Original Research Article

Bacteriocin production by clinical isolates of 'Non fermenting Gram negative bacilli'- a ray of hope in the post antibiotic era

Sonia Deb,Kabita Choudhury, Soma Sarkar,Shreya Mukherjee,Swagata Ganguly Bhattacharjee*, Nishith Kumar Pal

Department of Microbiology, N.R.S. Medical College and Hospital, Kolkata, India

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Abstract

Background: Bacteriocins are a kind of ribosomal synthesized antimicrobial peptides produced by bacteria, during their growth, triggered by competition for space and scarce resources. Non fermenting Gram negative bacilli (NFGNB), which have pivotal role in causing health care associated infection (HAI) need to establish themselves in the hospital environment pushing aside existing flora and do so probably by bacteriocin production.**Objective:**Present study was undertaken to explore bacteriocin production ability of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and their range of action on other bacteria.Setting and design: A prospective observational study.**Material and methods:**Fifty MDR-NFGNB isolates were tested for phenotypic detection of bacteriocin production against commonly encountered indicator hospital strains and control strains following some modification of Gratia and Fredericq's method.**Results:** All 27 (100%) *Pseudomonas aeruginosa* isolates, produced bacteriocin active against *Escherichia coli* and *Klebsiella pneumoniae*, 26(96.2%) and 21(77.7%) acted on *Proteus mirabilis* and *Enterococcus faecalis* respectively. Out of 23 *Acinetobacter baumannii*, 20(86.9%), 15(65.2%), 15(65.2%) and 12(52.1%) produced bacteriocin active against *Enterococcus faecalis*, *Escherichia coli*, Coagulase negative Staphylococcus species (CoNS) and *Klebsiella pneumonia* respectively. Bacteriocin produced by *A baumannii* had similar effect on both Gram positive and Gram negative bacteria.**Conclusion**: The antibacterial spectra of these bacteriocins raise a hope of using these peptides therapeutically in post-antibiotic era.

Key words: Bacteriocin, HAI, NFGNB.

Introduction

Long after discovery of penicillin (1928), many different antibiotics have been discovered and widely used for disease treatment,however, misuse of antibiotics around the world has raised concern[1,2]. Over the years, many types of multidrug-resistant (MDR) bacteria have been detected and an increasing number of antibiotics have reportedly become less effective[3-6].Drug resistance poses a serious threat to public health and is a major societal concern. In recent years there are reports of organisms resistant to most of the commonly used antibiotics including the last resort antibiotic colistin[7,8]. Therefore, novel antimicrobial substances that cause fewer side effects and less likely to become drug resistant are needed to replace traditional antibiotics[9].Bacteriocins, which can kill or inhibit bacterial strains closely-related or unrelated to the bacteria which produced bacteriocin, are a kind of ribosomal synthesized antimicrobial peptides (AMP) produced by bacteria, during their growth, triggered by scant nutrients in the environment and competition for space and resources[10]. Interestingly, bacteriocin does not harm the bacteria themselves by specific immunity proteins [10]. Although bacteriocins could be categorized as antibiotics, they are not. Major difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to strains of species related to the producing species and particularly to strains of the same species, whereas antibiotics have wider activity spectrum and even if their activity is restricted, do not show any preferential effect on closely related strains.In addition, bacteriocins are ribosomally synthesized and produced during primary growth phase; antibiotics are usually secondary metabolites[11].Non fermenting Gram negative bacilli (NFGNB) are emerging as important hospital acquired pathogens as they have a tendency to colonize various inanimate objects in hospital environment[12-14].These organisms play a pivotal role in causing health care associated infections (HAI) such as septicemia, pneumonia, urinary tract infection and surgical site infections [9]. NFGNB isolated from these patients are invariably multi drug resistant (MDR)[9,15].Recent reports of HAI (eg. ICUs, dialysis units, burn units, post-operative wards) shows rising trend of isolation of NFGNB, which had been a rare event about three decades back [16,17]. This reflects a changing flora in hospital environment which might have found in-roads on patients themself as well as health care management staff. It is not an easy task for a newly introduced bacterial genus to establish an ecological niche removing existing competitors. Moreover, few NFGNB are found to be quite sensitive to commonly used antibiotics[18,19]. Therefore it could be presumed that these bacteria might possess a special attribute i.e bacteriocin production, which could keep at bay existing environmental colonizers.Present endeavor tries to explore bacteriocin production ability of clinical isolates, namely *Pseudomonas aeruginosa* and *Acinetobacter baumannii*,two most commonly encountered non fermenters in hospital environment. If this phenomenon could be established, as a remote outcome, it might open up a new avenue of research using peptides of bacteriocin as antimicrobial agents.

