

Identification, antimicrobial susceptibility, and virulence factors of *Enterococcus* spp. strains isolated from Camels in Canary Islands, Spain

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Keywords

Antimicrobials,
Camels,
Enterococcus spp.,
Virulence.

Summary

This study investigated the presence of *Enterococcus* spp. strains in camel faeces, their virulence factors, and resistance to the antibiotics commonly used as therapy of enterococcal infections. One hundred and seventy three *Enterococcus* strains were isolated and identified to species level using polymerase chain reaction (PCR). Susceptibility to 11 antimicrobials was determined by disk diffusion method. Minimal Inhibitory Concentrations (MIC) of penicillin, ampicillin, vancomycin, teicoplanin, gentamicin, and streptomycin were all determined. Genes encoding resistance to vancomycin, tetracycline, and erythromycin as well as genes encoding some virulence factors were identified by PCR. *Enterococcus hirae* (54.3%) and *Enterococcus faecium* (25.4%) were the species most frequently isolated. None of the strains were resistant to vancomycin, teicoplanin, ampicillin or showed high level aminoglycoside resistance (HLAR). Strains resistant to rifampicin (42.42%) were those most commonly found followed those resistant to trimethoprim – sulfamethoxazole (33.33%). The genes *tetM*, *tetL*, *vanC1*, and *vanC2-C3* were detected in some strains. Virulence genes were not detected. Monitoring the presence of resistant strains of faecal enterococci in animal used with recreational purposes is important to prevent transmission of those strains to humans and to detect resistance or virulence genes that could be transferred to other clinically important bacteria.

Identificazione, suscettibilità antimicrobica e fattori di virulenza in ceppi di *Enterococcus* isolati da cammelli nelle Isole Canarie, Spagna

Parole chiave

Antimicrobico,
Cammello,
Enterococcus spp.,
Virulenza.

Riassunto

Questo studio ha esaminato la presenza, i fattori di virulenza e la resistenza agli antibiotici più comunemente utilizzati in terapia dei ceppi di *Enterococcus* spp. isolati dalle feci di cammelli. Centosettantatré ceppi di *Enterococcus* spp. sono stati isolati e identificati mediante reazione a catena della polimerasi (PCR). La resistenza nei confronti di 11 antibiotici è stata determinata con il metodo di diffusione su dischetto. Inoltre, sono state determinate le concentrazioni minime inibenti di penicillina, ampicillina, vancomicina, teicoplanina, gentamicina e streptomicina. I geni che codificano per la resistenza a vancomicina, tetraciclina, eritromicina e i geni codificanti per alcuni fattori di virulenza sono stati identificati mediante PCR. *Enterococcus hirae* (54,3%) e *Enterococcus faecium* (25,4%) sono risultate le specie più frequentemente isolate. Nessuno dei ceppi analizzati è risultato resistente a vancomicina, teicoplanina, ampicillina o ha mostrato un elevato livello di resistenza agli aminoglicosidi (HLAR). I ceppi resistenti alla rifampicina (42,42%) sono stati quelli più frequentemente isolati seguiti da quelli resistenti al trimetoprim sulfametossazolo (33,33%). I geni *tetM*, *tetL*, *vanC1* e *vanC2-C3* sono stati rilevati in alcuni ceppi mentre non sono stati rilevati i geni di virulenza. Monitorare la presenza di ceppi resistenti di enterococchi fecali in animali utilizzati a scopo ricreativo è importante per prevenirne la trasmissione all'uomo e per individuare geni di resistenza o virulenza che potrebbero essere trasferiti ad altri batteri clinicamente importanti.

