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SHORT COMMUNICATION

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

Katheryne Guerrero-Olmos[1], John Báez[1], Nicomédes Valenzuela[1], Joselyne Gahona[1], Rosa del Campo[2]* and Juan Silva[1]

[1]Departamento de Tecnología Médica, Universidad de Antofagasta, Antofagasta, Chile; [2]Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Madrid, Spain

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, *rpo*B, *phe*S, and *aac*(6)-*I* genes. Genetic diversity was measured by pulsed field gel electrophoresis (PFGE)-*Sma*I, 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–*Enterococcus faecalis* and non–*Enterococcus faecium* species. Some discrepancies were detected among the identification methods used, and the most reliable were the *rpo*B, *phe*S, 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*

*Correspondence to: Rosa del Campo, Hospital Universitario Ramón y Cajal, ES-28034 Madrid, Spain, Email: rosacampo@yahoo.com

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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 main occupation of Altiplano natives is camelid stockbreeding, and they exploit both their meat and their fleece. The highest mortality and morbidity period of these animals is among the newborn; in industrial countries, this affects only 2–3% (4–7), whereas in the Altiplano the mortality is higher (8). In Chile, camelids inhabit the regions of Arica and Parinacota (about 3,800 m above sea level) and consist of a population of 19,066 alpacas and 17,392 llamas (9).

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

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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, *rpo*B, *rpo*A, *aac*(6′)-*I*, and *phe*S 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 *efa*A, *agg*, *gel*E, *esp*, *cyl*A, 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 *Sma*I (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/cm^2, 14°C, and 22 h 5–35s. Band pattern analysis was performed constructing an UPGMA (unweighted pair group method with arithmetic mean) dendrogram based on Dice's coefficient by the Phoretrix 5.0 software (TotalLab, Newcastle upon Tyne, UK). Isolates were considered related if their PFGE banding patterns were $\geq$80% similar.

Table 1. Primers and conditions used in this study

Primer	Sequence (5′→3′)	Size	Reference
BAc08F	AGAGTTTGATCCTGGCTCAG	1,398	
Uni 1390R	GACGGGCGGTGTGTACAA		
rpoA-21-F	GACAGACCCTCACGAATA	533	Naser et al. (14)
rpoA-23-R	AGTTCATCATGCTGTAGTA		
aac-1	GGATAGCGGATGATTATCA	840	Del Campo et al. (15)
aac-2	TAAGAGTTTAATGAATAATTA		
pheS-21-F	CAYCCNGCHCGYGAYATGC	455	Naser et al. (14)
pheS-22-R	CCWARVCCRAARGCAAARCC		
ace-F	GTCTGTCTTTTCACTTGTTTCT	1,003	Rich et al. (16)
ace-R	GAGCAAAAGTTCAATCGTTGAC		
efaA-F	GACAGACCCTCACGAATA	705	Eaton and Gasson (17)
efaA-R	AGTTCATCATGCTGTAGTA		
cylA-F	TGGATGATAGTGATAGGAAGT	517	Eaton and Gasson (17)
cylA-R	TCTACAGTAAATCTTTCGTCA		
agg-F	AAGAAAAAGAAGTAGACCAAC	1,553	Eaton and Gasson (17)
agg-R	AAACGGCAAGACAAGTAAATA		
esp-F	TTGCTAATGCTAGTCCACGACC	933	Eaton and Gasson (17)
esp-R	GCGTCAACACTTGCATTGCCGA		
gelE-F	ACGCATTGCTTTTCCATC	419	Eaton and Gasson (17)
gelE-R	ACCCCGTATCATTGGTTT		

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

	Identification system					
	WIDER	MALDI-TOF	16S rRNA	*rpo*B	*phe*S	*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	–

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 pulsotype 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, *rpo*B, *phe*S, and *aac*(6′)-I genes, which were concordant in all of them.

