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Wild corvid birds colonized with vancomycin-resistant *Enterococcus faecium* of human origin harbor epidemic *vanA* plasmids

Veronika Oravcová[a], Luísa Peixe[b], Teresa M. Coque[c,d,e], Carla Novais[b], Maria V. Francia[f], Ivan Literák[a,g], Ana R. Freitas[b,*]

[a] Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic
[b] UCIBIO/REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, n° 228, 4050-313 Porto, Portugal
[c] Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Carretera Colmenar km 9.1, 28034 Madrid, Spain
[d] Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain
[e] Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain
[f] Microbiology, Marqués de Valdecilla University Hospital e Instituto de Investigación Marqués de Valdecilla (IDIVAL), Av. Valdecilla, 25, 39008 Santander, Cantabria, Spain
[g] CEITEC VFU, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic

A B S T R A C T

The most prevalent type of acquired vancomycin resistance in *Enterococcus faecium* (VREfm) is encoded by the *vanA* transposon Tn*1546*, mainly located on transferable plasmids. *vanA* plasmids have been characterized in VREfm from a variety of sources but not wild birds. The aim of this study was to analyse the genetic context of VREfm strains recovered from wild corvid birds and to compare their plasmid and strain characteristics with human strains. To achieve that, 75 VREfm isolates, including strains from wild birds recovered during wide surveillance studies performed in Europe, Canada and the United States (2010 − 2013), and clinical and wastewater strains from Czech Republic, a region lacking data about *vanA* plasmids, were analysed. Their population structure, presence of major putative virulence markers and characterization of *vanA* transposons and plasmids were established. VREfm from wild birds were mainly associated with major human lineages (ST18 and ST78) circulating in hospitals worldwide and were enriched in putative virulence markers that are highly associated with clinical *E. faecium* from human infections. They also carried plasmids of the same families usually found in the clinical setting [RCR, small theta plasmids, RepA_N (pRUM/pLG1) and Inc18]. The clinically widespread IS*1251*-carrying Tn*1546* type "F" was predominant and Tn*1546-vanA* was mainly located on pRUM/Axe-Txe (USA) and Inc18- or pLG1-like (Europe) plasmids. VREfm from hospitals and wastewaters carried Tn*1546-vanA* in different plasmid types including mosaic pRUM-Inc18 plasmids, not identified in wild birds. This is the first characterization of *vanA* plasmids obtained from wild birds. A similar plasmid pool seems to exist in different clonal *E. faecium* backgrounds of humans and wild birds. The isolation of VREfm strains from wild birds that belong to human *E. faecium* adapted lineages and carry virulence genes, Tn*1546* and plasmid variants widespread in the clinical setting is of concern and highlight their role as potential drivers of the global dissemination of vancomycin resistance.

1. Introduction

Antimicrobial resistance is a complex and multifaceted problem occurring at the animal-human-ecosystems interface. Animal and human health are intimately linked (*One Health* concept), with 6 out of 10 infectious diseases in humans being spread from animals and 75% of these originating from wildlife (Atlas and Maloy, 2014). Despite the increasing descriptions of clinically-relevant antibiotic resistance in bacteria from wild animals (Bachiri et al., 2017; Lozano et al., 2016; Oravcova et al., 2014a, 2014b), the role of wildlife in the emergence

* Corresponding author at: Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira n° 228, 4050-313 Porto, Portugal.
E-mail addresses: l19veronika@gmail.com (V. Oravcová), lpeixe@ff.up.pt (L. Peixe), mariateresa.coque@salud.madrid.org (T.M. Coque), casilva@ff.up.pt (C. Novais), mvictoriafrancia@scsalud.es (M.V. Francia), literaki@vfu.cz (I. Literák), afreitas@ff.up.pt (A.R. Freitas).

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and spread of antibacterial resistance across different regions might be neglected (Wang et al., 2017).

Vancomycin-resistant *Enterococcus faecium* (VREfm) are an example of a high priority pathogen to the World Health Organization (WHO, 2017) that can be found across different settings. Their unique ability to acquire and accumulate different adaptive traits, flexible genomes and plasmidomes, facilitating adaptation to harsh environments, colonization and spread, pose a relevant healthcare problem due to the few available therapeutic choices for treating infections caused by these organisms and the risk to transfer resistance to more pathogenic species as *Staphylococcus aureus* (Arias et al., 2010; Weigel et al., 2003; Werner et al., 2013). Since the first descriptions, VREfm have spread with surprising rapidity and are now associated with hard to treat invasive infections in hospitalized patients around the world (Cattoir and Leclercq, 2013). Although at lower rates, VREfm have been also detected in a variety of community sources such as foodstuffs, foodborne animals, environment and wildlife (Hammerum, 2012; Oravcova et al., 2013; Roberts et al., 2016). Available studies on wildlife identified VREfm linked to a diversity of clones, several associated with typically hospital sequence types (Oravcova et al., 2013; Oravcova et al., 2014a; Oravcova et al., 2017a; Roberts et al., 2016). Recent genomic data allowed recognizing two main clades in *E. faecium*: the clade A that is further subdivided in clade A1 comprising most epidemic hospital strains and clade A2 with strains of animals and sporadic human infections, and clade B that is mostly associated with community-based individuals (Lebreton et al., 2013; Guzman Prieto et al., 2016).

