The Determination of Virulence Factors among Fish Originated Enterococci

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Abstract: Enterococci are commensal organisms of human and animals, and may cause diseases in particular conditions. Several virulence factors are responsible in the production of diseases. The aim of this study was to isolate enterococci from fish and to determine virulence factors of the isolates. A total of 26 (13%) *Enterococcus faecalis* strains were isolated from live, moribund and dead fish collected from fish farms in Aegian Region. Cytolysin and gelatinase activities and aggregation substance production of these strains were examined.Cytolysin production was not detected in any of *E. faecalis* strains. Of 26 strains tested, 27% was found to produce aggregation substance. Gelatinase activity was found in 11.5% of strains. The presence of strains with important virulence factors in enterococci from fish was established. It was suggested that these strains have the potential of producing disease in human and animals.

Key words: Enterococcus, Fish, Virulence factors

Balık Kökenli Enterokoklarda Bazı Virülens Faktörlerinin Belirlenmesi

Özet: Enterokoklar insan ve hayvanlarda kommensal olarak bulunan ve özel koşullarda hastalığa neden olan bakterilerdir. Hastalığın oluşumunda birçok virülens faktörü rol oynamaktadır. Bu çalışmada balıklardan enterokok izolasyonu ve izole edilen enterokoklarda virülens faktörlerinin belirlenmesi amaçlanmıştır. Çalışma kapsamında Ege bölgesinde bulunan balık işletmelerinden toplanan balıklardan 26 (%13) adet *Enterococcus faecalis* izole edilmiştir. İzolatlarda sitolizin ve jelatinaz aktivitesi ile birlikte agregasyon substans üretimi incelenmiştir. İzolatların hiçbirisinde sitolizin aktivitesi tespit edilememiştir. İzolatların %27'sinde agregasyon substans üretimi ve %11,5'inde ise jelatinaz aktivitesi saptanmıştır. Sonuç olarak bu çalışmada; balık kökenli enterokoklarda önemli virülens faktörlerinin varlığı belirlenmiş ve bu suşların insan ve hayvanlarda hastalık oluşturma potansiyeli olduğu kanısına varılmıştır.

Anahtar kelimeler: Balık, Enterococcus, Virülens faktörleri

Introduction

Gram positive enterococci are considered responsible for most of the diseases that seen in aquaculture around the world. In this time, it is possible to encounter with the enterococcal infections throughout the world. Observations of tardy development, anorexia, inactivation, loss of gloss and necrosis in muscles, septicemia and mortalities were reported in enterococci infected fishes [5,11,13]. Enterococci are mostly found in intestine and can reach the number of 10^7 bacteria/g [2]. Apart from living organism, enterococci were isolated from surface water, river, sewage, soil and several food stuffs [16,30].

Although enterococci are approved to be part of normal bacterial flora and qualified as lower virulence microorganisms, especially their role in certain human diseases and mortality rate is remarkable. With the understanding the importance of enterococci in human diseases, enterococci were started to isolate from sporadic infections of some animals, particularly from nosocomial infections of small animals and pets [9,32]. A great majority of clinic infections are generated by *Enterococcus faecalis* and *E. faecium* [4]. Among all the nosocomial infections, enterococci are second most frequent pathogens [27]. Because of occurring in 80-90% of these cases, *E. faecalis* was approved the most prevailing enterococcus. That is followed by *E. faecium* with the frequency rate of 5-15% [4].

Despite the admitted low virulence of enterococci, accession of their infections to the serious extent and existence of high mortality rate are increased the attention to them [35]. There are several opinions about diffusion and contagion of virulent enterococcus strains. According to one opinion contagion is happened by strain's itself from patient to patient or from environment to patient. Pursuant to

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other opinion, spread of virulence and resistance genes -instead of strains- come into question [2, 29].

Aggregation substance (AS) is a surface protein that provides aggregation of donor and receiver enterococcus strains to get close contact during bacterial conjugation [7]. Most important characteristic of AS, except providing transfer of some virulence genes and antibiotic resistance genes with an efficient bacterial conjugation, is to supply binding enterococcus to eukaryotic cells and to play significant role in pathogenesis. AS also makes available the invasion of colon mucosa and the entry to intestinal epithelium cells by enterococci [20, 28].

