



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), *gelE* (gelatinase), *asa1* (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 *gelE* were the predominant virulence traits among all investigated enterococci isolates (80% each), followed by *ace*, *asa1* and *hyl* genes (50, 30 and 10%, respectively). Notably, *E. faecalis* and *E. faecium* isolates showed different patterns of virulence determinants; *esp*, *gelE*, *ace* and *asa1* 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*; *gelE*.

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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; Xia Z 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 *asa1* 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 (Giridhara Upadhyaya et al., 2009; 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 *gelE* gene that is capable of hydrolysing gelatin, collagen, casein, hemoglobin and other bioactive compounds (Lindenstrauss 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* (*sodA*) according to the procedures reported previously (Jackson et al., 2004; Zoletti et al., 2006). Moreover, enterococci isolates were analyzed for the presence of *asa1*, *esp*, *hyl*, *gelE* and *ace* virulence genes based on the protocols of several investigators (Creti et al., 2004; 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.

3. RESULTS

According to phenotypic criteria, 59 isolates (59%) were identified as *Enterococcus* sp. They all grew on bile aesculin agar and yielded the typical

pin pointed colonies of enterococci with a zone of black precipitate proving that they were tolerant to 40% bile and hydrolyzed esculin. All the isolates were presented microscopically as Gram-positive ovoid cocci arranged mostly in pairs or in short chains. Further confirmation of the recovered isolates revealed that they were catalase test negative, tolerant to high salinity and extreme pH (grew on BHI broth with 6.5% NaCl and at pH 9.6) and exhibited visible growth at 10 and 45°C.

The highest isolation rate of enterococci isolates was observed among mastitic milk samples (76%), followed by minced meat (60%), yoghurt (52%) and sausage (48%).

Ten enterococci isolates originated from mastitic cow milk (3), minced meat (3), yoghurt (2) and sausage (2) were further genotypically verified at the genus level through amplification of 16S rRNA gene. PCR identification with species-specific primers for *E. faecalis* and *E. faecium* revealed that the most prevalent species was *E. faecalis* (7/10, 70%), followed by *E. faecium* (2/10, 20%) and one unidentified isolate, even though 16S rRNA positive, it did not harbour any of the 2 species-specific genes investigated.

The frequencies and combinations of *esp*, *gelE*, *asa1*, *hyl* and *ace* virulence genes among the ten isolates were then assessed. It was evident that the oligonucleotide primer pairs targeting the genes under study successfully amplified the DNA extracted from tested enterococci isolates, generating the specific amplicon for each primer (Fig. 1). The detailed distribution of analyzed virulence genes among the tested species are shown in Table 2. In total, the *esp* and *gelE* were the predominant virulence traits among all investigated enterococci isolates (80% each), followed by *ace* gene (50%). On the other hand, *asa1* and *hyl* genes were found only in three and one isolates (30 and 10%, respectively). While none of the *E. faecalis* and *E. faecium* isolates harbored *hyl* gene, the unidentified isolate carried the *hyl* gene (100%). Interestingly, *E. faecalis* and *E. faecium* isolates showed different patterns of virulence determinants. The frequencies of *esp*, *gelE*, *ace* and *asa1* genes were higher in *E. faecium* than *E. faecalis* as both *E. faecium* isolates harbored *esp*, *gelE* and *ace* genes (100%) and one *E. faecium* carried *asa1* gene (50%).

Table 1. Oligonucleotide primer sequences used for virulence genotyping of enterococci isolates