Introduction

Enterococcus has emerged as a major cause of nosocomial infections in human and veterinary medicine (García-Migura et al. 2014, Bath et al. 2015). Traditionally, enterococcal infections are treated with penicillins, usually in combination with an aminoglycoside. Glycopeptides, especially vancomycin (VAN), are often used to treat enterococcal infections in patients with hypersensitivity to penicillins or in infections due to β -lactam resistant enterococci (Gold 2001). Clinical strains have become increasingly resistant to a broad range of antimicrobial agents, including aminoglycosides, β -lactams, and glycopeptides, limiting treatment options. Factors suspected to enhance the virulence of the bacterium include antibiotic resistance determinants, cytolytic toxin, gelatinase, aggregation substance, extracellular superoxide production and enterococcal surface protein (Mundy et al. 2000, Biendo et al. 2010). Cytolysin exerts activity against a broad spectrum of cell types, including a wide range of gram positive bacteria, eukaryotic cells such as human, bovine, and horse erythrocytes, retinal cells, polymorphonuclear leukocytes, and human intestinal epithelial cells (Cox et al. 2005). The enterococcal cytolysin is of interest because its activities boost enterococcal virulence in infection models. It has also been associated with patient mortality in epidemiological studies (Coburn et al. 2003). The production of cytolysin has also been shown to significantly worsen the severity of endocarditis (Chow et al. 1993). In 2002, Shankar and colleagues (Shankar et al. 2002) found a pathogenicity island (PAI) in vancomycin-resistant *Enterococcus faecalis*. It encodes several pathogenicity factors, among them the enterococcal surface protein (esp) conferring increased biofilm and colonization, a cytolysin with haemolytic, cytolytic, and antibacterial activity, the aggregation substance, a bile acid hydrolase, surface proteins and general stress proteins. The *E. faecalis* PAI products contribute in the successful establishment of certain enterococci as nosocomial pathogens (Laverde et al. 2011).

Reservoirs for antibiotic-resistant enterococci have not been completely determined although animals, humans, food and environment have been suspected as sources for some resistant clinical isolates (Descheemaeker et al. 1999, Lu et al. 2002, Van den Bogaard et al. 2002, Iversen et al. 2004, López et al. 2009).

This study investigated the presence of *Enterococcus* spp. in camel faeces, their virulence factors, and the resistance of these *Enterococcus* strains to antibiotics commonly used as therapy of enterococcal infections.

Materials and methods

Bacterial isolates and identification

Samples of faeces from camels were obtained directly and sent to Microbiology Laboratory (Las Palmas de Gran Canaria, Canary Islands, Spain) within 4 hours after collection. Samples were plated directly on M-Enterococcus Agar. Plates were incubated at 37°C and examined at 24 and 48 hours. Colonies from each sample, those showing the appearance of enterococci or those being gram positive cocci with negative reaction for catalase and growing in NaCl 6.5% were considered presumptive enterococci.

Identification of *Enterococcus* was made by polymerase chain reaction (PCR) (Jackson et al. 2004). The following strains from *Colección Española de Cultivos Tipo* (Spanish Type Culture Collection, CECT) were used as controls: *Enterococcus avium* CECT 968 (ATCC 14025), *Enterococcus durans* CECT 411 (ATCC 19432), *Enterococcus hirae* CECT 279 (ATCC 8043), *Enterococcus gallinarum* CECT 970 (ATCC 49573), *Enterococcus casseliflavus* CECT 969 (ATCC 25788), *Enterococcus faecium* CECT 410 (ATCC 19434), *Enterococcus malodoratus* CECT 971 (ATCC 43197) and *Enterococcus faecalis* ATCC 29212.

Antimicrobial susceptibility tests

Bacterial susceptibility to 11 antibiotics was determined using disk diffusion method: chloramphenicol (C), trimethoprim-sulfamethoxazole (SXT), fosfomicin (FOS), ampicillin (AM), penicillin (P), ciprofloxacin (CIP), erythromycin (E), rifampin (RA), tetracycline (TE), linezolid (LZD), and levofloxacin (LEV).

Minimal Inhibitory Concentrations (MICs) of penicillin, ampicillin, vancomycin (VAN), teicoplanin (TEC), gentamicin (GM), and streptomycin (SM) were determined on Mueller-Hinton agar (Difco, Detroit, Michigan, USA) containing double dilutions of the antibiotics¹. Concentrations ranging from 0.06 to 256 mg/L were used for penicillin (Laboratorios Normon, Madrid, Spain), ampicillin (Laboratorios Normon, Madrid, Spain), vancomycin and teicoplanin. Concentrations used for aminoglycosides ranged from 0.06 to 32768 mg/L for SM and to 4000mg/L for GM. High level resistance against streptomycin (Laboratorios Normon, Madrid, Spain) and gentamicin (Laboratorios Normon, Madrid, Spain) were defined as a MIC of >2000 and \geq 500 mg/L, respectively. Only 1 bacterial colony per plate was tested.

¹ Clinical and Laboratory Standards Institute (CLSI). 2007. Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document M100-S17.

