Absence of intestinal colonization by vancomycin-resistant enterococci in nonhuman primates

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The animal reservoirs of vancomycin-resistant enterococci (VRE) have important role in the epidemiology of the bacteria and resistant genes. The present work searched fecal samples taken off nonhuman primates for the presence of VRE. Resistance profiles, virulence traits, and genetic variability among enterococci isolates were also analyzed. The samples included Capuchin monkeys (Cebus apella, n=28) and Common marmoset (Callithrix penicillata, n=37) housed in the Primate Center of the University of Brasilia, Brazil. Most individuals were captive monkeys from the Central-West and South-East regions of Brazil (n=48). We collected rectal swabs and carried out selective isolation followed by multiplex Polymerase Chain Reaction (PCR) to identify species and resistance genes. No vanA or vanB-containing enterococci were found. The carriage rates ranged from 1.5% for the VanC-type E. casseliflavus and E. gallinarum until 12.3% (n=8) for Enterococcus faecalis. All E. faecalis isolates showed susceptibility to vancomycin, teicoplanin, ampicillin, gentamicin, and streptomycin. The virulence genes ace and esp were prevalent (100.0%, 87.5%). Multilocus variable number of tandem repeats (MLVA) revealed diversity in the number of repeats among E. faecalis isolates and targets, which was higher for espC, efa5, and efa6. We identified six different MLVA genotypes that were divergent from those described in human beings. Also, they were clustered into two genogroups that showed host-specificity for the species Cebus apella or Callithrix penicillata. In conclusion, no vanA- or vanB-containing enterococci were found colonizing those primate individuals. This finding suggested that the primate individuals investigated in our study are not directly involved in the epidemiological chain of high-level vancomycin-resistant genes vanA or vanB in Brazil. Our study also showed that E. faecalis isolated from nonhuman primates carry virulence traits and have ability to spread their lineages among different individuals.

INDEX TERMS: Enterococcus, vancomycin-resistance, MLVA, monkey.
ficiar espécies e genes de resistência. Não foram isolados enterococos contendo os genes vanA ou vanB. A porcentagem de enterococos variou de 1,5% para Enterococcus faecalis e 12,3% (n=8) para Enterococcus faecalis VanC. A totalidade dos isolados da espécie E. faecalis demonstrou sensibilidade aos antimicrobianos vancomicina, teicoplanina, ampicilina, gentamicina e estreptomicina. Os genes de virulência ace e esp foram prevalentes (100%, 87.5%). A análise em multilocus de repetições em tandem de número variável (MLVA) revelou diversidade no número de repetições entre os isolados de E. faecalis, que foi mais alta para espC, efa5 e efa6. Foram identificados seis diferentes genotipos de MLVA, divergindo daqueles já descritos em humanos. Os genotipos foram ainda agrupados em dois genogrupos, demonstrando especificidade de hospedeiro para as espécies Cebus apella ou Callithrix penicillata. Concluindo, não foram isoladas linhagens de enterococos contendo os genes vanA ou vanB colonizando as espécies de primatas analisadas. O presente estudo demonstrou que os isolados de E. faecalis obtidos de primatas não-humanos apresentam determinantes de virulência e possuem a habilidade de disseminar linhagens entre diferentes indivíduos.

TERMOS DE INDEXAÇÃO: Enterococcus, resistência à vancomicina, MLVA, macacos.

INTRODUCTION

Two decades after the first isolation reports, the epidemiology of vancomycin-resistant enterococci (VRE) remains not fully understood (Uttley et al. 1988, Cetinkaya et al. 2000, Nordmann et al. 2007). The bacteria colonize pets and food production animals and have shown remarkable ability to spread hospital-adapted lineages (Aarestrup et al. 2002, Klare et al. 2003, Willems et al. 2005, Rice 2006).

Most epidemiological studies in animals were focused on the food production chain and found relationships between the use of growth promoters and the rate of resistance development (Bager et al. 1997, Aarestrup et al. 2001). These studies also identified virulence genes in E. faecalis strains isolated from food (Franz et al. 2003, Moreno et al. 2006). However, resistance and virulence traits in enterococci isolated from wild animals were seldom reported (Livermore et al. 2001, Butaye et al. 2002, Poeta et al. 2005). This study was aimed to examine whether VRE colonize individuals from the species Cebus apella and Callithrix penicillata housed in a Primate Center from Brazil. We also analyzed genes for virulence and resistance, as well as the genetic variability among Enterococcus faecalis isolates.

MATERIALS AND METHODS

Individuals. We examined 65 fecal samples from Capuchin monkeys (Cebus apella, n=28) and Common marmoset (Callithrix penicillata, n=37) housed in the Primate Center of the University of Brasilia (CPUnB), Brazil, during July and August 2006. The samples included animals breed at the CPUnB (n=17) and captive monkeys from the Central-West and South-West Regions of Brazil (n=48) (Table 1). The animals were fed daily with tropical fruits (banana, orange, pineapple, mango, and others), vegetables (potato, carrot, tomato, pumpkin, cucumber, cabbage, cauliflower, spring greens, and others), and commercial cat food. Twice a week they received insect larva as a protein source. Water was provided ad libitum. We administered twice a year either Ivermectin or a combination of Pyrantel, Oxantel and Praziquantel as antiparasitics for the species C. apella and C. penicillata, respectively. Individuals eligible for this study received no antibiotic treatment within 12 months prior the sampling procedure.