Cytolysin is a toxin in protein structure and has a hemolytic characteristic that is found in *E. faecalis* strains. Enterococcal cytolysin is effective on some eukaryotic cells [26].

Experimental studies showed that cytolysin characteristic can be conveyed through strains by the way of conjugation [27].

As in other bacterial pathogens, proteolytic enzymes are presented among the virulence factors of enterococci [26]. Since for its identification gelatin is used, enterococcal protease -that is also known as gelatinase- is an extracellular enzyme that has metalloproteinase feature (zinc-endopeptidase) and 34,5 kDa in weight [22]. In the studies, its gelatinase feature was only determined in *E. faecalis* strains while it wasn't seen in *E. faecium* strains. Furthermore, gelatinase activity in *E. faecalis* strains was detected in a study that was carried out on animal and foodborne enterococci [10]. In an experiment that was conducted in strains isolated from bacteraemia cases, gelatinase was observed on 55% of *E. faecalis* strains while it was not produced in *E. faecium* strains [12].

In this study it was aimed to obtain detailed information about enterococci that cause economic losses and health problems in culture fishing by isolating them and determining main virulence factors of isolated strains.

Materials and Methods

Sample collection, isolation and identification of enterococci

In this study, 200 fish samples that were collected from different commercial fish farms establishment in Aegean region from Turkey. For isolation, samples were inoculated to selective Enterococcus M broth medium. The medium was incubated for 18-24 h at 37°C. Color change of medium was evaluated as an indicator of enterococci growth in the medium. Positive broth cultures were transferred to BBL™ Enterococcosel Agar (EA) for isolation of enterococci. Plates were incubated overnight at 37°C. At the end of the incubation period, black and dark brown colonies were considered as being an enterococci [21].

For the identification at the level of the genus, Gram staining, catalase tests on slide, gas reproduction from mannitol and reproduction at 45°C were made to a presumptive positive colonies.

The strains that gave positive results in these tests and were denominated as *Enterococcus* sp. have been observed for their various phenotypic and biochemical properties to identify them at species level [25]. In addition, certain types of the standard strains were used for control purposes.

Table 1. The oligonucleotide primers used in the study.

Primer names		Oligonucleotide sequences	
DD13	Forward	5'-CACCTGAAGAAACAGGC-3'	*E. faecalis*
DD3-2	Reverse	5'-ATGGCTACTTCAATTTCACG-3'	
FAC1-1	Forward	5'-GAGTAAATCACTGAACG-3'	*E. faecium*
FAC2-1	Reverse	5'-CGCTGATGGTATCGATTCAT-3'	
ENT1	Forward	5'-TACTGACAAACCATTCATGATG-3'	*Enterococcus* spp.
ENT2	Reverse	5'-AACTTCGTCACCAACGCGAAC-3'	

For genomic identification of enterococci by PCR, bacterial DNAs were extracted with boiling method and kept frozen at -20°C until used. The determination of enterococci at genus level was performed with using *Enterococcus* specific primer (Table 1) as described by Ke et al. [24]. For the identification of these enterococci either being *E. faecalis* or *E. faecium*, *ddl* gene targeted multiplex PCR was performed [8]. After amplification, the DNA fragments of 112-bp (*Enterococcus* spp.), 476-bp (*ddl* for *E.faecalis*) and 1091-bp (*ddl* for *E. faecium*) were separated by agarose gel electrophoresis and visualized under ultraviolet light.

Determination of virulence factors

Measurement of the AS of the enterococci was performed by clumping assay, as described by Chow et al. [6]. GelE activity of enterococci was tested in gelatine medium, as described by Su et al. [34]. And, the method described by Elsner at al. [12] was used for the detection of cytolysin activity.

