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A New Broad-Spectrum Peptide Antibiotic Produced by *Bacillus brevis* Strain MH9 Isolated from Margalla Hills of Islamabad, Pakistan

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Abstract The antimicrobial peptide from a bacterial strain is isolated from soil sample of Margalla Hills of Islamabad, Pakistan. The peptide is found to significantly inhibit the growth of both Gram-positive (*Staphylococcus aureus* ATCC 6538 and *Micrococcus luteus* ATCC 10240) and Gram-negative (*Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 14028) bacteria as compared to gramicidin as standard. The bacterium is identified as *Bacillus brevis* strain MH9 based on phenotype and phylogenetic analysis. The antibacterial polypeptide was produced optimally at 35 °C after 48 h of growth, precipitated by 50 % ammonium sulphate, and further purified using HPLC. The sequential steps of purification decrease the peptide contents with prominent antibacterial activity. The peptide composed of 11 amino acid was further characterized by FT-IR and NMR. Results suggested that the peptide molecule is a novel antibacterial agent that is effective against both Gram-positive and Gram-negative bacteria. This study may have important implications for new peptide antibiotic that could be a new addition to treat infections.

Keywords Antibiotic characterization · *Bacillus brevis* MH9 · Bacterial peptide antibiotic · Peptide purification

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Introduction

The bacterial infections and resistance to broad-spectrum antibiotics are progressively increasing, demanding identification and development of new bioactive compounds against these pathogens. Since the microorganisms are reservoir of various pharmacological active compounds (Smith et al. 1997; Spellberg et al. 2008) development of antibacterial drugs from natural sources form such microbes are emerging as new trends (Jacob and Zasloff 1994).

The genus *Bacillus* has been extensively used in microbial and pharmaceutical biotechnology for production of antibiotics and several other important secondary metabolites (Stachelhaus et al. 1995). Several strains of *Bacillus* reported to produce polypeptide antibiotics including bacitracin, polymyxin, gramicidin, and colistin (Berdy 2005) under various environmental stresses (Kleerebezem and Quadri 2001). These polypeptide antibiotics are usually closely related to each other (Stachelhaus et al. 1995) and differ from each other by one or a few amino acid residues (Katz and Demain 1977).

Reported antibacterial polypeptides produced by the genus *Bacilli* are found effective mostly against Gram-positive bacteria (Anderson et al. 2001; Muhammad et al. 2014). However, polymyxin, colistin, and circulin show actions against Gram-negative species while bacillomycin, mycobacillin, and fungistatin are active against molds and

yeasts (Anderson et al. 2001). *Bacillus brevis* produces gramicidin-C (Egorov 1999) which is active against Gram-positive bacteria and selectively acts against only Gram-negative *Neisseria* species (Bourinbaiar and Coleman 1997).

Here we report the production, purification, and characterization of a new peptide antibiotic produced by a newly isolated strain (MH9) of *B. brevis* that is highly effective against both the Gram-positive and Gram-negative bacteria.

Materials and Methods

Isolation, Screening, and Identification of Bacterial Strains

Strains of Mesophilic *B. brevis* were isolated from soil sample of Margalla Hills Islamabad, Pakistan by serial dilution method. Ten bacterial isolates were purified on nutrient agar medium at 37 °C using streak plate technique (Caccavo et al. 1994) and tested against indicator strains including *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 14028, *Micrococcus luteus* ATCC 10240, and *Staphylococcus aureus* ATCC 6538 using agar well diffusion assay (Sen et al. 1995; Awais et al. 2008). The agar well diffusion method was used to analyze the antibacterial production by the strain. Briefly the turbidity of 24 h old cultures of indicator strains was adjusted to 0.5 McFarland turbidity standard. The test strains was then applied on the surface of Muller Hinton agar plates (30 ml) using sterilized cotton swab and permitted to dry for 15 min. We used sterilized stainless steel borer for well formation (6 mm). About 100 µl of the cell-free culture supernatants was transferred into each well. The agar plates were then incubated at required temperatures and observed for the development of zones of inhibitions around each well.

