Pharmaceutical Sciences, September 2015, 21, 72-76
doi: 10.15171/PS.2015.20
http://journals.tbzmed.ac.ir/PHARM

Research Article

Evaluation of Bacteriocin Activities among Enterococcal Poultry Isolates from East Azarbaijan Iran

Vahid Javaherzadeh[1], Mahmood Jamshidian[1], Mohammadtaghi Zahraei[1], Arash Youseftabar[2], Morteza Milani[2], Maryam Hassan[3], Farzaneh Lotfipour[2*]

[1]*Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[2]*Gastrointestinal and Liver Disease Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*
[3]*Faculty of Pharmacy, Znjan University of Medical Sciences, Tabriz, Iran.*

Article info

Article History:
Received: 31 May 2015
Accepted: 6 June 2015
ePublished: 30 September 2015

Keywords:
Bacteriocin
Genes
Enterococci
Poultry

ABSTRACT

Background: The objectives of the study were to isolate and characterize the bacteriocin producing enterococci in 30 enterococcal poultry isolates from north-west of Iran and to determine the prevalence of bacteriocin structural genes among them. ***Methods:*** The obtained isolates were assessed for antibacterial action against 6 indicator strains. The PCR method was applied to detect previously identified enterocin genes. ***Results:*** Based on our results 4 (13.3%) of the examined poultry enterococcal isolates were considered as potential bacteriocinogenic strains, all of them identified to be *Enterococcus faecium* via phenotypic and genotypic examinations. At least one of the detected Bac^+ isolates had antibacterial activities against *Bacillus cereus, Listeria monocytogenes, Enterococcus faecalis and Enterococcus hirea* with no activity against *Escherichia coli* or *Staphylococcus aureus.* ***Conclusion****:* Genes encoding Enterocin A and enterocin LA50A/B are fairly distributed among the studied poultry enterococci.

Introduction

Antimicrobial peptides (AMPs) produced by a variety of microorganisms have gained lots of attention as novel antibacterial alternatives[1,2,3] to combat antibiotic resistance problem.[4,5] Bacteriocins have bactericidal action against closely related bacteria to producer strains.[6-8]

The bacteriocins have some properties which make them good candidates as new generation of antimicrobials: (i) their high potency, (ii) the narrow inhibition spectrum (iv) The high stability to many harsh conditions (iv) the AMPs are open to bioengineering.

Overall, Bacteriocins are categorized into three main groups.[9,10] Class I or lantibiotics (from lanthionine-containing bacteriocins) consist of small post translationally modified peptides (<5 kDa) and have the unusual amino acids lanthionine and methyl-lanthionine.[11] Class II include small (<10 kDa) heat-stable membrane active peptides which are the largest and most diverse group of bacteriocins among Gram-positive bacteria. They are typically cationic and amphiphilic and/or hydrophobic [9] which are further subdivided into three subclasses [10]: Class IIa consists of pediocin-like peptides with a strong antibacterial activity against listeria and enterococci.[12] Class IIb are included the bacteriocins whose their optimal action is rely on the action of two different peptides.[13] Other bacteriocin subgroups are the leaderless peptide and the circular bacteriocin.[8] Class III bacteriocins or bacteriolysins are big in size (>30 kDa) and heat-labile proteins.[14,15]

Biosynthesis of bacteriocins generally requires four types of genes, and these are often found in the same genetic locus: (1) the structural gene(s) encoding the bacteriocin itself (2) an immunity system. The AMP producer needs to encode an immunity mechanism to keep away from being killed by its own AMP. (3) A transport system. To be active outside the cell, the AMPs should be exported across the cell envelope. 4) Genes in charge for post-translational modifications (PTMs).

The mechanism of antibacterial action of bacteriocins differs deeply from classical antibiotics. In general, the bacteriocins can eradicate sensitive bacteria by two methods; in the first mechanism, they may target the cell envelope by producing pores or hindering cell wall biosynthesis which are observed among both Gram-negative and Gram-positive bacteriocins. In the second method, they may act intracellularly to inhibit essential enzymatic/metabolic processes such as DNA synthesis, transcription and translation which are found only among Gram-negative bacteriocins.

