Original Article

Impact of Putative Bacteriocins against Multidrug Resistant Clinical Isolates

1. Samyyia Abrar 2. Saba Riaz

1. PG Student, Deptt. of Microbiology and Molecular Genetics 2. Asstt. Prof. of Microbiology and Molecular Genetics, University of the Punjab, Lahore

ABSTRACT

Objective: To use one of such mechanism like bacteriocins, produced by Lactobacilli activities against pathogens.
Study Design: Analytical / observational study
Place and Duration of Study: This study was carried out at Gulab Devi Chest Hospital, Lahore from November 2012 to January 2013.
Materials and Methods: This study included 203 clinical samples. Multidrug resistant clinical isolates were selected on the basis of their MAR (Multiple antibiotic resistances) index, antibiotic susceptibility testing, methicillin resistant Staphylococcus aureus (MRSA) with oxacillin disc, double disc synergism and combination disc test. Plasmid isolation, conjugation was performed. Well-Diffusion assay was used for screening of putative bacteriocins produced by Lactobacillus strains against MDRs. Physiological characterization of antimicrobial compounds and protein estimation was analyzed.
Results: Twenty five strains were selected based on MAR index (>0.2). In which 6 MRSA and 19 extended spectrum beta-lacatmases (ESBL) producers were further proceeded for antimicrobial activity with putative bactericins. Plasmid was easily transferred their resistance by the process of conjugation. Five bacteriocins were obtained from Lactobacillus strains isolated from commercial products. These bacteriocins showed a strong anti-bacterial activity against selected MDRs. Decrease in zone sizes was observed when putative bacteriocins were treated with heat, SDS (Sodium dodecyl sulfate) and Protinase k. Putative bacteriocins produced by Lactobacilli exhibit significant antibacterial activity against MDRs. SA1 has high antibacterial activity with high protein content of 13mg/ml.
Conclusion: Putative bacteriocins produced by Lactobacilli exhibit significant antibacterial activity against selected MDRs. MDRs have ability to transfer their resistance to other bacteria. The peptidal component of these bacteriocins can be used as an alternative therapy. Proper hospital policies require minimizing the horizontal spread of MDRs. Hence, it is necessary to purify the antibacterial molecule out of putative bacteriocin for further analysis.
Key words: MDRs, Bacteriocins, Lactobacilli, Antibiotic Resistance, MAR, Antibacterial activity

Citation of article: Abrar S, Riaz S. Impact of Putative Bacteriocins against Multidrug Resistant Clinical Isolates. Med Forum 2016;27(2):28-32

INTRODUCTION

Antibiotics are becoming non-responsive against many bacteria from last three decades. This is because of excessive, indiscriminate use of drugs in agriculture, health sector and even in veterinary medicine.[1] This misuse of antibiotics is creating troubles in treating the different infections, hence increasing the rate of morbidity and mortality.[2] The un-prescribed utilization of the antibiotics is considered as major source behind the high level of drug resistance. Horizontal gene transfer has contributed a lot in the spread of such infections. Nosocomial and community acquired infections are the consequences of this uncontrolled usage of drug.[3] To combat with such issue, much work is being done on the use of natural products as antimicrobial agents.[4] The use of medicinal plants as antimicrobial therapy is very common and an old age practice. Most of the antibiotics available today come from natural origin particularly from microbial sources.[5] Microbial solutions to the microbial problems are one of the best possible solutions to this problem. Lactobacilli are the well-known friendly bacteria for their beneficial activities against pathogens. The antagonistic activity of lactic acid bacteria is debatable due to different substances such as hydrogen peroxide, diacetyl, lactic acid and bacteriocin.[6] The bacteriocins are antimicrobial peptides, naturally produced by Lactic Acid Bacteria (LAB). Several bacteriocins producing strains have been isolated from raw and fermented products.[7] Bacteriocins produced by these strains have been assessed for potential application as therapeutic agents. Anionic lipids are present in abundance in the cellular membranes; antibacterial peptides interact with those lipids, thereby initiating the pore formation in the membranes of susceptible cells.[8]

In the present work, the multidrug resistant strains (MDRs) were isolated and then biochemically characterized. Phenotypic and genotypic

Correspondence: Dr. Saba Riaz,
Asstt. Prof. of Microbiology and Molecular Genetics,
University of the Punjab, Lahore
Cell No.: 03214530648
E-mail: saba.mmg@pu.edu.pk

characterization was performed for confirmation of MDR. Putative bacteriocins was isolated from five Lactobacilli and checked for the nature of bacteriocins by different treatments.

