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Antimicrobial resistance and virulence genes in enterococci from wild game meat in Spain

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ABSTRACT

A total of 55 enterococci (45 *Enterococcus faecium*, 7 *Enterococcus faecalis*, and three *Enterococcus durans*) isolated from the meat of wild game animals (roe deer, boar, rabbit, pheasant, and pigeon) in North-Western Spain were tested for susceptibility to 14 antimicrobials by the disc diffusion method. All strains showed a multi-resistant phenotype (resistance to between three and 10 antimicrobials). The strains exhibited high percentages of resistance to erythromycin (89.1%), tetracycline (67.3%), ciprofloxacin (92.7%), nitrofurantoin (67.3%), and quinupristin-dalfopristin (81.8%). The lowest values (9.1%) were observed for high-level resistance to gentamicin, kanamycin, and streptomycin. The average number of resistances per strain was 5.8 for *E. faecium* isolates, 7.9 for *E. faecalis*, and 5.7 for *E. durans*. Genes encoding antimicrobial resistance and virulence were studied by polymerase chain reaction. A total of 15 (57.7%) of the 26 vancomycin-resistant isolates harboured the *van*A gene. Other resistance genes detected included *van*B, *erm*(B) and/or *erm*(C), *tet*(L) and/or *tet*(M), *acc*(6′)-*aph*(2″), and *aph*(3′)-IIIa in strains resistant to vancomycin, erythromycin, tetracycline, gentamicin, and kanamycin, respectively. Specific genes of the Tn*5397* transposon were detected in 54.8% of the *tet*(M)-positive enterococci. Nine virulence factors (*gel*E, *agg*, *ace*, *cpd*, *frs*, *esp*, *hyl*, *efaAfs* and *efaAfm*) were studied. All virulence genes, with the exception of the *frs* gene, were found to be present in the enterococcal isolates. At least one virulence gene was detected in 20.0% of *E. faecium*, 71.4% of *E. faecalis* and 33.3% of *E. durans* isolates, with *ace* and *cpd* being the most frequently detected genes (6 isolates each). This suggests that wild game meat might play a role in the spreading through the food chain of enterococci with antimicrobial resistance and virulence determinants to humans.

1. Introduction

Enterococci are Gram-positive bacteria of the gastrointestinal tract of humans and most animals (Aarestrup et al., 2000). Although enterococcal species are generally considered to be of low pathogenic potential, they have emerged as important causes of nosocomial- and community-acquired infections because of the capacity of these bacteria to acquire virulence traits (Silva et al., 2011).

Enterococci are intrinsically resistant to many antimicrobial agents, and have the ability to transfer antibiotic resistance to others via mobile genetic elements (such as plasmids or transposons), or through chromosomal exchange or mutations, posing a challenge in the treatment of infectious diseases (Hegstad et al., 2010). The problem of antimicrobial resistance in enterococci is not restricted to clinical settings alone, but also affects other

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environments. *Enterococcus* spp. have developed highly efficient mechanisms for the acquisition and distribution of antibiotic resistance genes. Thus, a high frequency of exchange between unrelated species (including the transfer of resistance genes to virulent strains) is to be expected in the habitats of these bacteria (for instance in the gastrointestinal tract or sewage water). In this way, *Enterococcus* spp., which have been recognized as reservoirs of antimicrobial resistance genes (Aarestrup et al., 2000), are considered important indicators of antibiotic resistance that may be used to track the evolution of antibiotic resistance in different environments (Capita and Alonso-Calleja, 2013).

Over the past decade, considerable concern has arisen in respect of the increase in antimicrobial resistance among commensal bacteria, including *Enterococcus* spp., in wild animal populations (Santos et al., 2013). Wild animals are of importance to antibiotic resistance in several different ways. Firstly, enterococci strains are sentinels of both livestock and human densities and its impact on the environment. Several human activities (e.g. farms, human and veterinary clinical settings, landfills, or waste-water treatment facilities) may bring about interactions with wildlife, which may have a direct association with the antibiotic resistance profiles of the intestinal bacteria of wild animals in a certain geographic location (Santos et al., 2013). Secondly, wild animals can be a reservoir of antibiotic-resistant bacteria and resistance genes, with the potential for long-distance dissemination throughout the environment. Lastly, wild animal populations are a possible source of antibiotic-resistant bacteria that may colonize or infect livestock and humans (Allen et al., 2010; Santos et al., 2013). In such a scenario, contact with species of game hunted for their meat which transfer multidrug-resistant enterococci to humans provides a biological mechanism for the increase in antibiotic resistance genes in human populations (Allen et al., 2010). The main concerns with wild game meat, in this context, are the possibility of the bacteria surviving as a consequence of insufficient processing and the potential for cross-contamination to other foods.

Studies of antimicrobial resistance and virulence traits in game animals or game meat have been performed in different countries (Literak et al., 2010; Poeta et al., 2007; Radhouani et al., 2014; Silva et al., 2011; Vargas et al., 2013). Nevertheless, this type of study has been very limited in Spain (Navarro-Gonzalez et al., 2013) and it would appear that no research of this sort has so far been performed in North-Western Spain. The objectives of this study were to determine the prevalence of resistance in *Enterococcus* spp. isolated from game meat in North-Western Spain, and to analyse their content of resistance and virulence genes.

