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Antimicrobial Activity of *Bacillus* Species Isolated from Soil against Human Pathogens

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Abstract: ***Aims: Emergence and spread of drug resistance microbial infection has become a major concern in disease control and treatment. So, it is of great priority to prevent MDR emergence and to develop new drug to cope up with this rising threat. The study was conducted to analyze the Bacillus species as a potential source of antibiotics with activity against resistant strains. Materials and Methods: 10 Soil samples from various locations were processed for the isolation of Bacillus spp. with antimicrobial activity. Identification was carried out based on the biochemical analysis according to Bergey's Manual of Determinative Bacteriology. Antimicrobial activity of the Bacillus spp. were demonstrated by agar well diffusion method against MRSA, Staphylococcus aureus, Escherichia coli, Klebsiella spp., Enterococcus spp., Salmonella spp. and Streptococcus spp. Results: 13 Bacillus isolates out of 41 were found showing antimicrobial effect with majority of them being B. larvae, B. firmus and B. laterosporus. All of the Bacillus isolates demonstrated inhibitory effect against the Gram's positive test organisms compared to 53.86% of isolates against the Gram's negative test organisms. Thus, majority of isolates were Gram positive inhibitors with 53.86% isolates demonstrating broad spectrum activity. Bacillus isolates were found to show effective inhibitory effect against MRSA compared to other test strains. Conclusion: Bacillus species can be a potential source of antibiotics with inhibitory effect against both Gram's positive and Gram's negative organisms along with potent effect against MRSA.***

Keywords: Antibiotics, Antimicrobial Activity, *Bacillus* species, Methicillin Resistant *Staphylococcus aureus*, Multi Drug Resistant

1. Introduction

The discovery of penicillin was a milestone, a new era in the treatment of infectious diseases, described as the "golden age" of antibiotic research (1940–1962) but it also created a new threat to human leading to the emergence of drug resistance microorganisms [1, 2]. Treatment of multiple drug resistant (MDR) infections are challenging and expensive, and few antimicrobial compounds, and still fewer antimicrobial agents using novel mechanisms of action, are available and in clinical development. Thus, bacterial pathogens that are resistant to multiple drugs represent a growing public health threat [3]. Resistance against antimicrobial agents in bacterial pathogens is a global challenge associated with high morbidity and mortality. Multidrug resistant patterns in Gram-positive and Gram-negative bacteria have resulted in difficult-to-treat or even untreat- able infections with conventional antimicrobials [4].

Methicillin Resistant *Staphylococcus aureus* (MRSA), Methicillin Resistant Coagulase negative Staphylococci, Glycopeptide intermediate sensitivity *S. aureus* (GISA), and Vancomycin resistant *Enterococcus* (VRE) are among the troublesome MDR strains that have been intensively studied and observed [5].Methicillin-resistant *Staphylococcus aureus* (MRSA)with first appearance in 1960s, have developed resistant not only to methicillin (which was developed to fight against penicillinase-producing *S. aureus*) but usually also against other classes of antibiotics including aminoglycosides, macrolides, tetracycline, chloramphenicol, and lincosamides [2].

The genus *Bacillus* is one of the largest and most ubiquitous and has gained notoriety with taxonomists for its extreme phenotypic diversity and heterogeneity [6]. *Bacillus* is a genus with varied medical and industrial application and the most predominant beneficial medical-related products produced by this genus is antibiotics. Most of these are peptide antibiotics that are synthesized either by non-ribosomal enzymatic processes or by ribosomal synthesis of linear peptides that then undergo posttranslational modification and proteolytic processing [7]. Antibiotics are secreted as secondary metabolites in the late logarithmic or early stationary phase of growth of batch cultures. Study have showed that *Bacillus* spp. are capable of generating as many as 169 of these secondary metabolites; for example, various strains of *B. subtilis* have been shown to produce 68 antibiotics while 23 such products from *B. brevis*. Some of the antibiotic secreted by *Bacillus* includes Bacitracin, Polymyxin, Gramicidin, Bacilysin, Pumilin etc [8, 9]. Hence *Bacillus* spp. can be an alternative to the prevalent drug resistance. This study aims in identifying potential *Bacillus* spp. having antimicrobial activity against human pathogens and MRSA.