Enterococcus faecalis ATCC 51299 (HLRG) and ATCC 29212 (susceptible to GM) were used as control strains. We used the PCR protocol described by Poeta and colleagues (Poeta *et al.* 2005) for the simultaneous detection of the genes encoding resistance to vancomycin, tetracycline, and erythromycin.

Virulence factors

To evaluate the pathogenic ability of isolates, the presence of virulence genes (*cyiA* and *cyiB*) was investigated by PCR.

Results and discussion

One hundred and seventy three *Enterococcus* strains were isolated. The distribution by species found in this study using PCR was: 54.3% *E. hirae*, 25.4% *E. faecium*, 5.8% *E. durans*, 4.62% *E. faecalis*, 4.1% *E. mundtii*, 2.9% *E. malodoratus*, 1.73% *E. casseliflavus*, and 1.15% *E. gallinarum*.

None of the strains were resistant to VAN, TEC, AM or showed HLAR. Only 11 isolates (6.3%) showed a MIC >8 mg/L for penicillin. Strains resistant to rifampicin (42.42%) were those most commonly found, followed by those resistant to trimethoprim sulfamethoxazole (33.33%). The gene *tetM* was detected in 9 strains and *tetL* was detected in 3 strains. Of the 11 (1 strain had both genes), 7 isolates were resistant to tetracycline. *VanC1* gene was detected in 2 strains of *E. gallinarum*. The gene *van C2-C3* was found in 2 strains, both of them identified as *E. casseliflavus*, a species of *Enterococcus* that intrinsically harbour this gene. A study conducted by Poeta and colleagues also reports finding these genes in the same species of *Enterococcus* isolated from wild animals (Poeta *et al.* 2005); *ermA*, *ermB*, *ermC*, *vanA* and *vanB* genes were not detected in any of the strains. Only 1 isolate was resistant to erythromycin.

A combination of penicillin and streptomycin is frequently used in Canary Islands to treat different infections in camels (Tejedor-Junco *et al.* 2009). Even if we did not find any strain of *Enterococcus* resistant to penicillin and only 1 strain showed HLRS (MIC > 512 mg/L), MICs values for these 2 antibiotics were close to resistance values. Only 11 strains showed a MIC > 8 mg/L for penicillin, but 26 isolates showed a MIC of 8 mg/L for this antibiotic. In addition to this, 36 strains showed MICs of 256 or 512 mg/L for streptomycin.

The resistance rates among *Enterococcus* strains isolated from camels were lower than those found among human strains isolated from hospital patients in a Canary Islands study (González-Martín *et al.* 2000). In this respect, it is worth mentioning that lately an outbreak of vancomycin-resistant *E. faecium* in kidney transplant patients has been described in a Hospital of Canary Islands (Montesinos *et al.* 2010), raising the need to check periodically vancomycin resistance in *Enterococcus* from animal samples.

Nucleotide sequence determination of the *E. faecalis* cytolysin operon revealed a complex determinant encoding 5 gene products, which are necessary and sufficient for cytolysin production (Biendo *et al.* 2010, Udo *et al.* 2011). The genes are *cyiLL*, *cyiLS*, *cyiM*, *cyiB*, *cyiA*, and *cyiI*; it has been suggested in the extant literature that β -haemolytic strains must have the whole set of *cyi* genes (Laverde *et al.* 2011). The presence of genes of *cyi* operon has been found more frequently in *E. faecalis* (most of them from clinical isolates), and less frequently in different enterococcal species and from different origins (Khan *et al.* 2005, Channaiah *et al.* 2010, Diarra *et al.* 2010, Olsen *et al.* 2012, Gonçalves *et al.* 2011, Silva *et al.* 2011, Champagne *et al.* 2011, Ramos *et al.* 2012, Dahlén *et al.* 2012). In the present study, no *cyiA* or *cyiB* genes were found in any of the strains, which is congruent with the strain being β -haemolytic. This negative result may be explained because *E. hirae* is the most frequent species (54.3%) and in other assays the yielded positive results for any of the *cyi* genes in this species has been too low (Semedo *et al.* 2003) or negative (Channaiah *et al.* 2010, Diarra *et al.* 2010, Champagne *et al.* 2011). Although our isolates yielded no positive results for any of the *cyi* genes tested, they may still harbour virulence determinants that were not investigated in this study.

