

ORIGINAL ARTICLE

High-level resistance to gentamicin: genetic transfer between *Enterococcus faecalis* isolated from food of animal origin and human microbiota

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Abstract

Aims: To investigate the *in vivo* gene transfer of high-level gentamicin resistance (HLRG) from *Enterococcus faecalis* isolated from the food of animal origin to a human isolate, using a mouse model of intestinally colonized human microbiota.

Methods and Results: *In vitro* study: The presence of plasmids involved in HLRG coding was investigated. After the conjugation experiment, the recipient strain, *Ent. faecalis* JH2-SS, acquired a plasmid responsible for HLRG [minimal inhibitory concentration (MIC) >800 µg ml⁻¹], in a similar position to the donor cells. *In vivo* study: Seven BALB/c mice were dosed with ceftriaxone (400 mg kg⁻¹) and then inoculated with a dilution of 1/100 of human faeces (HFc). After 72 h, *Ent. faecalis* JH2-SS (recipient) was inoculated and then, after a further 72 h, the animals were given *Ent. faecalis* CS19, isolated from the food of animal origin, involved in HLRG (donor). The presence of transconjugant strains in HFc was subsequently recorded on a daily basis until the end of the experiment. The clonal relationship between *Ent. faecalis* and *Escherichia coli* in faeces was assessed by RAPD-PCR. Both the *in vitro* and *in vivo* studies showed that the receptor strain acquired a plasmid responsible for HLRG (MICs >800 µg ml⁻¹), which migrated with a similar relative mobility value. Transconjugant strains were detected from 24 h after the donor strain inoculation and persisted until the end of the experiment.

Conclusions: The *in vivo* gene transfer of HLRG from *Ent. faecalis* strains, isolated from the food of animal origin, to human microbiota has been demonstrated in a mouse model.

Significance and Impact of the Study: The complexity found on the therapeutic responses of invasive infectious diseases caused by *Ent. faecalis* facilitates the assessment of food of animal origin as a resistant pathogen reservoir. In addition, this study may contribute to the understanding of antimicrobials' resistance gene transfer between *Ent. faecalis* strains from food and human GI tract.

Introduction

The relevance of the genus *Enterococcus* as an emerging pathogen associated with health care is based on (i) its natural resistance to conventional antimicrobial agents

(ATM) and (ii) its ability to acquire resistance from other bacteria, resulting in serious problems in terms of ATM therapy. Enterococci are intrinsically resistant to many ATM based in chromosomal genes, which typically are nontransferrable. Specifically, for aminoglycosides, the

low-level resistance reported as minimal inhibitory concentration (MIC) for gentamicin was 4–128 $\mu\text{g ml}^{-1}$ and for streptomycin, 64–512 $\mu\text{g ml}^{-1}$ (Leclercq 1994). Early in 1970, it was reported that the highest levels of resistance to both streptomycin (MIC >2000 $\mu\text{g ml}^{-1}$) and gentamicin (HLRG) (MIC >500 $\mu\text{g ml}^{-1}$) were found in *Enterococcus faecalis* strains. HLRG was mainly because of the presence of the bifunctional aminoglycoside-modifying enzyme, Aac6'-Aph2''. Other research confirmed that HLRG is prevalent in the strains of both *Ent. faecalis* and *Enterococcus faecium* (Moellering *et al.* 1970; Ferretti *et al.* 1986). More recently, it has been reported that HLRG in some strains of enterococci is linked to transposable elements with plasmidic localization or chromosomal integration (Predari *et al.* 1999; Ronconi and Merino 2000).

Enterococci are predominantly associated with mammals, although they have also been found in water and vegetables (Sparo and Mallo 2001; Foulquié *et al.* 2006). Moreover, enterococci may colonize raw food (milk and meat) through intestinal residues or environmental contamination.

