



HAL
open science

Molecular characterization of vancomycin-resistant enterococci and extended-spectrum beta-lactamase-containing *Escherichia coli* isolates in wild birds from the Azores Archipelago

Nuno Silva, Gilberto Igrejas, Pedro Rodrigues, Tiago Rodrigues, Alexandre Gonçalves, Ana Felgar, Rui Pacheco, David Gonçalves, Regina Cunha, Patrícia Poeta

► **To cite this version:**

Nuno Silva, Gilberto Igrejas, Pedro Rodrigues, Tiago Rodrigues, Alexandre Gonçalves, et al.. Molecular characterization of vancomycin-resistant enterococci and extended-spectrum beta-lactamase-containing *Escherichia coli* isolates in wild birds from the Azores Archipelago. *Avian Pathology*, 2011, pp.1. 10.1080/03079457.2011.599061 . hal-00723596

HAL Id: hal-00723596

<https://hal.science/hal-00723596>

Submitted on 11 Aug 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Molecular characterization of vancomycin-resistant enterococci and extended-spectrum beta-lactamase-containing Escherichia coli isolates in wild birds from the Azores Archipelago

Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2011-0044.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	03-May-2011
Complete List of Authors:	Silva, Nuno; University of Trás-os-Montes and Alto Douro, CECAV Igrejas, Gilberto; University of Trás-os-Montes and Alto Douro, Department of Genetics and Biotechnology Rodrigues, Pedro; University of the Azores, Department of Biology Rodrigues, Tiago; University of Porto, CIBIO Gonçalves, Alexandre; University of Trás-os-Montes and Alto Douro, Department of Veterinary Sciences Felgar, Ana; University of Trás-os-Montes and Alto Douro, Department of Genetics and Biotechnology Pacheco, Rui; University of Trás-os-Montes and Alto Douro, Department of Veterinary Sciences Gonçalves, David; University of Porto, CIBIO Cunha, Regina; University of the Azores, Department of Biology Poeta, Patrícia; University of Trás-os-Montes and Alto Douro, Department of Veterinary Sciences
Keywords:	Antimicrobial resistance, Enterococci, Escherichia coli, wild birds

For Peer Review Only

Cavp-2011-0044.R1

Molecular characterization of vancomycin-resistant enterococci and extended-spectrum beta-lactamase-containing *Escherichia coli* isolates in wild birds from the Azores Archipelago

Nuno Silva¹, Gilberto Igrejas², Pedro Rodrigues³, Tiago Rodrigues⁴; Alexandre Gonçalves^{1,2}, Ana Carolina Felgar^{1,2}, Rui Pacheco^{1,2}, David Gonçalves⁴, Regina Cunha³, Patrícia Poeta^{1*}

¹Centre of Studies of Animal and Veterinary Sciences, University of Trás-os-Montes and Alto Douro Vila Real, Portugal; Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. ²Institute for Biotechnology and Bioengineering, Centre of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. ³CIBIO - Research Center in Biodiversity and Genetic Resources, Department of Biology, University of Azores; Ponta Delgada, São Miguel, Azores, Portugal. ⁴CIBIO - Research Center in Biodiversity and Genetic Resources of the University of Porto; Vairão, Portugal

Short title: VRE and ESBL-containing *E. coli* in Azorean wild birds

***Corresponding author:**

Patrícia Poeta

FAX: 00351 259350629, Phone: 00351 259350466, e-mail: ppoeta@utad.pt

Received : 3 May 2011

Abstract

To study the prevalence of vancomycin-resistant enterococci (VRE) and extended-spectrum beta-lactamases (ESBLs) containing *Escherichia coli* isolates, and the mechanisms of resistance implicated, 220 faecal samples from wild birds were collected between 2006 and 2010 in the Azores Archipelago. Samples were spread in Slanetz-Bartley agar plates supplemented with 4 mg/L of vancomycin and in Levine agar plates supplemented with 2 mg/L of cefotaxime for VRE and ESBL-containing *E. coli* isolation, respectively. *vanA*-containing enterococcal isolates (four *Enterococcus faecium* and two *E. durans*) and *vanC-1 E. gallinarum* isolates were detected in six and seven faecal samples, respectively. VRE isolates showed ampicillin (n= 11), ciprofloxacin (n= 9), tetracycline (n= 6), erythromycin (n= 5), quinupristin/dalfopristin (n= 3) and high-level kanamycin resistance (n= 1). The *tet(L)* and/or *tet(M)* gene, was found in all tetracycline-resistant isolates and the *erm(B)* gene in all erythromycin-resistant isolates. Three *vanA*-containing *E. faecium* and two *E. gallinarum* presented specific sequences of the Tn5397 transposon. Four VRE isolates harboured the *ace* virulence gene. One faecal sample reveals one ESBL-containing *E. coli* isolate that belongs to the A phylogenetic group, showed a phenotype of resistance to beta-lactams and tetracycline and harbored the *bla*_{CTX-M-14}, *bla*_{SHV-12} and the *tet(A)* genes. To our knowledge, this is the first study to focus on defining the prevalence of VRE and/or ESBL-containing *E. coli* strains in wild birds from the Azores. The data recovered are essential to improve knowledge about the dissemination of resistant strains through wild ecosystems and their possible implications by transferring these resistances to other animals or to humans.

Introduction

Enterococcus spp. and *Escherichia coli* are common inhabitants of the gastrointestinal tracts of human and animals (Poeta *et al.*, 2005a; Sorum & Sunde, 2001).