Materials and methods

The present study was a prospective observational study undertaken in the Department of Microbiology, N.R.S. Medical College and Hospital, Kolkata for a period of six months from July 2018 to December 2018 after obtaining Institutional Ethical Clearance and

**Correspondence*
Dr. Swagata Ganguly Bhattacharjee
Professor and Head, NRS Medical College and Hospital, Department of Microbiology,Kolkata, West Bengal, India.
E-mail: swagatamedicine@gmail.com

patient consent where clinical isolates of NFGNB were phenotypically screened for production of bacteriocin against other commonly encountered hospital strains.Out of total 263 hospital NFGNB isolates, fifty (50) consecutive MDR-NFGNB, 27 *Pseudomonas aeruginosa* and 23 *Acinetobacter baumannii*, were tested for phenotypic detection of bacteriocin. In this study some modifications of Gratia[20] and Fredericq's[21] method for detection of bacteriocin production was done, based on the principle that bacteriocins can diffuse in solid or semi-solid culture media, which when subsequently inoculated with a suitable indicator strain, could inhibit growth of indicator bacteria. Present modification involves lawn culture with 1 Mac Farland turbidity of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* on Brain heart infusion (BHI) agar media in glass petridish and incubated aerobically at 37ºC for 48 hours. Lawn culture growth was then scraped off using the edge of a sterile glass cover slip leaving behind the secreted/excreted material of inoculated bacteria in the medium. To ensure sterility of agar surface on which indicator strains are to be inoculated, chloroform vapor was used to kill any residual bacterial growth by placing a sterile filter paper soaked in chloroform on the lid of the glass petri plate and keeping the agar surface downwards for 30 minutes. Use of chloroform vapor does not allow use of plastic petri dishes as chloroform dissolves plastic. Residual chloroform in the culture medium was sufficiently ventilated by aerating the culture plates in bio-safety cabinet for another 30 minutes.Next step, indicator bacterial suspensions including control strains were made and matched turbidity of 1 MacFarland standard. Ten (10) micro litre spot inoculation of these indicator bacteria were made in divided segments of the agar plate and incubated aerobically at 37ºC. After overnight incubation, the BHI plate was inspected for presence or absence of growth of indicator bacteria. Since bacteriocins can diffuse three dimensionally in semisolid agar, absence of growth of the indicator bacteria indicates susceptibility to secreted/excreted products of the bacteria (bacteriocin) in question.To exclude the effect of small sized inoculum (10 microlitre) used in this test, same process was repeated with 30 microlitre spot inoculation of the indicator bacteria in a separate agar plate, repeating the same procedure, using those strains which failed to grow in primary test plate.Results were analysed by simple observation method.Hospital isolates of *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus,* Coagulase negative Staphylococcus species (CoNS*)* and *Enterococcus faecalis* were taken as indicator strains. *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 16202 were taken as control indicator strains.

Statistical methods

After collection & compilation of data, statistical analysis was done.Descriptive statistics were calculated using Microsoft Excel 360. Categorical variables are expressed as number of patients and percentage of patients and compared across the groups using Pearson's Chi Square test for Independence of Attributes/ Fisher's Exact Test as appropriate.The statistical software SPSS version 20 has been used for the analysis. An alpha level of 5% has been taken, i.e. if any p-value is less than 0.05 (< 0.05) it has been considered as significant.