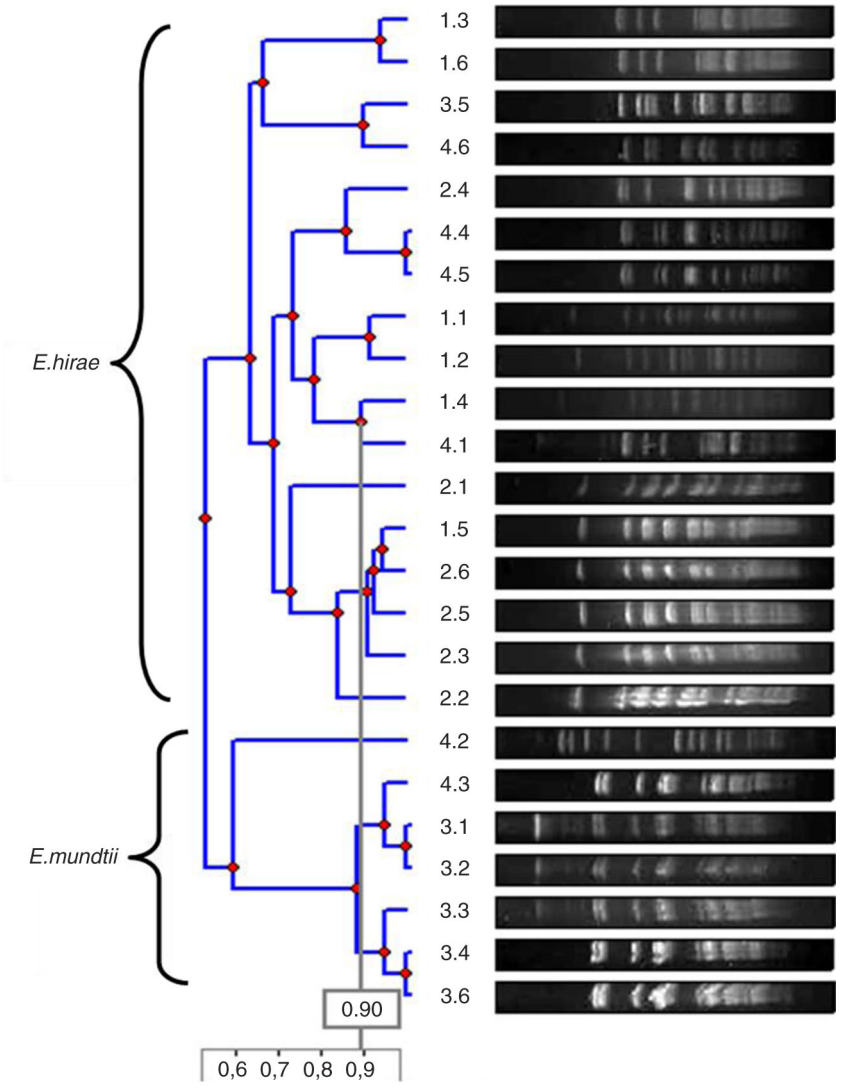

Fig. 1. Dendrogram of the genetic relationship of the most frequent *E. hirae* and *E. mundtii* pulsotypes.

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 camelids' 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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References

