**Virulence Genotyping of *Enterococcus* species isolated from meat and milk products**

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**Abstract**

Enterococci have recently emerged as nosocomial pathogens. Their ubiquitous nature determines their frequent finding in foods as contaminants. As little is known about their virulence potential, this study aimed to investigate the frequency of five potential virulence determinants in *Enterococcus* species isolated from various foodstuffs in Sharkia and Dakahlia Governorates, Egypt. A total of 59 enterococci isolates (59%) were recovered according to standard microbiological methods, with milk and meat being most contaminated (76 and 60%, respectively). Species-specific PCR of ten enterococci isolates identified by 16S rDNA revealed the presence of *E. faecalis*, *E. faecium* and unidentified enterococci in 70, 20 and 10% of the isolates, respectively. PCR screening for *esp* (enterococcal surface protein), *gel*E (gelatinase), *asa*1 (aggregation substance), *hyl* (hyaluronidase) and *ace* (collagen binding antigen) virulence factors showed that all the identified isolates were found to carry one or more virulence-encoding genes, with two or three being the most common pattern. The *esp* and *gel*E were the predominant virulence traits among all investigated enterococci isolates (80% each), followed by *ace*, *asa*1 and *hyl* genes (50, 30 and 10%, respectively). Notably, *E. faecalis* and *E. faecium* isolates showed different patterns of virulence determinants; *esp*, *gel*E, *ace* and *asa*1 genes were more prevalent in *E. faecium* than *E. faecalis*. Simultaneous presence of virulence markers was observed among the analyzed isolates. Therefore, the results of this study showed that food can play an important role in the spread of enterococci with virulence potential through the food chain to the human population.

*Keywords***:** *Enterococcus* species; foodstuffs; virulence; PCR; *esp*; *gel*E.

**1. Introduction**

Enterococci are a group of ubiquitous, Gram-positive, non-spore-forming, oxidase and catalase-negative and facultative anaerobic bacteria that are being the most controversial genus in the lactic acid bacteria (LAB). These bacteria are able to survive a wide array of hostile conditions and can persist in the environment for long periods of time (Van Tyne and Gilmore, 2014). They have the ability to grow in a wide range of temperatures (10–45 °C), as well as in a presence of 6.5% salt concentration.

*Enterococci* species are commonly found as normal constituents of the intestinal microbiota of humans and animals. They have been used widely over the last decade as a part of defined starter cultures in the food industry (Hammerum, 2012) as they contribute to the ripening and aroma development of certain cheeses or fermented sausages (Franz et al., 2011).

The two species *Enterococcus faecalis* and *Enterococcus faecium*, with the former being predominant, have gained significance in recent decades as leading opportunistic pathogens. They have been associated with various infections, including nosocomial infections, bacteremia, meningitis, surgical wound infection, endocarditis, and urinary tract infection (Hammerum, 2012 and Xia *et al.,* 2013).

One of the reasons that enterococcus infections are becoming more serious is their acquisition of various putative virulence determinants which have been found to render specific enterococci strains more apt to cause disease or worsen disease symptoms. Although the pathogenesis of these microorganisms remains unclear, a number of genes encoding for virulence factors in *E. faecalis* and *E. faecium* have been described. Aggregation substance (AS), a pheromone-inducible surface protein encoded by the plasmid *asa*1 gene has been reported to increase adherence and invasion of eukaryotic cells as well as promote biofilm formation (Chuang-Smith *et al.,* 2010). This protein is also required for cell-to-cell contact during conjugation and facilitates aggregation of donor and recipient bacteria for efficient transfer of transmissible conjugative plasmids (Upadhyaya *et al.,* 2009 and Schlievert *et al.,* 2010). Another enterococcal adhesin is the enterococcal surface protein (ESP), encoded by *esp* gene that participates in biofilm formation and mediates adherence and colonization of cells and abiotic surfaces (Paganelli *et al.,* 2012). Hyaluronidase, which is expressed by the *hyl* gene, acts on hyaluronic acid and is associated with virulence of enterococci in host tissue invasion (Fisher and Phillips, 2009). Gelatinase is an extracellular Zn- metalloprotease encoded by the chromosomal *gel*E gene that is capable of hydrolysing gelatin, collagen, casein, hemoglobin and other bioactive compounds (Lindenstrau *et al.,* 2011). Other cell surface protein is collagen binding protein (Ace) that mediates the association of bacteria to host cell matrix protein (Lebreton *et al.,* 2009).

