

## SCREENING AND IDENTIFICATION OF AQUATIC BACTERIOCINOGENIC BACILLUS STRAINS INHIBITING CLINICAL METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* AND VANCOMYCIN RESISTANT *ENTEROCOCCUS* FROM PAKISTAN

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### ABSTRACT

**Objective:** The purpose of the current study was to screen and identify novel bacteriocinogenic strains from marine, fresh and estuarine water bodies which can serve as alternatives to the antibiotics, as an antibiotic resistance is on the rise.

**Methods:** Altogether seven fresh, brackish, and marine water samples from various regions of Sindh were collected. All the aquatic bacterial strains were isolated by standard plate count and overlay methods. The bacteriocinogenic strains were screened by spot agar, stab and overlay, cross streak and agar well-diffusion techniques.

**Results:** Of the total 146 isolates, 41 (28%) were bacteriocinogenic, among which 28 (68%) were isolated from freshwater, 12 (29%) from marine and 1 (3%) from brackish water. The most significant bacteriocinogenic strains among the isolates were identified as *Bacillus coagulans*, *Bacillus simplex*, *Bacillus macerans* and *Bacillus circulans*. When tested against 17 significant pathogens, these strains could inhibit various clinical drug-resistant Gram-positive pathogens such as *Staphylococcus aureus* and methicillin-resistant *S. aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and vancomycin-resistant *Enterococcus* and *Corynebacterium diphtheriae*. Furthermore, *Escherichia coli* and *Klebsiella* spp. were observed to be sensitive against two bacteriocinogenic strains of *B. coagulans*. Furthermore, 59% of the aquatic isolates were sensitive against one of the bacteriocinogenic strain indicating competitive antagonism phenomenon.

**Conclusion:** This research study reveals the bacteriocinogenic potential of indigenous aquatic isolates against drug resistant clinical pathogens as well as against fish pathogens.

**Keywords:** Bacteriocin, Antagonistic, Methicillin-resistant *Staphylococcus aureus*, Vancomycin-resistant *Enterococcus*, Drug resistance.

### INTRODUCTION

The earth covers about 70% of its surface with water in the form of oceans, seas, rivers, estuaries, lakes, ponds, streams, canals, wetlands, etc. The aqueous environment is comprised of a diverse population of microscopic inhabitants that are capable of producing novel groups of bioactive compounds [1]. Nevertheless, the potential of marine and other aquatic environment has not been adequately explored.

The category of bacteriocins is the natural proteinaceous compounds produced by certain bacterial species, which can have broad-based applications. Bacteriocins are the ribosomally encoded proteins of bacteria that are efficient in antagonizing the organisms which have similar genetic origin as their producers [2]. In eighties, a number of publications both on colicin type and non-colicin type bacteriocins increased, but the attribution of nisin as generally regarded as safe (GRAS) status by Food and Drug Administration in 1988 unleashed interest in the bacteriocins produced by lactic acid fermenting bacteria. Bacteriocins are mostly produced by Eubacteria (true bacteria) such as *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Escherichia*, *Yersinia*, *Actinomycetes*, etc. Certain Archaeobacteria are also capable of producing distinct type of bacteriocins called "halocins" [3].

At present, there are relatively few reports in the literature of antibacterial peptides or proteins produced by aquatic bacteria. Bacteriocins like inhibitory substances (BLIS) have been described mainly from marine bacteria. Halocin H4 by *Haloferax mediterranei* was first reported by Rodriguez-Valera et al., 1982 and has been studied in details [4]. Halocin, H6 production from *Haloferax gibbonsii* was first reported by Torreblanca et al. in 1989. These organisms belong to the family Halobacteriaceae which are extremely halophilic archaeobacteria that inhabit aquatic

hypersaline environments. In 2004, Pirzada et al. isolated and studied a bacteriocinogenic strain ZM81, a Gram-positive pleomorphic rod, which was isolated from the open sea region of Karachi [5]. A novel, pH-tolerant and thermostable bacteriocin BL8, active against Gram-positive bacteria, was isolated from marine sediment *Bacillus licheniformis* [6]. Wilson et al. (2009) have isolated eight marine bacteria which produced anti-bacterial substances from a variety of marine invertebrates. Bacteria isolated from marine sponges also show antimicrobial properties.

