

Molecular screening of the *entA* gene of *Enterococcus faecium* isolated from Food and clinical sources

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Abstract

Background: The microbial production of substances that have the potency to suppress the growth of other microorganisms is probably one of the prevalent defense strategy developed in nature, microorganisms produce a variable bunch of microbial defense systems, which include antibiotics, metabolic by-products, lytic agents, bacteriocins and others.

Objective: The purpose of the present study was to isolate and identify *Enterococcus faecium* isolates then detecting its ability of carrying the gene responsible for enterocin production in this species.

Materials and methods: Out of 50 samples from different sources (food and clinical sources) were collected for the *Enterococcus faecium* isolation, and the isolated bacteria *Enterococcus faecium* (37) isolates were detected for their harboring of Enterocin A gene (*entA*), using conventional PCR technique.

Results: The identification revealed that 37(74%) isolates were considered as *Enterococcus faecium*, 20 isolates (54.05%) out of food samples (10 samples were collected from dairies, 7 from vegetables and 3 from fish samples), and 17 isolates 45.9% out of clinical samples (11 from stool and 6 from urine source). Genotypic Detection done by the amplification of the enterocin coding gene (*entA*), and the results revealed that all the isolates were harboring that gene despite of the phenotypical differences, that they amplified *entA* gene and the PCR product size (362 bp) was detected using agarose gel electrophoresis.

Conclusions: This study indicates the presence of *Enterococcus spp.* in food and clinical sources and the ability of these bacteria to produce antibacterial substances which is active against closely related clinical isolates.

Key words: Enterocin A, antibacterial, *entA* gene, *Enterococcus*.

Introduction

Enterococci are large genus of Gram-positive bacteria, catalase-negative, oxidase-negative, lactic acid producer (which is consider as a major end product of glucose fermentation), facultative anaerobic cocci generally looks like pairs or short chains (1). The genus *Enterococcus* which is belong to the family Enterococcaceae, one of the main families that belong to the order Lactobacillales and consider as one of the most important lactic acid bacteria, (entérocoque), that term was first coined and described by (2). *Enterococci spp.* are large genus of Gram-positive bacteria, non-spore-forming ovoid in shape, catalase-negative, oxidase-negative, bile salts tolerant and have the ability to hydrolyze esculin, generate lactic acid as its major end product of glucose fermentation, facultative anaerobic cocci which is usually appears in pairs or short chain, prevalent bacteria that have been isolated from different sources, could be in association with some plants, in the food products, or as a part of the gut microorganisms, as a commensal microorganisms in the gastrointestinal tract of human and animals (1)(3). *Enterococci spp.* are considered as LAB which are constitute of both commensal and opportunistic-pathogenic organisms and Due to their high durability towards many salts and acids, considered as highly appropriate to be a habitant in different food systems (4). *Enterococcus* species have been reported to produce bacteriocins which are ribosomally synthesized peptide or proteinaceous substances that inhibit the growth of food-borne and pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus* (5). *Enterococci spp.* produces a broader



range of bacteriocins which called enterocins, most of these enterocins have been isolated from *E. faecalis* and *E. faecium*, all were isolated from different sources such as dairy products, other food products including (fermented sausages, fish and vegetables), water, animals and human sources. The classification of bacteriocins was defined by (6). (Enterocin A) which is belongs to the class IIa bacteriocins, (isolated primarily from *Enterococcus faecium*) have an antimicrobial activity against important and pathogenic *Listeria* strains especially *L. monocytogenes* in fermented foods, and have a bioactivity over an extensive range of pH standards, temperature and NaCl levels, these possessions make it a possible applicant to be used as a food preservative and therapeutic antimicrobial agent (7).

Materials and Methods

Isolation of bacteria

Fifty samples (food and clinical sources) were collected randomly (from September to November 2019) the food samples were (row dairy products, leafy vegetables and fish) while the clinical samples were (urine and stool samples). All collected samples were inoculated on (Bile esculin-azide agar), incubated at 37°C for 24 hr. *Enterococcus spp.* have the ability to hydrolyze esculin in the presence of bile and convert the medium to a different color (8).