We would like to remark the importance of monitoring the presence of resistant enterococci in animals including the ones used for recreational purposes. In addition to this, faecal enterococci could contribute to horizontal spread of virulence and resistance genes to other clinically important bacteria. Epidemiological studies in different animals should be continued to monitor the presence and dissemination of resistance and virulence genes of enterococci in order to establish public health measures.

References

- Bath M.P., Patel A., Sahni B.A.K., Praharaj S.C.A.K., Grover C.N., Chaudhari S.C.N., Das N.K. & Kulkarni M. 2015. Emergence of multidrug resistant enterococci at a tertiary care centre. *Med J Armed Forces India*, **71**(2), 139-144. doi: 10.1016/j.mjafi.2014.08.007.
- Biendo M., Adjid'e C., Castelain S., Belmekki M., Rousseau F., Slama M., Ganry O., Schmit J.L. & Eb F. 2010. Molecular characterization of glycopeptide-resistant Enterococci from hospitals of the Picardy Region (France). *Int J Microbiol*, 2010: 150464. doi:10.1155/2010/150464.
- Champagne J., Diarra M.S., Rempel H., Topp E., Greer C.W., Harel J. & Masson L. 2011. Development of a DNA microarray for enterococcal species, virulence, and antibiotic resistance gene determinations among isolates from poultry. *Appl Environ Microbiol*, **77** (8), 2625-2633.
- Channaiah L.H., Subramanyam B., McKinney L.J. & Zurek L. 2010. Stored-product insects carry antibiotic-resistant and potentially virulent enterococci. *FEMS Microbiol Ecol*, **74** (2), 464-471.
- Chow J.W., Thal L.A., Perri M.B., Vazquez J.A., Donabedian S.M., Clewell D.B. & Zervos M.J. 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrob Agents Chemother*, **37**, 2474-2477.
- Coburn P.S. & Gilmore M.S. 2003. The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cell Microbiol*, **5** (10), 661-669.
- Cox C.R., Coburn P.S. & Gilmore M.S. 2005. Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. *Curr Prot Pept Sci*, **6**, 77-84.
- Dahlén G., Blomqvist S., Almståhl A. & Carlén A. 2012. Virulence factors and antibiotic susceptibility in enterococci isolated from oral mucosal and deep infections. *J Oral Microbiol*, 4:10855. doi: 10.3402/jom.v4i0.10855.
- Descheemaeker P.R.M., Chapelle S., Devriese L.A., Butaye P., Vandame P. & Goossens H. 1999. Comparison of glycopeptide-resistant *Enterococcus faecium* isolates and glycopeptide resistance genes of human and animal origins. *Antimicrob Agents Chemother*, **43**, 2032-2037.
- Diarra M.S., Rempel H., Champagne J., Masson L., Pritchard J. & Topp E. 2010. Distribution of antimicrobial resistance and virulence genes in *Enterococcus* spp. and characterization of isolates from broiler chickens. *Appl Environ Microbiol*, **76** (24), 8033-8043.
- Garcia-Migura L., Hendriksen R.S., Fraile L. & Aarestrup F.M. 2014. Antimicrobial resistance of zoonotic and commensal bacteria in Europe: the missing link between consumption and resistance in veterinary medicine. *Vet Microbiol*, **70** (1-2), 1-9. doi: 10.1016/j.vetmic.2014.01.013.
- Gold H.S. 2001. Vancomycin-resistant enterococci: mechanism and clinical observations. *Clin Infect Dis*, **15**, Suppl. 2, S133-145.
- Gonçalves A., Igrejas G., Radhouani H., López M., Guerra A., Petrucci-Fonseca F., Alcaide E., Zorrilla I., Serra R., Torres C. & Poeta P. 2011. Detection of vancomycin-resistant enterococci from faecal samples of Iberian wolf and Iberian lynx, including *Enterococcus faecium* strains of CC17 and the new singleton ST573. *Sci Total Environ*, **410-411**, 266-268.
- González-Martín M., de Miguel I., Cañas A. & Martín-Sánchez A.M. 2000. Resistencia a antibióticos en aislamientos clínicos del género *Enterococcus*. *Rev Esp Quimioter*, **813**, 412-416.
- Iversen A., Kühn I., Rahman M., Franklin A., Burman L.G., Olsson-Liljequist B., Torell E. & Möllby R. 2004. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. *Environ Microbiol*, **6**, 55-59.
- Jackson C.R., Fedorka-Cray P.J. & Barret J.B. 2004. Use of a Genus- and species specific multiplex PCR for identification of *Enterococci*. *J Clin Microb*, **42**, 3558-3565.
- Khan S.A., Nawaz M.S., Khan A.A., Hopper S.L., Jones R.A. & Cerniglia C.E. 2005. Molecular characterization of multidrug-resistant *Enterococcus* spp. from poultry and dairy farms: detection of virulence and vancomycin resistance gene markers by PCR. *Mol Cell Prob*, **19**, 27-34.
- Laverde J.A., Hendrickx A.P.A., Willems R.J., Top J., Sava I., Huebne J., Witte W. & Werner G. 2011. Intra- and interspecies genomic transfer of the *Enterococcus faecalis* pathogenicity island. *PLoS ONE*, **6** (4), e16720.
- López M., Saénz Y., Rojo-Bezares B., Martínez S., Del Campo R., Ruiz-Larrea F., Zarazaga M. & Torres C. 2009. Detection of *vanA* and *vanB2*-containing enterococci from food samples in Spain, including *Enterococcus faecium* strains of CC17 and the new singleton ST425. *Int J Food Microb*, **133**, 172-178.
- Lu H., Weng X.H., Yin Y.K., Pang M.Y. & Tang Y.W. 2002. *Enterococcus faecium*-related outbreak with molecular evidence of transmission from pigs to humans. *J Clin Microbiol*, **40**, 913-917.
- Montesinos I., Campos S., Ramos M.J., Ruiz-Garbajosa P., Riverol D., Batista N., Ojeda-Vargas M. & Sierra, A. 2010. Estudio del primer brote por *E. faecium vanA* en Canarias. *Enferm Infecc Microbiol Clin*, **28** (7), 430-434.
- Mundy L.M., Sahn D.F. & Gilmore M. 2000. Relationships between enterococcal virulence and antimicrobial resistance. *Clin Microb Rev*, **13**, 513-522.
- Olsen R.H., Schønheyder H.C., Christensen H. & Bisgaard M. 2012. *Enterococcus faecalis* of human and poultry origin share virulence genes supporting the zoonotic potential of *E. faecalis*. *Zoonoses Public Health*, **59** (4), 256-263.
- Poeta P., Costa D., Sáenz Y., Klibi N., Ruiz-Larrea F., Rodrigues J. & Torres C. 2005. Characterization of antibiotic resistance genes and virulence factors in faecal *Enterococci* of wild animals in Portugal. *J Vet Med B*, **52**, 396-402.
- Ramos S., Igrejas G., Rodrigues J., Capelo-Martinez J.L. & Poeta P. 2012. Genetic characterisation of antibiotic