Bacteriocins from aquatic habitats have also been seen to be effective against significant clinical pathogens. Longeon et al. (2004) reported BLIS from marine *Pseudoalteromonas* sp. X 153 having antibacterial activity against human pathogens collected from different substrates on the littoral of Brittany [7]. This antibacterial protein was reported to be 280 kDa size. Similarly, some reports about the antimicrobial properties of bacteria from *Bacillus pumilus* strain isolated from water having anti-methicillin resistant *Staphylococcus aureus* (MRSA) and anti-vancomycin resistant *Enterococcus* (VRE) are also reported [8]. Furthermore, bacteriocins and antimicrobial substances of moderately halotolerant bacteria from cenotes of Yucatan peninsula were active against six clinical pathogens [9].

In this era, the multi-drug resistant pathogenic microorganisms as well as the chemical food preservatives have become a potential threat to human health. The potential of novel bacteriocins from the aquatic environment has not been adequately explored. Therefore, it is the dire need of the time to look for the alternatives to the antibiotics as the antibiotic resistance is on the rise, and the choice of effective antibiotics is getting narrower. The purpose of the current study was to screen and identify novel bacteriocinogenic strains from different aquatic habitats like marine, fresh and estuarine water bodies.

## METHODS

### Sample collection

The aquatic water samples were collected from the River Indus, Keenjhar Lake, Arabian Sea (Cape Mount, Sandspit, Sea view sites) and Khapur Creek estuary of Sindh, Pakistan from February to January 2012 (Table 1).

### Isolation of the bacteria

All the samples were quantitatively and qualitatively analyzed by pour plate and spread plate methods using nutrient agar medium. Plates were incubated for 7-10 days at room temperature.

### Screening for bacteriocinogenic activity

In order to check the antimicrobial potential of the aquatic strains, the indicator strain of *Micrococcus luteus* was tested against the aquatic isolates by four different bacteriocin screening methods: Spot agar [10], stab and overlay [11], cross streak [12] and agar well diffusion methods [13]. The bacteriocinogenic strains were preserved in frozen glycerol vials.

### Identification of bacteriocinogenic strains

All the bacteriocinogenic strains were identified on the basis of colonial, microscopic, and biochemical characteristics with the help of Bergey's manual of systematic bacteriology Vol. 3: The firmicutes and the online service of advanced bacterial identification software. [http://www.tgw1916.net/bacteria\\_abis.html](http://www.tgw1916.net/bacteria_abis.html).

### Activity against pathogens

The most significant four bacteriocinogenic strains were tested against the pathogenic clinical strains of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *S faecalis*, *VRE*, *S. aureus*, *MRSA*, *Corynebacterium diphtheriae*, *Corynebacterium xerosis*, *Bacillus subtilis*, *M. luteus*, *Escherichia coli*, *Salmonella typhi* and *paratyphi B*, *Shigella dysenteriae*, *Proteus mirabilis*, *Klebsiella* spp. and *Pseudomonas aeruginosa* by cross streak method [12].

### Antagonism among aquatic microorganisms

For further determining the extent of antagonism among the aquatic isolates, the marine bacteriocinogenic strain ZP-55 was tested against 39 freshwater and marine isolates by cross streak method [12].

### Bacteriocin titre

The 24 hrs bacterial suspension was centrifuged twice (at 4000 rpm for 30 minutes and at 10,000 rpm for 15 minutes). The CFNS of each strain

was serially diluted by 10 folds in sterile nutrient broth. Antimicrobial activity was assayed of all the dilutions and undiluted supernatant against *M. luteus* as an indicator [14].

$$\text{AU / mL} = \frac{\text{Reciprocal of the highest dilution giving inhibition} \times 1000}{\text{Volume of bacteriocin}}$$

## RESULTS

In this study, aquatic samples from diversified marine, freshwater, and estuarine origins like River Indus, Keenjhar lake, Arabian sea (Cape Mount, Sandspit, Sea view sites) and Khapur Creek estuary of Sindh, Pakistan were collected and microbiologically analyzed during February-November, 2011 (Table 1). All the aquatic bacterial strains were isolated by standard plate count and overlay methods.