Enterococcus Identification

The collected samples in this study were isolated & identified depending on the morphological structures and biochemical tests (9). VITEK2 was used to enhance and confirm the results of identification. The identification of *Enterococcus* was accomplished by the inoculation of samples on (Bile-esculin-azide Agar) at 37 °C / overnight, *Enterococcus* converted the media to black coffee brown color around the colonies (due to the hydrolysis of esculin). (ABC streaking method) was performed on (brain-heart infusion agar) in order to make pure colonies, and then overloaded VITEK kit by the samples. *Enterococcus faecium* species recovered (74%) 37 samples), 54.05 % 20 isolates out of food samples (10 samples were collected from dairies, 7 from vegetables and 3 from fish samples), and 17 isolates) 45.9 % out of clinical samples (11 from stool and 6 from urine source).

Molecular Detection

DNA Extraction

DNA was extracted using genomic DNA mini kit (Geneaid) protocol for Gram-positive bacteria were used as following:

Sample Preparation Step:

A volume of 1.5 ml of bacterial broth was moved to a 1.5 ml micro centrifuge tube and centrifuged for 1 min for 16000 rpm then the supernatant was discarded.

Cell Wall Lysis Step:

A 200µl of Gram buffer (after ensuring the lysozyme was added and completely dissolved) was added to the sample in the 1.5 ml micro-centrifuge tube then resuspend the pellet by pipette , and incubated at 37°C for about 30 min. then a 20µl of Proteinase K (after ensuring the D DW was added) added to the sample in the 1.5 ml micro-centrifuge tube then mixed well by vortex and incubated at 60 °C for at least 10 min, after the incubation, 200µl of GB buffer was added to the sample and mixed by using vortex then was incubated at 70 °C for at least 10 min to guarantee the sample lysate is completely clear, throughout the incubation period the tubes were overturned every 3minutes.

DNA binding Step:

200µl of absolute ethanol was supplemented and mixed well by shaking dynamically, at that point transported to the GD column and centrifuged at 14000 rpm for 2min., after centrifugation the collection tube was discarded and the GD tube placed in a new one.

Washing Step:



W1 buffer (400 μ l) was added then centrifuged at 14000 rpm for 30 sec, and the flow through was ignored; 600 μ l of (washing buffer+ethanol) was added to the GD column and centrifuged at 14000 rpm for 30 sec, after centrifugation the flow through was discarded, and the GD column was centrifuged for about 3 min at 14000rpm.

Elution Step:

An elution buffer (50 μ l) was added to the GD column after it was transferred to a clean microfuge tube and let stand for about 3minutes then centrifuged at 14000-16000 rpm for 30 seconds.

Agarose Gel Electrophoresis

Agarose gel was prepared for the purity of DNA extraction detection, by liquefying 0.8g of agarose in 100ml of 1X TBE buffer, boiled then cooled after few minutes, 5 μ l of ethidium bromide, agarose was poured out into the gel jar after it was cooled to prevent bubbles formation, and numerous wells were cautiously made with comb at one side of the gel about 5-10 mm away from the end of gel; after the ultimate solidification the comb was wisely detached and the container was put in the electrophoresis tank. Before loading to the gel wells with samples; DNA was first mixed with a loading dye, 5 μ l of every DNA sample were mixed with 2 μ l loading dye, and then carefully transferred to the wells, and then after the closure of the electrophoresis tank with its lid an electric current was coordinated (70 v /1 hr.).

PCR Amplification of Enterocin A gene (*entA*)

The primers used to detect *Enterococcal* enterocinA coding gene (*entA*) in this study as shown in Table (1).

Table (1): Primers and their sequences that used in this study for the amplification of *entA* gene

Primer (<i>entA</i>)	Sequences	Size (bp)	Tm (°C)	Reference
Forward (<i>entA</i>)	AAA ATA AAT GTA CGG TCG ATT GG.	362	54.7	(4)
Reverse (<i>entA</i>)	CCA GCA GTT CTT CCA ATT TCA		55.7	

The primer was provided as a lyophilized form, and then was ran in a sterilized distilled water to offer the ultimate concentration of 100 pmol / μ l and stocked in the deep freezer till it used again in the PCR amplification. A conventional PCR was adapted to investigate the presence of this gene in the *Enterococcal* isolates.