MATERIALS AND METHODS

Two hundred and three clinical samples were analyzed during November 2012 to January 2013 in Gulab Devi Chest Hospital, Lahore. Samples include pus, sputum, blood, body fluids etc. Strain identification was done by using biochemical profiling and analytical profile index (API) according to the guidelines provided by Clinical and Laboratory Standards Institute (CLSI).[9]

Antibiotic susceptibility testing was performed using the modified Kirby-Bauer disk diffusion method according to CLSI guidelines. The antibiotic disks (HiMedia, Mumbai, India) usedwere Ampicillin (10µg), Piperacillin + Tazobactam (100/10 µg), Ceftriaxone (30 µg), Cefotaxime (30µg), Ciprofloxacin (5 µg), Norfloxacin (10 µg), Cephtazidime (30 µg), Amikacin(30 µg), Gentamycin (10 µg), Cotrimoxazole (5µg), Imepenem (10 µg), Meropenem (10 µg), Clindamycin (2 µg), Nitrofeurontoin (300µg), Oxacillin (1 µg), Vancomycin (30 µg) and Augmentin (10 µg). Multiple antibiotic resistance index (MAR) is helpful in analyzing health risk, and is used to check the antibiotic resistance. The MAR Index of an isolate is defined as "a/b", where "a" represents the number of antibiotics to which the isolate was resistant and "b" represents the number of antibiotics to which the isolate was subjected.

A disc of augmentin (20 µg amoxicillin + 10 µg CLA) was placed on the surface of Muller Hinton (MH), then discs of cefpodoxime (30 µg), CAZ (30 µg) and CTX (30 µg) were kept around it in such a way, that each disc was at distance ranging between 20 and 22 mm from the augmentin disc (centre to centre). The plate was incubated at 37°C overnight.

CAZ and CTX disc with and without clavulanic acid were placed on the agar at a distance more than 30mm. After that, plates were incubated at 37°C and observed zone of inhibition after 24hours. If the zone of inhibition increased upto 5 mm with the addition of clavulanic acid than the strain was reported to as positive for combination disc (CD) test. Plasmid isolation was done by rapid mini-prep isolation method. Finally the bands were visualized in 0.7% agarose gel with 0.5 mg/ml of ethidium bromide in 0.5 X TAE buffer.

Conjugation is the process by which DNA is transferred from one bacterial cell to another. The mechanism requires a direct contact between a donor and a recipient cell. Escherichia coli K12 and DH5α strains were used as donor strains. Donor and recipient were grown overnight at 37°C in N-broth supplemented with respective antibiotics. Conjugation mixtures were prepared by mixing Donor and Recipient in the ratio of 1:9, 1:1 and 9:1 respectively. Mating mixtures were incubated at 37°C for 18-24 hours. After incubation, 50µl of conjugation mixture was plated on N- Agar plates (containing respective antibiotics that were used as markers). Results were recorded after 18-24 hours of incubation at 37°C.

