SHORT COMMUNICATION

Molecular characterization and antibiotic resistance of Enterococcus species from gut microbiota of Chilean Altiplano camelids

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Background: Enterococcus is one of the major human pathogens able to acquire multiple antibiotic-resistant markers as well as virulence factors which also colonize remote ecosystems, including wild animals. In this work, we characterized the Enterococcus population colonizing the gut of Chilean Altiplano camelids without foreign human contact.

Material and methods: Rectal swabs from 40 llamas and 10 alpacas were seeded in M-Enterococcus agar, and we selected a total of 57 isolates. Species identification was performed by biochemical classical tests, semi-automated WIDER system, mass spectrometry analysis by MALDI-TOF (matrix-assisted laser desorption/ionization with a time-of-flight mass spectrometer), and, finally, nucleotide sequence of internal fragments of the 16S rRNA, rpoB, pheS, and aac(6)-I genes. Genetic diversity was measured by pulsed field gel electrophoresis (PFGE)-Smal, whereas the antibiotic susceptibility was determined by the WIDER system. Carriage of virulence factors was explored by polymerase chain reaction (PCR).

Results: Our results demonstrated that the most prevalent specie was Enterococcus hirae (82%), followed by other non-C. faecalis and non-Enterococcus faecium species. Some discrepancies were detected among the identification methods used, and the most reliable were the rpoB, pheS, and aac(6)-I nucleotide sequencing. Selected isolates exhibited susceptibility to almost all studied antibiotics, and virulence factors were not detected by PCR. Finally, some predominant clones were characterized by PFGE into a diverse genetic background.

Conclusion: Enterococcus species from the Chilean camelids’ gut microbiota were different from those adapted to humans, and they remained free of antibiotic resistance mechanisms as well as virulence factors.

Keywords: Enterococcus; antibiotic resistance; molecular identification; camelids; PFGE; virulence factors

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Latin American camelids are genetically related to those living in Asia or Africa, although they are much smaller, have no hump, and have adaptations that allow them to thrive at high elevations (1). Nowadays, they are divided into four species, two of them free-living (Lama guanicoe and Vicugna vicugna) and the other two domesticated (Lama glama and Lama pacos) (2, 3). The natural habitat of South American camelids is the Altiplano, the high Andean plateau extending through the countries of Bolivia, Peru, Argentina, and Chile. The Altiplano is a special ecosystem characterized by its high altitude and the scarcity of oxygen.

The major health diseases affecting camelids are the infectious diseases, including newborn diarrhea, acute respiratory processes, and bacteremia (10). Moreover, they have no access to veterinary control, and infrequent contact with foreign humans and antimicrobial drugs. In 1993, the first report of bacteremia in a llama by Enterococcus species (11) was described, and more recently neonatal
sepsis was characterized by Dolente et al. (12). The aim of this work is to characterize the *Enterococcus* species of the gut microbiota of Chilean Altiplano camelids, focusing on their identification by phenotypic and molecular methods.

**Material and methods**

**Samples collection**
A total of 50 rectal swabs were collected from 40 alpacas and 10 llamas in different farms of Putre (Parinacota). Samples were collected during 2 different days, maintained at room temperature using Copan Transystem swabs (Copan, Brescia, Italy), and transported to the Antofagasta University. Samples were pre-enriched on brain-heart infusion broth at 37°C for 18 h, and then seeded on M-Enterococcus and incubated at 37°C for 48 h. All media were purchased from Difco (Detroit, MI).

**Bacterial identification and antibiotic susceptibility**
From each plate, at least five different colonies were reseeded and identified to the species level by MALDI-TOF mass spectrometry (MS). Complementary phenotypic analysis as well as antibiotic susceptibility were performed by the WIDER semiautomatic system (Fco. Soria Melguizo, Madrid, Spain). Clinical and Laboratory Standards Institute (CLSI) criteria for antibiotic susceptibility breakpoints were applied (13). Whole DNA of all enterococci strains was obtained by resuspending one colony in 200 μl of distilled water, heating at 95°C for 10 min, centrifuging for 5 min at 13,000 rpm, and collecting the supernatant. Five μl of the supernatant were used as a template in the polymerase chain reaction (PCR). Enterococcal species were assigned after PCR amplification of 16S rRNA, *rpoB*, *rpoA*, *aac(6')-I*, and *pheS* genes; subsequent nucleotide sequencing in a ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, San Mateo, CA); and comparison with the BLAST database tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers and conditions are shown in Table 1.

**Virulence genes detection**
The presence of *efaA*, *agg*, *gelE*, *esp*, *cylA*, and *ace* genes was investigated by PCR using specific primers and conditions previously described (Table 1). Positive and negative control strains were included in each experiment.

**Genetic relationship**
Pulsed field gel electrophoresis (PFGE) genomic DNA was prepared in agarose plugs, digested with *SmaI* (New England BioLabs, Beverly, MA), and run on a CHEF-DR III (Bio-Rad Laboratories, Hercules, CA), as described previously (18). Electrophoresis conditions were 6 V/cm², 14°C, and 22 h 5°C1/2. Band pattern analysis was performed constructing an UPGMA (unweighted pair group method with arithmetic mean) dendrogram based on Dice’s coefficient by the Phoretix 5.0 software (TotalLab, Newcastle upon Tyne, UK). Isolates were considered related if their PFGE banding patterns were ≥80% similar.