Enterococci are intrinsically resistant to many antimicrobials and have the ability to acquire resistance to glycopeptides and aminoglycosides, among others, posing a challenge to therapeutic options (Aarestrup *et al.*, 2000). Multidrug-resistant enterococci have been increasingly identified as the most important pathogens responsible for nosocomial infections in human (Top *et al.*, 2007). Common enterococcal infections include those of the urinary tract, bloodstream, endocardium and wounds (Shepard & Gilmore, 2002). Although relatively rare, infections by *E. faecalis* (Chadfield *et al.*, 2004); *E. hirae* (Chadfield *et al.*, 2005) *E. durans* (Abe *et al.*, 2006) and *E. cecorum* (Stalker *et al.*, 2010) have been described in poultry, and in other birds like canaries and psittacine (Devriese *et al.*, 1990; 1995).

The increase in enterococci resistance to commonly used antimicrobials in both the public health and veterinary sectors is one of the major threats to health care worldwide (Heuer *et al.*, 2006). Different types of glycopeptide resistance and their biochemical mechanisms have been described in enterococci: acquired type (*vanA*, *vanB*, *vanD*, *vanE*, *vanG*, and *vanL*); and low-level intrinsic type (*vanC*, associated with the *Enterococcus gallinarum* and *E. casseliflavus/flavescens* species). Different virulence factors have been identified and include adhesins, sex pheromones, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin (Upadhyaya *et al.*, 2009).

The emergence of multi-resistant *E. coli* has been previously reported in humans and in different animal species, increasing public health concerns (Saenz *et al.*, 2004).

One of the most clinically relevant antimicrobial resistance mechanisms is the production of extended-spectrum beta-lactamases (ESBLs) (Paterson & Bonomo, 2005) by *Enterobacteriaceae*, specifically *E. coli* (Blanc *et al.*, 2006). The ESBLs have been increasingly reported in Europe since their first description in 1983 (Coque *et al.*, 2008). There is great concern, in recent years, about the emergence and wide dissemination of ESBLs among clinical *E. coli* isolates in hospitals, representing a major concern for the treatment of infectious diseases (Paterson & Bonomo, 2005). Most ESBLs are derivatives of TEM or SHV enzymes, but a new family of plasmid-mediated ESBLs, CTX-M, has also been reported worldwide in *E. coli* and other *Enterobacteriaceae* (Costa *et al.*, 2006; Livermore *et al.*, 2007; Bush & Jacoby, 2010).

The aim of the present study was to analyze the prevalence and study the phenotypic and genotypic characterization of antimicrobial resistance in VRE and ESBL-containing *E. coli* isolates recovered from faecal samples of 220 wild birds in Azores.

Material and Methods

Faecal samples and Bacterial isolates. Two hundred and twenty faecal samples of nine passerine species (178 samples) and two game birds (42 samples) species were included in this study (Table 1). All the bird species are resident in the Azores.

For passerine species, cloacal swabs (one per individual) were collected between February and June 2010 in all islands of the Azores Archipelago, under the study “Biogeography and Evolution of Azorean Passerines - An Integrative and Comparative Approach” performed by the Research Center in Biodiversity and Genetic Resources, University of the Azores. The birds were captured with the permission of the Azorean environmental authority, Secretariat for the Environment and the Sea, Directorate of the Nature Conservation Service. Briefly, the birds were captured using mist nets and faecal samples were recovered from cloacae with the aid of sterile swabs and gloves, and transported to the laboratory in a Carry-Blair medium before being processed. At the end, all the birds were released back into the environment.

For the game bird species, faecal samples (one per individual) were recovered from common snipe (*Gallinago gallinago*) and quail (*Coturnix coturnix conturbans*), under the project “Management of game species in the archipelago of the Azores” performed by the Research Center in Biodiversity and Genetic Resources, University of Porto. Faecal samples of the common snipe were collected from 2006-2009 during the hunting seasons in Flores and Pico Islands. In addition, the faecal samples of quail were collected during 2009 on Terceira and Graciosa Islands. Such hunting is supervised by the Regional Secretariat for Agriculture and Forestry (Azores), under the Regional Legislative Decree no.17/2007/A. After necropsy, intestinal contents were recovered

from the guts of the birds and stored individually in plastic sterile tubes containing a Carry-Blair medium and transported to the laboratory before they were processed.

The samples were seeded onto Slanetz–Bartley agar plates supplemented with 4 mg/L vancomycin (for VRE isolation) and onto Levine agar plates supplemented with 2 mg/L cefotaxime (for ESBL-containing *E. coli* isolation) and incubated at 37°C for 24 h. Colonies with typical enterococcal morphology (four per sample) were identified to the genus level by cultural characteristics, Gram-staining, catalase test and the bile-aesculin reaction. Species identification was confirmed by polymerase chain reaction (PCR) using primers and conditions for the different enterococcal species (Dutka-Malen *et al.*, 1995; Miele *et al.*, 1995; Robredo *et al.*, 1999). Additionally, colonies with typical *E. coli* morphology (four per sample) were selected and identified by classical biochemical methods (Gram-staining, catalase, oxidase, indol, Methyl-Red-Voges-Proskauer, citrate and urease), and by the API 20E system (BioMérieux, La Balme Les Grottes, France).