Altogether 41 (28%) bacteriocinogenic strains out of the total 146 isolates from fresh and marine water active against the indicator strain of *M. luteus* by applying four bacteriocinogenic activity monitoring methods i.e., spot agar, stab and overlay, cross streak and agar well diffusion methods were obtained (Fig. 1). Among these, 28 (68%) were isolated from freshwater, 12 (29%) from marine, and 1 (3%) from brackish water (Fig. 2). Majority of the bacteriocinogenic strains were the inhabitants of freshwater bodies and were Gram-positive. The significant producers belong to genus *Bacillus* and identified as *Bacillus coagulans*, *Bacillus simplex*, *Bacillus macerans* and *Bacillus circulans*.

When four of the selected bacteriocinogenic strains tested against 17 pathogens, these strains could inhibit various significant clinical and drug-resistant Gram-positive pathogens like *S. aureus* and *MRSA*, *S. pyogenes*, *S faecalis*, *S. agalactiae* and *VRE*, *C. xerosis*, and *Corynebacterium diphtheriae* (Fig. 3). Bacteriocinogenic activity was also observed against *B. subtilis* and *M. luteus*. Gram-negative clinical strains showed resistance except for *Klebsiella* spp. and *E. coli* which were sensitive to the freshwater producer strains *B. coagulans* (ZP-111) and *Bacillus* spp (ZP-110), respectively. While none of the tested strains could inhibit *S. typhi* and *paratyphi B*, *S. dysenteriae*, *P. mirabilis*, *Klebsiella* spp. and *P. aeruginosa* by cross streak method. The minimum concentration of the bacteriocin inhibitory to the sensitive organism was found to be 12,500 arbitrary units/mL for *B. coagulans* (ZP-111) while 1250 arbitrary units/mL for *Bacillus* spp. ZP-108.

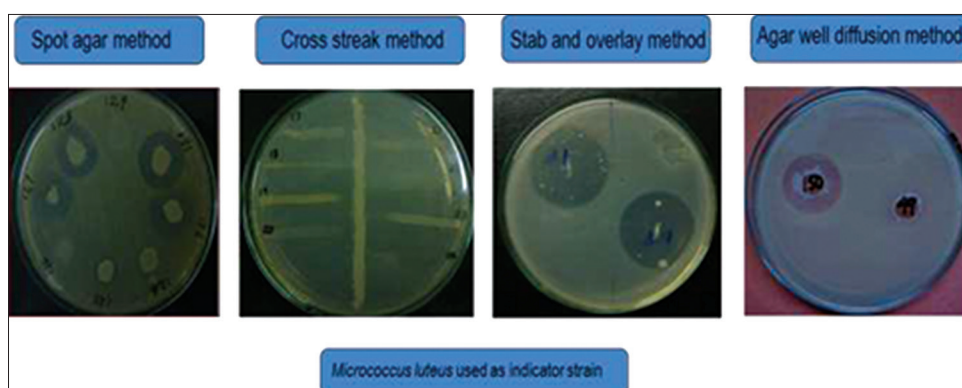
Furthermore, in order to observe the phenomenon of antagonism among aquatic isolates, one of the significant bacteriocinogenic strains was tested against various aquatic isolates. Our study indicated 23 out of 39 (59%) aquatic isolates showed sensitivity against a bacteriocinogenic strain of *B. coagulans* (Fig. 4).