For the GelE, *E. faecalis* OG1RF was used as a positive control, The plasmidless reference *E. faecalis* OG1X strain was used as a negative control, whereas 2 variants of *E. faecalis* OG1X containing either plasmid pAD1 or pCF10 were used as positive controls for the AS and *E. faecalis* OG1X (pAM944) was used as a positive control for the Detection of cytolysin production [15].

Reference strains

The reference strains of enterococci were kindly provided by Dr. D.B. Clewell (Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA) (Table 2).

Table 2. Standard *E. faecalis* derivates and contents of isogenic strains, plasmid/transposon, and virulence factors.

Strain/Isogen	Plasmid	Transposon	Virulence Factors
OG1X	None	None	None
OG1RF	None	None	GelE
OG1X(pAM9058)	pAD1	Tn917	AS
OG1X(pAM944)	pAD1	Tn917	Cytolysin
OG1X(pAM714)	pAD1	Tn917	Cytolysin + AS
OG1SSP	PCF10	Tn915	GelE + AS

Results

Isolation and identification

In this study, 26 (13 %) *Enterococcus* sp. were isolated from 200 fish samples and all of them were identified as *E. faecalis* according to the biochemically features. Also, identifications were confirmed by PCR and all of the isolates gave positive bands for *Enterococcus* spp. and *E.faecalis*. None of them gave a band for E.*faecium*. According to these results, all the strains were identified as E.*faecalis*.

Virulence factors

Aggregation substance (AS): Enterococcus strains isolated from fish were determined whether they produce aggregation substance against the stimulation of *E. faecalis* OG1X pheromone. In consequence of cluster test, 7 of inspected 26 *Entrococcus* strains (%27) were found to produce aggregation substance. OG1X (pAM714), OG1X (pAM9058) and OG1SS strains of *E. faecalis* that were used as control gave positive, and OG1X (pAM944) gave negative result to AS.

Cytolysin: As a result of hemolysis test that were performed in horse blood agar to determine cytolysin characteristic of fish originated enterococcus strains, none of the strains were observed to show cytolysin activity.

Gelatinase (GelE): In the gelatinase test, 3 of 26 (11.5%) inspected enterococcus strains were detected to produce gelatinase. OG1RF and OG1SS strains of *E. faecalis* that were used as control gave positive; OG1X(pAM714), OG1X(pAM944), OG1X(pAM9058) and OG1X strains gave negative results (Table 3).

Table 3. The distribution of virulence factors of *E. faecalis*

Serotypes	Virulence factors					
	AS		Cytolysin		GelE	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
E. faecalis (n=26)	7	19	-	26	3	23

Pos: Positive,
Neg: Negative,
AS: Aggregation substance,
GelE: Gelatinase

According to results, only 1 of 26 (3.84%) *E. faecalis* strain was found as positive for both AS and GelE. AS and GelE showed different positiveness results in other isolates, none of isolate was found positive to Cytolysin.

Discussion

Aquaculture has become an important sector nowadays. During the recent years it was seen that sporadic and epidemic cases in fish diseases have its source from Gram positive coccus. In taxonomic studies, different Gram positive coccus species causing fish diseases were indicated to be present such as *Streptococcus iniae, S. difficile, Lactococcus piscium, Vagococcus salmoninarum* and *Enterococcus seriolicida* [11]. Quite high level phenotypic heterogeneity was found between Gram positive coccus which show fish originated enterococcus or streptococcus properties. Eldar et al. [11] isolated *Enterococcus* species from trout pursuant to their phenologic and serologic properties.

In this study 26 *Enterococcus* sp. isolated from 200 fish samples and they were confirmed as *E. faecalis* based on all biochemical characteristics. Araujo et al. [1] reported that 42.2% of 64 *Enterococcus* strains isolated from rainbow trout were *E. faecium* while 35.9% of them were *E. hirae*.

Enterococci long time was assumed non-pathogenic since they were normally existed in humans and animal flora. Nevertheless, causing severe diseases and high lethality under certain conditions was revealed that they have notable virulence factors. In the studies conducted for this aim concluded that some properties of *Enterococci* might be related to virulency [26]. Aggregation substance is one of the most accentuated among these factors. Importance of AS were demonstrated either epidemiologic [12] or experimental studies [28,33]. According to determination of the studies; AS provides binding to mucosal epithelium cells and endocardium as well as invasion [20], increases the vitality in neutrophil [31,36] and inhibits the fusion of lysosomal vesicle and phagosome [17].