Samples were prepared by making dilutions of yoghurt, cheese and probiotic sachets. 50µl of 10^{-4}dilution of each sample was plated on MRS agar[10]. Biochemical tests were performed for the identification of strains and phylogenetic analysis was used for confirm identification

Isolated strains were characterized morphologically, microscopically as well as biochemically. Growth at different temperatures (15, 25 and 37ºC), acid tolerance test (pH 2, 3, 5, 7 and 9) and acid production was evaluated in which supplementation of media with different sugars normally present in diet was used. Minimal medium was supplemented with 4% sugars (glucose, lactose and sucrose).[11] Strains were inoculated in MRS broth. After specific incubation, broth culture from each tube was transferred to eppendorf. Supernatant was obtained by centrifugation at 10,000 rpm for 10 min. Addition of 5 mol l^{-1} of NaOH was used as neutralizing agent.[12] Cell free extract was then used to check inhibitory effect against characterized MDRs.[13] Wells were cut in swabbed plates and 100µl of the cell free supernatant of the Lactobacillus strains were placed into each well. Plates were incubated at 37ºC for 24 hours.[14] A clear zone of inhibition of at least 2mm was reported positive inhibitory activity.[15]

Putative bacteriocins were treated with SDS, high temperature and proteinase k enzyme [16].Cell free supernatant was treated with 20% SDS (sodium dodecyl sulphate) for 5mints, 5 µl of proteinase k for 2hrs and heated at 80ºC for 15 minutes. After treatment, 100µl of supernatant was placed in the agar wells and incubated at 37 ºC for 24 hrs. After incubation, zone sizes of treated and untreated extract were measure.

Protein content present in the antibacterial compounds of selected strains was estimated by the bradford method. Protein concentration was determined by comparing the curve with the standard curve of BSA.

16SrRNA sequencing was performed, phylogenetic analysis was established and tree was constructed by MEGA4 software. The sequenced data was refined and submitted to Gen Bank, and the accession numbers were obtained.

RESULTS

Two hundred and three clinical strains were analyzed. 135 member of Enterobacteriaceae were confirmed by API. Enterobacteriaceae confers resistance against ampicillin, amikacin, cephtazidime, gentamycin, augmentin, cotrimoxazole ceftriaxone, cefuroxime, ciprofloxacin and tazocin. Selected Staphylococcus aureus strains were resistant to augmentin, ampicillin,

cephradine, ciprofloxacin, gentamycin, ceftriaxone, cefuroxime, clindamycin, imepenum, oxacillin. When the gender based study was conducted it was observed that Escherichia coli resistance was higher in female. In Staphylococcus aureus, resistance in female was about 92% and only 23% in males. Overall resistance of antibiotics in Staphylococcus aureuswas 79% which was quite higher.MAR value ranges from 0.2 to 0.95 among different strains (Table 1).

Total 17 suspected ESBLs strains were tested. Among them 10 strains showed synergism of antibiotic CAZ with Amoxicillin. 4 strains showed Synergism of CTX with AMC. This indicated that 47% strains were determined ESBLs by this technique. Total 17 Suspected strains were tested for ESBLs character. 11 strains indicated positive test towards combination of CTX and CAZ with Clavunate. 65% were determined ESBLs positively by this test (Fig. 1). Plasmids of Selected strains were extracted and all of the tested strains possessed plasmid DNA. All the strains that harbor plasmid DNA were tested for conjugation and these strains indicated transfer of plasmids in the recipient strains. Total 15 strains were isolated on MRS Agar. Samples used were Yoghurt, cheese as well as commercial probiotic sachet. Out of 15 isolated strains 10 were from Yoghurt sample, 2 from cheese sample and 3 were obtained from probiotic sachet and they were named as SY1-10, SC1, 2 and SA1-5 respectively. Antibacterial compound was stable at high temperature treatment but significant decrease in zone sizes was observed when treated with 20% SDS and 5µl of Proteinase k (Fig. 2). This decrease in zone sizes indicated the proteinaceous nature of the compound.

Table No.1: Multiple antibiotic resistance (MAR) value of bacterial strains (n = 25)

MAR values	Number of strains (%)
0.2 – 0.4	16
0.41 – 0.6	20
0.61-0.8	36
0.81-1.0	28

DNA sequencing was done for selected strains. NCBI nucleotide blast was used to determine homology of consensus sequence. Phylogenetic tree was formulated. It was observed that the Blast results for strain SA1 and SA3 showed 99% similarity with Lactobacillus casei while SC1 and SY1 both showed 99 to 100% similarity with Lactobacillus paracasei. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.00532341 is shown. Accession no for strain SA1=KC967209, SA3=KC967210, SC1=KC967211 and SY1= KC967212 (Fig. 3).