Antimicrobial susceptibility test. Antimicrobial susceptibility was tested by the agar disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011). The susceptibility of the enterococcal isolates was tested for 11 antimicrobials: [vancomycin (30 µg), teicoplanin (30 µg), ampicillin (10 µg), streptomycin (300 µg), gentamicin (120 µg), kanamycin (120 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), quinupristin-dalfopristin (15 µg), and ciprofloxacin (5 µg)] by the disk diffusion method (CLSI, 2011). High-level resistance was evaluated for aminoglycosides. Minimal inhibitory concentrations (MICs) of vancomycin and teicoplanin were also determined by the agar dilution method (CLSI,

2011). *E. faecalis* strain ATCC 29212 and *Staphylococcus aureus* strain ATCC 25923 were used as quality controls.

Conversely, the susceptibility of the *E. coli* isolates was tested for 16 antimicrobials: [ampicillin (10 µg), amoxicillin-clavulanic acid (AMC) (20µg +10µg), cefoxitin (30 µg), cefotaxime (CTX) (30 µg), ceftazidime (CAZ) (30 µg), aztreonam (30 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), sulfamethoxazole-trimethoprim (1.25µg + 23.75µg), tetracycline (25 µg), and chloramphenicol (30 µg)]. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as quality control strains.

Agar plate-based screening for ESBL production was carried out by the double-disc diffusion test [using CTX (30µg), CAZ (30µg) and AMC (20µg +10µg)] (Bradford, 2001; CLSI, 2011).

Characterization of resistance genes. The isolates with resistance to one or more antimicrobials were selected for antimicrobial-resistance genes characterization. In VRE isolates, the vancomycin resistance genes (*vanA*, *vanB*, *vanC-1*, and *vanC-2*) were tested by PCR (Torres *et al.*, 2003) in all enterococcal isolates that showed resistance or reduced susceptibility to glycopeptides. The resistance genes for the other studied antimicrobials were also tested by PCR and were: *erm(A)*, and *erm(B)* (in erythromycin resistant isolates) (Torres *et al.*, 2003), *tet(M)* and *tet(L)* (in tetracycline-resistant isolates) (Aarestrup *et al.*, 2000; Torres *et al.*, 2003), *vat(D)* and *vat(E)* (in quinupristin/dalfopristin-resistant isolates) (Aarestrup *et al.*, 2000) and *aph(3')-IIIa* (in kanamycin-resistant isolates) (Torres *et al.*, 2003). In addition, the presence of Tn916- and Tn5397-specific sequences was also analyzed by PCR (Agerso *et al.*, 2006).

In the ESBL-containing *E. coli* isolates, the presence of genes encoding TEM, SHV, OXA, CTX-M, and CMY β -lactamases was studied by PCR using primers and conditions previously reported upon and sequencing (Jouini *et al.*, 2007). DNA sequences were compared with those included in the GenBank database as well as with those deposited at the website [http:// www.lahey.org/Studies/](http://www.lahey.org/Studies/), in order to determine the specific type of β -lactamase gene. The following genes were also studied by PCR: *tet(A)*, and *tet(B)*, *tet(C)*, *tet(D)* and *tet(E)* in tetracycline resistant isolates (Saenz *et al.*, 2004). Positive and negative controls were included in all PCRs.

Detection of virulence factors. The presence of genes encoding different virulence factors was tested by PCR in all enterococcal isolates recovered in this study using primers and conditions described previously (Eaton & Gasson, 2001; Mannu *et al.*, 2003; Poeta *et al.*, 2006). The tested genes, encoding virulence factors were: *gelE* (gelatinase); *fsr* locus (regulator of the expression of *gelE*); *cyl* operon (cytolysin); *cpd* (pheromone determinant); *hyl* (hyaluronidase); *agg* (aggregation substance); *esp* (extracellular surface protein); and *ace* (accessory colonization factor). Positive and negative controls were included in all PCRs (Poeta *et al.*, 2005b).

Detection of *E. coli* phylogenetic groups. The ESBL-containing *E. coli* isolates were classified in one of the four main phylogenetic groups A, B1, B2 and D, following the PCR strategy described previously based in the presence or absence of *chuA*, *yjaA*, or *tspE4.C2* genes (Clermont *et al.*, 2000).

Results

Detection of vancomycin-resistant isolates and characterization of antimicrobial resistance mechanisms. This is the first study reporting the presence of VRE in wild birds from the Azores. Figure 1 shows the distribution of these strains in the Azores. VRE were detected in 13 of the 220 wild bird faecal samples (6%), and four isolates per sample were identified to the species level and their antimicrobial susceptibility profiles determined. All the four VRE isolates from each positive sample exhibited the same enterococcal species and the same antimicrobial resistance profile and, for this reason, only one isolate per positive sample was maintained for further studies. Ten of the VRE were recovered from three passerine species (*E. rubecula*, *S. atricapilla* and *F. coelebs*) and the remaining 3 from quail (game specie) (Table 1). Six of these isolates presented an acquired mechanism of vancomycin resistance (*vanA* genotype) and the remaining seven an intrinsic mechanism of vancomycin resistance (*vanC-1* genotype). The six VRE isolates (2.8%) that exhibited an acquired mechanism of resistance were identified as *Enterococcus faecium* (four isolates) and *E. durans* (two isolates). The characteristics of these isolates are shown in Table 2. All the six isolates that exhibited a *vanA* genotype showed high-level resistance to vancomycin (MIC \geq 128 mg/l) and teicoplanin (MIC 64 mg/l). In these isolates, antimicrobial resistance was also detected for tetracycline (6 isolates), ciprofloxacin (6 isolates), ampicillin (5 isolates) erythromycin (4 isolates), quinupristin/dalfopristin (2 isolates) and kanamycin (1 isolates). The *tet(L)* and/or *tet(M)* genes, related to tetracycline resistance, was found in all tetracycline-resistant isolates, and the specific sequences of Tn916 and Tn5397 transposons were

detected in three of them. Moreover, all the erythromycin-resistant *vanA* isolates harboured the *erm(B)* gene.