In this study, AS production of *E. faecalis* strains isolated from fishes were examined by cluster test that based on aggregate generation of tested organisms after pheromone stimulation. AS was determined in 27% of inspected *E. faecalis* strains. There is a general opinion in literature that AS production is a property peculiar to *E. faecalis* and *E. faecium* species [22,26].

Parallelism between AS property and other virulence factors wasn't determined. Mundy et al. [26] reported that genes encoding the AS are independent or are located in plasmids carrying Cytolysin. Having cytolysin negative and AS positive phenotypes in this study indicated there might be only one plasmid in isolated strains.

Cytolysin is viewed as the most remarkable feature of virulence factors of *Enterococci* according to epidemiologic and experimental data [23,26]. In the epidemiological studies which shows the importance of AS; cytolysin characteristic were found at the higher rate in clinical isolates than in normal population isolates [18,19]. Playing significant role in pathogenicity by organ toxicity of cytolysin was also determined in the experimental endocarditis, endophtalmitis and peritonitis models [6,14]. Moreover, it was reported that cytolysin with its bacteriosin effect have a role in colonization by suppressing other bacteria [6].

In this study, cytolysin production of *E. faecalis* strains isolated from fishes was investigated by hemolysis test based on lysis of horse erythrocyte. Cytolysin activity was observed in none of the *Enterococci* strains. This result didn't change even the hemolysis tests repeated several times with control strains under different conditions. Although it wasn't found any study in literature supporting this result, cytolysis was accepted as atypical variants without denying existence of strains. Furthermore, the possibility of existence of this characteristic in subsequently observed *Enterococci* should not be forgotten by considering the previous AS sample. Because AS production was accepted long time as a characteristic that only belongs to *E. faecalis*; recently, it has been understood that *E. faecium* has that characteristic too.

Elsner et al. [12] determined cytolysin production in 16% of *E. faecalis* strains isolated from bacteraemiac humans. Eaton and Gasson [11] found cytolysin phenotype in 33% and 44% of *E. faecalis* strains that are clinically originated from humans and animals, respectively. But they stated the cytolysin frequency rate in human strains as 56%. This data indicates cytolysin production rate can change

from studies to studies and certain conditions need to be exist for gene expression.

As in other bacterial pathogens, proteolytic enzymes are introduced among the virulence factors of *Enterococci*. Relation between virulency and gelatinase property representing the protease was determined in the experimental animal models and epidemiological studies that show their higher existence in clinical isolates. In our study, proteolytic activity of *Enterococcus* strains was examined by a test that determine gelatinase which hydrolyzing gelatin. Gelatinase feature was observed in 11.5% of *E. faecalis* strains. Since the results didn't change in repeated tests with control, these strains were accepted as atypical gelatinase variants. Moreover, a relation between gelatinase characteristic and other virulence factors was not detected. Protease characteristic was found 54% of *Enterococcus* isolated from endocarditis cases and nosocomial infections, while this rate was 12% in healthy sources [22]. Eaton and Gasson [10] determined 56% gelatinase frequency in *E. faecalis* strains originated from foods and humans. These studies showed gelatinase activity is found at high rate in clinical strains and might also be exist in animal food originated strains. Carnerio et al. [3] investigated virulence factors in 87 *E. faecalis* strains isolated from fish and sea products. They reported 97% of these strains carry *gel*E gene, only 77% of them show gelatinase activity, beside 5% of these strains demonstrated cytolysin activity with hemolysis test.

In this study gelatinase activity was encountered relatively low in inspected strains. Nevertheless, existence of this factor was shown in fish originated strains. These virulence factors even if they are not related to deaths might contribute to pathogeny by providing the colonization of agent, binding to host cells and resistance to immune system. Consequently, existence of important virulence factors in fish originated *Enterococci* and occurrence of the strains' infection potential in human and animals were determined.

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