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Prevalence, antimicrobial resistance and virulence traits in enterococci from food of animal origin in Turkey

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ABSTRACT

The objective of this work was to investigate the antibiotic susceptibility, the mechanisms implicated and the potential virulence genes (gelatinase [*gel*E], cytolysins [*cyl*A, *cyl*M, *cyl*B], cell wall adhesins [*efa*Afs and *efaAfm*], enterococcal surface protein [*esp*], sex pheromones [*cpd*, *cob*, *ccf*], enhanced expression of pheromone [*eep*], aggregation substance [*agg*A]) in enterococci isolated from retail chicken and beef meat samples in Hatay, Turkey. Hundred-one (96%) isolates from chicken meat and sixty-three (63%) from minced meat isolates showed resistance to at least one of the 12 antimicrobial agents tested. The highest frequency of resistance was against tetracycline (89.5% and 53%), erythromycin (59% and 2%), ciprofloxacin (35.2% and 12%) and trimethoprim/sulfamethoxazole (34.3% and 7%) for isolates from chicken and beef samples, respectively. The *erm*B, *tet*M and *tet*L genes were predominantly detected by PCR. Five enterococci from chicken meat were found to be phenotypically resistant to vancomycin and carried the *vanA* gene. The presence of virulence genes including *gelE*, *ccf*, *cpd*, *efa*Afs, and *agg*A were frequently detected. The results of this study show that retail chicken and beef meat is source of concern for public health due to having high prevalence of antibiotic resistance and as well as harbouring virulence factors.

1. Introduction

Enterecocci are Gram-positive, facultative anaerobe bacteria that normally widespread in the intestine of animals and humans. In addition, enterococci are present in a variety of fermented meat and dairy products as a starter culture without affecting human health (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Moreover, some enterococci are able to produce bacteriocins called as enterocins which have already been reported to have antimicrobial activity against food spoilage bacteria such as *Listeria monocytogenes* (Ahmadova et al., 2013). Enterecocci have, however, been recognised as an emerging cause of nosocomial infections (Leavis, Bonten, & Willems, 2006) including bacteraemia, septicaemia, endocarditis and urinary tract infections (Hidron et al., 2008) which could be life threatening in immunocompetent and severely ill individuals.

Enterococci have an intrinsic antibiotic resistance to semi-synthetic penicillins, aminoglycosides (low level), vancomycin (low level resistant in *E. gallinarum*, *E. casseliflavus/E. flavescens*), lincosamides (mostly), polymyxines and streptogramins (*Enterococcus faecalis*) (Klare, Konstabel, Badstübner, Werner, & Witte, 2003). Enterococci can also develop acquired resistance to many other antibiotics by carrying various resistant traits through plasmids, integrongs and transposons (Hollenbeck & Rice, 2012). Enterococcal infections have been traditionally treated with glycopeptides antibiotics, mostly vancomycin, since it was approved for human use. However, because of extensive clinical use of vancomycin in hospitals, frequency of vancomycin resistance (Va^R) was dramatically increased (Kirst, Thompson, & Nicas, 1998). In addition to this extensive usage in hospitals, using growth promoters in livestock could potentially lead to the development of resistant strains. For example, in 1986, avoparcin, a glycopeptide analog, was approved to use as a growth promoter of food animals in Norway (Borgen et al., 2000). There was evidence to show an association between

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injudicious use of this class of antibiotics in food animals and a substantial rise in the prevalence of Va^R Enterococci (VRE) recovered from farm animals, foods of animal origin including chicken meat, pork, and beef and from infected humans. Resistance was found to be plasmid-borne and could be transferred to other enterococci (Flannagan et al., 2003). These authors suggested that use of avoparcin in livestock allowed for selection and persistence of resistant strains. Because of the increase prevalence of Va^R in *Enterococcus* isolates, the use of avoparcin as a feed additive in food animals was banned in 1997 in all European Union countries (Borgen et al., 2000).

Besides antibiotic resistance, enterecocci are able to produce potential virulence factors that may enhance their pathogenicity, in another word responsible for causing diseases (Biswas, Dey, Adhikari, & Sen, 2014). These include haemolysin, gelatinase, enterococcal surface protein (Esp), aggregation substance, serine protease, capsule, cell wall polysaccharide and superoxide (Elsner et al., 2000). For example, several studies suggest that haemolysin is important for *Enterococcus* infectivity in animals and humans (Chow et al., 1993; Johnson, 1994). Gelatinase has been shown to be an important virulence factor for aiding to endocarditis in an animal model (Thurlow et al., 2010). It has been shown that *E. faecalis* producing Enterococcal surface protein Esp is more persistence in urinary bladder in experimentally infected animals (Shankar et al., 2001). Enterococci from food of animal origin have been shown to produce these abovementioned virulence factors suggesting that these animal products could act as potential reservoirs for human infections.

There is little data about the incidence of microbial resistance of *Enterococcus* strains in foods of animal origin in Turkey. Therefore, monitoring antimicrobial sensitivity is not only necessary for choosing appropriate antimicrobial agents but also important to monitor antimicrobial resistance development. In this view, the aim of the current study was to investigate the prevalence of enterococci in retail meat samples (chicken and beef) and their antimicrobial resistance profile. The detection of virulence genes and antimicrobial resistance genes were also examined.