Enterococci with an intrinsic mechanism of vancomycin resistance were found in seven of the 220 wild bird faecal samples (3.2%). All seven enterococci were identified as *E. gallinarum* and contained the *vanC-1* gene (Table 2). They presented low level resistance to vancomycin (MIC 8 mg/l) and susceptibility to teicoplanin (MIC 1 mg/l). Two of the isolates exhibited resistance to tetracycline. The *tet(M)* [alone or associated with *tet(L)* gene] was observed in these isolates and both of them contained specific sequences of Tn5397 transposon. One *vanC-1* isolate resistant to erythromycin showed the presence of the *erm(B)* gene. Six *vanC-1* isolates were also ampicillin resistant, and three were additionally, resistant to ciprofloxacin and one to quinupristin/dalfopristin.

Detection of virulence factors genes. The *ace* was the unique virulence factor gene detected in our VRE isolates (two *E. gallinarum*; one *vanA-E. faecium* and one *vanA-E. durans*) (Table 2).

Detection of ESBL-producing *E. coli* isolates. This is also the first study reporting the presence of ESBL-producing *E. coli* isolates in wild birds from the Azores. *E. coli* isolates were detected on Levine-CTX plates from one of the 220 faecal samples of wild birds representing 0.5% of the analyzed samples. Four *E. coli* isolates were recovered from this sample and as all demonstrated the same antimicrobial resistance profile only one isolate was maintained for further studies. This *E. coli* isolate (AzP-38) was recovered from a passerine specie (*S. atricapilla*) on Graciosa Island (Figure 1). This

isolate showed a positive screening test for ESBL-production, belongs to the A phylogenetic group, showed a phenotype of resistance to beta-lactams and tetracycline and harbored the *bla*_{CTX-M-14}, *bla*_{SHV-12} and the *tet(A)* genes.

Discussion

In recent years, different reports have alerted us to the spread of VRE and/or ESBL-containing *E. coli* isolates from clinical, farm and wild animals in different European countries (Mallon *et al.*, 2002; Torres *et al.*, 2003; Bonnedahl *et al.*, 2010; Literak *et al.*, 2010; Pinto *et al.*, 2010). Although the percentage of *vanA* isolates from wild birds in the Azores was lower when compared with others studies performed in mainland Portugal in farm and wild animals (Poeta *et al.*, 2005a; Gonçalves *et al.*, 2010; Radhouani *et al.*, 2010b; Radhouani *et al.*, 2010c), our isolates showed a phenotype of multi-resistance. These differences might be explained by the use of antimicrobial drugs as prophylactic and therapeutic approaches in farm animals and also because the wild animals described in these previous studies inhabit areas closer to major urban centers and intensive livestock farms. It has been suggested that there may be a correlation between the level of antimicrobial resistance in faecal bacteria from animals and the level of contact of these animals with people (Skurnik *et al.*, 2006). A similar antimicrobial resistance pattern was previously observed in *vanA*-containing *Enterococcus* isolated from faecal samples of buzzards (Radhouani *et al.*, 2010b). However, in faecal cultures obtained from raptors in Illinois (USA) no VRE strains were detected (Marrow *et al.*, 2009).

Virulence factors have been detected in enterococci of food and animal origin (Eaton & Gasson, 2001); however only a few studies have focused on isolates of wild animals (Poeta *et al.*, 2005b; Radhouani *et al.*, 2010b; Silva *et al.*, 2011). The current study shows the presence of the *ace* virulence gene that encodes a protein which mediates the adherence to collagen (Kayaoglu *et al.*, 2008). In a previous study, the *ace* virulence gene was found in *E. faecalis* isolated from partridges (Silva *et al.*, 2011).

Although previous reports have demonstrated the presence of ESBL-producing *E. coli* in faecal samples of wild animals in mainland Portugal (Costa *et al.*, 2006; Poeta *et al.*, 2009; Pinto *et al.*, 2010; Radhouani *et al.*, 2010a) a recent study highlighted the absence of these strains in the song thrush (Silva *et al.*, 2010). In the last few years there has been an increase in the detection of ESBLs, mainly of the CTX-M group, in wild animals (Poeta *et al.*, 2009; Bonnedahl *et al.*, 2010; Pinto *et al.*, 2010; Radhouani *et al.*, 2010). In this study, the ESBL-containing *E. coli* isolate, classified in the A phylogroup, was more sensitive to the antimicrobial compounds tested than ESBL-positive *E. coli* isolates recovered from others wild animals in mainland Portugal (Costa *et al.*, 2006; Poeta *et al.*, 2009; Pinto *et al.*, 2010; Radhouani *et al.*, 2010a). Moreover, the beta-lactamase genes detected in our isolate (*bla*_{CTX-M-14} and *bla*_{SHV-12}) were also found in wild birds in Sweden (Bonnedahl *et al.*, 2010) and Poland (Literak *et al.*, 2010). How these wild birds acquired VRE and ESBL-containing *E. coli* is unclear. Although these species live in woodlands they are often observed on farms and in urban areas, and one reason that might explain the acquisition and dissemination of antimicrobial-resistant bacteria, even in the absence of direct antimicrobial pressure, is the use of antimicrobials in veterinary and human medicine and the consequent exposure of the wild birds to faecal material from farm animals or even from humans. Moreover, the

Azores it is a popular touristic destination for outdoor activities, including bird watching and the potential risk of contact between wild birds and tourists should not be discarded. On the other hand, although the birds described on this study are residents, the interaction with migratory birds from North America and Europe may provide a potential explanation for the introduction and dissemination of antimicrobial-resistant bacteria. The incidence of ESBL-producing *E. coli* in wild animals can have implications for the transfer of these resistances to other animals or even humans. Moreover, the resistant strains or genes could be transmitted to other wild animals, and this can represent a problem since the Azores have a rich diversity of wildlife many of them endemic, including species and subspecies of wild birds (Bannerman & Bannerman, 1966).

