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In Vivo Transfer of the *vanA* Resistance Gene from an *Enterococcus faecium* Isolate of Animal Origin to an *E. faecium* Isolate of Human Origin in the Intestines of Human Volunteers

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Transient colonization by vancomycin-resistant enterococci of animal origin has been documented in the intestines of humans. However, little is known about whether transfer of the *vanA* gene occurs in the human intestine. Six volunteers ingested a vancomycin-resistant *Enterococcus faecium* isolate of chicken origin, together with a vancomycin-susceptible *E. faecium* recipient of human origin. Transconjugants were recovered in three of six volunteers. In one volunteer, not only was vancomycin resistance transferred, but also quinupristin-dalfopristin resistance. This study shows that transfer of the *vanA* gene from an *E. faecium* isolate of animal origin to an *E. faecium* isolate of human origin can occur in the intestines of humans. It suggests that transient intestinal colonization by enterococci carrying mobile elements with resistance genes represents a risk for spread of resistance genes to other enterococci that are part of the human indigenous flora, which can be responsible for infections in certain groups of patients, e.g., immunocompromised patients.

Antimicrobials have been used to promote the growth of food animals since the 1950s. In the beginning of the 1990s, the presence of vancomycin-resistant enterococci (VRE) was reported in the environment and in production animals (6, 23). It was later shown that the growth promoter avoparcin, a glycopeptide very similar to vancomycin, selected for VRE in production animals (1, 5). In enterococci, there are several different genotypes of acquired glycopeptide resistance, of which the *vanA* genotype is the type encountered among food animals in Europe (3). The *vanA* gene cluster is typically located on transposons of the Tn*1546* type (3). Vancomycin is used for treatment of gram-positive infections, including multiresistant enterococcal infections in humans. The fact that avoparcin could select for VRE led in 1997 to the ban of this antimicrobial growth promoter in the European Union (EU). In 1999, the EU ban was extended to other antimicrobial growth promoters, i.e., tylosin, bacitracin, spiramycin, and virginiamycin, due to a risk for human health.

In 1999, several years after the ban of avoparcin, VRE could still be recovered from food animals and retail meat (8, 16). Whether these VRE of animal origin are capable of transferring their resistance genes to enterococci of human origin in a natural environment, e.g., the human intestine, however, is still not known. Transfer of resistance genes has been demonstrated between strains of *Enterococcus faecium* in the gastrointestinal tracts of streptomycin-treated mice (24), gnotobiotic rats (18), and gnotobiotic mice (26). However, these experiments were performed in animals with no or decreased colonization resistance. Whether transfer of resistance genes could happen in the intestine with normal colonization resistance, therefore, remains unknown. In human volunteers, Berchieri (7) and Sørensen et al. (28) showed transient colonization of the intestine by vancomycin-resistant enterococci of animal origin, but none of the studies was designed to study the possible transfer of resistance genes.

The present study was performed to determine whether resistance genes from an *E. faecium* isolate of animal origin could be transferred to a human *E. faecium* isolate in the intestines of human volunteers without any selective antimicrobial pressure.

MATERIALS AND METHODS

Study subjects. The study was conducted with six healthy human volunteers in October and November 2004. All subjects were more than 18 years old and had normal intestine functions. None of the subjects had received antimicrobials within 1 month prior to the study, and none took antimicrobials or other medicines during the study. None of the subjects was working in laboratories with vancomycin-resistant enterococci. All subjects received written and oral information about the study, and all provided written informed consent. The study protocol was approved by the scientific ethics committee for the Copenhagen and Frederiksberg municipalities [(KF)01-153/03].

Bacterial strains. A Danish *E. faecium* isolate of chicken origin (strain 9730129) harboring the *vanA*, *erm*(B), and *vat*(E) genes encoding resistance to vancomycin, erythromycin, and quinupristin-dalfopristin, respectively, was used as the donor strain (28). The strain was susceptible to penicillin, ampicillin, gentamicin, and linezolid. A rifampin- and fusidic-acid-resistant *E. faecium* strain of human origin (BM4105-RF) was used as the recipient strain (9).