***Corresponding Author:** Farzaneh Lotfipour, Tel: (+98) 41 33392580, Fax: (+98) 41 33344798, E-mail: lotfipoor@tbzmed.ac.ir*

Enterococci from lactic acid bacteria have a significant position in human and animal health both as nosocomial pathogens and potential probiotics.[16-18] Their pathogenicity occurs from virulence factors such as cytolysin, gelatinase, aggregation substance, extracellular surface proteins and other adhesions. Additionally, they can carry multiple antibiotic resistant genes and spread resistance to other bacterial strains which is another concern related to application of enterococci in food fermentation or as probiotics.[18-20] Most of the characterized bacteriocins from *Enterococcus* spp. belong to the group II bacteriocins.
Animal originated bacteriocinogenic strains from Iran, especially of *Enterococcus* species, are poorly described. In our previous work, we performed a survey on the prevalence of bacteriocins of *Enterococcus* spp. isolates originated from different dairy samples in North-Western Iran.[21, 22] In the present study we aimed to investigate the prevalence of *Enterococcus* spp. isolates from poultry samples in East Azarbijan province of Iran. The antibacterial activity of the isolates, presence of bacteriocin and certain virulence genes as well as antibiotic resistance pattern of bacteriocin producers has been studied.

Materials and Methods

Isolation and phenotypic characterization of the Enterococcus genus

Enterococci were identified according to Manual of Clinical Microbiology [23] as follow: feces samples collected from industrial poultry farms of East Azerbaijan provinces of Iran during 2012 to 2014 were inoculated in 20 mL of Bile Esculin Broth for 24 h at 37 ºC. Then, 20 µL of cultures which had turned black as a result of growth of the enterococcal cells, were collected and plated onto Bile-Esculin sodium azaid agar. Following the incubation at 37 ºC single black colonies were collected and kept in 30% glycerol at -20 ºC as well as -80 ºC as stocks. The isolates were also subjected for enterococcal phenotypic characterization tests. Growth at 10 °C and 45 °C for 7 days, growth in modified Brain Heart Infusion broth (BHI, Scharlau, Spain) at apparent pH 4 and 9 containing 6.5% NaCl and Esculin hydrolysis in the presence of 40% bile salts (bile-Esculin agar, Scharlau Spain) were examined. Strains were also tested for survival at 60 °C for 30 min.[24,25]

Screening for anti-bacterial activity of enterococcal isolates

The inhibitory activities of the isolates against a group of indicator strains (listed in table 1) were evaluated by agar spot method.[26] Brain Heart Infusion broth (Oxoid, Hampshire, England) supplemented with 1.5% agar (Bacto, Fischer, USA) as well as 5 mL of BHI agar 0.7% was applied as base and top agar respectively. The top agar medium was inoculated with 20 µL of an overnight culture of indicator microorganisms. A single colony of each *Enterococcus* spp. poultry isolates was spotted onto a previously seeded agar plates with indicator bacteria. Subsequently, plates were incubated at 30 °C in an upright position. Isolates that showed clear zones of growth inhibition wider than 6 mm were scored positive as potential bacteriocin producers (Bac^+) and selected for further studies.

Table 1. Indicator bacteria used agar spot test.

Staphylococcus aureus	PTCC[§] 1112
Listeria monocytogenes	PTCC 1249
Bacillus cereus	PTCC 1015
Escherichia coli	PTCC 1533
Enterococcus faecalis	PTCC 1237
Enterococcus hirea	PTCC 1239

[§]PTCC= Persian Type Culture Collection

Genotypic differentiation of enterococcal isolates by PCR

Total DNA was extracted by using QiaPrep MiniPrep kit (Qiagen) according to the manufacturers' protocol. The isolated DNA was used for PCR (Polymerase Chain Reaction) template in the genotypic identification. The target strains were genotyped by 16S rDNA using *Enterococcus* specific and species-specific primers[27,28] which are listed in Table 2. PCR amplification for genotypic identification of the isolates performed in 25 µL of reaction mixture containing 1 µL of each primer set, 5 µL of 10 × PCR buffer (Cinnagen), 2.5 mmol of $MgCl_2$, 1 U of *Taq* DNA polymerase 5 U/mL (Cinnagen), and 200 µmol of each deoxyribonucleoside triphosphate (Cinnagen). Deionised sterile water and DNA extracted from known *Enterococcus* species substituted as the DNA template as the negative and positive controls in each PCR reaction. PCR amplifications were performed with the following thermal cycling profile, 4 min at 95 °C and followed by 35 cycles consisting of: a denaturation step for 45 s at 95 °C, annealing for 30 s at 60 °C (for universal), 54 °C (for *Enterococcus*-specific primers), 56 °C (for species-specific primers) and extension for 1 min at 72 °C, followed by a single 5 min final extension step at 72 °C (Eppendorf AG, Hamburg, Germany). The PCR products were analyzed by 1.5% agarose gel electrophoresis (Bio-Rad Power Pac Basic Singapore) which was set at 70 V for 45 min in Tris-Acetate-EDTA (TAE) buffer. The gel staining procedure was 15 min in 0.5 µg/mL of ethidium bromide visualizated by ultraviolet light (312 nm) with (RAPD PCR).

