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Original Article

Detection of bacteriocin like substances from normal skin microflora as alternative to conventional antibiotics

Reazul Karim*, Nuruddin Mahmud, Mohammad Abdul Hakim

Department of Microbiology, University of Chittagong, Chittagong – 4331, Bangladesh

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**Corresponding author email:*
reazul.m.karim@gmail.com

Abstract

Gradual increase of antibiotic resistance is a global problem. In this study, we have developed an alternative approach as an alternative to conventional antibiotics from the natural source to solve the antibiotic resistance problem. Some normal microflora were isolated from healthy human skin, their antimicrobial efficacy were examined against some skin and intestinal pathogens initially by cross streak method and finally by disc and well diffusion method. Two normal microflora (e.g., *Bacillus licheniformis* and *Corynebacterium jeikeium*) were observed producing antimicrobial metabolites which were effective against *Klebsiella pneumoniae* subsp. *pneumoniae*, with maximum antimicrobial activity at 25^0C, 48h, pH 9 and 37^0C, 72h, pH 7 respectively. Only the antimicrobial metabolites produced by *Bacillus licheniformis* was detected as bacteriocin like substances which was further confirmed as antimicrobial peptide through papain treatment. Efficacy of crude bacteriocin like substances was compared with 10 commercially available antibiotics against *Klebsiella pneumoniae* subsp. *pneumoniae*. Of these, 4 antibiotics were found resistant but crude bacteriocin like substances along with 6 other antibiotics showed remarkable susceptibility. Therefore, more studies on the efficacy of this bacteriocin like substances needs to be done to fully understand its mechanism and potentiality as novel antimicrobials.

Keywords: Antimicrobial metabolites, Bacteriocin, Antibiotic resistance, Normal microbiota

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Introduction

The microorganisms which are the permanent residents of the human body are called normal microbiota. An adult human body consist of an enormous biomass of > 100 000 billion bacteria of > 400 different species, which generate intense metabolic activity, mainly in the colon, and play an important physiologic role in the host (Luckey, 1972).

The skin is our most exposed organ, responsible for providing a barrier to the external environment that can resist a wide range of challenges and respond appropriately to penetrating dangers. However, despite a potent cutaneous immune system, many different microbial communities thrive on the surface. More recently, it has been hypothesized that the skin commensal microbial communities not only co-exist despite our immune defense network but actually

modify immunity, therefore influencing normal skin health as well as participating in various dermatological conditions (Roth and James, 1988). The microbiota of the skin have therefore become the subject of much recent interest from the perspective of better understanding cutaneous disease and as a source for developing novel therapies for various diseases (James and Richard, 2013).Generally, normal microbiota play antipathogenic role by producingH_2O_2, acids or antimicrobial molecules. Some normal flora antagonize other bacteria through the production of substances which inhibit or kill non indigenous species. The intestinal bacteria produce a variety of substances ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins, which inhibit or kill other bacteria. Bacteriocins are bacterially produced peptides that are active against other bacteria and against which the producer has a specific immunity mechanism. (Cotter et al., 2005; Klaenhammer, 1993).Recent reports have revealed that some normal flora such as intestinal lactobacilli and bifidobacteria produce antimicrobial substances such as bacteriocin that are active against some pathogenic microorganisms. Another study showed that, α-haemolytic streptococci isolated from the nasopharynx are capable of inhibiting the growth *in vitro* of pathogens that frequently cause Acute otitis media (Bernstein et al., 1994 ; Brook and Gober, 2000 ; Tano et al., 1999). As, many pathogenic bacteria are developing resistance against conventional antibiotics day by day, search for novel antimicrobials from different sources, especially from natural sources is an attractive topic for research and bacteriocin could be an important antimicrobial agent against drug resistant pathogens. In this study, an alternative approach to solving antibiotic resistance problem was developed using normal skin microbiota in the production of bacteriocin like substances, of which their efficacy was compared with conventional antibiotics.

