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Determination of comparative minimum inhibitory concentration (MIC) of bacteriocins produced by enterococci for selected isolates of multi-antibiotic resistant *Enterococcus* spp.

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ABSTRACT

Introduction: The occurrence of multi-antibiotic resistance among enterococci is a prevalent clinical problem worldwide and continues to get serious due to the lack of efficient therapeutic options by the time. In this regards, prokaryotic antimicrobial peptides with bactericidal or bacteriostatic activity which are directed against bacterial strains closely related to producer strains looks one of the promising alternative to conventional antibiotics. ***Methods:*** The antibiotic susceptibility pattern of 20 clinical isolates of enterococci was evaluated and subsequently the isolates were screened for antibacterial activity against three different indicator strains. The efficacy of potential bacteriocinogenic isolates were assayed against multi-antibiotic resistant *Enterococcus* spp. by comparative minimum inhibitory concentration (MIC). ***Results:*** Antibiotic resistant pattern of enterococcal isolates demonstrated that multi-antibiotic resistant to conventional antibiotics were significantly high and the prevalent pattern of resistance was combination of gentamicin, streptomycin, chloramphenicol and vancomycin. In addition, the data from comparative MIC showed the noticeable activity of selected potential bacteriocinogenic strains against pathogenic enterococci. ***Conclusion:*** The present survey may address the potential applicability of antimicrobial peptides in alleviating the problems of antibiotic resistance.

Introduction

Enterococci are Gram-positive, non-sporeforming and facultative anaerobic cocci. They have important impact on human health due to their natural presence among gut microbiota, usage as probiotics and conversely their deleterious role in spoilage process of fruit juices and meat products.[1-3] Furthermore, among *Enterococcus* genus, *Enterococcus faecium* and *E. faecalis* are mostly responsible for serious relevant nosocomial infections such as urinary tract infections (UTIs), endocarditis, bacteremia, intra- abdominal and intra- pelvic abscesses.[3-7] Interstingly, many of these problems arise from the ability of enterococci to survive in adverse conditions (temperature ranging from 10 to 45 ºC, basic pH of 9.6 and growth in 6.5% NaCl), presence of several virulence determinants (cytolysin, gelatinase, aggregation substance, extracellular superoxide) and possess both intrinsic as well as acquired antibiotic resistance trait (vancomycin, streptogramins, isoxazolylpenicillins, monobactams and cephalosporins).[3,8-15] The rising prevalence of antimicrobial resistance trait among *Enterococcus* spp. has critical outcome on health care system due to increasing in mortality as a result of existence of severe infections such as endocarditis without any effective antimicrobial therapeutic agents.[16] Hence, emergence of antimicrobial resistance, particularly multi-antibiotic resistant bacterial strains and shortage of newer antimicrobial agents with different mechanism of action from current antibiotics would be a serious problem in the near future and consequently, development of novel alternative to conventional antibiotics is a necessity.[17-19]

Natural antimicrobial peptides (AMPs) such basically are considered as the most ancient and primitive mechanism of immunity in human, animal and all groups of organisms. Bacteriocins are gene-coded,

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ribosomally synthesized AMPs which are produced by prokaryotes and target narrow spectrum of susceptible bacteria.[10,20,21] These cationic small peptides are one of the promising classes of antibiotics that have the potential to serve as an alternative to conventional antimicrobial agents to treat infectious diseases and copes with the ongoing emergence of antimicrobial resistances against pathogenic bacteria.[22] Determination of minimum inhibitory concentration (MIC) of new agents with antimicrobial activity plays a crucial role in evaluating the efficacy of them. The purpose of this study was to assay the efficacy of AMPs from clinical isolated enterococci against multi-antibiotic resistant Enterococcus spp. by determining the comparative minimum inhibitory concentration of AMPs.

Materials and methods

Source of bacterial strains

The 20 enterococcal strains were isolated from blood and feces of hospitalized patients who had been referred to laboratories of West Azerbaijan provinces of Iran during 2007 to 2009.