2. Materials and Methods

Sample collection and Isolation

Soil samples were collected from different locations within and outside of Kathmandu valley, Nepal. Sample was drawn from the upper layer of the soil as the microbial activity is more profound in this region and microbial population is also maximum. Soil was taken from about 5-10 cm below the soil surface. About 10 g of soil was collected from each site and inoculated on Nutrient Agar after serial dilution.

The cultured plates were incubated at 28°C for 24 hours. The next day, cultured plates were observed for the visible growth of colonies. The colonies giving a clear zone around them during initial isolation were taken and defined as antibiotic producing colonies. Other clearly isolated colonies were also taken to test the antimicrobial activity.

Identification
Identification was performed according to the flow chart of Bergey's Manual of Determinative Bacteriology.

Extraction of crude antibiotic by tube culture
Colonies suspected to produce antibiotics were cultured in Nutrient Broth for about 3 days.Turbidity or visual change in the culture tubes were checked on daily basis and were shaken properly every day. When sufficient growth indicated by the formation of thick pellicle was observed, centrifugation was carried out at 6000 rpm for 20 minutes. The pellet of cells formed at the bottom of tube was discarded while to the supernatant, equal volume of ethyl acetate was added and again centrifuged at 6000 rpm for 20 minutes. The lower layer of liquid obtained was the crude antibiotic [10].

Screening of antibiotic activity of crude antibiotic
The antimicrobial activity of the crude antibiotics was tested against culture of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Streptococcus* spp., *Klebsiella* spp., *Salmonella* spp., *Enterococcus* spp. and MRSA by agar- well diffusion Method. The negative control for the test was agar well in which ethylene acetate was diffused.

Antimicrobial susceptibility testing of the test cultures
All 7 bacterial cultures used as a test strains were tested for antibiotic susceptibility test by Kirby Bauer disc diffusion method with Mueller-Hinton agar. Antibiotics susceptibility test was carried out according to guidelines in CLSI 2012. Zone of inhibition obtained was used as a reference for the determination of efficacy of crude extracts [10].

3. Results

10 samples were processed in the study of which 5 samples were chosen from 5 different location within the Kathmandu Valley and rest of the 5 samples were selected from outside the Kathmandu Valley. From total of 10 samples processed, 41 bacterial isolates were isolated and screened for the antibiotic producers. Out of 41 bacterial isolates screened for antimicrobial property, 13 isolates were observed to be antibiotic producers demonstrating antimicrobial activity while remaining 28 isolates were non-antibiotic producers.

Based on the result obtained from the Gram's staining, spore staining and biochemical analysis, the 13 antibiotic producing isolates were isolated and on biochemical analysis, maximum isolates were identified as *Bacillus licheniformis* (7.69 %), *B. thuringiensis* (7.69%), *B. laterosporus* (15.81 %), *B. subtilis* (7.69 %), *B. firmus* (15.81 %), *B. brevis* (7.69 %), *B. alvei* (7.69 %), *B. megaterium* (7.69 %), *B. coagulans* (7.69 %) and *B. larvae* (15.81 %).

13 screened antibiotic producers were tested against three Gram negative test organisms of which only 7 isolates demonstrated inhibitory effect against *Salmonella* species. No inhibitory effect was observed against *E. coli* and *Klebsiella* spp. Highest zone of inhibition was demonstrated by D5 and R1 – 13 mm. All 13 screened antibiotic producers demonstrated an inhibitory effect against 4 of the Gram-positive test organisms. Maximum antimicrobial activity was demonstrated against *Staphylococcus aureus* in which the isolate X3 produced 35 mm zone of inhibition.

All of the 13 *Bacillus* species isolated demonstrated an antimicrobial effect against the Gram positive test organisms of which 7 isolates demonstrated an effect against Gram negative test organism *Salmonella* spp. Thus, total of 7 isolates (53.86 %) *Bacillus* isolates had a broad-spectrum activity, rest of 6 isolates (46.15 %) with its activity specific against Gram positive organisms. In the above study all of the 13 antibiotic producers were found to show inhibitory effect against MRSA strain while only 9 isolates were found inhibitory to *S. aureus*. Among 13 isolated antibiotic producers, 4 isolates were observed inhibiting the growth of MRSA but not *S. aureus*.