## DISCUSSION

Bacteriocins from aquatic environments have not been studied extensively. At present, there are relatively few reports in the literature

**Table 1: Overview of samples and isolates collected from Sindh, Pakistan**

Type of samples	Location of samples	Average CFU/mL	No. of isolates
Estuarine	Khapur Creek	$59.0 \times 10^4$	29
Freshwater	River Indus Lake, Keenjhar	$1.2 \times 10^4$	80
Marine	Cape Mount, Sandspit Sea view	$2.7 \times 10^4$	37



**Fig. 1: Screening for the bacteriocinogenic strains by various activity monitoring methods**

of antibacterial peptides produced by aquatic bacteria, though few BLIS have been described from marine bacteria. It is, therefore, a need to look for the alternatives to antibiotics as the antibiotic resistance is on the rise and the choice of effective antibiotics is getting narrower.

This research study indicates the bacteriocinogenic potential of aquatic isolates against clinical pathogens as well as against indigenous aquatic isolates. The current study reports 41 (28%) bacteriocinogenic strains from fresh, marine, and estuarine water by applying four bacteriocinogenic activity monitoring methods, i.e., spot agar, stab and overlay, cross streak and agar well diffusion methods. Comparatively, the percentage of bacteriocinogenic/BLIS strains reported here is much higher than the previous studies. For instance, Uzair et al., 2006 reported 15% bacteriocinogenic strains from Baluchistan's coastal water isolates [15]. Among the bacteriocinogenic strains, majority were Gram-positive and the inhabitants of freshwater bodies. The producer strains were identified as those of *B. coagulans*, *B. simplex*, *B. macerans* and *B. circulans*. An interesting finding observed in this study was the detection of the higher numbers of bacteriocinogenic strains from the freshwater samples than the marine ones. Similar phenomenon also observed by Bost in 2004 which might be due to the competition among the diversified types of organisms in the freshwater bodies which requires more antagonistic traits [16].

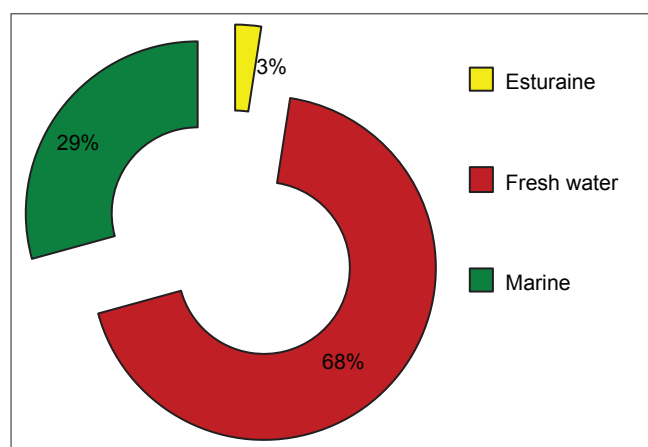


Fig. 2: Bacteriocinogenesis among aquatic isolates

Two of the *Bacillus* bacteriocinogenic strains indicated a narrow spectrum of antimicrobial activity since these inhibited only the Gram-positive bacteria while two of the strains ZP-110 and ZP-111 did show broad spectrum activity by inhibiting the strains of *E. coli* and *Klebsiella* as well. Many of the *Bacillus* species like *B. subtilis*, *B. cereus* etc., are well-known for their production of different antimicrobial compounds [17] such as antibiotics [18] and bacteriocins [19]. In many previous researches, member of *Bacillus* have been found to be possessing narrow spectrum of antimicrobial activity [20,21], although broad spectrum bacteriocins produced by *Bacillus* spp. have also been reported [22,23].

The multidrug-resistant strains of Gram-positive bacteria, VRE and MRSA, have made the clinical treatment of infections quite difficult. Importantly, in this study, we have observed three significant producing strains namely *B. coagulans* (ZP-73, 111) and *B. simplex* (ZP-110) that are capable of arresting the growth of VRE while the two strains of *B. coagulans* (ZP-55 and 73) are also quite effective against MRSA. The phenomenon of competitive antagonism was also observed among aquatic isolates as 59% of the aquatic isolates were sensitive against one of the indigenous bacteriocinogenic strain. This indicates that these compounds can possibly be used against fish pathogens and for aquaculture as well.