In vitro matings. Prior to the study, in vitro matings were performed to check the transferability of vancomycin resistance between the selected donor and recipient strains. Filter matings were performed according to the method of Clewell et al. (10) with the following modifications. One hundred microliters of overnight donor culture was mixed with 100 μl of overnight recipient culture. One hundred microliters of the mixed culture was dispensed onto a 47-mm sterile filter (0.45 μm; Millipore Corporation, Billerica, MA) placed on a 5%

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TABLE 1. Selective media used to detect donor strain, recipient strain, and transconjugants in transfer experiments

Antimicrobial-supplemented medium[a]	Day of collection of stool sample	Use
32 μg/ml vancomycin-supplemented Enterococcosel agar	0, 14, 35	As negative controls to make sure that none of the volunteers were colonized by vancomycin-resistant enterococci before and after the study
32 μg/ml vancomycin- and 32 μg/ml erythromycin-supplemented Enterococcosel agar	0 to 7, 14, 35	For detection of the donor strain and transconjugants
25 μg/ml fusidic acid- and 25 μg/ml rifampin-supplemented Enterococcosel agar	0 to 7, 14, 35	For detection of the recipient strain and transconjugants
25 μg/ml fusidic acid-, 25 μg/ml rifampin-, and 32 μg/ml vancomycin-supplemented Enterococcosel agar	0 to 7, 14, 35	For detection of transconjugants only

[a] Enterococcosel agar (BD Diagnostics, Sparks, MD); fusidic acid (Leo Pharmaceuticals, Ballerup, Denmark); erythromycin, rifampin, and vancomycin (Sigma-Aldrich Corp., St. Louis, MO).

blood agar plate (SSI Diagnostika, Copenhagen, Denmark). After the mixed culture was allowed to dry on the filter, the plates were incubated at 37°C. Transferability was determined after 24 h of incubation by placing the filter in 5 ml 0.9% saline, suspending the culture by vortex mixing, and plating it on antimicrobial-supplemented Enterococcosel agar plates (BD Diagnostics, Sparks, MD) (Table 1). The plates were incubated at 35°C for 48 h, and the colonies were counted.

Broth mating experiments were performed as previously described by Ike et al. (17) with the following modifications. Overnight donor culture (0.5 ml) and 0.5 ml of overnight recipient culture were each added to 5 ml of Todd-Hewitt broth (SSI Diagnostika, Copenhagen, Denmark). The mixture was then incubated at 37°C with gentle agitation for 4 hours. Portions of the mixed culture were then plated on antimicrobial-supplemented Enterococcosel agar plates (BD Diagnostics, Sparks, MD). The plates were incubated at 35°C for 48 h, and the colonies were counted. The mating experiments were repeated twice. Selected in vitro transconjugants were stored in 10% glycerin at −40°C for further investigation.

Preparation and ingestion of *E. faecium* suspensions. *E. faecium* suspensions were prepared as described by Sørensen et al. (28). The donor and the recipient strains were grown overnight at 35°C on 5% blood agar (SSI Diagnostika). A suspension of the recipient strain was prepared in 0.9% saline, and the concentration was adjusted to approximately 10^9 CFU per ml. One milliliter of this suspension was added to 250 ml of pasteurized whole milk to obtain a total count of 10^9 bacteria in 250 ml whole milk, which was immediately ingested by each of the volunteers. Three hours later, a suspension of the donor strain was prepared in the same way to a total count of 10^7 bacteria in 250 ml whole milk, which was immediately ingested by each of the volunteers. All bacterial concentrations were later confirmed by serial dilutions with colony counts.