Table 1: Distibution of antibiotic producer location wise

S.N.	Sample Soil	Antibiotic Producers	Non-Producers	Total Isolates
1	Kathmandu – 5 Samples	6	17	23
2	Outside Kathmandu - 5	7	11	18
	Total	13	28	41

Table 2: Identification of antibiotic producing isolates

S.N.	*Isolates*	*Identified Bacillus spp.*
1	A5	*B. licheniformis*
2	D4	*B. thuringiensis*
3	D5	*B. laterosporus*
4	E2	*B. subtilis*
5	E6	*B. firmus*
6	E7	*B. firmus*
7	R1	*B. brevis*
8	R2	*B. laterosporus*
9	P3	*B. alvei*
10	P4	*B. megaterium*
11	P5	*B. coagulans*
12	X1	*B. larvae*
13	X3	*B. larvae*

Table 3: Zone of inhibition demonstrated by antibiotic producing isolates against Gram negative strains

S.N.	*Isolates*	*Diameter of Zone of Inhibition (in mm)*		
		Salmonella spp.	*E. coli*	*Klebsiella spp.*
1	*B. licheniformis* (A5)	12 mm	-	-
2	*B. thuringiensis* (D4)	10 mm	-	-
3	*B. laterosporus* (D5)	13 mm	-	-
4	*B. subtilis* (E2)	-	-	-
5	*B. firmus* (E6)	12 mm	-	-
6	*B. firmus* (E7)	-	-	-
7	*B. brevis* (R1)	13 mm	-	-
8	*B. laterosporus* (R2)	11 mm	-	-
9	*B. alvei* (P3)	-	-	-
10	*B. megaterium* (P4)	-	-	-
11	*B. coagulans* (P5)	-	-	-
12	*B. larvae* (X1)	12 mm	-	-
13	*B. larvae* (X3)	-	-	-

Table 4: Zone of inhibition demonstrated by antibiotic producing isolates against Gram positive strains

S. N.	*Bacterial Isolates*	*Diameter of Zone of Inhibition (in mm)*			
		Streptococcus spp.	*Staphylococcus aureus*	*MRSA*	*Enterococcus spp.*
1	A5	-	24 mm	23 mm	16 mm
2	D4	-	14 mm	16 mm	14 mm
3	D5	-	-	17 mm	12 mm
4	E2	-	16 mm	16 mm	-
5	E6	-	16 mm	15 mm	13 mm
6	E7	-	-	20 mm	15 mm
7	R1	-	16 mm	14 mm	16 mm
8	R2	-	22 mm	15 mm	17 mm
9	P3	-	-	13 mm	10 mm
10	P4	-	15 mm	11 mm	15 mm
11	P5	-	14 mm	12 mm	13 mm
12	X1	-	-	16 mm	12 mm
13	X3	10 mm	35 mm	16 mm	-

Table 5: Classification of the antibiotic producers based on their antimicrobial activity

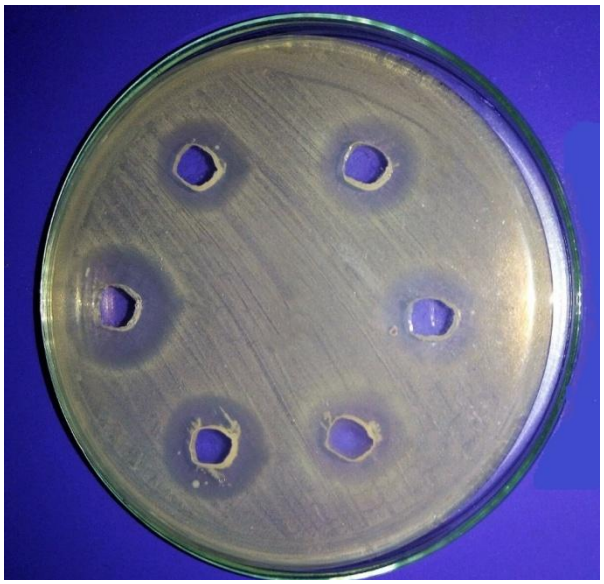
Antibiotic Producer's	Antimicrobial Activity Against		
	Gram Positive Test Strains	Gram Negative Test Strains	Broad Spectrum Activity
B. licheniformis (A5)	+	+	1
B. thuringiensis (D4)	+	+	1
B. laterosporus (D5)	+	+	1
B. subtilis (E2)	+	-	-
B. firmus (E6)	+	+	1
B. firmus (E7)	+	-	-
B. brevis (R1)	+	+	1
B. laterosporus (R2)	+	+	1
B. alvei (P3)	+	-	-
B. megaterium (P4)	+	-	-
B. coagulans (P5)	+	-	-
B. larvae (X1)	+	+	1
B. larvae (X3)	+	-	-
Total	13		7 (53.86 %)