Out of 15 isolated strains 10 appeared as non-motile, gram positive bacilli with negative results for catalase production as well as cytochrome oxidase production. Moreover, these strains showed maximum growth at 25º C and 37 ºC but growth was also observed at 15º C for some strains. All strains were stable at basic pH 5, 7 and 9butonly 8 strains were stable at basic as well as acidic pH (2 and 3). Acid production was also observed for some strains when different carbohydrate sources were used. Generally high production of acid was observed with 4% sucrose as a carbohydrate source, with the exception of SA2 (Table 2).

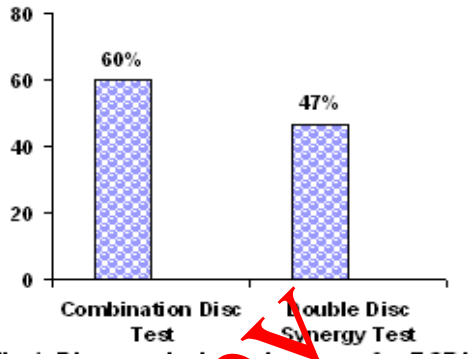

Figure No.1:Phenotypic detection tests for ESBLs

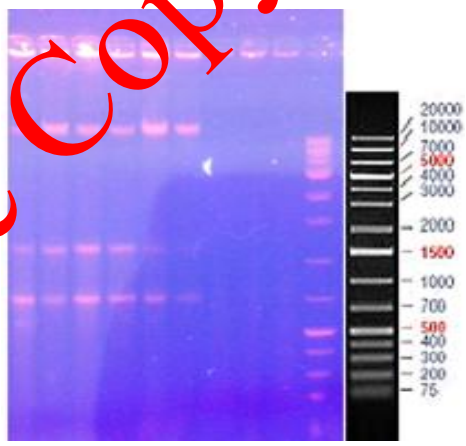

Figure No.2: Plasmid DNA extraction

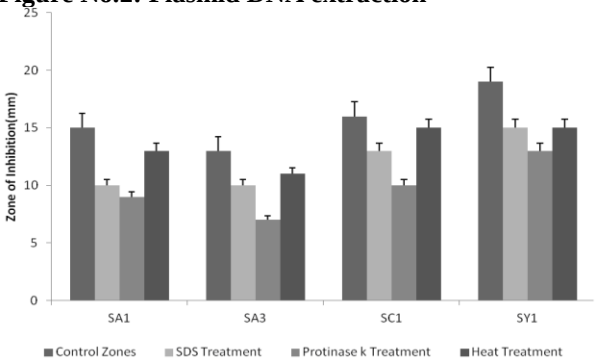

Figure No. 3: Effect of different treatment on antibacterial compound

All the characterized bacterial strains were observed for their growth inhibition activity against different MDRs. Only five strains showed significant activity against selected MDRs. Strains obtained from yoghurt SY1 were most effective against gram negative strains specifically Enterobacteriaceae. While, for gram

positive strains including Staphylococcus aureus and Streptococcus pyogenes, most effective strains were those obtained from cheese and probiotic sachet, SC1 and SA1.

Comparison of the optical density of antibacterial compounds with standard curve indicated that all the strains showed different protein contents. SY1 contain 10 mg/ml while SC1 contain 7mg/ml in its antibacterial compound (Table 2).

