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Antimicrobial susceptibility and genetic characterization of *Escherichia coli* recovered from frozen game meat

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ABSTRACT

The increasing number of antimicrobial resistant *Enterobacteriaceae* both in veterinary and human medicine, the dissemination of these bacteria in several environments and their possible repercussions on human health is causing concern. Game meat is usually seen as free of antimicrobial resistant bacteria. The objective of this study was to evaluate the current antimicrobial susceptibility status in generic *Escherichia coli* isolated from packed frozen game meat from a game handling establishment in Germany. A total of 229 *E. coli* isolates were obtained from cuts of red deer, roe deer and wild boar. The susceptibility to 12 antimicrobial agents was evaluated by a broth microdilution method according to ISO 20776-1:2006. Minimal Inhibitory Concentration (MIC) values were compared to breakpoints and cut-off values published by the EUCAST. Isolates showing MICs above the reference values were further studied for associated resistance determinants and phylogrouping by PCR. Overall, 16 *E. coli* isolates (7.0%) showed resistance (microbiological or clinical) to at least one antimicrobial agent tested. Clinical resistance was recorded to ampicillin (5/229) and chloramphenicol (4/229), whereas the MIC of 9 isolates exceeded the epidemiological cut-off value for doxycycline. One of the ampicillin-resistant isolates showed resistance to the β-lactam antibiotic derivatives tested, cephalosporines and aztreonam. Three of 9 non-wild-type isolates for doxycycline were positive for *tet* (B) genes. The ß-lactam-resistant isolate was found to harbour $bla_{CTX\text{-}M\text{-}1}$ gene. These data show a low prevalence of resistant *E. coli* in packed game meat compared to studies on conventional meat. Although isolates obtained in this study may also be originating from the processing environment and not necessarily from animals, based on our results, it is important to monitor the development of antimicrobial resistance in game animals and products in order to identify future threats for the consumers.

1. Introduction

The rise in antimicrobial resistance is causing concern both in human and animal medicine. In this context the use of antimicrobial substances in animals and humans is suspected to be the most probable cause for the emergence and persistence of resistance in different bacteria (World Health Organization, 2011). Within the last decade, studies regarding the presence of antimicrobial resistance among coliform bacteria in wildlife have shown an increase in the prevalence of multi-resistant micro-organisms in different wild animals (Literak et al., 2010; Navarro-Gonzalez et al., 2013; Zottola et al., 2013 Dias et al., 2015).

Meat products are potential vectors of commensal and pathogenic drug-resistant bacteria from animal reservoirs to humans (Hannah et al., 2009; Jakobsen et al., 2010). Game meat is likely to be more contaminated with enteric micro-organisms than meat from domestic animals, due to several highly variable factors during the harvesting process, for example, hunting practices or the conditions under which game carcasses are dressed (Gill, 2007). Early studies in Europe have shown that antimicrobial-resistant bacteria and transmissible resistance factors are already present in the enteric flora of wild ungulate populations, as reported for *E. coli* by Literak et al. (2010) in the Czech Republic, Navarro-Gonzalez et al. (2013) in Spain, or Dias et al. (2015) in Portugal; or as well, antibiotic-resistant Salmonella found in wild boars in Italy (Zottola et al., 2013). The last-mentioned studies on *E. coli* have shown that the prevalence of resistance in wild ungulates may be as high as 12.7% in some European regions. Indicator organisms, such as generic *Escherichia coli* or enterococci, have relevance for monitoring antimicrobial resistance in bacteria with zoonotic

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potential in meat products, as these bacteria are commonly found in animal faeces, and most resistance elements present in the microflora are harboured by these micro-organisms (EFSA, 2008). Although a recent Spanish study has shown that the fresh meat of wild boar and roe deer can be contaminated with resistant enterococci (Guerrero-Ramos et al., 2016), there are, to the best of our knowledge, no published studies about the presence of resistant bacteria in packed frozen game meat. Epidemiological data concerning to the antimicrobial susceptibility in indicator organisms (i. e. *E. coli*), isolated from frozen game meat, can be helpful to elucidate the current resistance situation and to evaluate emerging trends for consumers. This information is relevant due to the importance of these products to the European marketing of game meat (Hurlin and Schulze, 2007).

The objective of this study was therefore to assess the status of antimicrobial susceptibility in generic *E. coli* isolated from packed frozen game meat of red deer, roe deer and wild boar in Germany. Furthermore, the carriage of some important resistance determinants, including β-lactamase and tetracycline resistance genes, and the phylogenetic distribution of *E. coli* was investigated among resistant isolates.

2. Materials and methods

2.1. Sample collection

The study comprised 229 *E. coli* isolates recovered from 188 game meat samples between October 2011 and June 2013. Fifty-one meat samples of red deer (*Cervus elaphus*), 68 of roe deer (*Capreolus capreolus*) and 69 of wild boar (*Sus scrofa*) of a German game processing establishment were sent by the producer to the laboratory for microbiological analysis. The establishment participating in the present study is located in Southern Germany. In this establishment, national and imported meat of different game animals is processed, packed and commercialized. The hygiene of the production process is controlled through internal monitoring and external inspection procedures, in order to fulfil European standards of microbiological quality. Samples used for the purpose of this study were taken within the framework of internal quality controls in the game processing plant. Meat cuts were chosen at random from different batches which were ready for delivery at the final point of processing (vacuum packed and frozen below −18 °C). According to the producer, 182/188 of the meat samples were obtained from wild animals hunted within Germany. The remaining 6 samples were obtained from imported meat of red deer; however, further information, like the exact origin of every single sample, was not reported by the producer.