The importance of the presence of virulent enterococci in food is due to the possibility of virulence genes transmission to human microbiota through food chain. Thus, through the isolation, characterization, genotyping and screening of virulence genes among enterococci isolates from diverse samples collected in Sharkia and Dakahlia Provinces, Egypt, we aimed to assess the potential risk and the complex pathogenic process of these opportunistic microorganisms.

**2. Materials and methods**

2.1*. Samples*

A total of 100 samples were analyzed for *Enterococcus* sp.; 25 were obtained from each of milk from mastitic cow collected from different farms and yoghurt, minced meat and sausage collected from different supermarkets in Sharkia and Dakahlia Governorates. The collected samples were transported to in an ice box to microbiology laboratory for bacteriological examination within 2 h.

2.2*. Isolation and identification of enterococci*

Enterococci were cultured according to standard microbiological methods. The samples were inoculated onto surface of bile aesculin agar plates with sodium azide (Oxoid, Basingstoke, Hampshire, England, UK) and incubated at 37°C for 24-48 h. The characteristic pin pointed colonies growing on the agar with a zone of black precipitate and morphologically resembling enterococci were further subjected to presumptive identification based on Gram staining, catalase test and growth in brain-heart infusion broth (BHI) at pH 9.6, 10 and 45˚C and with 6.5% NaCl (Facklam and Collins, 1989). All isolates were kept in brain heart infusion broth with 30% glycerol at −70°C until further analysis.

2.3. *Virulence genotyping of enterococci isolates*

DNA extraction was carried out using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to manufacturers` instructions. PCR amplification was performed with a pair of primers targeting the 16S rRNA gene that is unique for the genus *Enterococcus* using the same method as previously described (Matsuda et al., 2009). *Enterococcus* species identification was carried out based on PCR amplifications of species-specific genes of *E. faecalis* (16S rRNA) and *E. faecium* (*sod*A) according to the procedures reported previously (Jackson *et al.,* 2004 and Zoletti *et al.,* 2006). Moreover, enterococci isolates were analyzed for the presence of *asa*1, *esp*, *hyl*, *gel*E and *ace* virulence genes based on the protocols of several investigators (Creti *et al*., 2004 and Vankerckhoven *et al*., 2004). The primer sequences, PCR cycling conditions and the respective molecular sizes of PCR amplified products are listed in Table 1. All PCR amplification reactions were performed in a singleplex PCR using a PTC-100 TM programmable thermal cycler (MJ Research Inc., Waltham, USA) with a final reaction volume of 25 μl consisting of 12.5 μl of DreamTaq TM Green Master Mix (2X) (Fermentas, Inc. Hanover, MD, USA), 0.1 μl of 100 pmol of each primer (SigmaAldrich, Co., St. Louis, MO, USA), 2 μl of the extracted DNA template and DNase/RNase-free water up to 25 μl. Appropriate positive and negative controls were included in all PCR assays. An aliquot of each amplified PCR product (5 μl) was electrophoresed on 1.5% agarose gel (Sigma-Aldrich, Co., St. Louis, MO, USA) containing 0.5 μg/ml ethidium bromide (Sigma-Aldrich, Co., St. Louis, MO, USA) using 1 X TBE buffer for 1 hour at 100V. The separated bands were visualized and photographed using an ultraviolet transilluminator (Spectroline, Westbury, New York, USA). A 100 bp DNA ladder (Fermentas, Inc. Hanover, MD, USA) was used as a molecular size marker to determine the molecular weights of the PCR products.