Detection of bacteriocin genes by PCR

Total DNA extracted from potential Bac^+ isolates (as previously described) was applied as template for PCR to search the presence of bacteriocin genes previously found between enterococci. The specific primer sets of enterocin A (*entA*), enterocin B (*entB*), enterocin P (*entP*),[29] bacteriocin 1071 (*bac1071*), enterocin L50A/B (*entL50A/B*), enterolysin A (*enlA*), cytolysin (*cyl*),[34] bacteriocin 31 (*bac31*), bacteriocin AS-48

(*bacAS-48*) [35] are shown in Table 2. PCR assay was performed using the primers (Table 2) and DNA templates in 25 µL reaction volume that contained 12.5 µL 2x PCR Master Mix (Cinnagen), 1 µL of each primer, 1 µL of template DNA and 10.5 µL water. Cycling parameters included 4 min initial denaturation at 94 ºC, followed by 40 cycles of 45 s at 95 ºC, 30 s at 56 ºC (for *entP*, *bacAS-48*, *bac31* and *entL50A/B*), 58 ºC in the case of (*entA*) and 60 ºC (for *entB*, *bac1071*, *cyl* and *enlA*) as annealing temperature, and 35 s at 72 ºC; this was followed by 10 min at 72 ºC. Amplified PCR fragments were resolved on 1% agarose gels. Specific enterocin sequences were verified by PCR product sequencing.

Table 2. PCR primers for detection of bacteriocin genes used in this study.

Primer	Sequence (5´-3´)	Fragment size(bp)	References
Universal amplification	f: AAYATGATIACIGGIGCIGCICARATGGA r: AYRTTITCICCIGGCATIACCAT	602	[27]
Enterococcus faecium	f: TTGAGGCAGACCAGATTGACG r: TATGACAGCGACTCCGATTCC	658	[27]
E. faecalis	f: ATCAAGTACAGTTAGTCTTTATTAG r: ACGATTCAAAGCTAACTGAATCAGT	941	[28]
Enterocin A	f: AAATATTATGGAAATGGAGTGTAT r: GCACTTCCCTGGAATTGCTC	126	[29]
Enterocin B	f: GAAAATGATCACAGAATGCCTA r: GTTGCATTTAGAGTATACATTTG	159	[29]
Enterocin P	f: TATGGTAATGGTGTTTATTGTAA r: ATGTCCCATACCTGCCAAAC	121	[29]
Enterocin L50A/B	f: TTGGGTGGCCTATTGTTAAA r: TCTATTGTCCATCCTTGTCCA	224	[30]
Bacteriocin 1071	f: ATGCTGTAGGTCCAGCTGC r: TTTCCAGGTCCTCCACCAGT	210	[31]
Bacteriocin AS-48	f: GAGGAGTATCATGGTTAAAGA r: ATATTGTTAAATTACCAA	339	[32]
Enterolysin A	f: CGCAGCTTCTAATGAGTGGT r: CATACACACTGCCATTTCCA	161	[33]
Cytolysin	f: TGGCGGTATTTTTACTGGAG r: TGAATCGCTTCCATTTCTTC	186	[33]

Results and Discussion

Isolation and phenotypic identification of the enterococcal strains

Thirty poultry isolates were phenotypically classified as *Enterococcus* spp., according to the results of the identification experiments. They are shown to be Gram-positive, non-motile, catalase negative cocci. They grew in modified BHI media containing 6.5% NaCl in the temperature of 10 °C to 45 °C for up to 7 days and endured in buffered media at pH 4 and 9. All isolates were able to hydrolyze Esculin in the presence of 40% bile salts and turned medium color to black.[23]

Screening of the inhibitory activity of the enterococcal strains

Isolates of *Enterococcus* spp. were assessed for production of antimicrobial substances. Four of 30 enterococcus isolates (13.3%) showed antimicrobial activity by producing a clear growth inhibition zone on BHI agar against at least one of the indicator strains as shown in figure 1.

At least one of the detected Bac^+ isolates had antibacterial activities against, *B. cereus*, *L.* monocytogenes, E. faecalis and E. hirea with no activity against E. coli or S. aureus (Table 3).

Figure 1. Antimicrobial activity of poultry enterococcal isolates (right to left: P1, P2, and P3) by producing a clear growth inhibition zone on BHI agar against *E. feacalis* indicator strain.

