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Antimicrobial resistance determinants in *Staphylococcus* spp. recovered from birds of prey in Portugal

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ABSTRACT

Antibiotic resistance among wild animals represent an emerging public health concern. The objective of this study was to analyze the staphylococcal nasal microbiota in birds of prey and their content in antimicrobial resistance determinants. Nasal samples from 16 birds of prey were collected, swabs were dipped and incubated into BHI broth [6.5% NaCl] and later seeded on manitol salt agar and oxacillin-resistance screening agar base media. Staphylococcal colonies were isolated from both media and were identified by biochemical and molecular methods. Susceptibility testing to 18 antimicrobial agents was performed by disk-diffusion method. Six of the 16 tested animals carried staphylococci (37.5%) and 7 isolates of the following species were recovered: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus sciuri rodentium*, *Staphylococcus cohnii urealitycum*, and *Staphylococcus gallinarum*. The *S. aureus* isolate was penicillin-resistant (with *blaZ* gene) but methicillin-susceptible and was ascribed to *spa*-type t012, sequence-type ST30 and *agr*-type III. The *S. epidermidis* isolate carried *blaZ*, *mecA*, *mrs*(*A*/*B*), *mphC*, *tet*(*K*), *drfA*, and *fusC* genes, *ica* operon, and was typed as ST35. The genes *ant6′-Ia*, *tet*(*K*), *tet*(*L*), *dfrG*, cat_{221}, cat_{194}, and cat_{223} were detected in *S. saprophyticus* or *S. gallinarum* isolates. Birds of prey seem to be a natural reservoir of *S. aureus* and coagulase-negative staphylococci resistant to multiple antibiotics. Due to the convergence between habitats, the contact between wildlife, other animals and humans is now more common and this involves an increased possibility of interchange of these microorganisms in the different ecosystems.

1. Introduction

Antimicrobial resistance remains a major threat to public health. *Staphylococcus* spp. are one of the keys to this problem. Often found in the natural microbiota of the skin, nose and mouth of humans and animals, these opportunist bacteria can cause a range of illnesses, from minor skin

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infections to life-threatening diseases (Walsh and Fanning, 2008). Staphylococci are facultative anaerobic Gram-positive coccal bacteria and the genus has at least 38 different species described, distributed in two major classes: coagulase-positive (ex: *Staphylococcus aureus*) and coagulase-negative staphylococci (CNS), like *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Staphylococcus sciuri*, among others. The epidemiology on *Staphylococcus* spp. has been changing since the 90's, highlighting methicillin-resistant *S. aureus* (MRSA), who is causing an important concern among population in general, being responsible of many nosocomial infections worldwide (Grundmann et al., 2006). However, CNS have been presented as emerging pathogens with clinical importance carrying resistance and virulence determinants (Kloos and Bannerman, 1994). In fact, there are several cases of people without contact with hospital environments and without risk factors for contracting an infection by these microorganisms that were diagnosed with MRSA and multiresistant CNS (Barbier et al., 2010; Lebeaux et al., 2012; Zaoutis et al., 2006). These bacteria are becoming prevalent in community-acquired infections. In recent years, staphylococci are found associated with companion animals, food-producing animals and food-products (Bagcigil et al., 2007; Kern and Perreten, 2013; Leonard and Markey, 2008; Podkowik et al., 2013). Nevertheless, little information is available about their prevalence in wild animals (Porrero et al., 2013). We must understand the epidemiology of these bacteria and recognize which are prevalent in hospitals, in the community and in the wild environment. Knowing which variants are associated with each focus of infection, to each animal species and within a particular environment may be important to carry out further control to the spread of these zoonotic bacteria and better understand its transmissibility. There is still no data on the prevalence of staphylococci in birds of prey and our study may add to our knowledge the prevalence and occurrence of multiresistant staphylococci in these animals.