Table 6: Antimicrobial susceptibility testing of pathogenic test strains- Gram negative

S.N.	Antibiotics	Zone of Inhibition	Inference
	Klebsiella spp.		
1	Gentamicin	11 mm	Sensitive
2	Ampicillin	8 mm	Resistant
3	Nalidixic Acid	19 mm	Sensitive
4	Erythromycin	5 mm	Resistant
5	Cefotaxime	31 mm	Sensitive
	Escherichia coli		
1	Ofloxacin	27 mm	Sensitive
2	Cefoxitin	10 mm	Resistant
3	Nitrofurantoin	18 mm	Sensitive
4	Amikacin	22 mm	Sensitive
5	Ampicillin	25 mm	Sensitive
	Salmonella spp.		
1	Ceftazidime	-	Resistant
2	Cefotaxime	11 mm	Resistant
3	Nitrofurantoin	16 mm	Intermediate
4	Gentamicin	25 mm	Sensitive
5	Nalidixic Acid	21 mm	Sensitive

Table 7: Antimicrobial susceptibility testing of pathogenic test strains – Gram positive strains

S.N.	Antibiotics	Zone of Inhibition	Inference
	Staphylococcus aureus		
1	Ciprofloxacin	40 mm	Sensitive
2	Erythromycin	34 mm	Sensitive
3	Ofloxacin	36 mm	Sensitive
4	Cefoxitin	23 mm	Sensitive
5	Nitrofurantoin	36 mm	Sensitive
	MRSA		
1	Ciprofloxacin	33 mm	Sensitive
2	Erythromycin	36 mm	Sensitive
3	Ofloxacin	35 mm	Sensitive
4	Cefoxitin	17 mm	Resistant
5	Nitrofurantoin	29 mm	Sensitive
	Streptococcus spp.		
1	Bacitracin	18 mm	Sensitive
	Enterococcus spp.		
1	Chloramphenicol	23 mm	Sensitive
2	Vancomycin	21 mm	Sensitive
3	Ampicillin	18 mm	Sensitive



Crude antibiotic extract demonstrating antimicrobial activities

4. Discussion

With an aim to isolate *Bacillus* species capable of antibiotic production with potential activity against MRSA, this study was carried out from November 2017 to January 2018 in the laboratory of St. Xavier's College. During this study a total of 10 soil samples were processed – 5 soil samples from within the Kathmandu valley while 5 samples outside the Valley.

Total of 41 isolates were obtained as the soil were processed and these were screened for the antibiotic production. Out of 41 isolates, 13 isolates (31.70 %) were screened as antibiotic producers and demonstrated its antimicrobial activity against various test organisms selected. On the contrary remaining 28 isolates (68.29 %) didn't exhibit any antimicrobial activity.

The study performed by Yilmaz et al in 2006 examined the antimicrobial activity of 29 *Bacillus* spp. Strains isolated from soil against tested bacteria of which 5 *Bacillus* spp. isolates showed antimicrobial activity, but other isolates did not show antimicrobial activity [11].

Based on the preliminary identification carried out according to Gram's staining and biochemical analysis, 13 isolates antibiotic producers were identified as, *Bacillus licheniformis* (7.69 %), *B. thuringiensis* (7.69%), *B. laterosporus* (15.81 %), *B. subtilis* (7.69 %), *B. firmus* (15.81 %), *B. brevis* (7.69 %), *B. alvei* (7.69 %), *B. megaterium* (7.69 %), *B. coagulans* (7.69 %) and *B. larvae* (15.81 %).