DNA, Primers and PCR premix (Accupower, Bioneer) were liquefied by using a vortex and centrifuge to get the contents to the bottommost of tubes, PCR mixture was put up in a full volume of 25 μ l included 5 μ l of PCR premix, 2 μ l of each primer and 4 μ l of DNA template have been used, the remnant of volume was done with a sterilized deionized distilled water. Negative control was contained all materials except DNA template, and instead distilled water was added, after centrifugation of the PCR tubes (in order to get the contents to the bottom) all were placed in into thermo-cycler PCR instrument where the DNA was amplified, and the program of amplification was indicated in Table (2).



Table (2): Thermal Cycler Program used to amplify the *ent A* gene

Name of cycle	Temperature	Time period
Initial denaturation	95 °C.	5 min.
Denaturation	95 °C	30 sec
Annealing	51.5 °C.	45 sec.
extension	72 °C	45 sec
Final extension	72 °C	5 min.

35 cycles

Determination of PCR product

After PCR amplification, gel electrophoresis was adopted to confirm the presence of amplification. gel was all set in 1.5 % concentration for the determination of the PCR specificity and sensitivity by thawing 1.5g of agarose powder in 100ml of 1X TBE buffer, and boiling , then the agarose was cooled out, then 5µl of Ethidium bromide was supplemented with mixing, agarose was added into the gel container after it was cooled to avoid bubbles development, and some wells were cautiously made with the comb at only one side of the gel about 5-10mm away from the end of gel; after ultimate solidification of the gel, the comb was carefully detached and the container was placed in the electrophoresis tank.

Five µl of the 100 bp DNA ladder was loaded in the first well or in the middle of other wells of gel, then 5µl of every PCR product was loaded in the wells of gel, followed by the closure of the electrophoresis tank was with its distinctive cover and the electric current was coordinated (70v / 1.5-2 hr.).

Results and Discussion

Fifty samples (food and clinical isolates) were collected for *Enterococcus faecium* identification. Thirty-seven samples (74%) were considered as *E. faecium* were specified relying on micro-scopical characteristics & biochemical tests as in Table (3).

Table(3) : Microscopical and biochemical tests of *Enterococcus* spp.

Test	Result
40% Bile	Tolerant
Esculin hydrolysis	Positive
Azide tolerance	Positive
Gram stain	Gram positive (cocci usually appears in pairs or short chain)
Catalase	Negative
Oxidase	Negative
Glucose fermentation	Lactic acid producers



Enterococcus identification was achieved by direct inoculating of samples on Bile esculin-azide agar at 37°C for 24hr., the positive result will transform the media to a dark brown color all over the place. (ABC streaking method) was accomplished to make pure colonies as shown in Figure (1).



Figure (1): *Enterococcus* spp. colonies on Bile-Esculin azide agar, the media was altered to dark brown color around the colonies or the whole media appears as black coffee brown color because of the hydrolysis of esculin by *Enterococcus*

Then VITEK system was depended for further species identification and all the 37 isolated Enterococcal species were *Enterococcus faecium*. *Enterococcus faecium* species recovered (74%) 37 samples, 54.05 % 20 isolates out of food samples (10 samples were collected from dairies, 7 from vegetables and 3 from fish samples), and 17 isolates 45.9 % out of clinical samples (11 from stool and 6 from urine source) Figure (2).

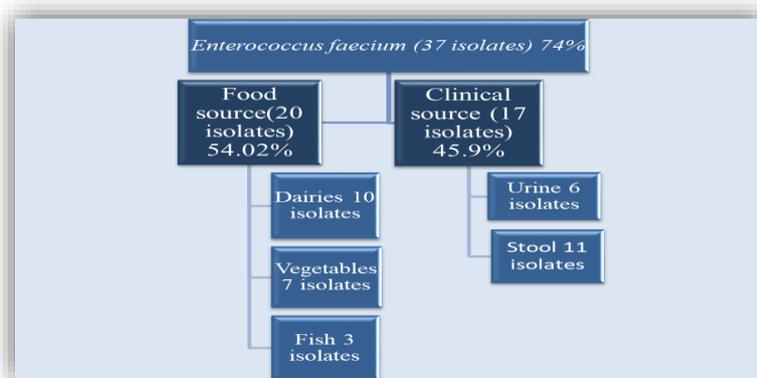


Figure (2): The isolated Enterococcal species with the isolation percentage of each species and its source of isolation

Genotypic Detection of Bacteriocin Production by *Enterococcus faecium*

DNA was extracted from thirty seven *Enterococcus faecium* isolates using genomic extraction kit and the extracted DNA samples were characterized to ensure their purity and quality using agarose gel electrophoresis, then the isolates were detected for their harboring of Enterocin A gene (*entA*), using conventional PCR technique, and the results revealed that all the isolates were ent A positive, that they amplified *entA* gene and the PCR product size (362 bp) was detected using agarose gel electrophoresis as shown in Figure (3).