Table No.2: Characterization of lactic acid bacteria

Strain name	Production of acid value			Growth at Different Temperatures				Growth at Different pH				Protein Content in Antibacterial Compound	
	Sucrose	Glucose	Lactose	15ºC	30ºC	37ºC	45ºC	6.8	5.0	3.0	2.0	OD at 595nm	Conc. (mg/ml)
SA1	40.18	34.65	37.22	+	++	++	+	++	++	+	+	1.45	4.0
SA2	40.81	45.23	45.04	-	++	++	+	++	++	-	+	1.40	2.0
SA3	36.24	36.41	34.67	+	++	++	+	++	++	++	++	1.43	3.0
SC1	42.70	39.94	35.60	+	++	++	-	++	++	-	-	1.50	7.0
SY1	43.42	36.12	40.36	-	-	++	-	++	+++	++	+	1.55	10
SY2	37.95	34.37	37.67	-	-	++	-	++	+++	++	+	1.53	8.0

DISCUSSION

A few years ago availability and discovery of new life saving drugs was considered as like vanishing the sorrows of the developing world. But, in western world bacterial strains are becoming highly resistant to the antibiotics that were used previously. Few decades ago, focus was particularly on MRSA (Methicillin Resistant Staphylococcus aureus) and vancomycin-resistant Enterococcus spp. Multidrug resistance in gram negative bacteria is going in an alarming situation.[17]

Here, higher resistance towards augmentin was reported (>90%) but in another study > 34.4% resistance has been reported. NOR had higher resistance profiles, similar results were observed in another study in which 18.5% resistance for this antibiotic among different clinical isolates.[18] This increase in bacterial resistance towards these antibiotics may be due to specific genetic makeup of the pathogens, antibiotics utilization including the widespread use of systemic antibiotics, less information about the utilization of antibiotics.[19] Horizontal gene transfer contributes towards the spontaneous spread of an epidemic which is predominantly due to mobile genetic elements. Other contributing factors may include improper dosage, misuse of antibiotics for non-bacterial infections, self-medication, extended duration of therapy, globalization as well as migration.[20]

Physiological characterization of isolated strains showed that these strains have ability to grow on large scale as they grow effectively at 25ºC and 37ºC growth was also observed at 15ºC. Another important criterion for these strains is to the tolerance of acidic environment of our GI tract that is as low as 1.5[21,22] and it is usually observed when a person is under fasting condition.[23] A probiotic source is considered as good if it remains stable at pH as low as 3. When these strains were grown at lower pH i.e. 2 and 3, reduction in their growth was observed but still they tolerated the extreme pH. High resistance to temperature and stability at low pH, makes these compounds more useful even inhuman gastrointestinal tract.[24] Protein content of the antibacterial compound effects directs to its antibacterial activity. This is an indirect indicator that the antibacterial compound is a protein product.

It has been predicted that in very near future antibiotic resistance will make healthcare professionals helpless towards effective therapies for bacterial infections. Consequently, there is an urgent need to search for unconventional antibiotics. Interest in peptide antibiotics has increased greatly during the past decade, as these are believed to be very potent and are biologically and chemically very diverse. They show higher activity and higher specificity towards their target. Moreover, they have few toxicological problems and their accumulation in organs is not observed quite often. There is few drug-drug interaction problems have been observed for peptide antibiotics.[25]

The antagonistic activity of the putative compounds secreted by the strains exerts either inhibitory or bactericidal activity. But, for the in vivo utilization of these bacterial strains it is necessary to know the process by which the bacteria is producing specific compound or compounds.

CONCLUSION

Putative bacteriocins produced by Lactobacilli exhibit significant antibacterial activity against selected MDRs. MDRs have ability to transfer their resistance to other bacteria. The peptidal component of these bacteriocins can be used as an alternative therapy. Proper hospital policies require minimizing the horizontal spread of MDRs. Hence, it is necessary to purify the antibacterial molecule out of putative bacteriocin for further analysis.

Acknowledgement: Authors wish to express their gratitude to staff of Gulab Devi Chest Hospital for their kind help in assistance of research.

Conflict of Interest: The study has no conflict of interest to declare by any author.