Meat samples were analysed as previously described by Mateus Vargas et al., 2013: in short, aliquots of homogenized meat samples were directly inoculated on diagnostic media Tryptone Bile X-Glucuronide (TBX)-Agar (Oxoid LTD., Hampshire, England). Following 24 h incubation at 44 °C, up to 5 colonies per sample showing typical growth on TBX-Agar were randomly selected and subsequently confirmed as *E.coli* using the API 20E system (bioMérieux, Marcy l'Etoile, France). Selective enrichment with antibiotics was avoided in order to obtain general information about the antimicrobial susceptibility status according to the recommendation for monitoring purposes of the European Food Safety Authority (EFSA, 2008). One to 2 isolates from each sample showing different biochemical profiles on API 20E were randomly selected for further analyses. This approach was used to enhance the probability of detection of resistant bacteria in a single sample while avoiding the analysis of copy strains, as already published by other authors (Mayrhofer et al., 2006).

2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the isolates was determined using a broth microdilution technique (MHK-Gram^{neg}, Bio-Rad Medical Diagnostics Ltd., Dreieich, Germany) according to manufacturer specifications and in accordance with standards published in ISO 20776-1:2006. Following 24 h aerobic incubation at 35 °C, plates were examined visually. Minimal Inhibitory Concentration (MIC) was determined as the lowest concentration of antimicrobial showing inhibition of bacterial growth and was interpreted according to the clinical breakpoints recommended by the European Committee on Antimicrobial Susceptibility (EUCAST, 2016a, b). Due to lack of clinical breakpoints for some substances like doxycycline, the epidemiological cut-off value (ECOFF-values) recommended by EUCAST was used to evaluate the results obtained from the susceptibility testing of this compound (EUCAST, 2016a, b). The ECOFF-values enable to distinguish between susceptible wild-type and the non-wild-type bacteria populations and can be used for the monitoring of resistance development in different environments (Kahlmeter et al., 2003). *E. coli* isolates were categorized as clinically or microbiologically resistant according to the clinical breakpoints or to ECOFF-values, respectively (EFSA, 2008). Clinical breakpoints were used for the following antimicrobial agents (abbreviation and breakpoints are indicated in parenthesis): Ampicillin (AMP; >8 μg/ml), piperacillin (PIP; >16 μg/ml), ticarcillin (TIC; >16 μg/ml), cefotaxime (CTX; >2 μg/ml), ceftazidime (CAZ; >4 μg/ml), cefepim (FEP; >4 μg/ml), aztreonam (ATM; >4 μg/ml), gentamicin (GEN; >4 μg/ml), chloramphenicol (CHL; >8 μg/ml), ciprofloxacin (CIP>1 μg/ml) and nitrofurantoin (NIT; >16 μg/ml). ECOFF-value ≤4 μg/ml was used for the interpretation of the MICs observed for doxycycline (DOX). The minimal inhibitory concentration for 50% (MIC_{50}) and 90% (MIC_{90}) of all strains tested were analysed in order to compare the MIC distributions of the population studied. *E. coli* ATCC 25922 was used for the quality control.

2.3. Analysis of resistance genes

Based on the antimicrobial susceptibility patterns, 16 *E. coli* isolates showing resistance (clinical or microbiological) to at least one antimicrobial substance were tested to detect the presence of antimicrobial resistance genes. Templates for the polymerase chain reaction (PCR) were prepared by a boiling method using protocols described by Daigle et al. (1994). Briefly, three to five colonies from a fresh culture incubated at 37 °C for 18–24 h on Standard I agar plate (Merck, KGaA, Darmstadt, Germany) were suspended homogenously in 200 μl of water and heated at 95 °C for 10 min. After centrifugation for 2 min at 10,000 rpm, 2 μl of the supernatant was extracted and used as template for PCR. The target genes were bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$, encoding extended-spectrum β-lactamases, and *tet* (A) und *tet* (B) for doxycycline. These genes were detected by PCR using conditions and primers as described in Table 1. Isolates testing positive for the $bla_{CTX\text{-}M}$ were further characterized in the different $bla_{CTX\text{-}M}$-groups by a multiplex PCR assay, according to Woodford et al. (2006) (Table 1). *E. coli* strain ATCC 25922 was used as quality control for extraction and phylogenetic assays.

2.4. Phylogenetic analysis

Resistant *E. coli* isolates were further analysed by triplex polymerase chain reaction using the genes *chuA, yjaA* and DNA fragment TspE4C2 (Higgins et al., 2007). This examination enables the assignment of the *E. coli* isolates to 4 phylogenetic groups (A, B1, B2, and D) (Clermont et al., 2000).