Collection and culture of stool samples. Stool samples were collected from the subjects 48 h prior to ingestion of the *E. faecium* suspensions, on a daily basis for the following 7 days (days 1 to 7), and on days 14 and 35. One gram of each stool sample was homogenized in 10 ml of 0.9% saline and plated on antimicrobial-supplemented Enterococcosel agar plates (BD Diagnostics) (Table 1). All plates were incubated at 35°C for 48 h, and then the colonies were counted. All in vivo transconjugants and selected in vitro transconjugants were stored in 10% glycerin at −40°C for further investigation.

Antimicrobial susceptibility testing. The donor strain, the recipient strain, and the transconjugants were tested for susceptibility to the antimicrobial agents erythromycin (0.5 to 32 μg/ml), gentamicin (2 to 2,048 μg/ml), linezolid (1 to 8 μg/ml), penicillin (2 to 64 μg/ml), synercid (0.5 to 16 μg/ml), and vancomycin (2 to 32 μg/ml) using a commercially prepared, dehydrated panel (Sensititre; Trek Diagnostic, United Kingdom) as previously described by Aarestrup et al. (2). The ATCC strain *E. faecalis* 29212 was used as a quality control strain to validate the MIC.

PCR. A sample DNA preparation of the enterococci was made by suspending a few overnight colonies in a tube containing 300 μl distilled water. The tube was heated at 95°C for 10 min and centrifuged at 20,000 × *g* for 5 min. Five microliters of the supernatant was used as a template. The transconjugants were tested for *vanA* with the primers previously described by Jensen et al. (20), for *erm*(B) with primers previously described by Jensen et al. (21), and for *vat*(E) with primers previously described by Werner and Witte (30).

A screening for point mutation at position 8234 of the *vanX* gene was done according to the method of Palepou et al. (27).

A PCR map of the entire Tn*1546* was made for the donor and for one in vitro transconjugant and all in vivo transconjugants with primers previously described by Willems et al. (31).

Pulsed-field gel electrophoresis (PFGE). Whole-cell DNA was prepared according to the method of Jensen et al. (20). A small slice of the agarose plug was digested with 30 U of the restriction enzyme SmaI (New England BioLabs Inc., Medinova Scientific A/S, Denmark) for a minimum of 4 h at 25°C. The digested DNA was electrophoresed in a 1% agarose gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) by use of a CHEF DRIII apparatus (Bio-Rad, Herlev, Denmark). The initial switch time was 5 s, the final switch time was 35 s, and the run time was 24 h at 6 V/cm. Lambda Ladder PFG Marker NO350S (New England BioLabs Inc.) was used as a molecular size marker.

RESULTS

None of the volunteers was colonized with vancomycin-resistant *E. faecium* prior to the study (day zero). The variations of the concentrations of the donor strain, the recipient strain, and the transconjugants in feces during the study are shown in Fig. 1. Concentrations of the donor strain reached a maximum of 2.8×10^4 to 8.0×10^6 CFU per gram feces on day 2 or 3, depending on the volunteer (Fig. 1A). On day 7, these concentrations ranged from less than 10 (detection limit) to 6×10^3 CFU per gram feces.

Concentrations of the recipient strain reached a maximum of 9.5×10^5 to 1.6×10^8 CFU per gram feces on days 1 to 4, depending on the volunteer (Fig. 1B). On day 7, these concentrations ranged from less than 10 CFU (detection limit) to 1.6×10^5 CFU per gram feces.

Transfer of the *vanA* gene from the donor strain to the recipient strain occurred in the intestines of three of the six volunteers. Transconjugants were recovered between day 2 and day 5 (Fig. 1B). In two volunteers, transconjugants were recovered in two consecutive stool samples 24 h apart. In the last volunteer, transconjugants were recovered in only one stool sample on day 5. On days 14 and 35, no vancomycin-resistant enterococci were detected in the stools of any of the volunteers (Fig. 1).

Prior to the study, in vitro filter mating experiments had shown a mean transfer frequency of 2×10^{-4} transconjugants/recipient and in vitro broth mating had shown a mean transfer frequency of 5×10^{-7} transconjugants/recipient.