Material and Methods

Sample collection

Samples were collected from healthy persons who did not have any record for taking antimicrobial drug for last six months. Different anatomical site i.e. forearm, forehead, leg, upper arm, under arm were considered for sample collection and Mannitol Salt Agar medium was used as selective media for isolation of skin microbiota. Considering colour and morphological variation, discrete colonies were transferred to pre-prepared nutrient agar slant and then purified through streak plate method.

List of pathogenic bacteria used for screening

Pathogens listed in Table 1 were collected from ICDDR, B, Chittagong Maa-O-Shishu Hospital and Microbiology Research Lab of Chittagong University.

Table-1: List of pathogens used for screening

Test pathogen	Strain No./Isolated from
Gram Negative	
1. *Klebsiella pneumoniae* subsp. *ozaenae*	Urinary tract infection
2.*Klebsiella pneumoniae* subsp. *pneumoniae*	Urinary tract infection
3. *Salmonella typhi*	AEI14296
4. *Escherichia coli*	ATCC 25922
5. *Pseudomonas aeruginosa*	ATCC 9027
Gram Positive	
6. *Staphylococcus aureus*	ATCC 6538
7. Staphylococcus aureus	Umbilical Swab
8. *Streptococcus pyogenes*	Pus
9. *Bacillus subtilis*	ATCC 6633

Screening of skin microbiota based on their antimicrobial activity

Skin microbiota were screened based on their antimicrobial metabolite production capability. For that, all isolated skin microbiota were initially screened on Muller Hinton Agar media through cross-streak method where skin microbiota were allowed to grow for 24h at 37^0C on straight line in 90 mm petri plate to facilitate the diffusion of antimicrobial metabolites. After 24 hours of incubation, all the pathogens were streaked across the right angles to the previously inoculated isolate and allowed to incubate at 37^0C for 24-72 hours. After incubation the plates were examined for growth inhibition of pathogens near the growth of skin microbiota. Based on clear growth inhibition, skin microbiota were detected for final screening. For that, selected skin microbiota were grown on test tube containing nutrient broth for 24 hours at 37^0C and cell debris were removed through 0.45µ cellulose nitrate membrane filter. Then the filtrate was used as crude metabolites and their antimicrobial efficacy were examined against pathogens by disc diffusion (Bauer et al., 1966) and well diffusion method (Magaldi et al., 2004). Muller

Hinton media was used for this purpose.

Identification of skin microbiota

After primary and secondary screening, normal skin microbiota, that exhibited significant results were chosen for identification using all the conventional biochemical tests along with morphological study mentioned in Bergey's Manual of Determinative Bacteriology, 8th ed. (Buchanon and Gibson, 1974) and 9th ed. (Holt et al., 2000) i.e**.** Gram-staining, starch hydrolysis, Voges Proskauer (V-P) Test, Production of hydrogen sulphide, Gelatin liquefaction test, Nitrate reduction test, Indole test Deep glucose agar test, Catalase reaction Methyl-red test, Fermentation test, Urease test Motility test, Oxidase test.

Growth parameter optimization for production of antimicrobial metabolites

Production of bacterial metabolites depends on different growth parameter i.e. pH of culture media, incubation period, incubation temperature. Therefore, optimization of antimicrobial metabolites production by the selected isolates were done.

Optimization of incubation period

To determine the optimum condition for antimicrobial metabolite production, each selected skin microbiota were inoculated into 4 sets of 50 ml nutrient broth and incubated for 24h, 48h, 72h and 96h respectively at 37^0C. Then crude antimicrobial metabolites were collected through the method used for final screening. Optimum activity of antimicrobial metabolites were determined by disc diffusion (Bauer et al., 1966) and well diffusion method (Magaldi et al., 2004).

Optimization of culture media pH

For pH optimization, 6 sets of 50ml nutrient broth were taken for each isolate and their pH was adjusted from pH 5 to pH 10. Then isolates were inoculated and incubated at 37^0C for initially determined optimum incubation period of each isolates. After incubation, crude antimicrobial metabolites collection and optimum antimicrobial activity were determined through the same method used for optimization of incubation period.