Previously, only *Lactobacillus* species were known to be GRAS to be used for preserving the edible items [24]. Now certain species of *Bacillus*, like *B. amyloliquefaciens* have proved to be safe enough to be used for the biopreservation of food [25]. Many species of the genus *Bacillus* are commercially available as biopesticides to control the number of plant diseases in the agricultural sector. All the bacteriocinogenic strains reported in this study, particularly those which can inhibit VRE and MRSA, additionally can have broad-based future applications. But despite having antagonistic activities against the pathogens, we still see no commercial production of bacteriocins in the form of medicines. Extensive studies are required to make full benefits of these natural substances and to explore all possible applications using bacteriocins or the combination of bacteriocins.

**CONCLUSION**

This research study signifies the bacteriocinogenic potential of indigenous aquatic isolates against drug-resistant clinical pathogens as well as against fish pathogens.

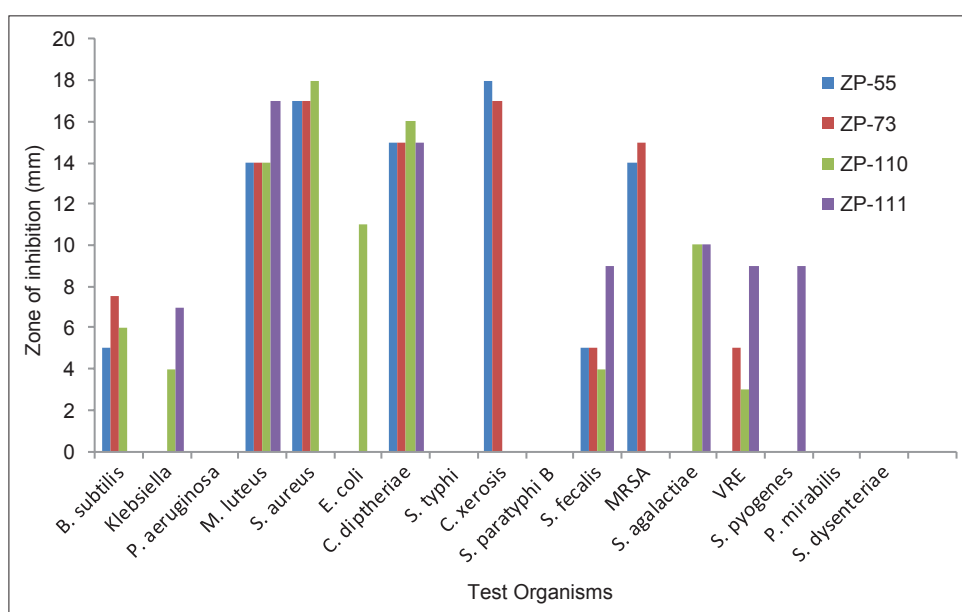
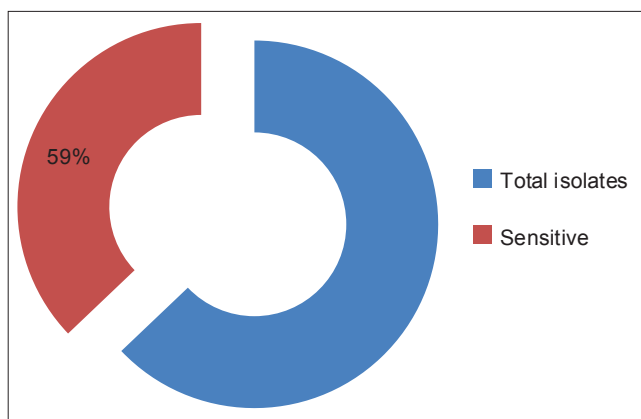


Fig. 3: Inhibition of clinical strains by bacteriocinogenic strains. Note: Bacteriocinogenic activity of four significant strains tested against 17 pathogens by cross streak method. Average values are plotted



**Fig. 4: Antagonism among aquatic isolates. Note: 23 out of 39 (59%) freshwater and marine isolates showed sensitivity against a bacteriocinogenic strain of *Bacillus coagulans* by cross streak method**

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