This study shows that bacteria resistant to antimicrobial drugs, or resistance genes, have already spread to the Azores, one of the most inhabited areas in the world indicating that even wild birds do not naturally come into contact with antimicrobial agents, these animals can be colonized by resistant bacteria and can act as a reservoir of resistance genes. Although the results presented here show a very low prevalence of ESBL-containing *E. coli*, a significant number of VRE with a multi-resistance phenotype was found in the faecal samples of wild birds. Further surveillance studies are needed to better understand the dissemination of VRE and ESBL strains in wild ecosystems and the evaluation of the potential risk of these resistant bacteria to human and animal health.

Acknowledgments

We thank the Directorate of the Nature Conservation Service and the Regional Secretariat for Agriculture and Forestry (Azores Government) for permission to capture birds, the different hunter's associations in the Azores, and a special thanks to Joana Micael and Roberto Resendes (University of Azores) for their help with the collection of samples. We also thank Prof Brian Morton for commenting on the revised manuscript of this paper.

For Peer Review Only

References

- Aarestrup F.M., Agerso, Y., Gerner-Smidt, P., Madsen, M. & Jensen L.B. (2000) Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagnostic Microbiology and Infectious Disease*, 37, 127-137
- Abe, Y., Nakamura, K., Yamada, M. & Yamamoto, Y. (2006). Encephalomalacia with *Enterococcus durans* infection in the brain stem and cerebral hemisphere in chicks in Japan. *Avian Diseases*, 50, 139-141.
- Agerso, Y., Pedersen, A.G. & Aarestrup, F.M. (2006). Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene *tet(M)* in enterococci from humans, pigs and poultry. *Journal of Antimicrobial Chemotherapy*, 57, 832-839.
- Bannerman, D.A. & Bannerman, W.M. (1966). *Birds of the Atlantic Islands. Vol III: A History of the Birds of the Azores*. Oliver & Boyd. Edinburgh and London.
- Blanc, V., Mesa, R., Saco, M., Lavilla, S., Prats, G., Miro, E., *et al.* (2006). ESBL- and plasmidic class C beta-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Veterinary Microbiology*, 118, 299-304.
- Bonnedahl, J., Drobni, P., Johansson, A., Hernandez, J., Melhus, A., Stedt, J., Olsen, B. & Drobni, M. (2010). Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. *Journal of Antimicrobial Chemotherapy*, 65, 1939-1944.

- Bradford, P.A. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews*, 14, 933-951, table of contents.
- Bush, K. & Jacoby, G.A. (2010). Updated functional classification of beta-lactamases. *Antimicrobial Agents and Chemotherapy*, 54, 969-976.
- Carneiro, C., Araujo, C., Goncalves, A., Vinue, L., Somalo, S., Ruiz, E., *et al.* Detection of CTX-M-14 and TEM-52 extended-spectrum beta-lactamases in fecal *Escherichia coli* isolates of captive ostrich in Portugal. *Foodborne Pathogens and Disease*, 7, 991-994.
- Chadfield, M.S., Christensen, J.P., Christensen, H. & Bisgaard, M. (2004). Characterization of streptococci and enterococci associated with septicaemia in broiler parents with a high prevalence of endocarditis. *Avian Pathology*, 33, 610-617.
- Chadfield, M.S., Christensen, J.P., Juhl-Hansen, J., Christensen, H. & Bisgaard, M. (2005). Characterization of *Enterococcus hirae* outbreaks in broiler flocks demonstrating increased mortality because of septicemia and endocarditis and/or altered production parameters. *Avian Diseases*, 49, 16-23.
- Clermont, O., Bonacorsi, S. & Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied and Environmental Microbiology*, 66, 4555-4558.
- Devriese, L.A., Uyttebroek, E., Ducatelle, R., Viaene, N., Derijcke, J. & Gevaert, D. (1990). Tracheitis due to *Enterococcus faecalis* infection in canaries. *Journal of the Association of Avian Veterinarians*, 4, 113-116.

- Devriese, L.A., Chiers, K., De Herdt, P., Vanrompay, D., Desmidt, M., Ducatelle, R. & Haesebrouck, F. (1995). *Enterococcus hirae* infections in psittacine birds: epidemiological, pathological and bacteriological observations. *Avian Pathology*, 24, 523-531.
- Dutka-Malen, S., Evers, S. & Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *Journal of Clinical Microbiology*, 33, 24-27.
- Eaton, T.J. & Gasson, M.J. (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology*, 67, 1628-1635.
- Gonçalves, A., Poeta, P., Silva, N., Araujo, C., Lopez, M., Ruiz, E., *et al.* (2010). Characterization of vancomycin-resistant enterococci isolated from fecal samples of ostriches by molecular methods. *Foodborne Pathogens and Disease*, 7, 1133-1136.
- Heuer, O.E., Hammerum, A.M., Collignon, P. & Wegener, H.C. (2006). Human health hazard from antimicrobial-resistant enterococci in animals and food. *Clinical Infectious Diseases*, 43, 911-916.
- Jouini, A., Vinue, L., Slama, K.B., Saenz, Y., Klibi, N., Hammami, S., Boudabous, A. & Torres, C. (2007). Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. *Journal of Antimicrobial Chemotherapy*, 60, 1137-1141.
- Kayaoglu, G., Erten, H. & Orstavik, D. (2008). Possible role of the adhesin ace and collagen adherence in conveying resistance to disinfectants on *Enterococcus faecalis*. *Oral Microbiology and Immunology*, 23, 449-454.

- Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., Bzoma, S. & Cizek, A. (2010). Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea Coast of Poland. *Applied and Environmental Microbiology*, 76, 8126-8134.
- Livermore, D.M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G.M., Arlet, G., *et al.* (2007). CTX-M: changing the face of ESBLs in Europe. *Journal of Antimicrobial Chemotherapy*, 59, 165-174.
- Mallon, D.J., Corkill, J.E., Hazel, S.M., Wilson, J.S., French, N.P., Bennett, M. & Hart, C.A. (2002). Excretion of vancomycin-resistant enterococci by wild mammals. *Emerging Infectious Diseases*, 8, 636-638.
- Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Dupre, I. & Sechi, L.A. (2003). Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *International Journal of Food Microbiology*, 88, 291-304.
- Marrow, J., Whittington, J.K., Mitchell, M., Hoyer, L.L. & Maddox, C. (2009). Prevalence and antibiotic-resistance characteristics of *Enterococcus* spp. isolated from free-living and captive raptors in central Illinois. *Journal of Wildlife Diseases*, 45, 302-313.
- Miele, A., Bandera, M. & Goldstein, B.P. (1995). Use of primers selective for vancomycin resistance genes to determine van genotype in enterococci and to study gene organization in *vanA* isolates. *Antimicrobial Agents and Chemotherapy*, 39-1772-1778.

- Paterson, D.L. & Bonomo, R.A. (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews*, 18, 657-686.
- Pinto, L., Radhouani, H., Coelho, C., Martins da Costa, P., Simoes, R., Brandao, R.M., *et al.* (2010). Genetic detection of extended-spectrum beta-lactamase-containing *Escherichia coli* isolates from birds of prey from Serra da Estrela Natural Reserve in Portugal. *Applied and Environmental Microbiology*, 76, 4118-4120.
- Poeta, P., Costa, D., Rodrigues, J. & Torres, C. (2005a). Study of faecal colonization by *vanA*-containing *Enterococcus* strains in healthy humans, pets, poultry and wild animals in Portugal. *Journal of Antimicrobial Chemotherapy*, 55, 278-280.
- Poeta, P., Costa, D., Saenz, Y., Klibi, N., Ruiz-Larrea, F., Rodrigues, J. & Torres, C. (2005b). Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. *Journal of Veterinary Medicine B, Infectious Diseases and Veterinary Public Health*, 52, 396-402.
- Poeta, P., Costa, D., Klibi, N., Rodrigues, J. & Torres, C. (2006). Phenotypic and genotypic study of gelatinase and beta-haemolysis activities in faecal enterococci of poultry in Portugal. *Journal of Veterinary Medicine B, Infectious Diseases and Veterinary Public Health*, 53, 203-208.
- Poeta, P., Costa, D., Igrejas, G., Rojo-Bezares, B., Saenz, Y., Zarazaga, M., *et al.* (2007). Characterization of *vanA*-containing *Enterococcus faecium* isolates carrying Tn5397-like and Tn916/Tn1545-like transposons in wild boars (*Sus Scrofa*). *Microbial Drug Resistance*, 13, 151-156.
- Poeta, P., Radhouani, H., Pinto, L., Martinho, A., Rego, V., Rodrigues, R., *et al.* (2009). Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing

- Escherichia coli* of different phylogenetic groups. *Journal of Basic Microbiology*, 49, 584-588.
- Radhouani, H., Pinto, L., Coelho, C., Goncalves, A., Sargo, R., Torres, C., Igrejas, G. & Poeta, P. (2010a). Detection of *Escherichia coli* harbouring extended-spectrum β -lactamases of the CTX-M classes in faecal samples of common buzzards (*Buteo buteo*). *Journal of Antimicrobial Chemotherapy*, 65, 171-173.
- Radhouani, H., Pinto, L., Coelho, C., Sargo, R., Araujo, C., Lopez, M., *et al.* (2010b). MLST and a genetic study of antibiotic resistance and virulence factors in *vanA*-containing *Enterococcus* from buzzards (*Buteo buteo*). *Letters in Applied Microbiology*, 50, 537-541.
- Radhouani, H., Poeta, P., Pinto, L., Miranda, J., Coelho, C., Carvalho, C., *et al.* (2010c). Proteomic characterization of *vanA*-containing *Enterococcus* recovered from Seagulls at the Berlengas Natural Reserve, W Portugal. *Proteome Science*, 8, 48.
- Robredo, B., Singh, K.V., Torres, C. & Murray, B.E. (1999). Identification to the species level by PCR of *Enterococcus hirae* and *Enterococcus durans*. *39th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)*. San Francisco.
- Saenz, Y., Brinas, L., Dominguez, E., Ruiz, J., Zarazaga, M., Vila, J. & Torres, C. (2004). Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrobial Agents and Chemotherapy*, 48, 3996-4001.
- Shepard, B.D. & Gilmore, M.S. (2002). Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. *Microbes and Infection*, 4, 215-224.