Among the 13 total antibiotic producing isolates 7 (53.86 %) were found to show inhibitory effect against *Salmonella* sp while none was found inhibitory to other two Gram negative test organisms – *Klebsiella* spp. and *E. coli*. In the study maximum inhibition was demonstrated by *B. brevis* and *B. laterosporus* with the inhibition zone of 13 mm diameter against *Salmonella* spp.

Bacillus genus is a large group and as many as 169 antibiotics as secondary metabolites have been reportedof which *Bacillus subtilis* have been reported to produce 68 antibiotics [8]. Thus, one of the major factors in determining the antimicrobial activity is the type of peptides secreted. Several mechanisms have been proposed to explain mode of action of peptide antibiotics but the exact mechanism is still unclear so mechanism probably depends upon the specific peptide, concentration and the bacterium. The major mode of action of polypeptides secreted by the *Bacillus* is the membrane permeability of the outer layer of the lipopolysaccharide (LPS) layer with some exceptions like Bacitracin that inhibits the cell wall synthesis [12].

In the case of our project, the crude antibiotics extracted from the *Bacillus* pp. was found non-inhibitory to *E. coli* and *Klebsiella* spp. with minimum activity against *Salmonella* spp. As mentioned earlier specific peptide, concentration and bacterium has major role in determining the antimicrobial activity and these factors must have contributed in the activity against Gram negative test organisms. Traditionally Gramicidin S has been considered as selective Gram positive inhibitors but the recent study has showed that if MIC measurements are done in correct fashion, Gramicidin S has an excellent activity against Gram negative organisms [13].

Development of resistance against the antimicrobial peptides (AMPs) could also assist to the low inhibitory effect against *Salmonella* spp. while no effect against other Gram's negative test organisms. Gram negative bacteria can inhibit natural AMPs in several ways. For example, firstly, proteolytic degradation leading to inactivation of AMPs. Secondly, resistance to AMPs can be attributable to different types of polysaccharides present in the bacterial cell envelop, such as capsule polysaccharide, biofilm-forming exopolysaccharides, and the O-polysaccharide of LPS. Thirdly, resistance to AMPs by modification of outer membrane of Gram-negative bacteria in which LPs play a crucial role. And finally, resistance is also possible by the pumping out or in of AMPs in the cell through the members of ABC transporters and the resistance nodulation-division efflux pump families [12].

All of the 13 *Bacillus* isolates demonstrated an inhibitory effect against the Gram positive test organisms. 11 isolates exhibited inhibitory effect against *Enterococcus* spp. with maximum inhibition demonstrated by *Bacillus laterosporus* (R2) while single isolate developed an inhibition against *Streptococcus* spp. 9 isolates exhibited an antimicrobial activity against *Staphylococcus aureus* while all the 13 isolates showed an inhibitory effect against MRSA. Based on the inhibitory effect observed against Gram positive and Gram's negative test organisms, the isolate *Bacillus* species were found to be more potent against Gram positive than Gram negative organisms. Similarly, 7 isolates produced an inhibition halo against Gram negative test organism – *Salmonella* spp. and all of the isolates produced an inhibition halo against Gram positive test organisms, 7 isolates (53.86 %) were found to show broad spectrum activity. 6 (46.15 %) isolates were found to be specific Gram positive inhibitors while none of the isolates were specific Gram negative inhibitors.

Majority of the AMPs are effective against Gram positive rather than Gram negative with activity that causes cell permeability affecting the cytoplasmic membranes. AMPs are cationic with positive charge that specifically bind with the teichoic acid of cell wall that facilitates the entry of the AMPs and finally affects the cytoplasmic membrane. Resistance against the AMPS are also not much as shown by the Gram negative organisms, this mediates in effective activity against Gram positive organisms. Thus, these factors can be considered to have a role in determining the maximum inhibitory activity against Gram positive test organisms.

In a study similar like this where the activity of the antibiotic producers was only tested against Gram positive bacteria, out of 51 bacterial colony isolated, 3 bacteria (5.88%) showed antibacterial activity against Gram positive bacteria, and also had good activity against MRSA [14]. In the study *B. larvae* was found to be a potent Gram positive inhibitor. Study performed by Abdulkar and Waliyu in 2012 found out *B. lentus* as a potent antibiotic producer that exhibited an effective inhibitory effect to the growth of Gram positive bacteria [15].