Results

All 27 *Pseudomonas aeruginosa* placed in the study produced bacteriocins which acted on *Escherichia coli* and *Klebsiella pneumoniae* indicators. Many produced bacteriocins which inhibited indicators like *Proteus mirabilis, Enterococcus faecalis, Staphylococcus aureus,* other *Acinetobacter baumannii* to the extent of 26(96.2%), 21(77.7%), 17(62.9%), 16(59.2%) respectively (Table 1). No inhibitory effect was observed against any local isolate of *Pseudomonas aeruginosa* indicators, although control indicators *Acinetobacter baumannii*(ATCC 16202) and *Pseudomonas aeruginosa* (ATCC 27853) were inhibited by all(100%).Out of 23 *Acinetobacter baumannii*, 20(86.9%), 15(65.2%), 15(65.2%), 12(52.1%) could inhibit *Enterococcus faecalis, Escherichia coli*, Coagulase negative Staphylococcus species (CoNS), *Klebsiella pneumoniae* respectively. In addition, *A baumannii* could inhibit *Pseudomonas aeruginosa, Staphylococcus aureus* and *Proteus mirabilis* to the extent of 10(43.4%), 10(43.4%) and 4(17.3%) respectively (Table 1). All 23 *A. baumannii* inhibited control strains *Pseudomonas aeruginosa*(ATCC 27853) and *Acinetobacter baumannii*(ATCC 16202).The same procedure was repeated with 30 micro litre spot inoculation of the indicator bacteria in a separate agar plate, repeating the same procedure, using bacterial isolates who failed to grow in primary test plate and it yielded similar results i.e absence of growth, supporting the fact that inhibition of growth in primary test plate was not due to inoculum effect.

To determine whether the substance produced was bacteriostatic or bactericidal, subculture was done from the spot inoculation surface in a sterile nutrient agar media which showed no growth after 24 hours aerobic incubation, indicating bactericidal activity of bacteriocins produced.Also as there is no direct contact between the producer and the indicator strains, phage particles cannot reach the indicator bacteria. Therefore, growth inhibition due to phages could be excluded.

Table 1:Result of bacteriocin production by *Pseudomonas aeruginosa* and *Acinetobacterbaumannii*

Sl no.	Indicator strains (Hospital isolates)	Bacteriocin production			
		Pseudomonas aeruginosa (n= 27)		*Acinetobacterbaumannii* (n=23)	
1	*Escherichia coli*	27	100%*	15	65.2%**
2	*Klebsiellapneumoniae*	27	100%*	12	52.1%
3	*Proteus mirabilis*	26	96.2%*	4	17.3%
4	*Pseudomonas aeruginosa*	0	0%	10	43.4%
5	*Acinetobacterbaumannii*	16	59.2%	0	0%
6	*Staphylococcus aureus*	17	62.9%*	10	43.4%
7	CoNS	15	55.5%	15	65.2%**
8	*Enterococcus faecalis*	21	77.7%*	20	86.9%**
9	P *aeruginosa* ATCC 27853	27	100%*	23	100%**
10	A *baumannii* ATCC 16202	27	100%*	23	100%**

*p-value< 0.01(significant), **p-value< 0.01(significant)

Table 2:Proposed cocktail model no.1 from this study

Indicator bacteria	*Pseudomonas aeruginosa* isolates						
	No.1	**No.2**	**No.5**	**No.9**	**No.13**	**No.18**	**No.25**
E coli	**NG**	NG	NG	NG	NG	NG	NG
K pneumoniae	NG	**NG**	NG	NG	NG	NG	NG
P mirabilis	NG	NG	**NG**	G	NG	NG	NG