Optimization of incubation temperature

Three sets of 50ml nutrient broth were taken for each isolates and optimum pH was adjusted for each isolates respectively. Then incubated at 27^0C, 37^0C and 45^0C for initially determined optimum incubation period of each isolates. After incubation, crude antimicrobial metabolites collection and optimum antimicrobial activity were determined through the same method used for optimization of incubation period.

Determination of bacteriocin like substances from antimicrobial metabolites

Generally, bacterial strain exhibited antimicrobial activity through the production of H_2O_2, acids or antimicrobial proteinaceous molecules known as bacteriocin. To determine whether our selected isolates produced bacteriocin like substances or not we applied the techniques described by Schillinger and Luke (Schillinger and Luke, 1989). Selected isolates were grown in Brain Heart infusion Broth (Chaimanee et al., 2009) and after incubation crude metabolites were collected by centrifugation at 5000 rpm for 10 minutes at 4^0C and cell residue were removed by filtering through .45µ Cellulose nitrate membrane filter. To remove the effect of acid, pH was adjusted to 6.5 with 5N NaOH and 5N HCl. Impact of H_2O_2 was removed by incubation with 5 mg/ml catalase at 37^0C for one hour. 20mM phosphate buffer was used for serial dilution (2 fold dilution) of crude metabolites. The antimicrobial metabolites detected as bacteriocin like substances, then subjected to bacteriocin assay. Fresh culture of indicator organism *Klebsiella pneumoniae* subsp. *Pneumoniae* was mixed with Brain Heart Infusion soft agar and spread over the Pre solidified Brain Heart Infusion agar plate. 8 mm well were cut into the agar plate and 100 µl of crude antimicrobial metabolites was placed in that well. Then incubated at 37^0C for 24 hours and activity of bacteriocin/ml (AU/ml) was determined by the equation AU/ml $=2^n \times 1000$ µl /amount of sample per well (µl). Here n indicates number of highest dilution that exhibited minimum zone of inhibition against indicator organism.

Confirmation of bacteriocin like substances through the determination of antimicrobial peptide

Bacteriocins are subset of antimicrobial peptides ribosomally synthesized in bacteria. Hence, for further confirmation of antimicrobial metabolites as bacteriocin, we tried to detect antimicrobial peptide in detected crude bacteriocin like substances sample. For this study, pH of crude culture filtrate was adjusted at 6.5 and incubated with 1.0 mg/ml papain for 1 hour. To eliminate enzyme activity the sample was then heated at 100^0C for 3 minutes. Then bacteriocin assay

was done both for papain treated and untreated sample against control against indicator organism (Narayanapillai et al., 2012).

Comparison of bacteriocin like substances activity with conventional antibiotics

To compare the detected bacteriocin like substances efficacy with conventional antibiotics, both the crude bacteriocin like substances and conventional antibiotics were tested for susceptibility to the pathogens against which detected bacteriocin showed antimicrobial activity. For this test, disc diffusion method (Bauer et al., 1966) was used. Suspension of Pathogen were spread on Mueller-Hinton agar plates and antibiotic disc and Standard discs containing specific amount of antibiotic were placed on the surface of the agar plate. Dried and sterilized filter paper discs (4mm in diameter) were then treated with an amount of 50 µl crude bacteriocin sample and also placed in Mueller-Hinton agar plate. The plate kept at 4^0C for 30 minutes and then incubated at 37^0C for 24h. Antibiotic disc used for this test were Amoxicillin 30 µg, Erythromycin 15 µg, Cefixime5 µg, Ampicillin 25 µg, Gentamycin 10 µg, Imipenem10 µg, Penicillin G 10 µg, Rifampicin 5µg, Cefradine30µg, Cifrofloxacin15 µg. The manufacturer of all antibiotic discs were Oxoid.