1. Wheeler JC. Evolution and present situation of the South-American camelidae. Biol J Linn Soc 1995; 54: 271–95.
2. Kadwell M, Fernandez M, Stanley HF, Baldi R. Genetic analysis reveals the wild ancestors of the llama and the alpaca. Proc R Soc Lond 2001; 268: 2575–84.
3. Marín JC, Zapata B, González BA, Bonacic C, Wheeler JC, Casey C, et al. Sistemática, taxonomía y domesticación de alpacas y llamas: nueva evidencia cromosómica y molecular. Rev Chil Hist Nat 2007; 80: 121–40.
4. D'Alterio GL, Knowles TG, Eknaes EI, Loevland IE, Foster AP. Postal survey of the population of South American camelids in the United Kingdom in 2000/01. Vet Rec 2006; 158: 86–90.
5. Davis R, Keeble E, Wright A, Morgan KL. South American camelids in the United Kingdom: population statistics, mortality rates and causes of death. Vet Rec 1998; 142: 162–6.
6. Sharpe MS, Lord LK, Wittum TE, Anderson DE. Pre-weaning morbidity and mortality of llamas and alpacas. Aust Vet J 2009; 87: 56–60.
7. Wright A, Davis R, Keeble E, Morgan KL. South American camelids in the United Kingdom: reproductive failure, pregnancy diagnosis and neonatal care. Vet Rec 1998; 142: 214–15.
8. Ameghino E, De Martini J. Mortalidad de crías de alpacas. Boletín de divulgación del Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA). Lima, Perú: Universidad de San Marcos; 1991, pp. 71–80.
9. Instituto Nacional de Estadística (INE) (2007). VII Censo Nacional Agropecuario y Forestal. Santiago, Chile: Instituto Nacional de Estadística.
10. Martín C, Pinto J, Chris E, Vázquez C, Dolores M. Camélidos Sudamericanos: estado sanitario de sus crías. RCCV 2010; 4: 37–50.
11. Burkhardt J, Janovitz E, Bowersock T, Higgins R. Septicemic *Enterococcus* infection in an adult llama. J Vet Diagn Invest 1993; 5: 106–9.
12. Dolente BA, Lindborg S, Palmer JE, Wilkins PA. Culture-positive sepsis in neonatal camelids: 21 cases. J Vet Intern Med 2007; 21: 519–25.
13. Clinical Laboratory Standards Institute (2012). Performance standards for antimicrobial susceptibility testing: twenty-second informational. Supplement M100–S22. Wayne, PA: CLSI.
14. Naser SM, Dawyndt P, Hoste B, Gevers D, Vandemeulebroecke K, Cleenwerck I, et al. Identification of lactobacilli by *phe*S and *rpo*A gene sequence analyses. Int J Syst Evol Microbiol 2005; 57: 2777–89.
15. Del Campo R, Galán JC, Tenorio C, Ruiz-Garbajosa P, Zarazaga M, Torres C, et al. New *aac*(6′)-I genes in *Enterococcus hirae* and *Enterococcus durans*: effect on {beta}-lactam/aminoglycoside synergy. J Antimicrob Chemother 2005; 55: 1053–5.
16. Rich RL, Kreikemeyer B, Owens RT, LaBrenz S, Narayana SV, Weinstock GM, et al. Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. J Biol Chem 1999; 274: 26939–45.
17. Eaton TJ, Gasson MJ. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol 2001; 67: 1628–35.
18. Murray BE, Singh KV, Heath JD, Sharma BR, Weinstock GM. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J Clin Microbiol 1990; 28: 2059–63.
19. Facklam RR, Collins MD. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. J Clin Microbiol 1989; 27: 731–4.

20. Goh SH, Facklam RR, Chang M, Hill JE, Tyrrell GJ, Burns EC, et al. Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridization to chaperonin 60 gene sequences. J Clin Microbiol 2000; 38: 3953–9.
21. Benagli C, Rossi V, Dolina M, Tonolla M, Petrini O. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of clinically relevant bacteria. PLoS One 2011; 6: e16424.
22. Fang H, Ohlsson AK, Ullberg M, Ozenci V. Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical *Enterococcus* isolates. Eur J Clin Microbiol Infect Dis 2012; 31: 3073–7.
23. Han D, Unno T, Jang J, Lim K, Lee SN, Ko G, et al. The occurrence of virulence traits among high-level aminoglycosides resistant *Enterococcus* isolates obtained from feces of humans, animals, and birds in South Korea. Int J Food Microbiol 2011; 144: 387–92.
24. Marrow J, Whittington JK, Mitchell M, Hoyer LL, Maddox C. Prevalence and antibiotic-resistance characteristics of Enterococcus spp. isolated from free-living and captive raptors in Central Illinois. J Wildl Dis 2009; 45: 302–13.
25. Radhouani H, Silva N, Poeta P, Torres C, Correia S, Igrejas G. Potential impact of antimicrobial resistance in wildlife, environment and human health. Front Microbiol 2014; 5: 23.
26. Lanthier M, Scott A, Lapen DR, Zhang Y, Topp E. Frequency of virulence genes and antibiotic resistances in *Enterococcus* spp. isolates from wastewater and feces of domesticated mammals and birds, and wildlife. Can J Microbiol 2010; 56: 715–29.
27. Poeta P, Costa D, Igrejas G, Rodrigues J, Torres C. Phenotypic and genotypic characterization of antimicrobial resistance in faecal enterococci from wild boars (*Sus scrofa*). Vet Microbiol 2007; 125: 368–74.
28. Radhouani H, Igrejas G, Carvalho C, Pinto L, Gonçalves A, Lopez M, et al. Clonal lineages, antibiotic resistance and virulence factors in vancomycin-resistant enterococci isolated from fecal samples of red foxes (*Vulpes vulpes*). J Wildl Dis 2011; 47: 769–73.