Some strains of *Ent. faecalis* may be considered probiotics with beneficial effects to the host (Castro *et al.* 2007). In contrast, other *Ent. faecalis* strains may have some pathogenicity determinants and also the capability of acquiring and transferring ATM resistance genes. The latter play a pivotal role in several invasive diseases including intra-abdominal and pelvic abscesses, endocarditis and occasionally meningitis or pneumonia (Gentile *et al.* 1995; Maschieto *et al.* 2004). Interestingly, ATM multiresistant strains of enterococci used in human medicine have been isolated from nonhospital environments such as poultry faeces (Hayes *et al.* 2004). In this context, controlled *in vitro* studies have been developed to evaluate the gene transfer to intestinal microbiota (Clewell 1990; Donabedian *et al.* 2003). However, *in vivo* studies to confirm the *in vitro* evidence for resistance gene transfer are required. In this context, the main goal of this research was to demonstrate the transfer of HLRG genes from *Ent. faecalis* isolated from the food of animal origin to mice colonized with human microbiota.

Materials and methods

Bacterial strains

Donor strains: Nine strains of *Ent. faecalis* (CS17–CS26) were isolated from the food of animal origin (CS17–CS21 – ground meat beef, CS22–CS25 – artisanal crafted sausages and CS26 – cattle cheese), containing HLRG gene (MIC gentamicin >800 $\mu\text{g ml}^{-1}$). These strains were provided by the Laboratory of Pharmacology, Faculty of Veterinary Medicine, UNCPBA, Argentina. Recipient

strain: Human *Ent. faecalis* JH2-SS strain (obtained from the Michael S. Gilmore collection, Oklahoma University, USA) without plasmids, but resistant to streptomycin (MIC: 500 $\mu\text{g ml}^{-1}$) and spectinomycin (MIC: 500 $\mu\text{g ml}^{-1}$) by spontaneous mutation, was used in the *in vitro* assay (Yagi and Clewell 1980).

Gene transfer experiments

In vitro assays. The presence of plasmids involved in HLRG coding and plasmid curing was assessed using an assay based on the protocol previously described by Ruiz-Barba *et al.* (1991) and Sparo *et al.* (2006).

Plasmid curing. Briefly, after culture in brain heart infusion (BHI) broth, aliquots of 10 ml *Ent. faecalis* from the food of animal origin (MIC gentamicin >800 $\mu\text{g ml}^{-1}$) were adjusted to a viable count of 10^5 CFU ml^{-1} . Then, after the addition of novobiocin (Sigma-Aldrich, USA) at several concentrations between 0.125 to 16 $\mu\text{g ml}^{-1}$ and pronase (Sigma-Aldrich) at a final concentration of 0.5 g l^{-1} , cultures were incubated for 72 h at 35°C. Cells incubated without added novobiocin were used as a control. After incubation, cells treated with the highest concentration of novobiocin displaying visible growth were diluted in sterile saline and plated onto BHI agar. After overnight incubation at 35°C, replicate plating was carried out to BHI agar and BHI agar containing gentamicin (500 $\mu\text{g ml}^{-1}$). Colonies that grew only in BHI agar were picked and transferred to BHI broth. These bacteria were stored at –20°C in BHI infusion containing 20% v/v glycerol until used in the conjugation experiments. Plasmid profiles of *Ent. faecalis* strains from the food of animal origin were analysed before and after the curing experiments as described below.

Conjugation. For conjugation assessment, a 1 : 10 ratio of donor/recipient cells was used. Strains from the food (donors) and human *Ent. faecalis* JH2-SS (recipient) were mixed in BHI broth. After incubation for 5 h at 35°C, streptomycin (300 $\mu\text{g ml}^{-1}$; Sigma-Aldrich) was added and the incubation continued for a further 9 h at 35°C. Samples were then centrifuged at 2600 g (4°C) by 10 min, and the resultant sediments were suspended in 0.5 ml of BHI broth. Dilutions of this suspension were made in BHI agar supplemented with streptomycin (300 $\mu\text{g ml}^{-1}$) and gentamicin (500 $\mu\text{g ml}^{-1}$). All samples were incubated for 48 h at 35°C in order to determine the viable counts (CFU ml^{-1}). Plasmid content and genetic identity were determined as described in the following sections. Three control groups were set up to eliminate possible alternative gene-transfer mechanisms as follows: (i) *Ent. faecalis* isolates from the animal food (donor) were treated with chloroform and DNase (Sigma-Aldrich) before being mixed with recipient cells,

(ii) recipient bacteria were incubated with supernatants from the donor strain cultures and (iii) donor and recipient bacteria were co-cultured in BHI broth containing gentamicin ($500 \mu\text{g ml}^{-1}$) and streptomycin ($300 \mu\text{g ml}^{-1}$). Conjugation frequency was calculated as the number of transconjugant cells (expressed as CFU ml^{-1}) divided by the number of donor cells, where transconjugant cells were considered as those which exhibited growth in BHI agar supplemented with gentamicin and streptomycin.