Bacteriocin assay against standard indicator bacteria by spot-on-lawn method

Antimicrobial activities of the isolates were detected by agar spot method in duplicate.[23]The isolated enterococci were spotted into Brain Heart Infusion broth (Oxoid, Hampshire, England) supplemented with 1.5% agar (Bacto) and incubated for 18 h at 37 °C. The colonies were overlaid with 5 mL of BHI agar 0.7% seeded with 20 µL of an overnight culture of indicator microorganisms *Listeria innocua* LMGT 2785, *Enterococcus faecalis* PTCC 1237 and *E. hirea*PTCC 1239.The strains were obtained from PTCC (Persian Type Culture Collection) and LMGT (Norwegian University of Life Sciences) collections. Subsequently, plates were incubated at 37°C in an upright position. After overnight incubation isolates that formed growth inhibition zones were considered as potential bacteriocin producers (Bac^+). At the same time, to investigate the protein entity of antimicrobial compounds, the sensitivity of antimicrobial activity to proteinase K was tested.

Partial purification of bacteriocin-containing supernatant

The overnight culture of the bacteriocinogenic enterococci was centrifuged (10000×g, 15 min, 4 °C) and then the peptide fraction of cell free supernatants of potential Bac^+ strains were precipitated by 40% ammonium sulphate by centrifugation at 15,000×g for 30 min (4 ºC).[24] The salted-out protein pellets were dissolved in a small volume of distilled water and then the suspension filter sterilized through 0.25 µm membrane filters.

Antimicrobial susceptibility test

Antimicrobial susceptibility of the isolated organisms was determined by disc diffusion method using the Kirby-Bauer technique[22] and as recommended by NationalCommittee for Clinical Laboratory Standards (NCCLS).[25]The surface of the Mueller-Hinton agar containing plates were uniformly inoculated by 2 µl bacterial suspension with equivalent turbidity to 0.5 McFarland standards. The applied antibiotic-containing disks (Himedia) were ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), streptomycin (10 µg) and vancomycin (30 µg). Zones of inhibition were measured after incubation for 24-48 hours at 37°C. Results were graded as Resistant (R) Intermediate (I) and Sensitive (S) according to the reference of particular antibiotic.

Determination of MIC by microtiter plate method

The comparative minimum inhibitory concentration (MIC) of bacteriocin in supernatants and partial purified ones was determined by monitoring growth of an indicator strain in a 96 well microtiter plate.[26] *L. innocua* was applied as an indicator microorganism and multi-antibiotic resistant enterococci were subjected to evaluate the bacteriocin activity. In the first step, 100 µl broth media was added to each well in the microtiter plate then, 100 µl of a fraction to be tested for antimicrobial activity was added to the wells of column 1. In the next step, two-fold dilution of the sample was done from column 1 to column 2 and then continuing down 7 columns (the 8th was as a control column). Subsequently, overnight culture of indicator strain and resistant enterococci were diluted in fresh broth media to 1/20, prior to adding of 100 µl to each well. Finally, the microtiter plate was incubated under conditions appropriate for the relevant indicator, and amount of growth was measured by reading optical density at 620 nm in a microtiter plate reader. The bacteriocin amount that cause growth inhibition of indicator strain by 50% under the assay conditions was defined as one bacteriocin unit (BU).

Results and discussion

Multiple antimicrobial resistances of the pathogenic bacteria have been reported frequently recently.[27-31] It is of great concern in human medicine worldwide since it is a serious problem in the treatment of infectious diseases.[32,33] Enterococci are one the predominant pathogens which become resistant to many commonly used antimicrobials due to acquisition of transferable antibiotic resistance genes. Therefore, development of new and more efficient antimicrobial agents seems inevitable. In this regard, different strategies are proposed[22,34-37] and antimicrobial peptides such as bacteriocins are one of the most interesting candidates under intensive investigation.[38-40] In many respect enterococci play an important role in human health not only because they are from an important group of

generally recognized as safe (GRAS) bacteria but also due to their importance as one the major cause of nosocomial infections.[41] Interestingly, bacteriocin production is one of the prominent features of enterococci which improve their competitiveness in nature.[42]

The 20 isolates of enterococci were screened for antimicrobial production using *L. innocua*LMGT 2785, *Enterococcus faecalis*PTCC 1237 and *E. hirea*PTCC 1239 as indicators. Four isolates from 20 *Enterococcus* isolates (D1, D4, S8 and O9) were found highly active against indicators. Inhibition zones were sensitive to proteolytic enzyme. In the next step the residual bactericidal activity of partially purified bacteriocins were also tested against at least two indicators by microtiter plate method.