Antimicrobial susceptibility testing showed that all, in vitro as well as in vivo, transconjugants were resistant to vancomycin (MIC > 32 μg/ml). Moreover PCR confirmed the presence of the *vanA* gene in all transconjugants. Two in vivo transconju-

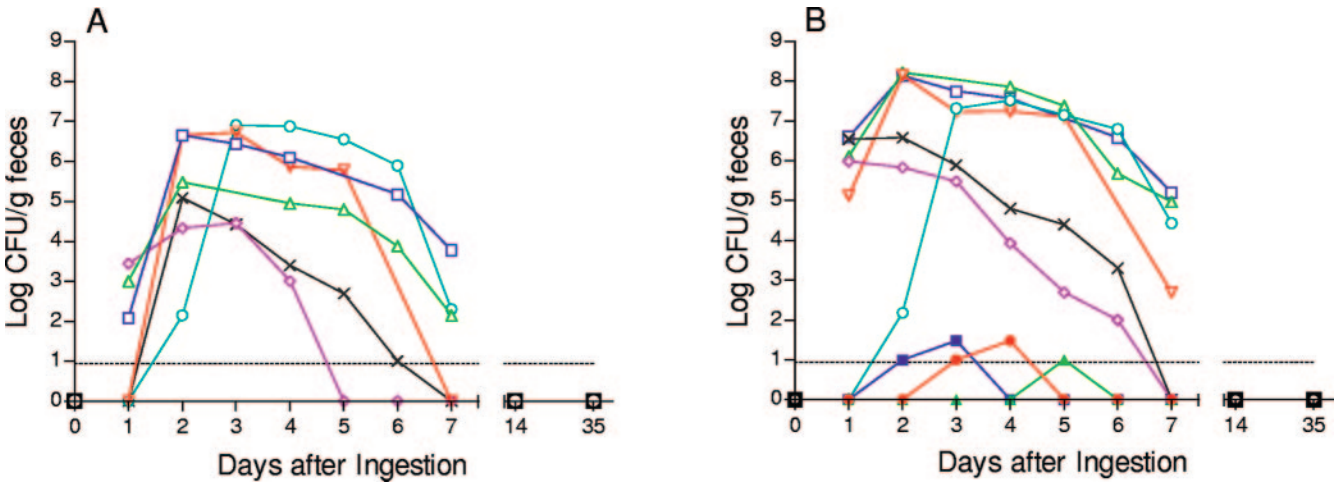

FIG. 1. Fecal excretion of strains by the six volunteers. (A) Donor strain (open symbols). (B) Recipient strain (open symbols) and transconjugants (solid symbols). Each curve shows the results for one volunteer. Each volunteer is represented by one color. Transconjugants were excreted by only three volunteers. Results from stool samples obtained within 48 h before ingestion of the bacteria are plotted as day zero. The minimal detectable level is shown by the dashed line. The solid black squares with open centers (days 0, 14, and 35) represent the superposition of results from all six volunteers.

gants from one volunteer and eight in vitro transconjugants from the broth mating experiments were also resistant to erythromycin (MIC $>$ 32 μg/ml) and quinupristin-dalfopristin (MIC $>$ 16 μg/ml), and PCR confirmed the presence of the *erm*(B) and *vat*(E) genes, respectively, in these transconjugants. The point mutation at position 8234 in the *vanX* gene was the G variant (a poultry variant) in both the donor and the transconjugants.

The PCR maps of Tn*1546* were exactly the same for the donor strain and all the transconjugants.

PFGE typing of the in vitro and in vivo transconjugants showed a profile similar to that of the recipient with only one band difference. The in vivo transconjugants from human volunteers had the same profile as transconjugants from the in vitro mating experiments.

DISCUSSION

This study shows that transfer of the *vanA* gene from an *E. faecium* isolate of animal origin to a human *E. faecium* isolate is possible in the intestines of humans. To our knowledge, this is the first time that transfer of resistance genes between enterococci in the intestines of humans has been shown.