A baumannii	NG	G	G	**NG**	NG	G	G
S aureus	G	NG	NG	**NG**	G	G	NG
CoNS	NG	NG	G	G	**NG**	NG	G
E faecalis	G	NG	NG	G	NG	**NG**	NG

Table 3:Proposed cocktail model no.2 from this study

Indicator bacteria	*Pseudomonas aeruginosa* isolates						
	No.1	**No.2**	**No.5**	**No.9**	**No.13**	**No.18**	**No.25**
E coli	**NG**	NG	NG	NG	NG	NG	NG
K pneumoniae	NG	**NG**	NG	NG	NG	NG	NG
P mirabilis	NG	NG	**NG**	G	NG	NG	NG
A baumannii	NG	G	G	**NG**	NG	G	G
S aureus	G	NG	NG	**NG**	G	G	NG
CoNS	NG	NG	G	G	**NG**	NG	G
E faecalis	G	NG	NG	G	NG	**NG**	NG

Discussion

All 27 *Pseudomonas aeruginosa* isolates produced bacteriocin acting on *E. coli* and *K. pneumoniae* while no effect on other *P aeruginosa* isolates supports the fact that bacteriocins can kill or inhibit bacterial strains closely-related to the bacteria which produced bacteriocin but does not harm the bacteria in question. Against Gram positive bacteria, maximum effect was seen against *E faecalis* (77.7%).In our study, bacteriocin production by *A baumannii* had similar effects on both Gram positive and Gram negative bacteria as evidenced by the fact that 65.2% *A baumannii* isolates produced bacteriocin acting on both *E coli* and CoNS. This observation raises a question whether *A baumannii* residing in hospital environment could undergo mutational change and thus acquire genes for bacteriocin production effective against both Gram positive and Gram negative organisms. It opens up an avenue for further research into genetics of Acinetobacter in the days to come.Literature search shows sparse reports of bacteriocin production by Acinetobacter although pyocin production by Pseudomonas is an established fact[22,36,37]. Current endeavour successfully establishes this ability by a sample of 23 hospital isolates of *A baumannii*. Bacteriocin (pyocin) production by current sample of 27 *P aeruginosa* shows effectiveness for inhibition of growth of commonly isolated hospital strains, thus holding a good prospect for peptide purification in quest of new antimicrobial agents in the current scenario of post antibiotic era.Results of bacteriocin production by *A baumannii* and *P aeruginosa* is not uniformly effective on the current indicator strains used in the study. Inspite of the fact, the result shows that if a cocktail of bacteriocins is used concurrently, resultant bacteriocin peptides in combination might prove effective against the common hospital isolates from HAI. A proposed cocktail model from this study is shown in table 2 and table 3. The resultant peptide purified should be hospital specific due to specificity of bacteriocin.

Study limitations: Genotypic characterisation of the bacteriocin produced could not be done.Use of bacteriocins with the aim to treat or control infectious diseases seem to be cost-effective,non-toxic[23-29]and stable to heat,[10]thus becoming one of the weapons against microorganisms in post antibiotic era. Relatively narrow killing spectrum of bacteriocins and their antimicrobial mechanisms distinct from traditional broad-spectrum antibiotics mean that they may be used to replace traditional antibiotics[30,31]. For example, bacteriocins- Epidermin and Gallidermin have been used to treat dermatologic infections[32].

Pumilicin 4 is shown to have antimicrobial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA)[33]. Nisin, which is FDA approved, has been commercialized in more than 48 countries and is the only bacteriocin that has been approved for application in food industry for preservation of meat and meat products[34]. Therefore, bacteriocins are considered to be promising alternatives to traditional antibiotics[35].

This exciting new era of bacteriocin research may lead to new inventions and new applications. With rapid rate at which genome sequences are becoming available, genome mining becoming easier, and with the latest techniques in gene synthesis and protein expression, we can look forward to novel bacteriocins with very dedicated applications against multi drug resistant organisms in the current scenario of post antibiotic era.

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