INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 4, p. 372-379, 2017

RESEARCH PAPER OPEN ACCESS

Antibacterial activity of bacteriocin isolated from *Lactobacillus acidophilus* against throat infections causing bacteria

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Key words: Bacteriocin, antibiotic resistant, respiratory tract, infections.

http://dx.doi.org/10.12692/ijb/10.4.372-379 Article published on April 30, 2017

Abstract

The present study was designed to illustrate the antibacterial activity of bacteriocin against antibiotic resistant bacteria isolated from throat of patients with upper respiratory tract infections. Throat swabs of 439 patients were collected from local population of District Sargodha and employed in the study. Bacteriocin was conjugated antibiotic and isolated from *L. acidophilus*. It was purified using ammonium sulphate precipitation method. It was found stable at different temperatures and specific pH range. First, antibiotic susceptibility was checked against widely used antibiotics (Ciprofloxacin, Levofloxicin, Amoxicillin and Erythromycin). Then, bacteriocin sensitivity assay was performed using disc diffusion technique and agar well diffusion assay. Other than commonly reported strains; three strains (*Moraxella catarrhalis, Neisseria meningitides* and *Alcaligenes sargodhrensis*) were associated with the upper respiratory tract infection in the local population of District Sargodha. *Alcaligenes sargodhrensis* was novel strain and identified using 16S rRN Aribotyping. All strains were resistant to selected antibiotics at certain concentrations. Bacteriocin was effective against all test antibiotic resistant strains. This study suggested that the developing resistance in respiratory tract pathogens against cultural antibiotics can be minimized with the alternative strategies such as bacteriocins.

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Introduction

The upper respiratory tract infections (URTIs) are most communal infections, prevailing in every age group. The main infections include; pharyngitis, nasopharyngitis, tonsillitis, otitis media and sinusitis. These infections are primarily triggered by viruses and then propagated by bacterial pathogens infecting the major organs. The *H. influenzae*, *S. pyogenes*, *M. catarrhalis*, *S. aureus*, and *S. pneumonia are involved in* URTIs. The respiratory tract infections are more prevalent and leading cause of death in children in developing countries (Ndip *et al.*, 2008). In US about 2 to 4 million people are affected annually by respiratory infections.

Antibiotic resistance is the emerging challenge for the developing world. The pathogenic bacterial are developing the resistance against common and repeated use of general antibiotics. Ampicillin resistance has been reported in respiratory pathogens (Goldstein *et al.*, 2009). The epidemic rate of development of antibiotic resistance in several pathogenic bacterial strains has varying perspectives. The drug resistant bacteria like MRSA (Methicillin Resistant *Staphylococcus aurous)*, *S. pneumonia*, *M. tuberculosis* and multidrug resistant bacillus had devastating medical history worldwide. Over viewing the span of effect and significant impact on mortality and morbidity of multidrug-resistant pathogensposes substantial hazard to global health (Spellberg *et al.*, 2008).

Antibacterial resistance limits the proficient prevention and management of rising infections due to bacteria. The developing realms such as Pakistan are lacking in health care services for community acquired bacterial diseases. The inappropriate treatment and nonprescribed administration of antibiotics worsen the condition hence abandoned use of antibiotics is escalating day by day (Abdullah *et al.*, 2012; Khan *et al.*, 2015 a,b). The individuals pay for low quality medicines and subsequently mistreatment them due to be deficient edification and low monetary significance. As a result of this non judicial use of antibiotics in health care institutes and community, antibiotic resistant agents are thriving. Moreover the treatments prospects were limited by augmented resilient to modern antimicrobial drugs. This scenario also adds to in the finances of hospitalization and management approach to deal severity of infections. The phase when antimicrobial drugs stop working against the bacterial infections is the most alluring condition to deal with. Due to the resistant pathogens, the failure in organ surgeries and transplantation also reported. Such outbreak leads to complete adversity in countries like Pakistan. There is no barrier of boundaries for these contagions and this could exist to other countries too. To evade the deficiency of the blessing of wonder drugs there is a need of regulation for proper administrations of antibiotics (Hussain, 2015).