Enterococci are found in milk and meat products at numbers ranging from 10^2 to 10^5 per gram (12). In this study, we chose an inoculum of 10^7 bacteria, which corresponds to eating 100 g of a milk or meat product.

The maximum concentration reached by the donor strain is in accordance with a previous study by Sørensen et al. (28). Colonization of the donor strain was only transient, which confirms the results of previous studies on the ingestion of enterococcus-contaminated food (11, 25, 28).

In vivo conjugation occurred in three out of six volunteers. The transconjugants showed the same PFGE profile as the recipient, with only one band difference, which makes them very closely related to the recipient (29) and confirms that in vivo conjugation did occur. The three volunteers in whom conjugation occurred between the ingested strains were all heavily colonized with both the donor strain and the recipient strain. Conversely, the other three volunteers showed lower maximum concentrations (two volunteers) or a delayed increase in concentration (one volunteer) of these strains. This could indicate that in order for transfer to occur, high numbers of both the donor and recipient strains must be present at the same time. Colonization by the transconjugants was only transient; however, our study was carried out without any selective antimicrobial pressure. The duration of colonization and the transfer frequency would probably have been higher in the presence of an antimicrobial. This hypothesis, however, was not tested in our study.

At present, several years after the EU ban on avoparcin, VRE can still be found in poultry (8, 16) and in healthy humans (13, 28) in Denmark. Before the ban, VRE were found in large amounts in poultry and pigs (3, 4), and intestinal colonization by VRE after eating chicken or pork was probably frequent among humans. Our study suggests that transfer of the *vanA* gene probably did take place in the intestines of humans before the EU ban took place. Even though avoparcin is no longer used in Europe, transfer of resistance genes from bacteria of animal origin to bacteria of human origin still represents a potential problem. In Japan, Kariyama et al. (22) found the same Tn*1546* transposon variant in an *E. faecium* isolate from a Japanese patient as was found in pigs from Europe, thus indicating transfer of Tn*1546* of porcine origin to a human *E. faecium* isolate. A study by Jensen (19) has shown a 2-base pair variant at position 8234 in the *vanX* gene of Tn*1546*. Jensen found a G variant and a T variant. The G variant was found in isolates from poultry, whereas the T variant was found in isolates of porcine origin. The donor and transconjugants belonged to the same variant (G variant). The PCR maps of the entire Tn*1546* were exactly the same for the donor strain and all the transconjugants. Both of these experiments indicate that it was the transposon from our donor strain that was transferred to the transconjugants.

In the United States, large amounts of virginiamycin are still

used in poultry production, and a high prevalence of virginiamycin resistance is found among *E. faecium* isolates from animals and retail meat (14, 15). Virginiamycin resistance is encoded by the *vat*(E) gene, which confers complete cross-resistance to quinupristin-dalfopristin (Synercid), an agent used to treat infections with VRE and methicillin-resistant *Staphylococcus aureus*. In one volunteer in the present study, the *vanA* gene was transferred together with *vat*(E) and *erm*(B). This transfer happened as a cotransfer with *vanA* and indicates that cotransfer of several resistance genes at the same time is possible. In the future, specifically designed studies should aim to further demonstrate the possibility of in vivo transfer of other resistance genes, such as *vat*(E).

In conclusion, our study demonstrated that transfer of resistance genes from bacteria of animal origin to bacteria of human origin in the intestines of humans is possible. Even though colonization was only temporary, it suggests that the passage of enterococci carrying mobile elements with resistance genes in the intestines of humans represents an opportunity for the spread of these resistance genes to the human indigenous intestinal flora. These resistant strains can probably cause infections, especially in immunocompromised patients, and due to their resistance, these infections will be difficult to treat. These findings support the recommendation for the discontinuation of the use of antimicrobial agents as growth promoters in animals.

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