Plasmid content. The Qiagen® (Plasmid Midi kit; Qiagen, CA, USA) method modified as previously described (Sparo *et al.* 2006) was utilized to analyse the plasmid content. The same procedure was also used with *Escherichia coli* strain V517 to provide a plasmid content reference range with the following values: 56.4, 7.5, 5.3, 4.0, 3.1, 2.8 and 2.2 kbp.

RAPD-PCR of enterococci strains. The RAPD-PCR of *Ent. faecalis* CS17–CS26 and *Ent. faecalis* JH2–SS strains was performed according to Sparo *et al.* (2008). The primers used were D8635 (5' GAG CGG CCA AAG GGA GCA GAC 3') and M13 (5' GAG GGT GGC GGT TCT 3'). The clonal relationship was set up by the software RAPDISTANCE 1.04 Software Package (Australian National University). The strain isolated from the food of animal origin with lowest conjugation frequency per donor cell was used for the HLRG *in vivo* transfer study.

In vivo assay. Replacement of the intestinal microbiota. Seven 8-week-old BALB/c mice (approximately 25 g live body weight) were dosed intraperitoneally twice, 24 h apart, with 400 mg kg^{-1} ceftriaxone (Roche, Vaud, Switzerland), in order to decrease the coliform population in the intestine. Sixty hours after this ATM treatment, mice were inoculated by the intragastric (IG) route with human faeces (HFc) diluted 1/100 in sterile deionized water. HFc was obtained from a 6-month-old child, still on milk, who had not received any previous ATM treatment and had no detectable enterococci population, on the evidence of screening in selective and nonselective cultures (BHI agar and bile-esculin-azide agar, respectively). For the analysis of coliform populations, mice were sampled at 24 and 0 h before treatment, then again 24 and 48 h after treatment with ceftriaxone. Further samples were taken after HFc inoculation at the end of the experiment (15 days). Coliform and enterococcal viable counts (CFU g^{-1} faeces) were determined in EMB-Levine agar and bile-esculin-azide agar, respectively, by the plate count method.

Intestinal tract implantation of recipient and donor strains. Seventy-two hours after inoculation of HFc, mice were dosed orally with the recipient strain, *Ent. faecalis* JH2–SS (1.5×10^8 CFU), and the subsequent presence of this strain in faeces was investigated at 24 and 48 h after

treatment. At 72 h, the donor isolate that demonstrated the lowest conjugation frequency *in vitro* was inoculated IG (1.5×10^8 CFU). Then, for the following 8 days, faeces were collected to investigate the presence of donor, recipient and transconjugant *Ent. faecalis* strains. Finally, the distribution of these strains was determined in intestinal tissue removed from the mice at postmortem following humane sacrifice.

Enterobacteria and enterococci strain characterization. Enterobacterial phenotyping was performed using the API-20E (bioMérieux, France) system, whilst enterococci were identified according to the method previously described by Sparo *et al.* (2006, 2008).

RAPD-PCR studies. The clonal relationship of *E. coli* was determined by RAPD-PCR following the protocol reported by Cave *et al.* (1994). The primer used was the oligonucleotide 1274, 5'-AAG AGC CCG-3'. The RAPD-PCR, technique described on the *in vitro* assays, was used for the genetic characterization of *Ent. faecalis* transconjugants.

Results

In vitro assay

In *Ent. faecalis* strains with HLRG recovered from the food of animal origin, bacterial development was observed in cultures containing novobiocin concentrations of up to $4 \mu\text{g ml}^{-1}$. Plasmid curing was observed in all strains assayed which grew in BHI agar medium without gentamicin but did not develop in gentamicin-supplemented medium (Fig. 1, lanes 2 and 3 – *Ent. faecalis*