Target gene	Specificity	Oligonucleotide primer sequence (5'→3')	PCR amplification cycles	Amplicon size (bp)	References
16S rRNA	genus <i>Enterococcus</i>	ATCAGAGGGGATAACACTT ACTCTCATCCTTGTTCTTCTC	95°C 15 min; 40 x: 94°C 20 s, 55°C 20 s, 72°C 50 s	337	Matsuda <i>et al.</i> , 2009
16S rRNA	<i>E. faecalis</i>	GTTTATGCCGCATGGCATAAGAG CCGTCAGGGGACGTTTCAG	95°C 2 min; 36 x: 95°C 30 s, 60°C 1 min, 72°C 1 min; 72°C 2 min	310	Zoletti <i>et al.</i> , 2006
<i>sodA</i>	<i>E. faecium</i>	GAAAAACAATAGAAGAATTAT TGCTTTTTGAATTCTTCTTTA	95°C 4 min; 30 x: 95°C 30 s, 55°C 1 min, 72°C 1 min	215	Jackson <i>et al.</i> , 2004
<i>esp</i>	Enterococcal surface protein	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	95°C 15 min; 30 x: 94°C 1 min, 56°C 1 min, 72°C 1 min	510	Vankerkhoven <i>et al.</i> , 2004
<i>gelE</i>	Gelatinase	AGATGCACCCGAAATAATATA GCACGCTATTACGAACATATGA	72°C 10 min	213	
<i>asa1</i>	Aggregation substance	TAAGAAAGAACATCACCACGA ACAGAAGAGCTGCAGGAAATG		375	
<i>hyl</i>	Hyaluronidase	GACTGACGTCCAAGTTTCCAA		276	
<i>ace</i>	collagen-binding protein	GGAATGACCCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	95°C 5 min; 30 x: 95°C 60 s, 58°C 60 s, 72°C 60 s; 72°C 10 min	616	Creti <i>et al.</i> , 2004

x: Number of cycles

Table 2. Overall distribution of species and virulence genes of enterococci isolates

Species (n, %)	Presence of virulence genes (%)				
	<i>esp</i>	<i>gelE</i>	<i>ace</i>	<i>asa1</i>	<i>hyl</i>
<i>E. faecalis</i> (7, 70)	6 (85.7)	5 (71.4)	3 (42.9)	1 (14.3)	0
<i>E. faecium</i> (2, 20)	2 (100)	2 (100)	2 (100)	1 (50)	0
Unidentified (1, 10)	0	1 (100)	0	1 (100)	1 (100)
Total (10)	8 (80)	8 (80)	5 (50)	3 (30)	1 (10)

Table 3. Genetic linkage of virulence factor-related gene clusters among *Enterococci* species from different sources

Source (n)	Species	Virulence gene profile
Milk from mastitic cow (3)	<i>E. faecalis</i>	<i>ace, asa1</i>
	<i>E. faecium</i>	<i>esp, gelE, ace, asa1</i>
	Unidentified	<i>gelE, asa1, hyl</i>
Minced meat (3)	<i>E. faecalis</i>	<i>esp, gelE, ace</i>
	<i>E. faecalis</i>	<i>esp, gelE</i>
	<i>E. faecium</i>	<i>esp, gelE, ace</i>
Yoghurt (2)	<i>E. faecalis</i>	<i>esp</i>
	<i>E. faecalis</i>	<i>esp, gelE</i>
Sausage (2)	<i>E. faecalis</i>	<i>esp, gelE, ace</i>
	<i>E. faecalis</i>	<i>esp, gelE</i>

