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Research Article

COMPARISON OF ANTIMICROBIAL SUSCEPTIBILITY OF BACTERIOCINS FROM LACTIC ACID BACTERIA WITH VARIOUS ANTIBIOTICS AGAINST *GARDNERELLA VAGINALIS*

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ABSTRACT

This study was conducted to evaluate antibacterial activity of the bacteriocins associated with lactic acid bacteria isolated from vaginal swabs of healthy ladies of reproductive age group. The spreading of bacterial antibiotic resistance and the demand for products with fewer chemicals create the necessity of exploring new alternatives, in order to reduce the abusive use of therapeutic antibiotics in bacterial vaginosis. In this context bacteriocins are indicated to prevent the growth of *G. vaginalis* bacteria in vagina and more natural way, which is convenient for health and accepted by the community. According to their properties, structure, molecular weight (MW), and antimicrobial spectrum, bacteriocins are classified in three different groups: lantibiotics and nonlantibiotics of low MW, and those of higher MW. Antibiotic susceptibility testing of *G. vaginalis* has shown that penicillin, ampicillin, erythromycin, clindamycin, and vancomycin are effective *in vitro*. The culture media was optimized at different pH.

Keywords: Antibacterial activity, Bacteriocins, G. vaginalis.

INTRODUCTION

Gardnerella vaginalis is a normal constituent of the vaginal flora and is one of the organisms associated with bacterial vaginosis.[1,2] *Gardnerella vaginalis*, a Gram variable bacillus first described by Leopold in 1953[3], has been implicated as the predominant organism in bacterial vaginosis[4].The name *Haemophilus vaginalis* was first proposed by Gardner and Dukes[4] because of the organism' colonial morphology and biochemical profile. Because the organism morphologically resembled diphtheroid bacilli in gram stained preparations, it was subsequently named Corynebacterium vaginale[5]. Lactic Acid Bacteria (LAB) are Gram-positive, catalase-negative, non-motile, non-spore forming, aciduric bacteria. They are found in carbohydrate-rich materials, especially fermented foods[6]. They are of great interest in recent times because of their ability to produce antimicrobial substances like bacteriocin, hydrogen-peroxide and organic acids[7]. Bacteriocin is the most potent of all the antimicrobial compounds produced by Lactic Acid Bacteria (LAB). Bacteriocins are ribosomal synthesized peptides which are generally only active against closely related bacterial species[8]. In general these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity. Depending on the producer organism and classification criteria, bacteriocins can be classified into several groups[9-12] in which classes I and II are the most thoroughly studied. Class I, termed lantibiotics, constitutes a group of small peptides that are characterized by their content of several unusual amino acids[13] .The class II bacteriocins are small, nonmodified, heat stable peptides[14].

MATERIALS AND METHODS

Isolation and Identification

100 vaginal swabs of healthy ladies of reproductive age group were collected from gynecologist (GMCH Sec-32, Chandigarh). They were transferred aseptically to sterilized saline (NaCl) solution and transferred in MRS broth (pH 6.5) at 37°C for 18-20 h. Supernatants of overnight grown cultures were isolated and analyzed for detection of bacteriocin activity. A LAB isolate was purified by repeated streaking and purity was checked by Gram staining. Later on it was identified on the basis of 16SrDNA sequencing as *Lactobacillus fermentum*.

Preparation of culture supernatant

The bacteriocin producing lactic acid bacteria were grown in MRS broth at 37°C for 18-20 hours. The lactobacilli culture was taken from culture plate which is grown in MRS broth media , mixed with distilled water in tube then centrifuged at 13,000 rpm for 10 minutes and boiled for 20 min then the supernatant was taken.

Bacteriocin Assay

Bacteriocin activity was detected by Agar well diffusion method. 20µl culture supernatant was transferred to the wells in Casman' media plates supplemented by selective media and 5% defibrinated blood. The plates kept for 2 h at 4°C and then incubated at 37°C for overnight and examined for the presence of inhibition zones around the wells expressed in millimeter (mm). *Gardnerella vaginalis* ATCC14018 was procured from American Type Culture Collection for demonstrating the anti- *Gardnerella* activity. Culture was revived and maintained in Casman's medium supplemented by *Gardnerella* supplement and 5% w/v defibrinated human blood. Bacteriocin activity assay was performed using spot-on-lawn method[15] and agar well diffusion method[16] but agar well diffusion method has shown good results.