All of the detectable antimicrobial activities were produced only on the solid surfaces (agar plates) and no antimicrobial activity could be found in broth medium after growth (Table 3). Similar observations regarding the antimicrobial activity on solid and liquid media have also been reported in previous studies.[36,37] These conflicting findings might be explained by the effect of growth conditions such as pH, medium composition and temperature on regulation of bacteriocin production [30,36,38] as well as frequent involvement of bacteriocin-like peptides which can act as peptide

pheromone in the quorum-sensing regulatory mechanism of bacteriocin gene expression[8,39-41] might explain these conflicting results.

Table 3. The inhibitory spectra of Bac$^+$ strains against selected indicator bacteria.

Isolate Name	Inhibition zone diameter (mm) with indicator strains					
	***E. feacalis* PTCC 1237**	***E. hirea* PTCC 1239**	***Li. mono.* PTCC 1249**	***S. aureus* PTCC 1112**	***E. coli* PTCC *1533***	***B. cereus* PTCC *1015***
P1	14	-	12	-	-	14
P2	12	12	12	-	-	12
P3	12	-	16	-	-	12
P4	10	-	-	-	-	-

Genotypic identification of enterococcal isolates by PCR

Genus and species identification was accomplished by PCR using the universal bacterial 16S rDNA, *Enterococcus* species specific primers. All of our Bac$^+$ enterococcal isolates (4 isolates) were identified as *E. faecium* (Fig. 2). However, some of previous studies showed that in *Enterococcus* isolates, the bacteriocinogenic phenotype was more dominant in isolates of *E. faecalis* than *E. faecium* [35, 42].

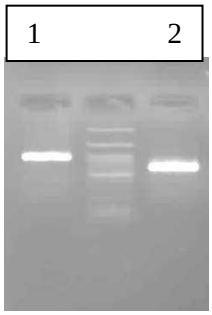

Figure 2. Agarose gel electrophoresis of PCR amplification with specific primers for a selected isolate (P1). (Lanes 1) Bacterial universal 16S rDNA (602 bps); (Lane 2) 100-bp DNA ladder; (lane 3) *E. faecium* (658 bps).

Screening of structural bacteriocin encoding genes in enterococcal isolates

The isolates with positive antibacterial activity on agar spot test were screened by PCR for the genes encoding the known bacteriocins. The results are shown in Table 4. *EntA* were found as the most frequent gene encoding bacteriocins, at an incidence of 100% among bac$^+$ strains (Fig 3). Also *entL50A/B* (87%) and *cyl* (67%) were detected frequently. Interestingly, *entB*, *entP*, *bac31*, *bac1071* and *bacAS-48* were not detected in any of the Bac$^+$ isolates. According to the reports in this regard, *entA* and *entL50A/B* and cyl are the most prevalent bacteriocin genes described.[43] The PCR results (Table 4) suggest that all of Bac$^+$ isolates contained multiple structural bacteriocin genes. Two different structural genes were found in all four isolates.

According to results of earlier studies the structural gene *entA* is widely distributed in ruminal enterococci.[44,45] Our results indicate that the incidence of *entA* and also *entL50A/B* are frequently found in enterococcal isolates from dairy origin and are in line with these findings. Additionally, according to our previous study, same genes of bacteriocin have been found in human blood and fecal derived enterococci; it is rational to believe that because of the extensive distribution of enterococcal contamination in nature they are likely derived from the same environment and most probably from intestine of various animals.

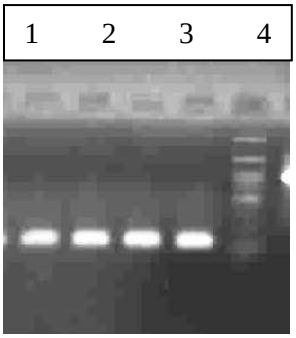

Figure 3. Agarose gel electrophoresis of PCR products from enterocin A (155 bp)screening.
a) Lanes 1 to 4: Bacteriocin producer enterococci; 5: 100-bp DNA ladder.

Conclusion

In conclusion, this work has shown that more than 13% of tested poultry isolates of enterococci displayed antimicrobial activity against closely related species and to be considered as potential bacteriocinogenic strains. All of the positive isolates were identified to be *Enterococcus faecium* via phenotypic and genotypic examinations. *EntA* and *entL50A/B* were detected frequently among the positive strains. Further study on the safety to human, virulence factors and antibiotic resistance pattern are needed in future studies.

Conflict of Interest

The authors report no conflicts of interest.

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