Drivers of vancomycin resistance in *E. faecium* are mainly associated with *vanA*-Tn*1546* and *vanB2*-Tn*5382/*Tn*1549* genetic clusters worldwide, which are often located in chimeric plasmids of narrow-host-range RepA_N (pRUM/pLG1) and/or broad-host-range Inc18 types (Freitas et al., 2016). To date, most studies providing information on vancomycin-resistant plasmids include clinical isolates (Freitas et al., 2013; Freitas et al., 2016; Sivertsen et al., 2014; Valdezate et al., 2012; Wardal et al., 2014), with only a few dedicated to foodborne animals (Freitas et al., 2011; Garcia-Migura et al., 2011; Rosvoll et al., 2010). Previous studies have described that wild birds can be colonized with VREfm strains (Oravcova et al., 2013, 2014a, 2014b, 2016, 2017a, 2017b; Roberts et al., 2016) but the molecular characterization of their subcellular genetic elements (transposons and plasmids) has never been provided.

Even though wild birds are not exposed to use of antimicrobial agents directly, there are several evidences they can acquire and disseminate antibiotic-resistant bacteria through the environment and thrive in human environments (Allen et al., 2010; Wang et al., 2017). Our goal was to characterize the genetic context of VREfm strains obtained from wild birds during wide surveillance studies in different continents and to compare them with available data from outbreak/prevalent VREfm clinical strains (Freitas et al., 2016). *vanA* plasmids of VREfm strains from hospital and urban wastewaters in Czech Republic, from where several wild bird's samples originate, were also characterized as no data on vancomycin-resistant plasmids from this country are available. Our results will allow establishing the potential of wild birds to carry and spread epidemic vancomycin-resistant plasmids.

2. Materials and methods

2.1. Selection of VREfm strains

The *vanA* isolates from wild corvids included in this study (n = 23) were obtained during previous surveillance studies analyzing winter samples of wild bird's roosting places and included: i) 1073 samples of rook (*Corvus frugilegus*) feces from 8 EU countries (2010 − 2011) (Oravcova et al., 2013); ii) 99 and 287 samples of rooks (*Corvus frugilegus*) and ravens (*Corvus corax*), respectively, in Slovakia (2013) (Oravcova et al., 2016); iii) 590 samples of American crows (*Corvus brachyrhynchos*) from different U.S. regions (2012) (Oravcova et al., 2014a); and iv) 400 and 49 samples of American crows and ravens, respectively, from Canada (2012 − 2013) (Oravcova et al., 2014b). From each study, we selected representatives of different clones and antibiotic resistance patterns to provide maximal diversity.

Representative (different clones and antibiotic resistance profiles) isolates of VREfm obtained from the surveillance of hospitalized patients (n = 26), the hospital environment (n = 2) and urban treated wastewaters (n = 24) in Brno, Czech Republic (2012–2013) were also included given the lack of data about *vanA* plasmids in this country (Oravcova et al., 2017b). The sequence types were determined by MLST (www.pubmlst.org) in previous studies (Oravcova et al., 2013, 2014a, 2014b, 2016, 2017b) and the population genetic analysis was in this study performed using goeBURST and Bayesian Analysis Population Structure (BAPS) software as described (Tedim et al., 2015; Willems et al., 2012).

2.2. Extended virulence profiling

A set of putative virulence markers (PVM), recently highlighted for the safety assessment of *E. faecium* strains and as reliable indicators of an increased risk for Public Health (Freitas et al., 2018), were tested in a subset of isolates (n = 38) representing different corvids and other sources, as well as different clones and *vanA* plasmid types, to illustrate the virulence content of our strain collection. They corresponded to 12 genes coding for surface adhesins (*esp*, *sgrA*, *ecbA*, complete *acm*) and pili proteins (*fms20*, *fms14*, *ebpA* and *fms16*), or others enhancing colonization (*ptsD*, *orf1481*, *hyl*) or plasticity (IS*16*). The presence of *fms20*, *fms14*, *ebpA* and *fms16* genes correlates well with the presence of the four complete pili gene cluster described in *E. faecium* (PGC-1, PGC-2, PGC-3 and PGC-4, respectively; Freitas et al., 2018).

2.3. Characterization of glycopeptide resistance genetic context

The backbone structure of Tn*1546* (*vanA*) was determined by PCR mapping and further sequencing of fragments with unusual size (Novais et al., 2008). Estimation of plasmid content (number and size) of wild-type strains was determined by PFGE of S1-digested genomic DNA accordingly Barton et al. (Barton et al., 1995; Freitas et al., 2016). Plasmids were categorized according to the presence of sequences associated with replication (replication initiator proteins, RIPs), mobilisation (relaxases, RELs) and stability (toxin-antitoxin systems, TAs), which were detected by PCR typing schemes and further hybridization of S1-digested genomic DNA with specific probes (Freitas et al., 2016). We referred to the rep sequences according to the numeric nomenclature used by Jensen et al. (2010) and our own (Freitas et al., 2016). Hybridization experiments were performed by using the Gene Images AlkPhos Direct labeling and detection system (Amersham GB, GE Healthcare Life Sciences UK Limited). The relationship among plasmids of similar size and RIP-REL content from the different sources was established by RFLP using *Cla*I and *Eco*RI (Promega, USA) enzymes after plasmid extraction, as previously described (Freitas et al., 2013).

3. Results

3.1. VREfm wild bird strains belonged to major human lineages and carry putative virulence markers associated with a public health risk

VREfm from wild birds were clonally diverse and belonged to clade A1 (n = 12/23, 52%, 5 STs) and clade A2 (n = 11/23, 48%, 8 STs), while the great majority of VREfm from hospital and wastewaters belonged to clade A1 (n = 49/52, 94%, 8 STs) and a few to clade A2 (n = 3/52, 6%, 2 STs) (Fig. 1). VREfm wild bird isolates from clade A1 grouped into clonal lineage 18 (67%, ST18 and ST917) and clonal lineage 78 (33%, ST121, ST749 and ST750), and that from clade A2 split into BAPS subgroups 2.1b (ST671), 2.3a (ST92, ST640), 3.1 (ST448, ST752), 3.2 (ST6, ST555) and 3.3b (ST751). No isolate from