- Silva, N., Felgar, A., Goncalves, A., Correia, S., Pacheco, R., Araujo, C., Igrejas, G. & Poeta, P. (2010). Absence of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates in migratory birds: song thrush (*Turdus philomelos*). *Journal of Antimicrobial Chemotherapy*, 65, 1306-1307.
- Silva, N., Igrejas, G., Vaz, J., Araujo, C., Cardoso, L., Rodrigues, J., Torres, C. & Poeta, P. (2011). Virulence Factors in Enterococci from Partridges (*Alectoris rufa*) Representing a Food Safety Problem. *Foodborne Pathogens and Disease*.
- Skurnik, D., Ruimy, R., Andremont, A., Amorin, C., Rouquet, P., Picard, B. & Denamur, E. (2006). Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *Journal of Antimicrobial Chemotherapy*, 57, 1215-1219
- Sorum, H. & Sunde, M. (2001). Resistance to antibiotics in the normal flora of animals. *Veterinary Research*, 32, 227-241.
- Stalker, M.J., Brash, M.L., Weisz, A., Ouckama, R.M. & Slavic, D. (2010). Arthritis and osteomyelitis associated with *Enterococcus cecorum* infection in broiler and broiler breeder chickens in Ontario, Canada. *Journal of Veterinary Diagnostic Investigation*, 22, 643-645.
- Top, J., Willems, R., Blok, H., de Regt, M., Jalink, K., Troelstra, A., Goorhuis, B. & Bonten, M. (2007). Ecological replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*. *Clinical Microbiology and Infection*, 13, 316-319.
- Torres, C., Tenorio, C., Portillo, A., Garcia, M., Martinez, C., Del Campo, R., Ruiz-Larrea, F. & Zarazaga, M. (2003). Intestinal colonization by *vanA*- or *vanB2*-

containing enterococcal isolates of healthy animals in Spain. *Microbial Drug Resistance*, 9 Suppl 1, S47-52.

Torres, C. and Zarazaga, M., 2007. BLEE en animales y su importancia en la transmision a humanos. *Enfermedades Infecciosas y Microbiología Clínica*. 25 Suppl 2, 29–37.

Upadhyaya, G.P.M., Ravikumar, K.L. & Umapathy, B.L. (2009). Review of virulence factors of enterococcus: an emerging nosocomial pathogen. *Indian Journal of Medical Microbiology*, 27, 301-305.

Peer Review Only

Fig. 1 Distribution of VRE and ESBL-containing *E. coli* recovered from wild birds in Azores Islands.

Black open circles, *vanC-1*-containing *E. gallinarum*; Black open boxes, *vanA*-containing *E. durans*; Black filled boxes, *vanA*-containing *E. faecium*; Black filled triangle ESBL-containing *E. coli*.