2. Methodology

Samples from the nasopharynx were collected from 16 birds of prey (*Buteo buteo*, *Strix aluco* and *Corvus corone*) when admitted into CRAS (wild birds' recovering center from the Veterinary Medical Hospital at the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal). Swabs were inoculated into BHI broth containing 6.5% NaCl and incubated for 24 h at 37 °C; then, 150 μL was seeded on MSA (manitol salt agar) and ORSAB (oxacillin-resistance screening agar base, supplemented with 2 mg/L oxacillin, from Oxoid®). Presumptive *Staphylococcus* spp. colonies obtained in both types of media were isolated and identified by biochemical (Gram staining, DNase, catalase) and molecular methods. A multiplex PCR of 16S rDNA, *mecA*, and *nuc* genes was used for identification of *S. aureus* and detection of methicillin resistance, and a PCR of *sod*A gene with sequencing was used to identify CNS (Supplementary Table). Only isolates with different species and phenotypes of resistance for each sample were maintained and further studied. Susceptibility testing to 18 antimicrobial agents was performed by disk-diffusion test for the following antibiotics (μg/disk): mupirocin (5), tetracycline (30), trimethoprim-sulfamethoxazole (1.25 + 23.75); vancomycin (30); teicoplanin (30); erythromycin (15); clindamycin (2); gentamicin (10); tobramycin (10); penicillin G (10U); linezolid (30); cefoxitin (30); oxacillin (1); chloramphenicol (30); ciprofloxacin (5), and kanamycin (30) (CLSI, 2013). The criteria of the French Society for Microbiology (SFM) were used for susceptibility testing of fusidic acid (10 μg/disk) and streptomycin (10 μg/disk).

DNA extraction was performed using lysostaphin [1 μg/mL], proteinase K [2 mg/mL] and Tris–HCl [0.1 M/pH 8]. The presence of resistance genes was studied by PCR and sequencing. The obtained DNA amplicons were sequenced on both strands and gene homology searches were performed using BLAST analysis. Positive and negative controls were used from the bacterial collection of the University of La Rioja, Spain.

The presence of the *ica* operon was studied on the *S. epidermidis* isolate. The genetic lineages of *S. aureus* were determined by *spa*-typing and *agr*-typing. Also, Multilocus Sequence Typing (MLST) on *S. aureus* and *S. epidermidis* isolates was performed (all primers and conditions are indicated in supplementary table). For MLST profiling and analysis we used data from www.mlst.net and eBurst analysis was performed to assemble the isolates into the respective clonal complex (CC).

3. Results

Six of the 16 tested animals carried staphylococci in the nasal samples (37.5%) and 7 isolates were recovered from the positive animals (1 isolate/sample but one sample with two isolates). Five of these isolates were recovered from MSA and two from ORSAB media and the following species were identified: *S. saprophyticus* (2 isolates), *S. aureus*, *S. epidermidis*, *Staphylococcus sciuri rodentium*, *Staphylococcus cohnii urealitycum*, and *Staphylococcus gallinarum* (Table 1). The *S. aureus* and *S. epidermidis* isolates were recovered from the same animal (*Buteo buteo*). The *S. aureus* isolate was penicillin-resistant (carrying the *blaZ* gene) but showed methicillin-susceptibility and was ascribed to *spa*-type t012, sequence-type ST30 (included in clonal complex CC30) and *agr*-type III. The *S. epidermidis* isolate showed a multiresistance phenotype (carrying *blaZ*, *mecA*, *mrs*(*A*/*B*), *mphC*, *tet*(*K*), *drfA*, and *fusC* genes, associated with resistance to 7 different families of antimicrobials); this *S. epidermidis* also carried the *ica* operon (Table 1) and was typed as ST35 (included in CC2). The genes *ant6′-Ia*, *tet*(*K*), *tet*(*L*), *dfrG*, cat_{221}, cat_{194}, and cat_{223} were detected in *S. saprophyticus* or *S. gallinarum* isolates.

4. Discussion

S. aureus and CNS are usually referred as part of the natural microbiota of humans and animals and are capable of establish a commensal relationship with them. These bacteria can be very important in epidemiological studies due to their heterogeneity in pathogenesis and habitat preferences (Otto, 2010). In this study, *S. aureus* and CNS were identified in wild animals, birds of prey to be exact,