For the duration of the current decades the rapid development of resistance was resulted due to the ease of availability of antibiotics, which also increases the misuse of antibiotics. Abdulhak *et al.* (2011) stated that there are about 77.6% of the pharmacies sales out the antibiotics without any doctoral instructions and about more than 90% of such antibiotics are for cure of throat infections and diarrhoea. One more study conducted in Karachi demonstrates that about 84% of the general practitioners are involved in nonjudicious use of antibacterial drugs (Naz *et al.*, 2008). These problems have led to amplified snags in the management and control of antibiotic resistant bacteria. Several studies have shown that antibiotic resistance varies with the geographical position and time (Ndip *et al.*, 2002).

Lactobacillus is one of the members of Lactic acid producing bacteria (LAB) (Satokari *et al.*, 2005). It is naturally present or added with intent in raw milk, yogurt and all fermented food items (Vernoux *et al.*, 2003). Numerous LAB exhibits copious antimicrobial activities due to presence of organic and inorganic elements. The potential to synthesize antimicrobial agents like bacteriocin is one of the major characters of several LAB. In recent years, due to their probable efficacy as usual preservatives, increases importance of these agents. There is growing user concern to create link between diet and fitness. Such response of

consumers has many optimistic health effects and the part of probiotics (LAB) was supported by recent scientific studies (Soomro *et al.*, 2002). Bacteriocins and bacteriocin like substances are produced by *Lactobacilli sp*. showing antibacterial and antifungal activity (Simova *et al.*, 2009). Bacteriocins are reported to be small heterogeneous family of peptides produced by several probiotic bacterial strains (Cotter *et al.*, 2005).

Bacteriocins also used as the safe alternatives of chemical preservatives in food industry. It also adds more shelf life in the food items prone to pathogenic contamination (Abee *et al.*, 1995). There are several bacteria producing bacteriocins, active against some gram negative strains (Messaoudi *et al.*, 2011). The main difference is that bacteriocin are synthesized ribosomal during primary growth phase, on the other hand, antibiotics have broad range and usually are metabolites produced during secondary growth phase (Beasley and Saris, 2004).

The major antibacterial elements obtained from bacteria include bacteriocin (primary metabolite have narrow spectrum) and antibiotics (secondary metabolite, usually broad spectrum). The antibiotics are employed to deal various bacterial infections. Conversely to antibiotics, bacteriocins are peptides with antibacterial potential and produced by numerous bacterial groups. Bacteriocins are considered as antibiotics by traditional description. In fact these are different compounds (Alam, 2010).

The speedy rise and extend of antibiotic resistant bacteria have increased the concern to search for an alternate strategy to treat infections. Pathogens and commensally bacterial agents have developed resistance against broad spectrum antibiotics. Alternate elucidation is being provided by bacteriocin and bacteriocin like substances. Thus bacteriocins can be used as an appropriate agent against antibiotic resistant bacteria.

In this study, the bacteriocin was isolated from *L. acidophilus*. This strain can ferment sugars like fructose, glucose, gelatos, mannose, sucrose, maltose, lactose, salicin, trehalose, amygdaline and cellobiose (Gomes and Malcata, 1999). Effect of isolated bacteriocin against antibiotic resistant respiratory pathogens was also studied.

Materials and methods

Sampling of Test Strain

Antibiotic resistant bacteria were isolated using throat swabs of 391 out of 439 patient samples. All patients were confirmed for upper respiratory tract infection with baseline characteristics. All procedures of isolation and culturing were in accordance with the declaration of Helsinki and ethical criteria were taken before sampling.

Culture Media

Blood agar and chocolate agar was used for isolation of test strain. Blood agar or nutrient agar (100ml) was prepared following standard protocol and sterilized by autoclaving at 121°C for 15 minutes. When the agar was cooled to 50°C, sterile sheep blood was added and mixed well, avoiding bubbling.

Then 15ml of media was poured in to sterile Petri plates and let them to solidify at room temperature for 1 hour. To prepare chocolate agar, blood agar was heated till red blood cells lazed and the medium becomes brown in colour. It was then dispensed to sterile Petri dishes and solidifies and room temperature for 1 hour.

Biochemical characterization

The biochemical characterization was performed with several tests. Sugar fermentation test was performed with sugars (glucose, manitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygadlin and arabinose). The other biochemical tests include ONPG, ADH, LDC, CIT, H_2S, URE, TDA, IND, VP and GEL test. Susceptibility testing was performed against ampicillin, erythromycin, ciprofloxacin, moxifloxacin and levofloxacin (Nawaz *et al.*, 2009; Riaz *et al.*, 2010).