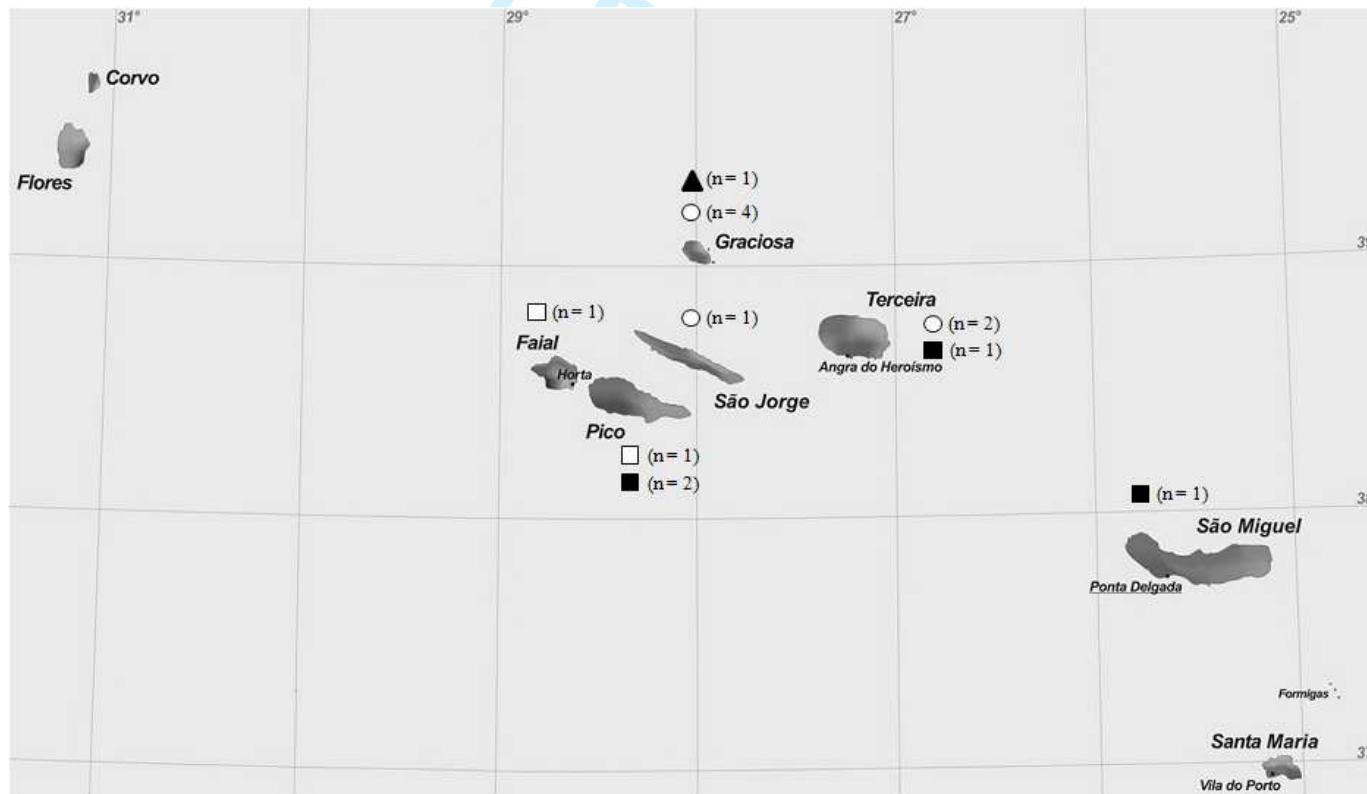


Table 1. Animal species in which VRE and ESBL-containing *E. coli* isolates were recovered.

Animal species	Number of tested animals	Azores Islands									VRE*	ESBLs**
		Santa Maria	São Miguel	Terceira	Graciosa	São Jorge	Faial	Pico	Flores	Corvo		
Passerine species												
European goldfinch (<i>Carduelis carduelis</i>)	9	-	2	-	7	-	-	-	-	-	-	-
European greenfinch (<i>Carduelis chloris</i>)	2	-	2	-	-	-	-	-	-	-	-	-
European robin (<i>Erithacus rubecula</i>)	18	-	7	-	2	2	5	2	-	-	2	-
Common chaffinch (<i>Fringilla coelebs</i>)	46	-	15	6	6	-	7	6	3	3	3	-
Goldcrest (<i>Regulus regulus</i>)	17	1	2	3	-	2	4	3	2	-	-	-
Atlantic canary (<i>Serinus canaria</i>)	13	2	2	-	6	1	1	1	-	-	-	-
Common starling (<i>Sturnus vulgaris</i>)	3	-	-	-	-	-	-	-	2	1	-	-
Blackcap (<i>Sylvia atricapilla</i>)	52	-	7	2	11	2	11	10	5	4	5	1
Common blackbird (<i>Turdus merula</i>)	18	-	6	2	2	2	4	-	1	1	-	-
Game Species												
Common snipe (<i>Gallinago gallinago</i>)	22	-	-	-	-	2	-	17	3	-	-	-
Quail (<i>Coturnix coturnix conturbans</i>)	20	-	-	10	10	-	-	-	-	-	3	-

*Number of animals containing VRE

**Number of animals containing ESBL-positive *E. coli* isolates

Table 2. Phenotypes and genotypes of resistance of the VRE isolates recovered from wild birds in Azores Islands.

Sample ID	Bird specie	Sample collected (island)	<i>Enterococcus</i> species	MIC (mg/L)*		Vancomycin resistance gene detected	Resistance phenotype for other antimicrobials**	Genes or transposon sequences detected	Virulence factors
				Vancomycin	Teicoplanin				
AzP-07A	<i>E. rubecula</i>	São Miguel	<i>E. faecium</i>	>128	64	<i>vanA</i>	ERY-TET-CIP-AMP	<i>erm(B)-tet(L)-tet(M)-Tn916- Tn5397</i>	-
AzP-75D	<i>S. atricapilla</i>	Faial	<i>E. durans</i>	>128	64	<i>vanA</i>	ERY-TET-CIP-AMP	<i>erm(B)- tet(L)</i>	-
AzP-101A	<i>S. atricapilla</i>	Pico	<i>E. faecium</i>	>128	64	<i>vanA</i>	ERY- TET CIP-AMP	<i>erm(B)-tet(L)-tet(M)-Tn916- Tn5397</i>	-
AzP-104C	<i>S. atricapilla</i>	Pico	<i>E. faecium</i>	>128	64	<i>vanA</i>	ERY-TET-CIP QD-AMP	<i>erm(B)-tet(L)-tet(M)- Tn916- Tn5397</i>	<i>ace</i>
AzP-118D	<i>F. coelebs</i>	Pico	<i>E. durans</i>	>128	64	<i>vanA</i>	TET -CIP-KAN	<i>tet(L)</i>	<i>ace</i>
AzP-157D	<i>F. coelebs</i>	Terceira	<i>E. faecium</i>	>128	64	<i>vanA</i>	TET-CIP-QD-AMP	<i>tet(L)</i>	-
AzT-01C	<i>C. coturnix conturbans</i>	Graciosa	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	AMP	-	-
AzT-06A	<i>C. coturnix conturbans</i>	Graciosa	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	AMP	-	-
AzT-18C	<i>C. coturnix conturbans</i>	Terceira	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	CIP-AMP	-	-
AzP-17D	<i>S. atricapilla</i>	Graciosa	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	ERY-TET-	<i>erm(B)-tet(L)-tet(M)-Tn5397</i>	-
AzP-38C	<i>S. atricapilla</i>	Graciosa	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	TET-CIP-AMP	<i>tet(M)- Tn5397</i>	-
AzP-156B	<i>F. coelebs</i>	Terceira	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	AMP	-	<i>ace</i>
AZP-162C	<i>E. rubecula</i>	São Jorge	<i>E. gallinarum</i>	8	1	<i>vanC-1</i>	CIP-QD-AMP	-	<i>ace</i>

*MIC, minimal inhibitory concentration.

**ERY, erythromycin; QD, quinupristin-dalfopristin; TET, tetracycline; CIP, ciprofloxacin; AMP, ampicillin; K, kanamycin.