Screened bacterial isolates were morphologically characterized as described by Buchanan and Gibbons (1974). For phylogenetic characterization, genomic DNA was extracted from the bacteria using Wizard genomic Kit (Promega, Madison, USA) according to the manufacture's instruction and PCR was performed to amplify the 16 s rRNA gene using primers 5′F (5′-AAGTCGAGCGGA CAGATGG-3′) and 3′R (3′-GGGGAGGGTCATTGGAAC TGG-5′). PCR was carried out in a T-Personal combi PCR machine (Biometra, Germany # 2106284) with the following programs: 3 min denaturation at 95 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C, and a final extension step of 3 min at 72 °C. PCR products of the correct sizes were purified using JET quick PCR products purification spin/250 kit (GENOMED, Germany). Sequencing of PCR products was performed and analyzed in both directions using an ABI Prism 310 automated DNA sequencer using BigDye Terminator cycle sequencing kit (PE Applied and Biosystem USA). The 16S rDNA gene sequence of bacterial strain MH9 was compared with other bacterial sequences by NCBI Basic Local Alignment Search Tool (BLASTn) for their pair wise identities. Neighbor-joining method (Saitou and Nei 1987) was used for phylogenetic tree construction and analysis using MEGA 4.0 (Tamura et al. 2007).

Production of Antibiotic by *Bacillus brevis* MH9 at Optimum Conditions

Bacillus brevis MH9 was grown as described by Mendo et al. (2000). The medium for the production of antibiotic was composed of L-glutamic acid: 5 g/l; KH_2PO_4: 0.5 g/l; K_2HPO_4: 0.5 g/l; $MgSO_4$: 0.2 g/l; $MnSO_4$: 0.01 g/l; NaCl: 0.01 g/l; $FeSO_4$: 0.01 g/l; $CuSO_4$: 0.01 g/l; $CaCl_2$: 0.015; and Glucose: 1 %. The pH was adjusted to 7.5. Antibiotic was produced in a 500 ml shake-flask at 35 °C and 150 rpm agitation rate. Finally, the culture was centrifuged (10,000 rpm, 10 min, 4 °C) to separate the bacterial cells and the supernatant was stored at 4 °C (Bushra et al. 2007; Muhammad et al. 2009; Muhammad and Ahmed 2015). Optimization factors for the production of antibiotic was studied at different set of parameters: incubation time (24–144 h), agitation rate (100–150 rpm), pH (4–9), temperature (25–45 °C), glutamic acid concentration as a source of nitrogen (0.25–2 %), and carbon concentration (0.25–3 %). The antibacterial activity was confirmed using agar well diffusion assay (Sen et al. 1995).

Purification of Peptide Antibiotic

Precipitation of the peptide antibiotic was carried out using ammonium sulphate (Wingfield 2001; Charles et al. 2008). The culture supernatant was treated with powdered ammonium sulphate (20, 40, 50, and 60 % saturation). After sufficient shaking, the solution was placed on ice for 1 h and then the precipitates were collected by centrifugation at 10,000 rpm for 20 min at 4 °C. The pellet was re-suspended in 15 ml of 0.05 M potassium phosphate buffer at pH 7.5. Dialysis was carried out against the same buffer for 24 h in dialyzing bag and the pellets were freeze dried. The resultant pellets were solubilized and finally purified using isocratic elution of 40 % (v/v) acetonitrile and 0.1 % (v/v) tri-fluoroacetic acid through preparative reverse phase column chromatography HPLC (C_{18} column; 5 µm; 4.6 mm × 150 mm; Agilent Technologies, USA) at a flow rate of 1.0 ml/min. The collected fractions were assayed for antibiotic activity against Gram-positive *S. aureus* and Gram-negative *E. coli* using agar plate well diffusion assay

(Sen et al. 1995; Awais et al. 2008). Peptide antibiotic samples were lyophilized and stored at −20 °C for further analysis.

Comparative Antibacterial Activity Analysis

Antibacterial activity of purified peptide sample obtained from *B. brevis* MH9 was compared to gramicidin (Sigma Aldrich) taken as standard by agar well diffusion assay.

Characterization of Peptide

The total protein content of the peptide antibiotic was estimated according to the method as described by Lowry et al. (1951). In order to determine the purity and molecular weight of the peptide, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10 % polyacrylamide and 5 % of stacking gel (Lee et al. 1987) were used. Samples were prepared by dissolving in equal proportion of sample buffer and loaded on the wells along with molecular weight standard (spectra multicolor, page ruler low range unstained protein ladder). Half of the gel was stained with Coomassie blue dye (Lee et al. 1987) while the other half was directly assayed for antimicrobial activity following the protocol of Bhunia and Johnson (1992) to identify the peptide antibiotic on SDS-PAGE.