Specimen collection, isolation of enterococci and species identification. The rectal swabs (n=65) were inoculated in the selective media BBL Enterococcusel broth (Becton Dickinson and Co., Sparks, MD) supplemented with vancomycin 8µg/mL. After 24-48h of incubation at 35°C, the positive samples were seeded onto sheep blood agar plates. The colonies showing typical morphology and positive phenotypic testing for Enterococci were submitted to multiplex PCR as described earlier (Titze-de-Almeida et al. 2004a; Table 2). This PCR identifies the species E. faecalis, E. faecium, E. gallinarum (vanC1), E. casseliflavus (vanC2/3) and, in addition, the resistance genes vanA and vanB (Xavier et al. 2006).

Virulence genes detection and multilocus variable number of tandem repeat analysis - MLVA. We used a previously described MLVA scheme to analyze genetic polymorphisms in seven different repeat regions (Titze-de-Almeida et al. 2004b). The method was also used to type the isolates and to identify the virulence genes ace and esp that code for microbial surface proteins with function of cellular adhesins. To obtain the DNA, the strains were cultured at 37°C in brain heart infusion broth; 1.0 mL of this media was then centrifuged at 13.000 G for 3 min. The pellets were diluted in 100µL of sterile milli-Q water, boiled for 10 min., and centrifuged at 13.000 G for 3 min. To prepare the PCR mixture, we added 5µL of this supernatant, 25pmol of the previously described primers aceB, espC, espA, efa5, efa3, efa5 and efa6 (Titze-de-Almeida et al. 2004b; Table 2), 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl2, 0.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), and 1.2U of Taq DNA polymerase. We used the following PCR program: initial preheating step at 94° for 2 min; initial denaturation step at 94°C for 4min; 35 cycles of amplification (denaturation 94°C for 1 min., annealing at 55°C for 1min, extension at 72°C for 2min); and a final extension at 72°C for 5min. The PCR products were electrophoresed through a 1% agarose gel stained with ethidium bromide for 1 hour at 60V. The gel was photographed under UV light.

Antimicrobial susceptibility testing. The disk diffusion susceptibility testing was used to determine antimicrobial resistance to vancomycin, teicoplanin, and ampicillin, according to the NCCLS guidelines (NCCLS, 2002). High-level resistance to gentamicin and streptomycin (Oxoid, Basingstoke, UK) was also tested.

RESULTS

Sixty-five rectal samples from Capuchin monkeys and Common marmoset from the Primate Center of the University of Brasilia were examined for the presence of VRE. No vanA- or vanB-containing enterococci were found in this study. The most prevalent species was Enterococcus faecalis (n=8, 12.3%), followed by E. gallinarum (n=1, 1.5%, vanC1), and E. casseliflavus (n=1, 1.5%, vanC2/3).

Six of the seven MLVA loci showed genetic variability (Table 3). The number of repeats differed strongly among the isolates.

and targets, which varied from one repeat in espA until nine in espC (Table 4). Except for aceB, all loci showed any negative repeat results. The variability in the number of repeats was higher for espC, efa5, and efa6, in comparison with aceB, espA and efa2.

Six different MLVA genotypes were found and they were clustered in four genogroups of isolates sharing 6-7 identical number of repeats (Table 3). Two of them composed major genogroups of isolates. The genogroup I comprised enterococci with MLVA types 1 and 2, all of them isolated from C. penicillata individuals. Within this genogroup, three isolates showed identical number of repeats for all the seven alleles tested (M1- M3); the remaining one (M4) differed only in efa5 target. MLVA types 3 and 4 were clustered in the genogroup II. This genogroup contained two isolates from the species C. apella that differed only in espC repeats.

Genes coding for the virulence traits aceB and espC were identified in E. faecalis isolates, as part of the MLVA method. All isolates harboured aceB and seven of the eight isolates carried espC.

The disk diffusion testing showed antimicrobial susceptibility to vancomycin, teicoplanin, ampcillin, gentamicin, and streptomycein.

**DISCUSSION**

The present study examined rectal samples from the nonhuman primates Cebus apella and Callithrix penicillata for the presence...
Enterococcal species prevalence varied greatly according to the host species studied. Poeta et al. (2005) examined faecal samples \( (n=77) \) from various wild animals including birds of prey, owls, foxes, wild rabbits, European genets, forest wildcats, salamanders, storks, magpies, deer, vipers, otters, wolves, moufflon, badgers, partridge, hedgehog, pigeon, ferret, quails and wild boar. In that study, Enterococcus faecalis (52.1%) was the most prevalent species, followed by E. faecium (32.1%), E. hirae (10%), E. casseliflavus (2.8%), and E. gallinarum (1.4%). In contrast, the prevalence of E. faecalis was relatively low (1.5%) in Sus scrofa wild boars, in which E. faecium (50%) and E. hirae (40.3%) were prevalent (Poeta et al. 2007).