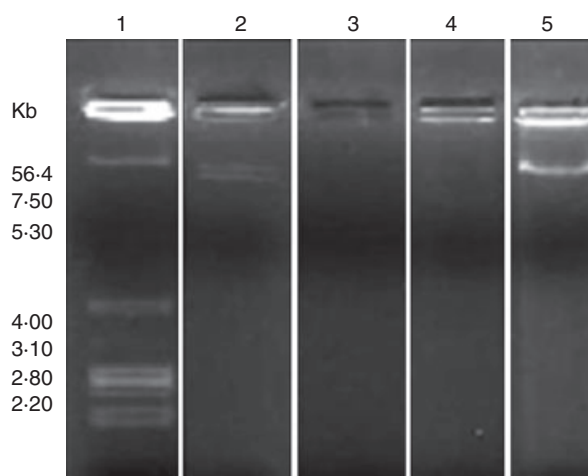


Figure 1 *In vitro* conjugation plasmid transference. Lane 1: plasmids of *Escherichia coli* V517, lane 2: *Enterococcus faecalis* CS19, lane 3: *Ent. faecalis* CS19 cured, lane 4: *Ent. faecalis* JH2–SS, lane 5: *Ent. faecalis* JH2–SS transconjugated.

CS19 strain). However, *Ent. faecalis* strains that were not exposed to novobiocin retained the plasmid and successfully grew in gentamicin-supplemented agar. After the conjugation experiment, the recipient strain *Ent. faecalis* JH2-SS migrated with a relative mobility value (Rf) similar to that of the donor cells, indicating that plasmid incorporation was at a similar position (Fig. 1, lane 5 – *Ent. faecalis* CS19), and the plasmid acquired by this strain was responsible for HLRG resistance (MIC >800 µg ml⁻¹). The frequency of transference of the plasmid by conjugation ranged from 2.9×10^{-5} to 2.6×10^{-3} per donor cell. Of the strains isolated from the food of animal origin, *Ent. faecalis* CS19 showed the lowest frequency of plasmid transfer.

In vivo assay

The enterobacterial population of the inoculated HFc contained *E. coli* 10⁸ CFU g⁻¹ faeces. Before ceftriaxone administration, the enterobacterial population in mouse faeces was as follows: *E. coli* 10⁴–10⁵ CFU g⁻¹ faeces, *Enterobacter* spp. 10³–10⁴ CFU g⁻¹ faeces and *Proteus* spp. 10²–10³ CFU g⁻¹ faeces. After ceftriaxone administration, the faecal enterobacterial population changed as follows: *E. coli* was not detected, *Enterobacter* spp. 10²–10³ CFU g⁻¹ faeces and *Proteus* spp. 10³–10⁴ CFU g⁻¹ faeces. Interestingly, 24 h after the administration of HFc, mice were colonized with *E. coli* (10⁵–10⁶ CFU g⁻¹ faeces). These changes on mice microbiota are illustrated in Fig. 2. Clonal heterogeneity of *E. coli* in mice faeces before inoculation of HMc was evidenced by the different electrophoretic profiles obtained by RAPD-PCR. However, a random selection of 8–10 colonies from EMB-Levine agar cultures of mouse faeces sampled 9 days after HFc inoculation indicated that the *E. coli* population had a genetic profile identical to that of the implanted human *E. coli*. Colonization by the latter persisted in all the HFc-treated mice until day 10 (±1) postinoculation. The recipient (JH2-SS) and the donor (CS19) *Ent. faecalis* strains were detected in mouse faeces from 24 h postinoculation and both persisted until the end of the experiment (Day 10; see Fig. 3). Transconjugant strains were detected at 24 h postdonor strain inoculation and, again, these persisted for the duration of the experiment, with numbers

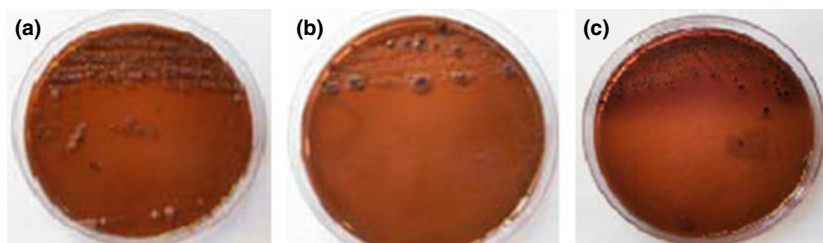


Figure 2 Mice faeces microbiota isolation in EMB-Levine agar pre-ceftriaxone (a), 24 h after two doses of ceftriaxone, the microbiota changed (b), and 24 h postadministration of HFc, mice intestine with human-microbiota colonization (c). HFc, human faeces.