REFERENCES

1. McDermott PF, Walker R, White DG. Antimicrobials: modes of action and mechanisms of resistance. Int J Toxicol 2003;22:135-43.
2. White GD. Antimicrobial resistance in pathogenic Escherichia coli from animals. Emerg Infec Dis 2006; 44:145–66.
3. Pfaller MA, Jones RN, Marshall SA. Inducible amp C beta-lactamase producing gram-negative bacilli from blood stream infections: frequency, antimicrobial susceptibility, and molecular epidemiology in a national surveillance program (SCOPE). Diag Micr Infec Dis 1997; 28:211-9.
4. Riaz S, Faisal M, Hasnain S. Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum β-lactamase (ESBL) producing Escherichia coli and Klebsiella species in Pakistan. Afr J Biotechnol 2011; 10:6325-31.
5. Saleem M, Nazir M, Ali MS, Hussain H, Lee YS, Riaz N, et al. Antimicrobial natural products: an update on future antibiotic drug candidates. Nat Prod Rep 2010; 27:238–254.
6. Remiger A, Eijsink MA, Ehrmann. Purification and partial amino acid sequence of plantaricin 1.25ααα and 1.25βββ, two bacteriocins produced by Lactobacillus plantarum TMW1.25. . App Microbiol 1999; 86:1053-8.
7. Rodriguez E, Gonzalis B, Gaya P, Nuñez M, Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. Int Dairy J 2000;10:7-15.
8. Moll GN, Konings WN, Driessen JM. Bacteriocins: mechanism of membrane insertion and pore formation. Anton van Leeuw J Microb 1999;76:185–98.
9. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; Twenty-first informational supplement; M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA, 2011.
10. De Man JC, Rogosa M, Sharpe ME. A medium for the cultivation of Lactobacilli. J Appl Bacteriol 1960; 23:130-35.
11. Bosch M, Molina JN, Audivert S, Bonachera MA, Alemany AS, Fuentes MC, Cuñé J. Isolation and characterization of probiotic strains for improving oral health. Arch Oral Biol 2011; 2691:413-24.
12. Nawaz SK, Riaz S, Hasnain S. Screening for anti-methicillin resistant Staphylococcus aureus (MRSA) bacteriocin producing bacteria. Afr J Biotechnol 2009; 8:365-8.
13. Schillinger U, Lucke FK. Antibacterial activity of Lactobacillus sake isolated from meat. Appl Environ Microb 1989; 55:1901-6.
14. Ivanova I, Kabadjova P, Pantev A, Danova S, Dousset X. Detection, purification and partial characterization of a novel bacteriocin, substance produced by Lactoccouslactis subsp. lactis b14 isolated from Boza-Bulgarian traditional cereal beverage. Biocatalysis 2000; 41: 47-53.
15. van Reenen CA, Dicks LMT, Chikindas ML. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by Lactobacillus plantarum. J Appl Microb 1998; 84: 1131–7.
16. Rajaram G, Manivasagan P, Thilagavathi B, Saravanakumar A. Purification and Characterization of a Bacteriocin Produced by Lactobacillus lactis Isolated from Marine Environment. In. J F Sci Tech 2009; 2: 138-44.
17. Rice LB. The clinical consequences of antimicrobial resistance. Curr Opin Microbiol 2009; 12: 476–81.
18. Guay DR. An update on the role of nitrofurans in the management of urinary tract infections. Drugs 2001; 61: 353–64.
19. Harbarth S, Samore MH. Antimicrobial resistance determinants and future control. Emerg Infect Dis 2005; 11:794–801.
20. Sharma S. Antibiotic resistance in ocular bacterial pathogens. Indian J Med Microbiol 2001;29: 218-22.
21. Lin WH, Hwang CF, Chen LW and Tsen HY. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microbiol 2006;23: 74-81.
22. Huang Y, Adams MC. In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Int J Food Microbiol 2004; 91: 253-60.
23. Fernandez MF, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J Appl Microbiol 2003; 94: 449-55.
24. Russel JB, Mantovani HC. Thebacteriocins of ruminal bacteria and their potential as an alternative to antibiotics. J Mol Microbiol Biotechnol 2004;4:347–55.
25. Rehman S, Khanum, A. Isolation and characterization of peptide(s) from Pisum Sativum having antimicrobial activity against various Bacteria. Pak J Bot 2001; 43: 2971-78..