The primary motive of this study was to demonstrate an inhibitory effect against a multi-drug resistant strain – MRSA. Antimicrobial resistance is a major global health concern, and, of the Gram positive bacteria, drug-resistant *Staphylococcus aureus* is a serious threat. *S. aureus* causes a wide range of infections commonly involving the skin, soft tissue, bone, joints, and infections associated with indwelling catheters or prosthetic devices. In addition, *S. aureus* is a leading cause of bacteremia in industrialized nations [16]. Thus, it is of upmost importance to discover new drug to tackle MDR strains.

In the study all the isolated antibiotic producers were found to demonstrate inhibitory effect against MRSA strain. Highest inhibitory effect was marked by *B. licheniformis* that produced up to 23 mm zone of inhibition followed by *B.*

firmus producing 20 mm zone of inhibition, least effect was demonstrated by *B. megaterium* exhibiting 11 mm zone of inhibition followed by *B. coagulans* producing 12 mm zone of inhibition. Similarly, 9 isolates with maximum effect demonstrated by *B. larvae* (X3) with 35 mm inhibitory halo against *S. aureus* was observed. On comparative analysis of result obtained for MRSA and *S. aureus* it was found that of all isolated strains only 9 were inhibitory for S. aureus while all the strains were inhibitory to MRSA.

Exact mechanism for this is not well known but some factors can be pointed out for this result. Development of resistance towards AMPs, Variation in the bacterial strains and presence of other compounds other than crude antibiotics can be considered as points for non-inhibitory effect against *S. aureus* by some crude extracts on contrary to total inhibitory activity by all the isolates against MRSA.

In a research similar to the above study three isolates – *B. subtilis*, *B. megaterium* and *B. licheniformis* isolated from an Egyptian soil was found to demonstrate considerable inhibitory effect against the MRSA producing zone of inhibition greater than 20 mm (Shaaban et al 2015) [17. In the research conducted by Chalasani et al., the cell free modified tryptone soya broth (pH 7.4 ± 0.2) of *Bacillus subtilis* URID showed significant antimicrobial activity against multidrug-resistant strains of *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes* and *Enterococcus faecalis* [18].

Despite these challenges to the development of *Bacillus*-derived AMPs as alternatives for conventional antibiotics, the emergence of drug-resistant bacteria has necessitated a constant search for new AMPs. More studies focusing on AMPs are urgently required to bring these potential antibiotics into practical use and to fully realize their commercial and biotechnological applications. Microbial peptides have the potential to combat the escalating problem of single-drug and multi-drug-resistant infectious pathogens in the foreseeable future and may constitute a new generation of antibiotics [12].

5. Conclusion

A total of 13 *Bacillus* isolates were screened as antibiotic producers out of 41 isolates isolated from soil samples. 13 isolates - *Bacillus licheniformis* (7.69 %), *B. thuringiensis* (7.69%), *B. laterosporus* (15.81 %), *B. subtilis* (7.69 %), *B. firmus* (15.81 %), *B. brevis* (7.69 %), *B. alvei* (7.69 %), *B. megaterium* (7.69 %), *B. coagulans* (7.69 %) and *B. larvae* (15.81 %) demonstrated inhibitory effect against test organisms selected for the study. From the study isolated *Bacillus* was found to be potent Gram positive inhibitor with all the isolates showing effect against Gram positive test organisms. Inhibitory effect against Gram negative organisms - *E. coli* and *Klebsiella* spp. was not observed while minimum effect was observed against *Salmonella* spp. Of total, 53.86 % isolates were found to have broad spectrum activity while 46.15 % were observed to be specific Gram positive inhibitors. In context of MRSA, all the isolated strains demonstrated an inhibitory effect where maximum inhibition was demonstrated by *B. licheniformis* showing zone of inhibition of 23 mm diameter. Thus, we can conclude that the *Bacillus* can be considered one of the possible alternative in the sector of new drug development to tackle the crisis arising due to multi-drug resistant strains.

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