultry in Korea (10.9%). In addition, all high-level gentamicin resistant enterococci showed co-expression of HLSR[17]. Interestingly, the most frequent HLGR gene detected in human enterococci is *aac(6´)-Ie–aph(2``)-Ia*[28].

Recently, our group reported the presence of HLGR in *E. faecalis* from food of animal origin, as well as *in vivo* horizontal transfer of this resistance between food and human enterococci[1,2]. Therefore, horizontal transfer of resistance determinants between enterococci from different origin could not be ruled out as source of antimicrobial resistance.

In Argentina, in human enterococci, antimicrobial resistance has been studied. *E. faecalis* has been reported as the most prevalent enterococcal species expressing HLGR in isolates recovered form hospitalized patients[29].

Vancomycin-resistant *E. faecalis* were not recovered during this investigation. In 2007, vancomycin resistance was found in 20% of poultry enterococci from Slovakia, mainly in *E. faecalis*[30]. Chan *et al*.[16] also found *aac (6´) -Ie-aph (2´´)-Ia* and *vanA* genes in *E. faecalis* (4%) recovered from Korean poultry. In Europe, previous studies have shown the relevance of these genetic determinants of HLGR and glycopeptide resistance in human enterococci[28,31].

Nowadays, *E. faecium* is also considered another enterococcal species with clinical relevance[26]. In addition, previous reports also found that *E. faecium* was the second commonest enterococcal isolates[16,17].

HLGR was observed in vancomycin-resistant *E. faecium* (20%). In poultry enterococcal isolates from China, *aac(6)-aph(2")* gene was detected in *E. faecium* although HLGR was not confirmed. Furthermore, in Australian range-meat chickens and indoor-meat chickens, it was reported the recovery of gentamicin-resistant *E. faecium*[16,17].

Along this investigation, glycopeptide-resistant *E. faecium* were recovered (7.1%). VanA phenotype is encoded by gene *vanA*, typically carried in plasmid-harbored transposons. The most frequent glycopeptide resistance genotype in clinical enterococci is *vanA*[32]. Nowadays, there is an increasing concern about *vanA* plasmid-mediated transfer to methicillin-resistant *Staphylococcus aureus*[15,33]. In Chinese poultry, lower vancomycin resistance prevalence (1.3%) was detected for *E. faecium*, found in poultry drinking water. Recently, In Argentina, on the contrary, VanA *E. faecium* have been recovered from food of animal origin[1,16].

In a local nation-wide survey about resistance in clinical enterococci most of the *E. faecium* strains carried *vanA* gene (98%), expressing HLGR (77.2%) and high-level streptomycin resistance (95.8%) as well[32].

In countries like the United States and in the European Union the use of antimicrobials in husbandry is documented. Aminoglycosides (gentamicin) are employed in poultry[5,6].

However, glycopeptides, such as avoparcin, were banned due to its linkage with spread of vancomycin-resistant enterococci. After the ban it was observed a significant decrease of glycopeptide resistance in Danish poultry isolates[9]. However, in Argentina, the lack of consistent information about antimicrobial use in poultry husbandry does not allow to rule out this practice as a source of glycopeptide or aminoglycoside resistance in poultry enterococci. It is important to note that in the two studied farms, antimicrobials were frequently administered.

This study reports the presence of horizontally transferable antimicrobial resistance determinants (HLGR, *vanA*) in *E. faecalis* and *E. faecium* recovered from poultry in the Province of Buenos Aires, Argentina. The existence of these enterococcal reservoirs in farm animals such as poultry should be taken into account as a potential source of antimicrobial resistance genes, strengthening their potential spread to humans through the food chain.

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