Molecular characterization of unknown strain

The 16S ribotyping was performed for one strain with the help of First BASE Laboratories SdnBhd (604944-X) Malaysia.

Determination of growth curves

For finding the optimum temperature, 3 test tubes were (5 sets each) used. Each tube had 5ml of nutrient medium which was inoculated with 50µl of bacterial at 15°C, 25°C, 35°C, 45°C and 55°C for 24 hours. The bacterial growth was determined by observing OD at 600nm. For optimum pH, nutrient broth with various pH i.e. 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 was used in 3 sets of test tubes. The broth was inoculated with growing bacterial cells (50µl) and incubated at 37°C for 24 hours. OD was noted at 600 nm. For preparation nutrient broth, medium was inoculated with 50µl each bacterial strain. The tubes were incubated in shaking incubator at 37°C and 150rpm. The optical density (O.D) was noted at 600nm after every 2 hours (Riaz *et al*., 2010).

Screening of bacteriocin producing strain

Bacteriocin producing bacteria were isolated (*Lactobacillus acidophilus*) according to their morphological and biochemical characters (Basmajian, 1976; Nawaz *et al*., 2009). The used tests were: Gram reaction; catalyse test, cyto-chrome oxidise test and carbohydrate fermentation test (De Man *et al*., 1960; Tatman-Otkun *et al*., 2005).

Characterization of bacteriocin

For molecular characterization SDS PAGE was performed following standard protocol (EZ™ Pre stained protein ladder marker; cat # PM001; size ~12kda to ~160kda; bright reference bands: ~27kda to ~100kda). Bacteriocin was also characterized on the basis of effect of temperature and pH. Effect of temperature were monitored by incubating the cell free culture of bacteriocin at 50°C, 75°C and 100°C for five minutes. Disc diffusion assay was performed to detect the activity of bacteriocin (Moghaddam *et al*., 2006). The effect of pH on bacteriocin activity was determined using HCl and NaOH.

Results

Out of total 439 collected specimens 391 shown growth on selective media. The 65% samples were taken from females and 35% were from males. Three different strains (*Moraxella catarrhalis, Neisseria meningitides* and *Alcaligenes sargodhrensis*) were found in samples. Tributary test was performed for distinguishing *Moraxella catarrhalis* from *Neisseria* species and other *Moraxella* species.

To distinguish *Neisseria meningitides* and *Neisseria gonorrhoea* growth on blood agar plate was observed. *Alcaligenes sargodhrensis* did not resemble to any of the available keys of identification hence it was subjected to 16S rRNA ribo-typing and sequencing. Result reveals that strain is 93% similar to *Alcaligene specialist*. The 7% difference in sequence of third strain predicted it was novel strained and named as *Alcaligenes sargodhrensis*. All identified strains were resistant to selected antibiotics. The growth of test strains was found maximum at 35°C to 37°C and at pH 6.5 to 7 (Fig. 3, 4).

Table 1. Antibiotic sensitivity test of selected antibiotic against bacterial strain isolated from patients infected with upper respiratory tract infection.

Bacterial strains (391)	Antibiotic sensitivity result									
	Moxifloxacin 400mg		Ciprofloxacin 500mg		Levofloxicin 500mg		Amoxicillin 500mg		Erythromycin 250mg	
	Resist.	Sen.	Resist.	Sen.	Resist.	Sen.	Resist.	Sen.	Resist.	Sen.
M. catarrhalis N=317 (81.07%)	0.00 (0/317)	100% (317/317)	17.35% (55/317)	82.64% (262/317)	53.33% (169/317)	46.68% (148/317)	80.12% (254/317)	19.87% (63/317)	76.02% (241/317)	23.97% (76/317)
N. meningitidis N=63 (16.11%)	0.00 (0/63)	100% (63/63)	0.00 (0/63)	100% (63/63)	0.00 (0/63)	100% (63/63)	7.93% (5/63)	92.06% (58/63)	1.58% (1/63)	98.41% (62/63)
A. sargodhrensi	0.00 (0/11)	100% (11/11)	9.09% (1/11)	90.90% (10/11)	9.09% (1/11)	90.90% (10/11)	54.54% (6/11)	45.45% (5/11)	27.27% (3/11)	72.72% (8/11)

s
N=11(2.81%)

Resist. : Resistant; Sen.: Sensitive.