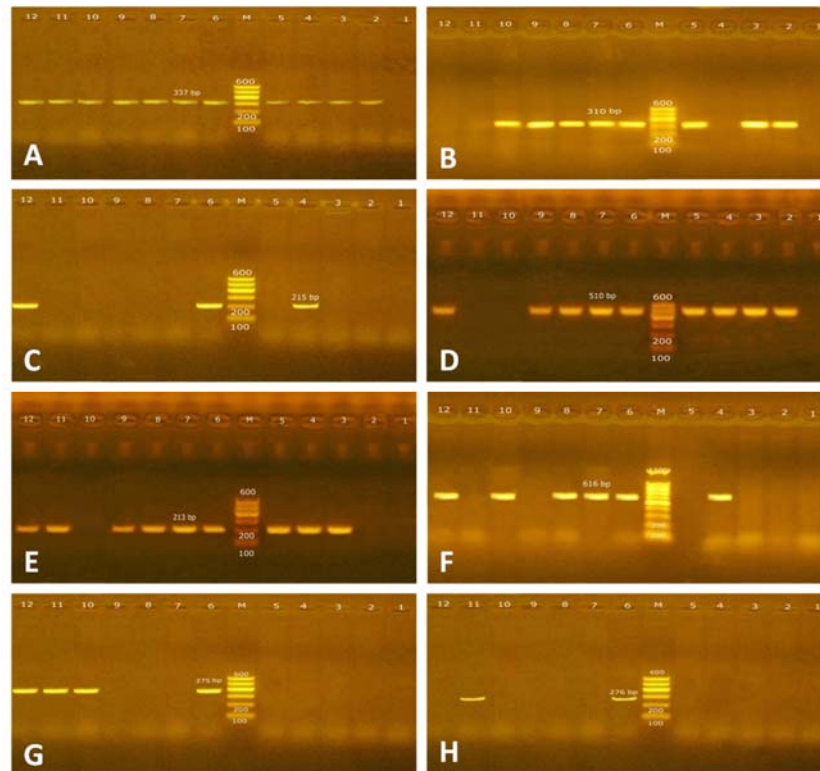


Figure 1. Agarose gel electrophoresis of PCR amplified products of *Enterococcus* 16S rRNA gene (A), *E. faecalis* 16S rRNA gene (B), *E. faecium* *sodA* gene (C) and *esp* (D), *gelE* (E), *ace* (F), *asa1* (G) and *hyl* (H) virulence genes. Lane M: DNA molecular size marker (100 bp), lane 1: negative control, lane 6: positive control, lanes 2,3: *E. faecalis* from yoghurt samples, lane 4: *E. faecium* from minced meat sample, lanes 5,7: *E. faecalis* from minced meat samples, lanes 8,9: *E. faecalis* from sausage samples, lane 10: *E. faecalis* from milk sample, lane 11: unidentified *Enterococcus* species from milk sample, lane 12: *E. faecium* from milk sample. The size in base pairs (bp) of each PCR product is indicated for the bands.

The virulence gene profile of *Enterococci* species isolated from different sources revealed that all the isolates carried at least one and concomitantly up to as many as four virulence markers, but none of them was positive for all the investigated virulence genes. In general, 90% of the isolates harboured between two and four virulence determinants, with majority of them carrying predominantly two and three virulence markers concomitantly (Table 3). Moreover, two

4. DISCUSSION

Enterococci are natural inhabitants of the intestinal tract of many animals. The ubiquitous nature of the enterococci and resistance to adverse environmental conditions take account for their ability to colonize different habitats and to become the predominant contaminant microbiota in many foods (Giraffa, 2002). Despite the increasing importance of enterococci as opportunistic pathogens, their virulence factors are still poorly understood. Therefore, this study determines the frequency of virulence factors in enterococci isolates from different sources in Sharkia and Dakahlia Governorates.

In the present study, the overall incidence level of enterococci (59%) from the analyzed food samples was close to that reported earlier in Brazil (52.5%) (Gomes et al., 2008). With reference to the prevalence of *Enterococcus* spp in different samples, the higher prevalence rate of enterococci was obtained from milk samples (76%), which is higher than those of previous studies reported the prevalence of enterococci in bovine mastitis in Turkey (10.97%) (Kuyucuoglu, 2011) and Pakistan (3.17%) (Ali M.A. et al., 2011). Variation in prevalence of mastitis might be due to the different regions, therapeutic practices, management conditions and presence of microorganisms in environment. The prevalence of enterococci from minced meat samples in our study (60%) is exactly the same as that obtained in another study conducted in Egypt (Abdeen et al., 2016). The incidence of enterococci in yoghurt samples herein (52%) is higher than that obtained in an earlier report in Egypt (31.43%) (Sadek et al., 2014). Among the food samples tested, 48% of sausage samples were positive for enterococci. This is nearly similar to the incidence rate of enterococci from sausage samples in Italy (41.3 %) (Pesavento et al., 2014). Generally, the high incidence of enterococci in foods may be attributed to the fact that enterococci are comparatively heat resistant, salt tolerant, grow at wide range of temperatures,

virulence genes were found to coexist preferentially among *E. faecalis* isolates.

Altogether, the 10 isolates were divided into 6 molecular genotypes based on the different combinations of virulence genes. Overall, linkage between these isolates consisted of a shared common combination of the *esp* and *gelE* genes only or with *ace* gene with the predominance of both *esp*⁺, *gelE*⁺, *ace*⁺ and *esp*⁺, *gelE*⁺ genotypes in the studied enterococci isolates (30% each). low pH and more resistant to drying (Giraffa, 2002).

All isolates in the present study expressed the criteria which are generally considered to define enterococci as was previously reported (Buyukyork et al., 2014). They were Gram-positive, catalase negative, grew at 10 and 45 °C, in broth with 6.5% NaCl or at pH 9.6, tolerated bile and hydrolyzed esculin.