Production by Adsorption and Desorption

Bacteriocin was obtained and purified by pH dependent adsorption and desorption on to producer cells, Protocol relies on the property of several bacteriocins to adsorb to the producer cells at neutral pH, and their release after being treated with a highly acidic salt solution. Culture of *Lactobacillus fermentum* inoculated initially in MRS broth was grown to late log phase (18 h, incubated at 37 °C). The culture broth was then heated in boiling water bath and was allowed to cool. The pH was adjusted to 6.5 and kept for overnight stirring. Cells were harvested by centrifugation for 20 minutes at 9000 rpm and culture pellet were washed twice in 5 mM sodium phosphate buffer (pH 6.5) and the pellet was re-suspended in 10 ml of 100 mM NaCl solution that had adjusted pH 1.5 with 5% (v/v) phosphoric acid. The suspension was stirred overnight and cells were harvested by centrifugation at 9000 rpm for 30 min. Cells were stored at -20°C. Activity of bacteriocin was confirmed by well-diffusion assay showing inhibition of 22mm against *G. vaginalis* ATCC14018[17].

Bacteriocin Purification by Cation Reverse-Phase (RP) HPLC

This analytical technique has been shown to be extremely valuable for the analysis of these antimicrobial peptides, since bacteriocins are generally resistant to different organic solvents used as mobile phases and the high pressures employed through the chromatographic technique.

Composition MRS Broth (de Mann Ragossa Sharpe enrichment medium)

Ingredient	Quantity (g/l)
Peptone	10.0
Meat extract	5.0
Yeast extract	5.0
D(+)-Glucose	20.0
Dipotassium hydrogen phosphate	2.0
Diammonium hydrogen citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Tween-80	1ml
Agar (added in bottom agar plates)	10.0
pH	6.5 ± 0.2
47.2 gms per litre	

Casman' medium

Ingredient	Quantity (g/l)
Proteose peptone	10.00
Tryptone	10.00
Beef extract	3.00
Dextrose	0.50
Corn starch	1.00
Sodium chloride	5.00
Nicotinamide	0.05
p-Amino benzoic acid(PABA)	0.05
Selective supplement	1ml
Defibrinated blood	5% v/v

***G. vaginalis* ATCC 14018 Selective Supplement**

An antibiotic selective supplement recommended for the isolation of *Gardnerella vaginalis.*

Gentamycin sulphate	2.00mg
Nalidixic acid	15.00mg
Amphotericin B	1.00mg

Rehydrated with 2ml water of sterile distilled water. Mixed well and aseptically added to 500ml of sterile, molten Casman's media with 5% defibrinated blood.

Table 1: Inhibition of *G. vaginalis* ATCC14018 by various Antibiotics and Bacteriocin6B of *L. fermentum*

Sr. No.	Name of antibiotic	Diameter of Inhibition zone (mm)			
		20ug/ml	40ug/ml	60ug/ml	80ug/ml
1	Metronidazole	-	13	15	16
2	Metronidazole H	-	12	14	15.5
3	Penicillin	12	17	20	22
4	Tinidazole	15	16.5	18	23
5	Ofloxacin	15.5	16	19	24
6	Amoxicillin +Clavulanic acid	14	16	17.5	19
7	Cefaxime	-	17	19	22
8	Amoxicillin	15	16	19	20
9	Ciprofloxacin	13	15	16	18
10	Erythromycin	-	-	-	13
11	Co-trimoxazole	-	-	-	14
12	Tetracycline	9	12.5	13	14.5
13	Azithromycin	-	-	-	-
14	Miconazole	-	-	-	-
15	Rifampicin	14	16	17.5	20
16	Bacteriocin 6B	8	13	18	24

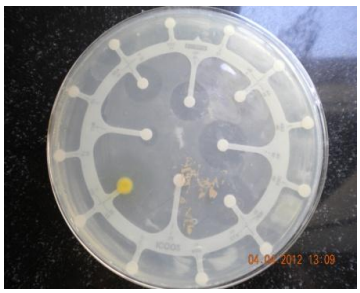

Fig1: Showing the Zone of Inhibition of various Antibiotics with Moderate, Sensitive and Resistant zones against Gram Positive.

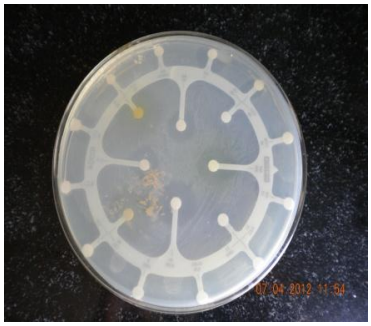

Fig2: Showing the Zone of Inhibition of various Antibiotics with Moderate , Sensitive and Resistant zone against Gram Negative Bacteria.