- resistance and virulence factors in *vanA*-containing enterococci from cattle, sheep and pigs subsequent to the discontinuation of the use of avoparcin. *Vet J*, **193** (1), 301-303.
- Semedo T., Almeida M., Martins P., Silva M.F., Figueiredo J.J., Tenreiro R. & Barreto M.T. 2003. Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the *cyl* operon in enterococci. *J Clin Microbiol*, **41** (6), 2569-2576.
- Shankar N., Baghdayan A.S. & Gilmore M.S. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature*, **417**, 746-750.
- Silva N., Igrejas G., Vaz J., Araújo C., Cardoso L., Rodrigues J., Torres C. & Poeta P. 2011. Virulence factors in enterococci from partridges (*Alectoris rufa*) representing a food safety problem. *Foodborne Pathog Dis*, **8** (7), 831-833.
- Tejedor- Junco M.T., Lupiola P., Caballero M.J., Corbera J.A. & Gutierrez C. 2009. Multiple abscesses caused by *Salmonella enterica* and *Corynebacterium pseudotuberculosis* in a dromedary camel. *Trop Anim Health Prod*, **41**, 711-714.
- Udo E.E. & Al-Sweih N. 2011. Frequency of virulence-associated genes in *Enterococcus faecalis* isolated in Kuwait Hospitals. *Med Princ Pract*, **20**, 259-264.
- Van den Bogaard A.E., Willems R., London N., Topenicillin J. & Stobberingh E.E. 2002. Antibiotic resistance of faecal enterococci in poultry, poultry farmers and poultry slaughterers. *J Antimicrob Chemother*, **49**, 497-505.