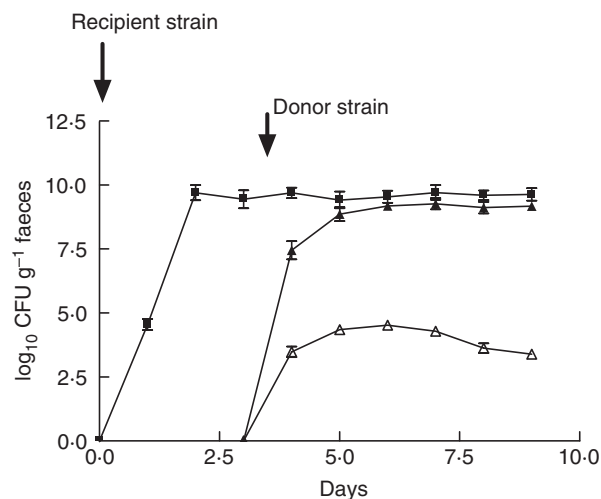


Figure 3 Bacterial counts in faeces of mice colonized with human faecal microbiota. Day 0, mice were inoculated with 10⁸ CFU *Enterococcus faecalis* JH2-SS (recipient strain), and on day 3, mice were inoculated with 10⁸ CFU *Ent. faecalis* CS-19 (donor strain). (■) *Enterococcus faecalis* JH2-SS; (▲) *Ent. faecalis* CS-19 and (Δ) *Ent. faecalis* transconjugants. Data are the mean of log₁₀ CFU g⁻¹ faeces. The error bars represent the standard deviation.

ranging from 10³ to 10⁴ CFU g⁻¹ faeces (Fig. 3). Similar numbers (10³ CFU g⁻¹ tissue) of transconjugant strains were also observed in mice intestines obtained by post-mortem at the end of the experiment. Figure 4 illustrates the similar electrophoretic patterns obtained for the wild-type (lane 1) and transconjugated (lane 2) *Ent. faecalis* JH2-SS strains and the differing profile of the donor strain (*Ent. faecalis* CS19) (lane 3).

Discussion

The emergence of high-level gentamicin resistance (HLRG) in clinical medicine in 1979 had a substantial negative effect on the treatment for severe enterococcal infections and was rapidly followed by many reports of nosocomial infection in the 1980s (Zervos et al. 1987).

Antibiotic pressure may not only affect the microbiota, but also promote the transfer of elements encoding resistance to the particular drug. The transfer (by mobile genetic elements) of genes conferring ATM resistance

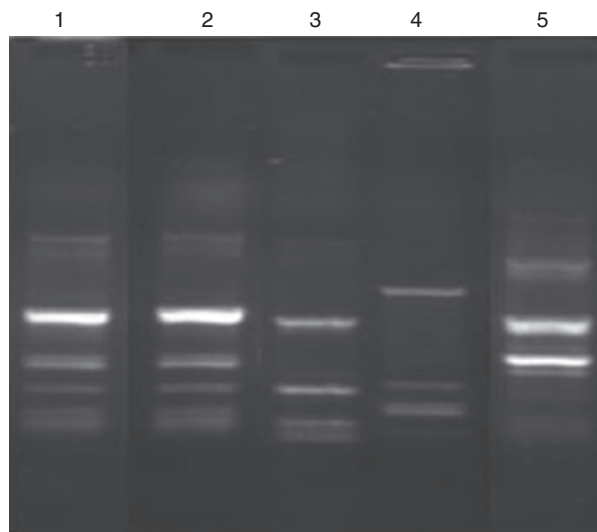


Figure 4 RAPD-PCR *Enterococcus faecalis* strain from faeces. Lane 1: *Ent. faecalis* JH2-SS; lane 2: *Ent. faecalis* JH2-SS transconjugated; lane 3: *Ent. faecalis* CS19; lane 4: *Enterococcus faecium* ATCC 19434 (control 1); lane 5: *Ent. faecalis* CS26 (control 2).

from the strains obtained from food samples has been reported (Hayes *et al.* 2004). However, plasmid conjugation provides a more efficient way for horizontal gene transfer and is generally considered the most relevant mechanism for the increase in bacteria multiresistant to conventional ATMs (Kurenbach *et al.* 2003).