Results and Discussion

Table-2: Activity of crude antimicrobial metabolites against pathogen

	Zone of inhibition (mm) against pathogen *Klebsiella pneumoniae* subsp. *pneumonia*			
	crude antimicrobial metabolites in well diffusion method		crude antimicrobial metabolites in disc diffusion method	
Isolate	**100µl**	**50µl**	**80µl**	**50µl**
Isolate FA	16	11	12	10
Isolate UpA	20	14	18	13

Screening of selected isolates for antimicrobial metabolite production

A total of 10 bacterial colonies were isolated from 30 persons based on their colour and morphological variation. Initial screening was done by cross streak method and total four isolates exhibited activity against six pathogens. In secondary screening, two isolates (UpA isolated from upper arm) and (FA isolated from forearm) showed significant activity against the same pathogen *K. pneumoniae* subsp. *pneumoniae* (Table 2)

Identification of selected skin microbiota

Skin microbiota UpA and FA were chosen for identification through conventional biochemical tests described in "Bergey's Manual of Determinative Bacteriology", 8th ed. (Buchanon and Gibson, 1974) and 9th ed. (Holt et al., 2000**).** Biochemical and morphological test result (Table 3) indicated that the characteristics of isolate UpA was closely related with strain *Bacillus licheniformis* and FA was closely related with strain *Corynebacterium jeikeium*

Growth parameter optimization for production of antimicrobial metabolites

An attempt was made to optimize the cultural condition for antimicrobial metabolite production**.** The isolates of *Bacillus licheniformis* and *Corynebacterium jeikeium* produced metabolites with maximum antimicrobial activity against *K. pneumoniae* subsp. *pneumoniae* after 48 and 72 hours of incubation, respectively (Figure 1). Also, *Bacillus licheniformis* exhibited its maximum activity at culture condition with pH 9 while *Corynebacterium jeikeium* produced its optimum metabolites at pH 7 (Figure 2). In case of incubation temperature *B. licheniformis* released metabolites with highest activity against *K. pneumoniae* subsp. *pneumoniae* at 25^0C. A temperature of 37^0C was optimum for the isolate of *Corynebacterium jeikeium* to produce most effective metabolites against *K. pneumoniae* subsp. *pneumoniae* (Figure 3). Similar attempt was made by Narayana and Vijayalakshmi (2008) where they tried to optimize the culture condition for antimicrobial metabolite production by *Streptomyces albidoflavus.* They found that *Streptomyces albidoflavus* produced optimum metabolite after 120 h of incubation at 35^0C when pH was adjusted to 7.

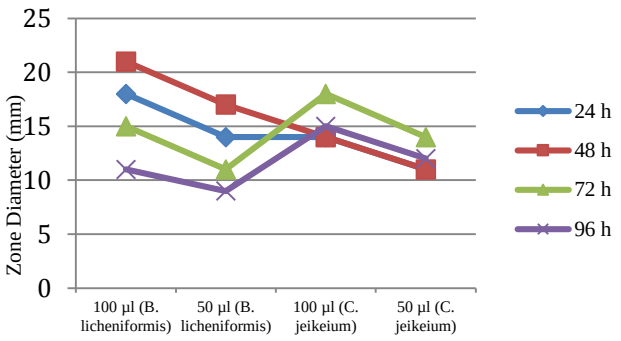

Figure-1: Incubation period optimization for antimicrobial metabolites production.

Table-3: Cultural, morphological and biochemical characteristics of selected isolate