2. Materials and methods

2.1. Sample collection, isolation and identification of Enterococcus species

A total of 200 samples of chicken (100) and beef (100) were collected from butcher shops and supermarkets in and around Hatay province in Turkey. All samples were collected in sterile plastic bags, stored in ice packs and transported immediately to the laboratory within 2 h for microbiological analysis. Each sample was screened for the presence of *Enterococcus* spp. using previously published protocols with some modifications (Hayes et al., 2003; Klibi et al., 2013). Meat samples (25 g) were placed in sterile plastic bags containing 225 mL buffered peptone water and mixed with stomacher for 3 min. Rinsate samples (50 mL) were then incubated at 37 °C for 24 h. Following incubation, 10 μl was subcultured into Enterococcosel Broth and further incubated at 37 °C for 24 h. After enrichment, a loopful of broth culture was aseptically streaked on VRE agar and VRA agar including 6 mg/L vancomycin. Plates were incubated at 37 °C for 24 h after which one colony per sample with typical enterococci morphology was then transferred onto blood agar plates in order to obtain pure culture. These isolates were then subjected to Gram staining and catalase test. Identification of the isolates was done by 16S rRNA sequencing. Bacterial 16S rRNA was amplified by using universal primers 16S 20 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1390 (5′-GAC GGG CGG TGT GTA CAA-3′) (Sghir, Antonopoulos, & Mackie, 1998; Suau et al.,1999). The PCR products were sequenced and analysed with the BLAST program available at the National Center for Biotechnology Information (NCBI).

2.2. Antibiotic sensitivity testing

Antimicrobial susceptibility was determined using disc diffusion method according to the Clinical Laboratory Standards Institute (CLSI, 2012) recommendations. Mueller Hinton Agar (MHA) was used for susceptibility testing and the plates were incubated in incubator at 37 °C for 20–24 h. The isolates were screened for susceptibility to 10 antibiotics including penicillin (P; 10 U/disc), ampicillin (AMP; 10 μg/disc), vancomycin (VA; 30 μg/disc), teicoplanin (TEC; 30 μg/disc), erythromycin (E; 15 μg/disc), tetracycline (TE; 30 μg/disc), ciprofloxacin (CIP; 5 μg/disc), chloramphenicol (C; 30 μg/disc), gentamycin (CN; 10 μg/disc) and trimethoprim/sulfamethoxazole (SXT; 1.25–23.75 μg/disc). Antibiotics tested in this study were selected based on their usage in veterinary practice among those classified as “critically important” (P, AMP, VA, TEC, CIP, CN and E) or “highly important” (SXT, C and TE) in human medicine (WHO, 2011). The minimum inhibitory concentration (MIC) values for vancomycin and teicoplanin were determined for Va^R strains using E-Test (Oxoid UK).

2.3. Screening of antibiotic resistance genes

The presence of genetic determinants in isolates showing antimicrobial resistance by disc assay conferring resistance to macrolide and tetracycline (*erm*A, *erm*B, *mef*A/E, *tet*K, *tet*L, *tet*M and *tet*O)) (Malhotra-Kumar, Lammens, Piessens, & Goossens, 2005), to aminoglycosides (*aac(6)-Ie-aph(2)-Ia*, *aph(2)-Ib*, *aph(2)-Ic*, *aph(2)-Id*, *aph(3)-IIIa*, *ant(4)-Ia* (Vakulenko et al., 2003) and chloramphenicol (*cat)* (Aarestrup, Agrees, Gerner-Smith, Madsen, & Jensen, 2000) was determined by PCR. The presence of vancomycin resistance genes (*vanA*, *vanB*, *vanC1/2*, *vanD*, *vanE*, *vanG*) was also analysed as previously described (Depardieu, Perichon, & Courvalin, 2004). Antibiotic resistance genes, primer sequences and lengths of products are listed in Table 1.

2.4. Detection of genetic determinants related to virulence

The presence of the genes responsible for the expression of gelatinase (*gel*A), cytolysin (*cyl*A, *cyl*M and *cyl*B), cell wall adhesins (*efaAfs* and *efaAfm*), enterococcal surface protein (*esp*), sex-pheromones (*cpd*, *cob*, *ccf* and *eep*), and the aggregation substance (*aggA*) were investigated in all enterecocci isolates (Eaton & Gasson, 2001; Marques & Suzart 2004; Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999). Virulence markers and PCR primers are listed in Table 2.

2.5. Statistical analysis

Pearson's chi–square ($\chi 2$) test was used to determine if there were significant differences ($P < 0.05$) in frequency of antimicrobial resistance profiles, resistance genes and virulence traits among Enterecocci isolates obtained from different meat species.

3. Results

A total of 205 isolates were obtained from chicken ($n = 105$) and beef samples ($n = 100$). Three different species including *E. faecalis* ($n = 103$), *E. hirae* ($n = 1$) and *Enterococcus faecium* ($n = 1$) were isolated from chicken meat, while only *E. faecalis* was identified from beef samples. A total of five VRE, including four *E. faecalis* and one *E. faecium* isolates, were isolated from one-hundred chicken meats.