N 2nd part, Bacteriocin producing Lactobacillus was isolated strain from fermented milk. Morphological and biochemical characterization showed that the stain was *Lactobacillus acidophilus*.

Molecular characterization by SDS PAGE revealed that the isolated bacteriocin size is 13kda (Fig. 1). The zone of inhibition was found maximum against *N. meningitides* and minimum against *A. sargodhrensis* (Table 2, Fig.2). Further, the Bacteriocin was stable on all temperature and pH ranges.

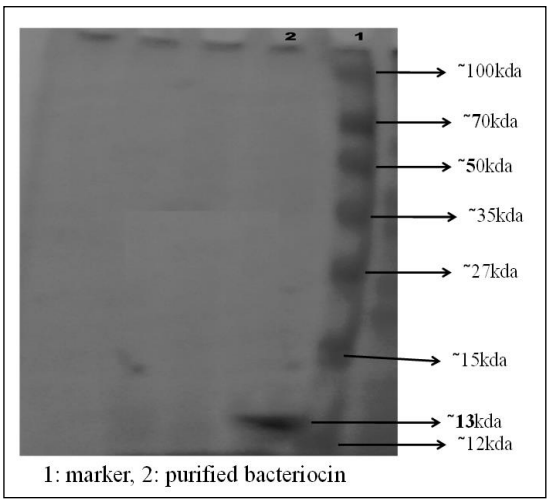

Fig.1. SDS PAGE results of pure bacteriocin isolated from L. acidophilus.

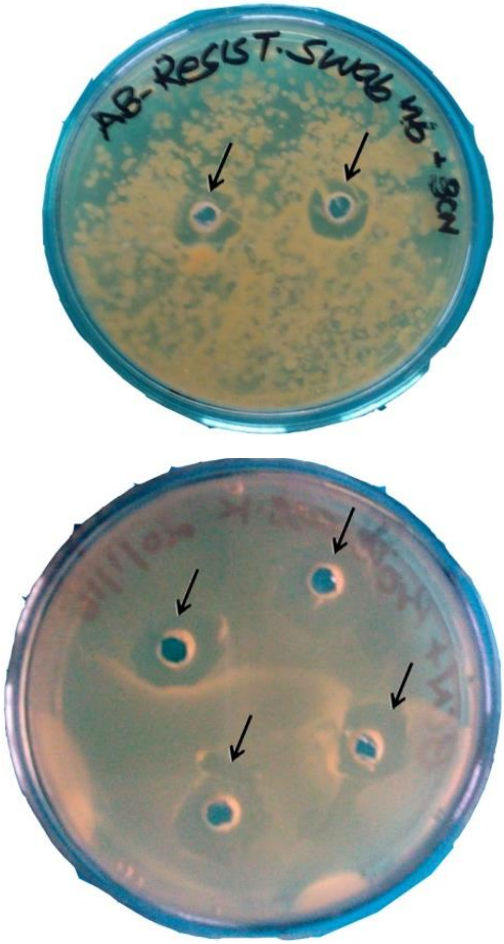

Fig. 2: Bacteriocin zone of inhibition against *N. meningitidis, A. sargodherensis* and *Morexella catarrhalis* (The arrows represent the zone of inhibitions).

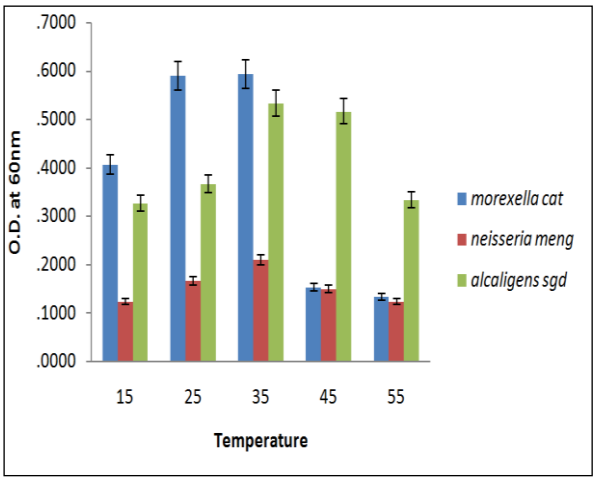

Fig. 3. Effect of temperature on growth of test strains.