**Table 1.** Primers and conditions used in this study

| Primer     | Sequence (5'→3')                   | Size   | Reference       |
|------------|------------------------------------|--------|-----------------|
| BAc08F     | AGAGTTTGATCCTGGCGTCTCA            | 1,398  |                 |
| Uni 1390R  | GACGGGCGGCGTGTGACA                |        |                 |
| rpoA-21-F  | GACGAGCCCTCAGAGA1A                | 533    |                 |
| rpoA-23-R  | AGTTCCATCATGCTGAGTA               |        |                 |
| aac-1      | GGATAGCGATGATTAC                 | 840    | Del Campo et al. (15) |
| aac-2      | TAAGAGGTTTAATAATGTTA             |        |                 |
| pheS-21-F  | CAYCCNGCAGCAGYATGC               | 455    | Naser et al. (14) |
| pheS-22-R  | CCWARCCRAARGCAAARCC               |        |                 |
| ace-F      | GCTCGTCTTTCCACTTGTCTT            | 1,003  | Rich et al. (16) |
| ace-R      | GAGCAAAAGTTCACTGGTGAC            |        |                 |
| efaA-F     | GACGAGCCCTCAGAGA1A                | 705    | Eaton and Gasson (17) |
| efaA-R     | AGTTCCATCATGCTGAGTA               |        |                 |
| cylA-F     | TGGATGATGAGTAGATAGGAAGT          | 517    | Eaton and Gasson (17) |
| cylA-R     | TCTACAGTAATCTTTGGTCAG            |        |                 |
| agg-F      | AAGAAAAAAAGAAGTAGACCAAC          | 1,553  | Eaton and Gasson (17) |
| agg-R      | AAACGCACGACGACAGTGAATAA          |        |                 |
| esp-F      | TTGCAAGCTGATGGTACCCAGG           | 933    | Eaton and Gasson (17) |
| esp-R      | GCGTCAACACTTGCGATGCCGA           |        |                 |
| gelE-F     | AGCGATTGCTTTCCATC                | 419    | Eaton and Gasson (17) |
| gelE-R     | ACCCGGTATCATGTTT                 |        |                 |
Results and discussion

After selective culture of the 50 rectal swabs from 40 llamas and 10 alpacas, a total of 57 unrelated colonies presenting compatible morphology with *Enterococcus* were finally selected. Initially, up to five morphologically different colonies were analyzed from each sample, but finally only one colony by species and pulotype was further selected. The most frequently human-related species, *Enterococcus faecalis* and *Enterococcus faecium*, were not found, and some discrepancies were detected in their identification (Table 2).

According to material previously described (14), the species was finally assigned by the 16S rRNA, *rpoB*, *pheS*, and *aac(6')-I* genes, which were concordant in all of them.

### Table 2. Bacterial identification obtained by the different systems used in the 57 isolates

| Identification system | WIDER | MALDI-TOF | 16S rRNA | rpoB | pheS | aac(6')-I |
|-----------------------|-------|-----------|----------|------|------|-----------|
| *E. hirae*            | 41    | 46        | 47       | 47   | 47   | 47        |
| *E. mundtii*          | 12    | 7         | 6        | 6    | 6    | –         |
| *E. casseliflavus*     | 3     | 3         | 3        | 3    | 3    | –         |
| *E. gallinarum*       | 1     | 1         | 1        | 1    | 1    | –         |

Fig. 1. Dendrogram of the genetic relationship of the most frequent *E. hirae* and *E. mundtii* pulatypes.
The worst result was obtained with the semiautomated WIDER system, based on phenotypical and biochemical tests. MALDI-TOF technology misidentified only the Enterococcus hirae isolates, which were assigned to Enterococcus mundtii. Nevertheless, MALDI-TOF could be actually considered as a reliable method for enterococcal identification (19–22).

E. hirae was the most frequent species detected (82.5%), followed by E. mundtii (10.5%), Enterococcus casseliflavus (5.2%), and, finally, Enterococcus gallinarum (1.7%). Previous reports of gut microbiota from wild animals, including geese, free-living raptors, and other animals, confirmed the presence of these species (23–25).

Antibiotic resistance for penicillin, ampicillin, amoxicillin/clavulanate, daptomycin, levofloxacin, erythromycin, linezolid, minocycline, and nitrofurantoin was not detected in any enterococcal isolate, and glycopeptide resistance was confirmed in three E. casseliflavus isolates (CMI = 8 µg/ml for vancomycin and susceptibility to teicoplanin). Five E. hirae isolates and one E. casseliflavus isolate exhibited resistance to quinupristin/dalfopristin. The E. gallinarum isolate exhibited resistance to fosfomycin and rifampin. Virulence genes were not amplified in any enterococcal strains. Dissemination of antibiotic resistance mechanisms and virulence genes has been demonstrated in enterococcal isolates from wild animals (25–28), although in our case, the Altiplano remains a remote region with scarce antibiotic exposition.

As to the genetic variability of these isolates by the PFGE analysis, eight unrelated band patterns for the E. hirae isolates and three for the E. mundtii isolates were identified (Fig. 1). Pulsotype F was the most frequent, representing 57% of all E. hirae isolates, suggesting a common source of this clone or animal-to-animal transmission. Although the animals belonged to different farms and are separated at night, during the day they roam freely and can have contact.

In conclusion, Enterococcus spp. from the Chilean camelds’ gut microbiota were different from those adapted to humans (E. faecalis and E. faecium), and remained free of antibiotic resistance mechanisms as well as virulence factors. Genetic studies demonstrated the existence of several lineages in each species, suggesting a non-clonal situation. Although Enterococcus might be a major cause of bacteremia in newborn animals (11, 12), our results pointed out that potentially pathogenic isolates are not present in the gut of these animals.

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