The role of enterococci in foods is still unclear and the knowledge about the genetic information of these bacteria is of a great interest. In this sense, the identification of *Enterococci* species and even their distribution in foods is of crucial importance to understand the epidemiological importance of this genus. In this study, out of 59 enterococci isolates, 10 were identified at the genus and species levels; 7 were *E. faecalis* (70%), 2 were *E. faecium* (20%) and one isolate (10%) could not be identified with the 2 primers used. Virulence of *Enterococcus* species may be linked to a particular species, with *E. faecalis* and *E. faecium* being the most relevant members of this genus with regard to clinical aspects (Franz et al., 2001). Despite, a relatively small number of isolates molecularly analyzed in this study, *E. faecalis*, followed by *E. faecium* were the dominant species in the investigated foodstuffs. This is supported by previous reports in the Czech Republic (Trivedi et al., 2011) and Egypt (Abdeen et al., 2016).

Studying the enterococcal virulence is complex, because the essential factors for pathogenicity have not yet been described. Indeed, virulence in this genus has been typically considered a multifactorial process, with the participation of several genes and their products. In the present study, we surveyed 10 enterococci isolates for the presence of *esp*, *gelE*, *asa1*, *hyl* and *ace* virulence genes. In total, all the isolates were found to carry one or more virulence genes and majority of them (90%) harbored between two and four virulence determinants. This is corroborating the results of previous reports in the Czech Republic (Trivedi et al., 2011), where 88% of enterococci isolates from foodstuffs carried one or more virulence genes.

Moreover, the incidence of multiple virulence genes present at the same time was high in enterococci isolates from food samples in Germany (93.3%) (Anderson et al., 2015). Generally, *esp* and *gelE* were the predominant virulence traits among our investigated isolates (80% each), followed by *ace* gene (50%). These prevalence rates are higher than those recorded in Brazil, where *gel*, *ace* and *esp* genes were recovered from 56.62, 36.99 and 1.37% of enterococci isolates from Brazilian foods, respectively (Gomes et al., 2008). However, a previous investigation in Egypt reported the predominance of *esp* and *ace* virulence genes among enterococci isolates from minced meat samples (Abdeen et al., 2016). On the other hand, *asa1* and *hyl* genes were found only in 30 and 10% of the isolates, respectively corroborating results of previous reports in the Czech Republic (Trivedi et al., 2011), where the presence of *hyl* and *asa1* genes were found less frequently in the tested enterococci isolates from foodstuffs (19 and 15%, respectively).

Both *E. faecalis* and *E. faecium* isolates showed different patterns of virulence determinants. Higher prevalence rates of genotypic virulence markers (*esp*, *gelE*, *ace* and *asa1*) were detected in *E. faecium* isolates when compared to *E. faecalis* from food contradicting the results previously reported in United Kingdom and Brazil (Eaton and Gasson, 2001; Gomes et al., 2008). Data from a recent study in the Czech Republic (Trivedi et al., 2011) regarding the presence of *hyl* gene in both *E. faecalis* and *E. faecium* isolates are contradictory with our results and with those previously reported (Gomes et al., 2008), where *hyl* gene was absent in both species analyzed

As a whole, our study supported the findings of a previous report in Brazil (Bittencourt de Marques and Suzart, 2004), where the high percentage of virulence genes in enterococci isolates is noteworthy as majority of the isolates harbored between two and three virulence determinants, but none of them was positive all the investigated virulence genes. Overall, both genotypes *esp*⁺, *gelE*⁺, *ace*⁺ and *esp*⁺, *gelE*⁺ were predominantly found in the enterococci isolates studied and the linkage between these isolates consisted of a shared common combination of the *esp* and *gelE* genes only or with *ace* gene as was previously proved (Vidana et al., 2016). How these virulence factors interact and mediated disease needs to be further addressed.

5. CONCLUSION

The results of the present study clearly revealed that enterococci, predominantly *E. faecalis* and *E. faecium* are common contaminants in Egyptian foods, with milk and meats being most contaminated. The higher frequency of putative virulence determinants among these isolates raises evidences of the detrimental aspects of enterococci in foods as they may act as reservoirs of virulence factors, enabling the dissemination of these genes to the human microbiota through the food chain. Therefore, significance and use of enterococci in foods still represent a challenge and should come under tighter control.

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