Parameter	Isolate UpA	Isolate FA
Colony Character		
Colony diameter	3mm	3 mm
Surface	Rough	Smooth
Color	Off white	Off White
Form	Irregular	Irregular
Elevation	Flat	Raised
Mergin	Rough	Lobate
Morphology and staining property		
Shape	Long rod	Coccoid
Size	4.48×1.76 µm	1,85-2.4 µm
Form	Single/In pair or chain	Single/In pair or cluster
Gram Staining	Gram positive	Gram positive
Biochemical Test		
Motility	+	-
Deep glucose agar	Aerobic	Aerobic
Oxidase	+	+
Citrate utilization	-	+
Indole	-	-
Methyl red	+	+
Voges-Proskauer	-	+
H_2S production	+	+
Urease	-	-
Starch hydrolysis	-	-
Nitrate reduction	+	+
Gelatin hydrolysis	+	+
Catalase	+	+
Fermentation		
Fructose	+	-
Arabinose	-	-
Sucrose	-	-
Lactose	+	+
Mannitol	-	-
Glucose	+	+
Salicin	-	-
Maltose	+	+
Cellubiose	+	+
Raffinose	-	+
Galactose	+	-
Inulin	+	-

Note +: positive growth, - : Negative Growth

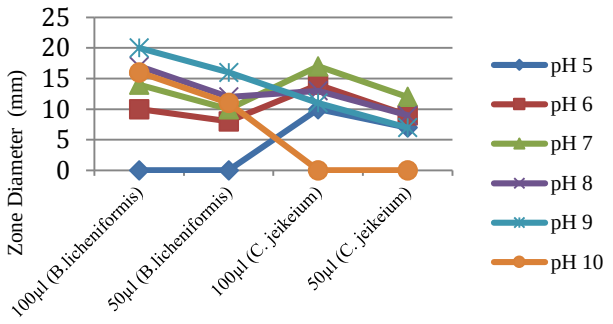

Figure-2: pH optimization for antimicrobial metabolites production.

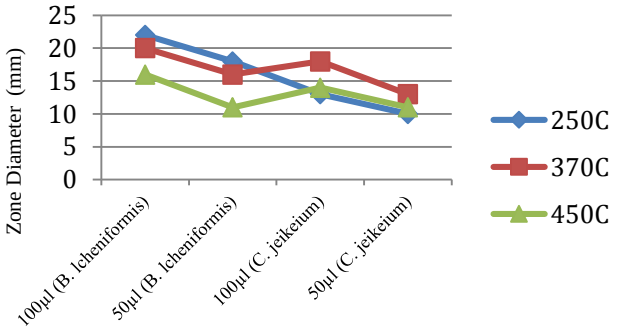

Figure-3: Temperature optimization for antimicrobial metabolites production.

Determination of bacteriocin like substances from antimicrobial metabolites and confirmation through antimicrobial peptide detection

We tried to detect whether the antimicrobial metabolites produced by the selected isolates was bacteriocin like substances or not. For this purpose, the isolates were grown in Brain Heart infusion Broth and the crude metabolites were collected by centrifugation at 5000 rpm for 10 minutes at 4^0C followed by filtration through o.45µ cellulose nitrate filter paper. Activity of organic acid and H_2O_2 was removed by pH adjustment and catalase enzyme respectively. After bacteriocin bioassay, only *B. licheniformis* exhibited zone of inhibition against indicator organism *K. pneumoniae* subsp. *pneumoniae* and the bacteriocin activity was found 80AU/ml (Figure 4). Another isolate *C. jeikeium* did not show any activity against indicator organism suggesting that its' metabolite that previously showed inhibitory effect may be due to other substances rather than bacteriocin i,e. hydrogen peroxide or organic acid. In related study, the growth of pathogens *Streptococcus agalactiae* and

Staphylococcus. dysgalactia was inhibited by the bacteriocin produced by *L. fermentum* and *Streptococcus bovis*in which the activity was 40 AU/ml (Chaimanee et al., 2009). Since bacteriocin are known as subset of antimicrobial peptide, we opted to verify whether the detected antimicrobial metabolites contain antimicrobial peptide or not. Hence, the pH of crude metabolites were adjusted at 6.5 and incubated with 1.0 mg/ml papain for 1 hour. To eliminate any enzyme activity, the samples were heated at 100^0C for 3 minutes and bacteriocin assay was done to determine bacteriocin activity. We found that the sample treated with bacteriocin did not show any antimicrobial activity. On the other hand, papain free crude bacteriocin showed 80 AU/ml activities against indicator organism which was a clear indication that the antimicrobial metabolites contain antimicrobial peptide (Figure 4).