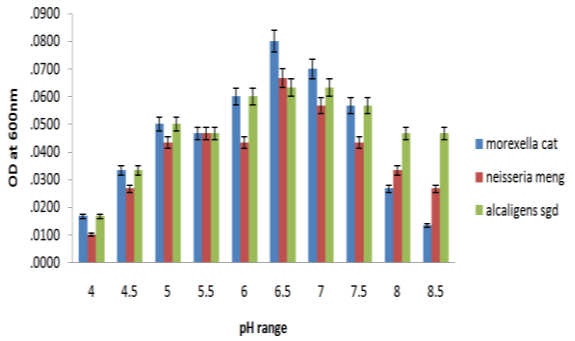

Fig. 4. Effect of pH on growth of test strains.

Table 2. Sensitivity of bacteriocin to heat and different pH values.

Bacterial Strain	Resistance to heating (°C)			Resistance to boiling			Sensitivity to different pH				
	50	75	100	15	30	60	3	5	7	9	11
L. acidophilus	R	R	R	R	R	R	R	R	R	R	R

*R: Resistant.

Table 3. Antibacterial activity of bacteriocin against test strains.

S. no	Test Strain	Replicates	Zone of inhibition (mm)
1	*M. catarrhalis*	3	0.40±0.10
2	*N. meningitidis*	3	0.60±0.45
3	*A. sargodhrensis*	3	0.23±0.15

(Mean ±S.D)

Discussion

The present study aimed to isolate the antibiotic resistant bacteria from local patients of upper respiratory tract infection. Three different bacterial strains were observed in addition to normal pathogenic micro flora of throat. Two strains were identified as *M. catarrhalis*and *N. meningitidis*. Third strain was identified through 16SRNA ribo-typing. It was identified as *A. sargodhrensis*.

Using those strains antimicrobial effects of bacteriocin were also investigated. The bacteriocin was isolated from *L. acidophilus* due to well reputed inhibitory effects (Noro and Yang, 1995). Previously, it was also reported for the production of Lactacin B or F (Klaenhammer, 1993). Results of this study revealed that purified bacteriocin showed anti-bacterial activity against pathogenic and anti-biotic resistant strains of upper respiratory tract infection.

There are several studies showing the ant pathogenic effects of bacteriocin isolated from *L. acidophilus*. Experiments associated to the optimization show that *L. acidophilus* can be grown simply in lab and industrial scale due to endurance of strain at broad range of pH and temperatures.

Several bacteriocins showed different behaviour at different temperatures and pH range. For instance, bacteriocins isolated from vaginal micro flora show the stability at pH ranges from 4.5 to 7.0 however susceptible at pH 9 (Karaoğlu *et al.*, 2003). Likewise, different types of bacteriocin isolated from *L. bulgaricus* and *L. acidophilus* were stable at pH ranges from 3 to 10. Similarly, bacteriocin from *L. Helveticas* was reported to be stable at pH ranges from 3 to 9 (Moghaddam *et al.*, 2006). In present study, bacteriocin from *L. acidophilus* was stable at pH range 3 to 11 which shows high stability under wide range of pH.

Stability of bacteriocin at different temperature values is also useful in characterization. For instance, bacteriocin obtained from *L. acidophilus, L. bulgaricus*and *L. Helveticas* was reported to be stable at 50°C, 70 °C and 80°C while bacteriocins produced by *L. acidophilus* and *L. bulgaricus*is reported to be stale at 100°C (Moghaddam *et al.*, 2006). The bacteriocin obtained in the present study was found stable at all temperatures under experiment.

Conclusion

The project designed illustrates the developing antibiotic resistance in bacterial infection of upper respiratory tract.

Moreover, the bacteriocin isolated from Lactobacilli is an economical and easily obtained source that may prove to be helper of wonder drugs. It can be concluded that bacteriocin isolated from *L. acidophilus* can be tested for the treatment of upper respiratory tract infections caused by antibiotic resistant bacterial strains such as *M. catarrhalis* and *N. meningitides* along with novel strain i.e. *A. sargodhrensis*. This was a preliminary study showing the possibility of using the bacteriocin as alternate strategy. On the basis of this conclusion, advance research project can be commended for the control of antibiotic resistant in respiratory infections.

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