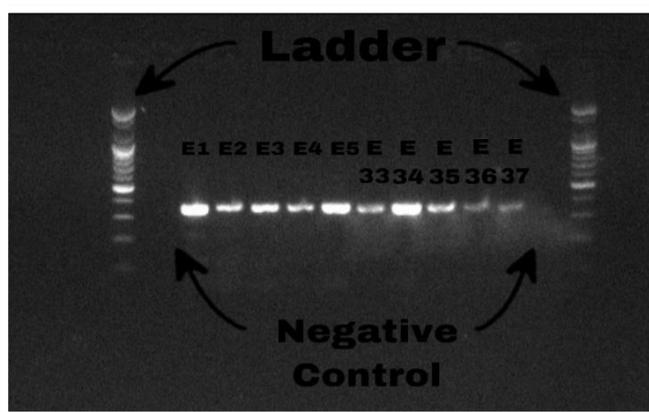


Figure (3): agarose gel electrophoresis of amplified PCR product of *entA* gene (362 bp), (agarose 1.5 %, 1X TBE buffer, ladder 100bp)

From Figure (3) the positive results of *Enterococcus faecium* can be noticed, the figure represent ten positive isolates that belongs to *E. faecium* (both sources, food and clinical, E1-E5 isolated from food sources and E33-E37 isolated from clinical sources). Many researchers reported the presence of *ent A* gene in *Enterococcus spp.* especially *E. faecium* (10). This study also stated that there is no correlation could be found between the presence of enterocin structural genes and the origin of the strain. In this study *E. faecium* was the most predominant species in bacteriocin production detection, another studies suggested that *E. faecalis* percentage in bacteriocin production is higher than *E. faecium* (11). Bacteriocins characterize a promising function to regulate bacterial infections, alone or with other antibiotics as a grouping, and also to reduce the probability of food contamination with further microorganisms and may give a promising role to aid in preservation of various types of foods, usually been used as food preservatives, whichever added or formed by starter cultures during fermentation processes (12). (13) Which stated that the *Enterococcus* contain an antibacterial substances called enterocins that inhibit the growth of closely related foodborne organisms. Antibacterial activity of *Enterococcus spp.* Isolates against other bacterial isolates from different sources may be due to several factors one of which is the enterocins which consider as the main antibacterial substance produced by *Enterococcus spp.*, antibacterial activity of food-borne *Enterococcus spp.* Against other pathogenic isolates may give a possible role as sources of bacteriocin preparations to aid in preservation of many types of foods (14). In the current study *E. faecium* was the most predominant species in bacteriocin production detection (phenotypic and genotypic detection), the variation in inhibitory spectra may be due to several antagonistic factors, among which the production of the known enterocins, such as enterocin A and B, and there might be an association between enterocins genes such as *entB* and *entA* it is possible that both enterocins may act synergistically when expressed in the same culture(15). The use of a specific bacteriocin depends on many characteristics such as effectiveness of pathogen, PH and heat stability, tolerance and effectiveness in acidic environments, its activity in a combination with some chelating agents and organic acids (16). There are new possibilities how to take advantages of such peptides for the benefit of man and animals. Bacteriocin production has become an important property of probiotic bacteria and the use of bacteriocins to fight certain pathogens may have a brilliant future, for many reasons it is meaningful to separate bacteriocins of gram-positive and gram-negative bacteria, based on chemical structure, molecular weight and thermal stability (17).

Conclusion

Lactic acid bacteria constantly gain a great interest among an increasing number of researches owing to their huge applications both in food and in pharmaceutical industries. This study indicates the presence of *Enterococcus spp.* in food and clinical samples and the ability of these bacteria to produce antibacterial

substances which is active against closely related clinical isolates. Enterocins are one of those antibacterial substances that produced by *Enterococcus spp.* those substances may play a possible role in the food industry as natural preservatives, these applications of enterocins against pathogenic and food spoilage microorganisms have been established to be effective and consider as safe.

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