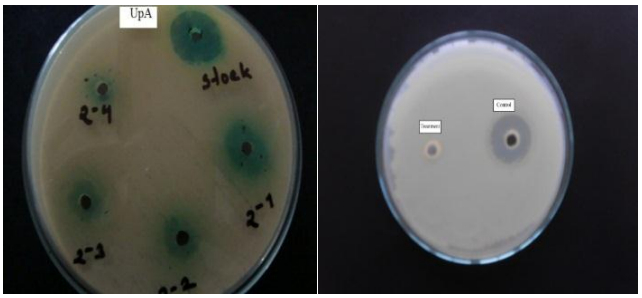

Figure-4: **Detection and confirmation of bacteriocin**

Comparison of bacteriocin like substances activity with conventional antibiotics

To compare the efficacy of detected bacteriocin like substances and conventional antibiotics, antibiotic susceptibility test was done using the pathogens against which detected bacteriocin like substances showed antimicrobial activity. In this test, the pathogen *K. pneumoniae* subsp. *pneumoniae* showed resistance against antibiotics- Penicillin G, Ciprofloxacin, Ampicillin and Amoxicillin, but was susceptible against crude bacteriocin along with other antibiotics-Gentamycin, Imipenem, Rifampicin, Cefradine, Cefixime and Erythromycin (Figure 5 & Figure 6). Golami et al. (2018) also performed a similar research work where he compared efficacy of Cronobacteriocin DGH2 and Enterobacteriocin DGH4 with conventional antibiotics against *Xanthomonas citri* subsp. *citri*.

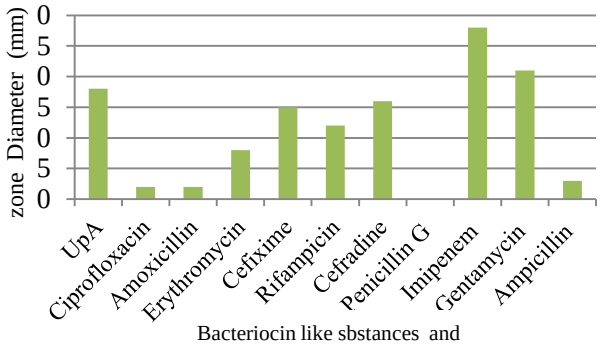

Figure-5: Comparative Study of bacteriocin and available commercial antibiotics

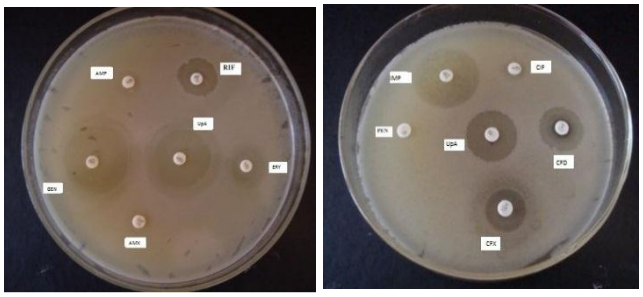

Figure-6: Comparison of Bacteriocin like substances with conventional antibiotics

Conclusion

Development of novel antimicrobials are desperately needed to control the increasing problem related to antibiotic resistance development. Along with synthetic antibiotics, natural sources can be a viable source of novel antimicrobials. In this study, we determined the potentials of normal skin microbiota as a source of novel antimicrobials. The results showed two skin microbiota exhibiting remarkable antimicrobial activity and antimicrobial metabolites. Of these, one skin microbiota was detected as bacteriocin like substances. Further research work like purification of antimicrobial metabolites and human trial can establish these antimicrobials as an effective weapons for combating against antibiotic resistance problem.

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Contribution of Authors

Karim R: Conducted the research work and wrote the manuscript.
Mahmud N: Designed and supervised the research work and helped to write the manuscript.
Hakim MA: Supervised the research work and manuscript writing.