2. Materials and methods

2.1. Samples and strains

Enterococcal isolates were recovered from 55 samples of meat obtained from wild game. These were roe deer (*Capreolus capreolus*; 35 samples), wild boar (*Sus scrofa*; 14 samples), wild rabbit (*Oryctolagus cuniculus*; two samples), pheasant (*Phasianus colchicus*; two samples) and wild pigeon (*Columba palumbus*; two samples) which had been hunted in the autonomous regions of *Castilla y León* and *Galicia* in the North-West of Spain.

The skinless animal legs were used for sampling. Each sample was prepared by excising a piece of 5 cm^2 in area and approximately 3 mm in thickness from the surface of the meat with a sterile knife blade and a template. The exact surface location for sampling was selected at random. The samples were placed in a sterile stomacher bag containing 45 mL of sterile 0.1% (wt/vol) peptone water (Oxoid Ltd., Hampshire, U.K.) and homogenized (Masticator IUL, Barcelona, Spain) for two minutes. Decimal dilutions in sterile 0.1% (wt/vol) peptone water were prepared from this homogenate, and volumes of 1 mL were pour-plated in duplicate in kanamycin aesculin azide agar (KAA, Oxoid) plates and incubated for 24 h at 42 °C.

For each sample, from three to five typical colonies were identified on the basis of Gram staining, catalase production, growth at 10 °C and at 45 °C and growth in the presence of 6.5% NaCl. Gram-positive cocci able to grow in these conditions were inoculated into microtubes of API 20 Strep (bioMérieux, Marcy L'Étoile, France) in accordance with the manufacturer's instructions. Data interpretation was performed using the Analytical Profile Index (API) database (V7.0) with the apiweb™ identification software (bioMérieux). Species identification was confirmed by polymerase chain reaction (PCR) assays using specific primers and conditions for detection of $ddl_{E.\ faecalis}$ (*Enterococcus faecalis*), $ddl_{E.\ faecium}$ (*Enterococcus faecium*), *van*C1 (*Enterococcus gallinarum*), *van*C2-*van*C3 (*Enterococcus casseliflavus*), *mur*G (*Enterococcus hirae*) and *mur*-2 (*Enterococcus durans*) genes (Table 1).

DNA of the isolates was extracted using the commercial system InstaGene Matrix DNA extraction kit (Bio-Rad Laboratories, Hercules, CA, USA) and following the manufacturer's instructions. The DNA purity and concentration was determined using the NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR reactions were performed in a total volume of 25 μL, using 0.5 μM of each primer (Isogen Life Science, Barcelona, Spain), 0.2 mM of deoxynucleoside triphosphates (dNTP's) mix (GeneAmp® dNTP blend, Thermo Scientific), 1× of PCR incomplete buffer (Bioron GmbH, Ludwigshafen, Germany), 3 mM of $MgCl_2$ (Bioron), 1.25 U of taq DNA polymerase (Bioron) and 5 μL of extracted solution of enterococcal DNA.

DNA amplifications were performed in a Mastercycler (Eppendorf Ibérica S.L.U., Madrid, Spain). PCR products were separated by horizontal electrophoresis through 1% (wt/vol) agarose (Bioron) gels in 1× TAE buffer, stained with GelRed (Biotium Inc., Hayward, CA, USA) diluted at 1:10,000, visualized and photographed on a UV transilluminator (Gel Doc™ EZ System; Bio-Rad). The size of each PCR product was estimated using standard molecular weight markers (1-kb DNA ladder; Bioron). Negative controls (samples without DNA template) and positive controls (samples with DNA from the collection of strains of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal) were included in all PCR assays. Only one isolate of a given species per positive sample was further studied by means of the tests listed below.

2.2. Antimicrobial susceptibility testing

Enterococcal isolates were screened for susceptibility to a panel of 14 antimicrobial drugs on Mueller-Hinton agar (Oxoid) by a disc diffusion method (CLSI, 2008). The following discs were used: ampicillin (AMP; 10 μg), vancomycin (VA; 30 μg), teicoplanin (TEC; 30 μg), erythromycin (E; 15 μg), tetracycline (TE; 30 μg), ciprofloxacin (CIP; 5 μg), nitrofurantoin (F; 300 μg), rifampicin (RD; 5 μg), fosfomycin (FOS; 200 μg), chloramphenicol (C; 30 μg), quinupristin-dalfopristin (QD; 15 μg), gentamicin (CN; 120 μg), kanamycin (K; 120 μg), and streptomycin (S; 300 μg). All the antibiotic discs were obtained from Oxoid with the exception of the kanamycin discs, which were prepared in our laboratory. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as reference strains for antibiotic disc control. After incubation at 35 °C for 18–24 h, inhibition halos were measured and scored as sensitive, intermediate (reduced susceptibility) or resistant according to the CLSI guidelines. Only the category of high-level resistance (HLR) was considered for gentamicin, kanamycin, and streptomycin (CLSI, 2014).