The method used for enterococci isolation may also affect the species prevalence results. In a previous study (VanC1, 13.0%) and E. gallinarum (12.3%) were absent from 77 wild animal samples (mammals, birds, and other) from Portugal natural parks (Poeta et al. 2005). Rice et al. (2003) also failed to find VRE in a surveillance study on 14 different species of domestic and wild animals, including cow, deer, duck, goat, horse, llama, pig, rabbit, sheep, squirrel, dog, turkey, and goose. In a previous study, we also found absence of VRE in cloacal samples from poultry raised on non-intensive production farms in Brazil (Xavier et al. 2006). In the present study, no vanA- or vanB-containing enterococci were found in the bank voles (Clethrionomys glareolus) that live in the same region. High-level vancomycin resistant enterococci were also absent from 77 wild animal samples (mammals, birds, and other) from Portugal natural parks (Poeta et al. 2005). Rice et al. (2003) also failed to find VRE in a surveillance study on 14 different species of domestic and wild animals, including cow, deer, duck, goat, horse, llama, pig, rabbit, sheep, squirrel, dog, turkey, and goose. In a previous study, we also found absence of VRE in cloacal samples from poultry raised on non-intensive production farms in Brazil (Xavier et al. 2006). In the present study, no vanA- or vanB-containing enterococci were found in Capuchin monkeys and Common marmoset housed in the Primate Center of the University of Brasilia, Brazil.

Few surveillance studies in wild animals have tested antimicrobial resistance to the clinically important antibiotics teicoplanin, ampicillin, gentamicin, and streptomycin. A previous study that analyzed 140 enterococci isolated from wild animals in Portugal revealed susceptibility to teicoplanin and ampicillin; resistance to gentamicin and, streptomycin occurred in only two isolates from an owl (Poeta et al. 2005). Low resistance rates to kanamycin, streptomycin, and ampicillin (9.0%, 6.7%, 3.7%), and susceptibility to gentamycin was found in samples from Sus scrofa (Poeta et al. 2007). In our study, E. faecalis strains isolated from the nonhuman primates Cebus apella and Callithrix penicillata were all susceptible to teicoplanin, ampicillin, gentamicin, and streptomycin. The low prevalence of antimicrobial resistance in enterococci isolated from wild animal species contrasts with those data concerned to food animals. This fact corroborates the selective pressure theory, in which the volume of antibiotic use would affect the rate of resistance development (Aarestrup et al. 2001).

In our study, we also examined whether enterococci colonizing wild monkeys carry the virulence genes ace (adhesin of collagen) from E. faecalis) and esp (enterococcal surface...
protein (Shankar et al. 1999, Nallapareddy et al. 2000, Mundy et al. 2000). Both factors are involved in microbial adhesion to host tissue; Ace binds extracellular matrix proteins and Esp is involved in microbial infection and biofilm formation, which exact role remains controversial (Shankar et al. 1999, Nallapareddy et al. 2000, Willems & Bonten 2007). In a previous study from a broad range of wild animals, the prevalence of ace and esp was relatively low (9.6%, 4.1%) compared to values found in our study (100% and 87%, respectively). The prevalence of esp found in our study was also higher than those observed in E. faecalis isolates recovered from human (44-46%) and food (33%) samples (Eaton & Gasson 2001, Titze-de-Almeida et al. 2004b).

The MLVA revealed different levels of genetic variability among the loci studied, which was previously described in medical isolates (Titze-de-Almeida et al. 2004b). espC, efa5, and efa6 showed three different alleles, contrasting with aceB, espA, and efa2 that presented only two different alleles (Table 4). The six MLVA types found in nonhuman primates (Table 3) differed from those previously described in human beings (Titze-de-Almeida et al. 2004b). These genotypes were classified in major clusters named genogroups I (n=4) and II (n=2) and others composed by single isolates, genogroups III and IV. This clustering characteristic was also observed in previous work from human beings, where isolates formed major genogroups (MLVA types 9 and 37; n=13 and n=14, respectively) and minor genogroups composed by unique isolates, MLVA types 1, 2, 4-8 (Titze-de-Almeida et al. 2004a, Willems et al. 2005, Titze-de-Almeida et al. 2006, Willems & Bonten 2007). In our study, the genetically related isolates M1-M4 from the genogroup I have colonized monkeys originated from geographically distant regions which includes the South-east (M1), Central-West (M2, M4), and CPUnB (M3) (Table 1 and 3). We should remark that these primates live in distinct cages in CPUnB. However, the cage structure allowed physical contact between neighbors. In addition, the animals were treated by the same technicians, which could contribute for the lineages spreading.

Indeed, host-specificity recently described in enterococci suggests the microbial lineages evolved in parallel with the host evolution (Willems et al. 2000). In our study, we also found host-specificity among nonhuman primate enterococci isolates; the MLVA genogroups I and II shared no isolates from different primate species.

In conclusion, we found absence of VRE colonizing individuals from the species C. apella and C. pericillata housed in CPUnB. E. faecalis isolated from these primates showed host-specificity, ability to harbour virulence genes, and the spreading behavior already reported in other animal species.

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