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Assessment of antibiotic resistance of *Escherichia coli* isolates and screening of *Salmonella* spp. in wild ungulates from Portugal

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Abstract

Antibiotic resistance is an emerging global problem. Wild animals are rarely exposed to antibiotics and therefore low levels of antibiotic resistance are expected. However, the growing interactions of these animals with humans and livestock may have a huge impact on their bacterial flora. This study aimed to assess the levels of antibiotic resistance in *Escherichia coli* isolated from widespread wild ungulates in Portugal. The interpretation of inhibition zone diameters was performed according to clinical breakpoints and epidemiological cut-offs, determined with the normalized resistance interpretation (NRI) method. For clinical breakpoints, 16% of the isolates were resistant to at least one antibiotic, including ampicillin (10%), tetracycline (9%), streptomycin (5%) co-trimoxazole (4%), amoxicillin/clavulanic acid (1%) and cefoxitin (1%). The levels of resistance detected in *E. coli* strains isolated from wild boar were statistically different for ampicillin and co-trimoxasol. According to NRI cut-offs, 10% of the population showed a non-wild-type phenotype against at least one antibiotic, also including tetracycline (9%), co-trimoxazole (6%), streptomycin (4%), ampicillin (2%) and amoxicillin/clavulanic acid (1%). Considering this parameter of comparison, no statistically different levels of resistance were identified between *E. coli* recovered from the three wild ungulates. Screening of *Salmonella* spp., which can be potentially pathogenic, was also performed, revealing that its prevalence was very low (1.5%). The study demonstrated that wild ungulates from Portugal are also reservoirs of antibiotic-resistant bacteria.

Keywords: Wildlife; Fecal samples; Antibiotic resistance; Normalized resistance method; Clinical breakpoint; ECOFF

1. Introduction

Antibiotic resistance (ABR) was recently identified by the World Health Organization as a major problem in terms of the environment and public and animal health [1]. Antibiotics are widely used in diverse settings, including human and veterinary medicine, agriculture, aquaculture and the food industry. This ubiquitous application results in their constant release into soil and water, mainly through wastewater treatment plant effluents, leakage from waste-storage containers, agricultural waste and application of biosolids to fields [2,3]. Consequently, antibiotic pollution exerts a selective pressure in environmental reservoirs of resistance, which are a combination of natural, animal and human resistance [4]. This type of pollution facilitates the development and dissemination of ABR. As such, understanding this biological process will require a holistic approach, in both clinical and natural environments [5]. Several studies have reported antibiotic-resistant bacteria in various animal species, from healthy pets to wild animals (e.g. the fox *Vulpes vulpes*, the Iberian lynx *Lynx pardinus* and the wild boar *Sus scrofa*) [6–9]. Proximity to humans is predicted to influence the antibiotic resistance profile of microbiological communities of wild animals [10] and this association has been demonstrated. For instance, mountain gorillas in contact with humans carried more

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antibiotic-resistant enteric bacteria than those living in areas further away from anthropogenic influences [11]. Interestingly, ABR has also been described in more remote locations having little contact with humans. One such example is the presence of *Escherichia coli*-resistant isolates recovered from Arctic birds [12]. *E. coli* is commonly found in the intestinal tract of a wide variety of animals and humans. This intestinal bacterium can be easily disseminated in different ecosystems and can be used as an indicator of the selective pressure exerted by the use of antibiotics [13]. According to the Centers for Disease Control and Prevention (CDC), approximately 75% of the recently emerging infectious diseases affecting humans are of animal origin [14]. In addition, the European Food Safety Authority (EFSA) and the European Center for Disease Prevention and Control (ECDC) reported a total of 5550 food-borne outbreaks between 2005 and 2009, caused mainly by *Salmonella* spp., viruses and bacterial toxins. *Salmonella* species (spp.) colonize a wide range of domestic and wild animals, and have been isolated from the intestinal content of several wildlife species (e.g. the white-tailed deer *Odocoileus virginianus*, rabbits *Oryctolagus cuniculus* and wild boar) [15].