Eight anti *Gardnerella vaginalis* Lactic acid bacteria strains (Table2) obtained from the vaginal swabs of women were tested for their in *vitro* susceptibilities to 15 antibiotics and bacteriocin 6B. The in *vitro* inhibitory activity of 15 antimicrobial agents and Bacteriocin 6B was also tested against *Gardnerella vaginalis*. Zone of inhibitions were determined by Disc diffusion method**.** In disc diffusion method 1% selective media agar plates were prepared and seeded with 30µl of 24 hour old indicator culture in 0.75% soft agar and then spread gently on selective agar plate. Icosa discs containing different antibiotics were placed on to the petriplates as enlisted in Table 1. Plates were incubated at 37°C for 24 hours and the inhibition zones were recorded by antibiotic scale.

Optimization of Culture Media

The culture media was optimized by studying the growth of Lactic Acid bacterial isolate *L. fermentum* at different pH. The production of lactic acid was confirmed at different pH by measuring the absorbance of the supernatants at UV-Visible Spectrophotometer. At pH between 6.5-7.0, the growth was maximum.

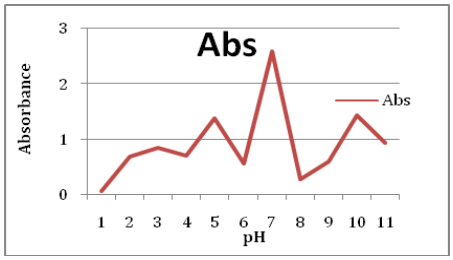

Fig3: Showing effect of pH on the growth of Lactic Acid bacterial isolate *L. fermentum*

CONCLUSION

G. vaginalis was sensitive to Metronidazole, Metronidazole H, Penicillin, Tinidazole, Ofloxacin**,** Amoxicillin + Clavulanic acid, Cefaxime, Tetracycline and Rifampicin. All the LAB isolates were resistant to Miconazole and Azithromycin. Cefaxime was the most active cephalosporin, inhibiting all isolates at 40mcg/ml. Most of the isolated strains were susceptible to Metronidazole and Hydroxy metabolite of Metronidazole. Tinidazole was highly active inhibiting all isolates at 20mcg/ml. Zones of inhibition of Bacteriocin 6B were recorded between concentration 60ug/ml to 80ug/ml as shown in fig.4. Maximum inhibition zone was observed in bacteriocin 6B, which was selected for further studies and produced and purified further Activity units per ml were calculated after purifying bacteriocin by Yang et al. (1992) method of adsorption and desorption and the results indicate that after purification it is effective even in small concentrations.

Table 2: Inhibition of G*. vaginalis* ATCC14018 by various human vaginal lab isolates

Sr. No.	Human Vaginal (HV) LAB isolates	Zone of Inhibition (mm)
1	HV6A	23.0
2	HV6B	24.0
3	HV54 A	18.0
4	HV54 B	-
5	HV59 A	22.5
6	HV59 B	-
7	HV59 C	18.0
8	HV59 D	17.5
9	HV69 A	22.0
10	HV75 A	19.0

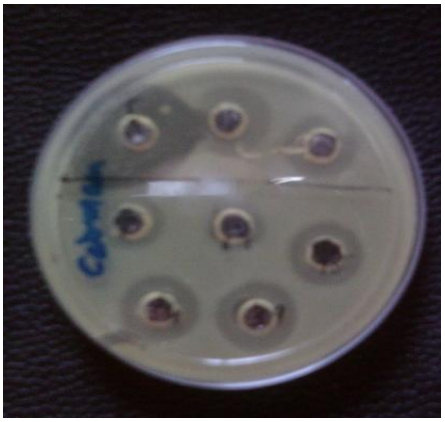

Fig 4. **Showing the inhibition zones for various concentration of crude Protein(Bacteriocin)**

Fig 5: **Showing the inhibition zones for various concentrations of Purified Bacteriocin6B**

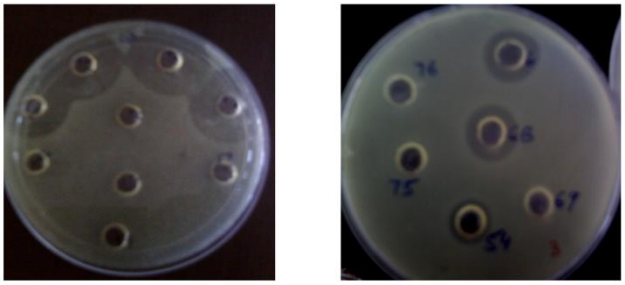

Fig 6: Comparison of different Antibiotics with Bacteriocins

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