Higher levels of intestinal colonization by *Ent. faecalis* are a frequent precursor of human invasive infections, although other predeterminants may be important in patients with comorbidity factors. One reason for this may be the failure of local or systemic use of ATM to remove *Ent. faecalis* owing to its natural antibiotic resistance (i.e. to cephalosporins). The use of broad-spectrum ATM contributes to the development of a selective genetic pressure on the intestinal microbiota, resulting in the increased emergence of multiresistant bacteria. The human GI tract is a massive reservoir of bacteria with the potential of acquiring and transferring resistance to conventional ATMs. In addition, there is the possibility that bacteria isolated from the food of animal origin may act as a pool of resistance. Recently, it has been reported that 15% of enterococci isolated from the faeces of hospitalized patients exhibit HLRG (Ronconi and Merino 2000).

The *in vitro* findings detailed in this paper are consistent with a previous study (Clewell 1990) in which the author noted the ability of enterococci to exchange genetic information by conjugation and with another (Huycke *et al.* 1992) study which, based on studies in a hamster model, reported that such exchange processes may occur in the gastrointestinal tract.

The design of *in vivo* models to assess the gene transfer has proved challenging but such an approach is needed in order to provide definitive evidence of genetic exchange between enterococcal strains from the food of animal origin and humans. An *in vivo* model that reproduced the interaction of the food strains with the human GI tract microbiota, which involved colonizing gnotobiotic mice with human colonic microbiota, was reported by Hirayama (1999). More recently, a model for assessing the transfer of vancomycin resistance from gen *vanA* obtained from an animal isolate to one from clinical human source in the intestine of mice treated with cefuroxime has been reported by Lester and Hammerum (2010). There have been consistent reports on the *in vivo* transfer of glycopeptide resistance along with the transfer of gen *vanA* and other resistance genes from porcine to human *Enterococcus faecium* at high frequency in the digestive tract of gnotobiotic mice (Moubareck *et al.* 2003; Dahl *et al.* 2007). However, there have been no previous *in vivo* reports for the transfer of HLRG in an environment comparable to the human intestine. In the present study, a model for genetic transfer in nonsterile mice colonized with human GI microbiota was established. The results obtained suggest that the gastrointestinal environment is important for gene flux both within and between transient and resident bacterial species. Moreover, the colonization of the GI tract by indigenous *Ent. faecalis* strains containing HLRG implies the possibility of translocation.

Donabedian *et al.* (2003) evaluated gentamicin-resistant enterococci isolated from humans, food and farm animals and provided evidence for the spread of gentamicin-resistant enterococci from animals to humans through the food supply. They showed that enterococci isolated from animals and humans possess the same aminoglycoside resistance genes. However, it is significant to determine the transferability of these resistance genes because the dissemination of genes can occur by horizontal transfer.

The current experiments provide the first evidence of the possibility of HLRG gene transfer between enterococci from the food of animal origin to human enterococci in a similar environment with immune-competent mice colonized with human microbiota.

Furthermore, phenotyping of the most common enterococci in the intestine of human volunteers has revealed that the enterococcal microbiota of the human intestine often changes suggesting that, in general, enterococci do not colonize for extended periods of time (Gelsomino *et al.* 2003). However, the transitory colonization of resistant enterococci strains such as those with HLRG, as confirmed by the *in vivo* results described in this paper, may be sufficient to allow gene transfer. In the current studies, it is relevant to note that colonization of the intestine with human microbiota persisted for at least

10 days and therefore offers a useful *in vivo* technique for studying the genetic transfer in a human bowel model (Fig. 3).

In conclusion, the *in vivo* genetic transfer of HLRG from strains isolated from the food of animal origin to human faecal microbiota has been confirmed within a mouse model. The complexity found in the therapeutic responses of invasive infectious diseases associated with *Ent. faecalis* allows the assessment of food of animal origin as a reservoir for resistant pathogenic bacteria. In addition, this study may contribute to the understanding of ATM resistance gene transfer between *Ent. faecalis* strains from food and the human GI tract.

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