Aminoglycosides, ampicillin and glycopeptide resistant are of most significance in the enterococci.[43]In this study, antibiotic susceptibility patterns of 20 isolates of enterococci were evaluated. Interestingly, as Table 1 demonstrated clinically isolated enterococci were generally susceptible to ampicillin (60 %) and vancomycin (65 %). By contrast, ciprofloxacin (70 %) and aminoglycosides (streptomycin (75 %) and gentamicin (90 %)) resistant were highly prevalent among Enterococcus. Furthermore, many of isolates showed resistant to erythromycin and chloramphenicol (55 %). Surprisingly, chloramphenicol, ciprofloxacin and erythromycin intermediate resistant were noticeable in the current study. Antibiotic resistances of enterococci have been emerged mostly in recent years[41] and as the results of this survey indicated vancomycin resistance was not high but alarming and this gives rise to concern because of the limited therapeutic choices in treatment of severe enterococcal infections in near future.[3] However, aminoglycosides resistance was high among clinical enterococcal isolates due to the high frequency of both intrinsic and acquired resistance of enterococci.[3] In particular, multiple antibiotics resistant to conventional antibiotics were remarkable and the prevalent multi-antibiotic resistant which was recorded was for the combination of gentamicin, streptomycin, chloramphenicol and vancomycin (data not shown).

Table 1. Antibiogram of *Enterococcus* strains (n = 20)

Antibiotics	Resistant	Intermediate	Sensitive
Ampicillin	8 (40 %)	0 (0 %)	12 (60 %)
Chloramphenicol	11 (55 %)	5 (25 %)	4 (20 %)
Ciprofloxacin	14 (70 %)	3 (15 %)	3 (15 %)
Erythromycin	11 (55 %)	7 (35 %)	2 (10 %)
Gentamicin	18 (90 %)	1 (5 %)	1 (5 %)
Streptomycin	15 (75 %)	0 (0 %)	5 (25 %)
Vancomycin	7 (35 %)	0 (0 %)	13 (65%)

Comparative MIC of both bacteriocin-containing supernatant and partially purified ones were tested against *L. innocua*as an indicator and eight multi-antibiotic resistant enterococcal strains by microtiter plate method. As observed in Table 2 of eight strains, two were resistant to all four bacteriocin producer strains and two other were only sensitive toward S8. Furthermore, sample number 11 and 38 demonstrated acceptable sensitivity to all four tested Bac^+ strains however; all four Bac^+ enterococci were highly active toward strains 18 and 49. One of sensitive strains has shown enhanced tolerance to bacteriocins after overnight incubation. Based on these data, it can be assumed that most of the clinical enterococcal isolates are naturally sensitive to enterococcalbacteriocins.

Table 2. The comparative minimum inhibitory concentration (MIC) of the bacteriocin-containing supernatant and partially purified ones**.**

Sample number	Activity (BU)							
	Culture Supernatant				Partially Purified Bacteriocin			
	D1	D4	S8	O9	D1	D4	S8	O9
L. innocua	640	640	80	640	10200	10200	5120	10200
11	40	40	80	-	640	640	1280	20
13	-	-	20	-	-	-	320	-
17	-	-	80	-	-	-	1280	-
18	160	160	160	20	2560	2560	2560	320
37	-	-	-	-	-	-	-	-
38	40	40	20	20	640	640	320	320
39	-	-	-	-	-	-	-	-
49	640	640	160	20	10200	10200	160	320

Conclusions

In conclusion, enterococci can play significant role in human life both as useful probiotics and possible source of new antimicrobial agents as well as harmful deadly multi antibiotic resistance pathogens. Unfortunately, due to limited therapeutic options and the limited success in development of new agents with noticeable antibacterial activity, the elevating researches in this regard are inevitable. Interestingly, this detailed examination regard to the evaluation of comparative MIC of partially purified bacteriocins from enterococcal strains against the eight multi-antibiotic resistant enterococci originated from clinical human samples has demonstrated that the majority of the isolates were sensitive to at least one Bac+ enterococcal strains. It therefore seems that

bacteriocins have the potential to serve as a suitable alternative to conventional antibiotics. However, further study on their applicability is a necessity.

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Conflict of interest
The authors report no conflicts of interest in this work.

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