Small wild mammals (e.g. rats) have been used in studies investigating the impact of human activities on the development of ABR in the environment and in associated wildlife; however, due to their limited home range, they are unlikely to be involved in the spread/dissemination of resistant bacteria [16]. On the other hand, in the last few decades, wild ungulates have increased dramatically both in number and range, with a current wide global distribution in Europe, and Portugal is no exception [17,18]. Therefore, wild ungulates might be an excellent model species for investigating ABR: i) they have increased in number during the last few decades; ii) they have extremely wide home ranges compared with small mammals; iii) they are unlikely to be treated with antibiotics; iv) their habitat overlaps with that of livestock and humans; and v) ABR can be investigated in a geographic gradient. Thus, studies investigating the occurrence of ABR in wild ungulates and their association with potentially pathogenic agents are needed. Nevertheless, in Portugal, available information is restricted to wild boar and to a few northern populations [19]. The present study aims to fill this gap by: i) assessing antibacterial susceptibility levels of *E. coli* populations; and ii) evaluating the incidence of *Salmonella* spp. in samples of wild ungulates (roe deer *Capreolus capreolus*, red deer *Cervus elaphus* and wild boar) in different geographic areas of Portugal.

2. Materials and methods

2.1. Origin and description of the samples

A total of sixty-seven fecal samples from different geographic locations in Portugal were obtained by the Wildlife Research Unit (UVS) (University of Aveiro) from October 2013 to April 2014. Fresh samples of red deer (n = 42) and roe deer (n = 4) were collected from natural environments. Samples of wild boar (n = 21) were collected from hunted animals, directly from the rectum. After collection in sterile containers, samples were kept in a field cooler up to 2 h and then stored at 4 °C until processing. Sampling locations (Fig. 1.) were: i) Montesinho Natural Park (northeast Portugal), characterized by a weak density of humans and livestock; ii) Lousã (central Portugal), characterized by high human density and average livestock density; and iii) Herdade de Vale Feitoso – Idanha-a-Nova (southeast Portugal), characterized by low human density, but high livestock density. Once in the laboratory, 1 g of each fecal sample was homogenized and diluted in 10 mL of buffered peptone water (BPW; Merck) under aseptic conditions.

2.2. Isolation and selection of E. coli isolates

After addition of BPW, serial dilutions of each sample were prepared and cultured on MacConkey agar plates (Oxoid) overnight at 37 °C. Ten lactose-positive colonies with the *E. coli* phenotype were randomly selected from each sample and grown on Chromocult® coliform agar (Merck), overnight at 37 °C. Blue-to violet-colored colonies were considered as *E. coli* and therefore subjected to further PCR confirmation by amplification of *gadA/B* and *uidA* genes using *E. coli* ATCC® 25922 as the positive control [20]. PCR amplification was performed in a final volume of 12.5 µl containing 3 mM $MgCl_2$, 0.2 mM dNTPs, 1× Green GoTaq®

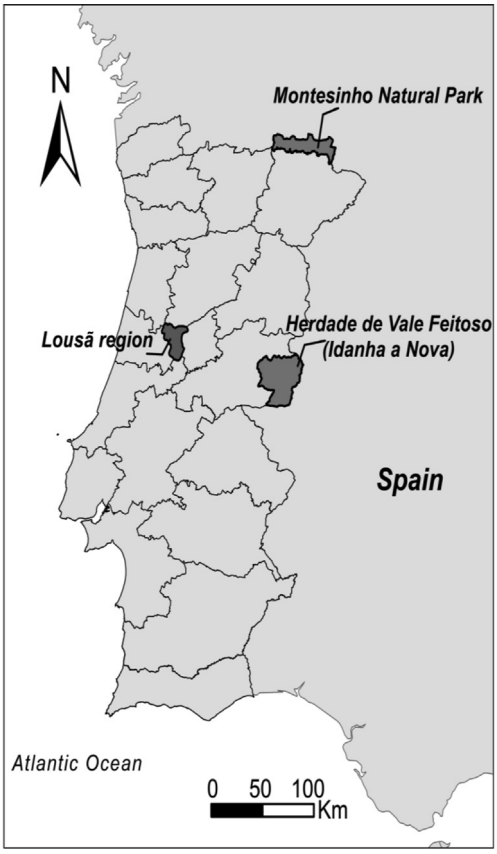

Fig. 1. Map of Portugal representing the geographic areas from which fecal samples were collected.