Amino Acid Analysis

Amino acid analyzer LC3000 was used for residual analysis of the peptide and FT-IR and NMR were used for the characterization. The FT-IR spectroscopic analysis was carried out using an Alpha FT-IR (Bruker, Germany) in the 4000–500 cm^{-1} region. Peptide samples for NMR study were prepared by taking 10 mg in 0.5 ml D_2O and spectra were analyzed in Bruker 300 MHz spectrometer equipped with 5 mm of probe-head for ^1H analysis.

Results

Finding Antibacterial Activity

The active metabolites produced by ten different isolates exhibited different degree of antibacterial activities. Among these ten isolates, the strain MH9 showed most significant results (zone of inhibition ranging from 18–28 mm) against both the Gram-positive and Gram-negative test bacteria. Most notably, the MH9 strain inhibited *S. aureus* growth with a zone of inhibition of 28 mm (Table 1).

Identification of Bacterial Strain

Morphologically the bacterial strain MH9 was found to be Gram-positive motile and spore forming bacilli arranged in pairs. Bacterial colony on nutrient agar plate was circular, flat and whitish in color indicating *Bacillus* specie. According to 16 s rRNA gene based phylogenetic analysis, the isolate is *B. brevis* (Accession number: X60612.1).

Phylogenetic tree with the sum of branch length 0.002 was constructed by neighbor-joining method and evolutionary relationship of sequence of query strain MH9 was determined in relation to reference sequences from NCBI (Fig. 1).

Evaluation of Peptide Antibiotic

The peptide antibiotic produced by *B. brevis* MH9 at optimized conditions (Fig. 2) was precipitated with 50 % saturated solution of ammonium sulphate. The *B. brevis* MH9 indicated optimum level of peptide antibiotic production at 35 °C, 7.5 pH, 1 % glutamic acid, and 1.5 % glucose concentration after 48 h of incubation. The precipitated peptide antibacterial compound was isolated and purified from the supernatant. The concentration of peptide sample was estimated and its antibacterial activity was measured. The details of these analyses are represented in Table 2. It was observed that additional purification steps increase the purity of the peptide, but reduce the yield of the peptide. The HPLC chromatogram of active fractions of peptide samples showed retention time of 24.04 min at 254 nm (Fig. 3).

Comparative Analysis of Antibacterial Activity

The purified peptide sample showed prominent antibacterial activity with a zone of inhibition of 34 and 28 mm against *S. aureus* and *E. coli* respectively as compared to gramicidin as standard which indicated 22 and 2 mm zone of inhibition respectively against both test bacterial strains (Fig. 4).

Characterization of the Peptide Antibiotic

The amino acid components of the purified peptide antibiotic fraction from MH9 revealed that it contains 11 amino acid residues (Table 3). SDS-PAGE showed a single band at position below 1.6 kDa marker (~1.4 kDa) (Fig. 5). The FT-IR spectrum of peptide antibiotic showed absorption peaks at the regions of 794 cm^{-1} (C=C), 1257 cm^{-1} (C–O), 1700 cm^{-1} (N–H), 1940 cm^{-1} (O–N–O), 2350 cm^{-1} (–C=N), 2810 cm^{-1} (=C–H), 3430 cm^{-1} (–N–C–H), 3490 cm^{-1} (H–O–H), and in the region of

Table 1 Antibacterial activity of isolated bacterial strains against reference bacterial species

Bacterial strains	Zone of inhibitions against test bacterial species (mm)*			
	Staphylococcus aureus ATCC 6538	*Micrococcus luteus* ATCC 10240	*Salmonella typhi* ATCC 14028	*Escherichia coli* ATCC 87064
MH1	4	2	0	0
MH2	2	5	0	0
MH3	0	0	0	0
MH4	0	0	0	0
MH5	6	2	0	2
MH6	0	0	0	0
MH7	4	4	2	2
MH8	4	6	0	2
MH9	28	25	18	22
MH10	4	6	0	0

* Zone of inhibitions using agar well diffusion assay

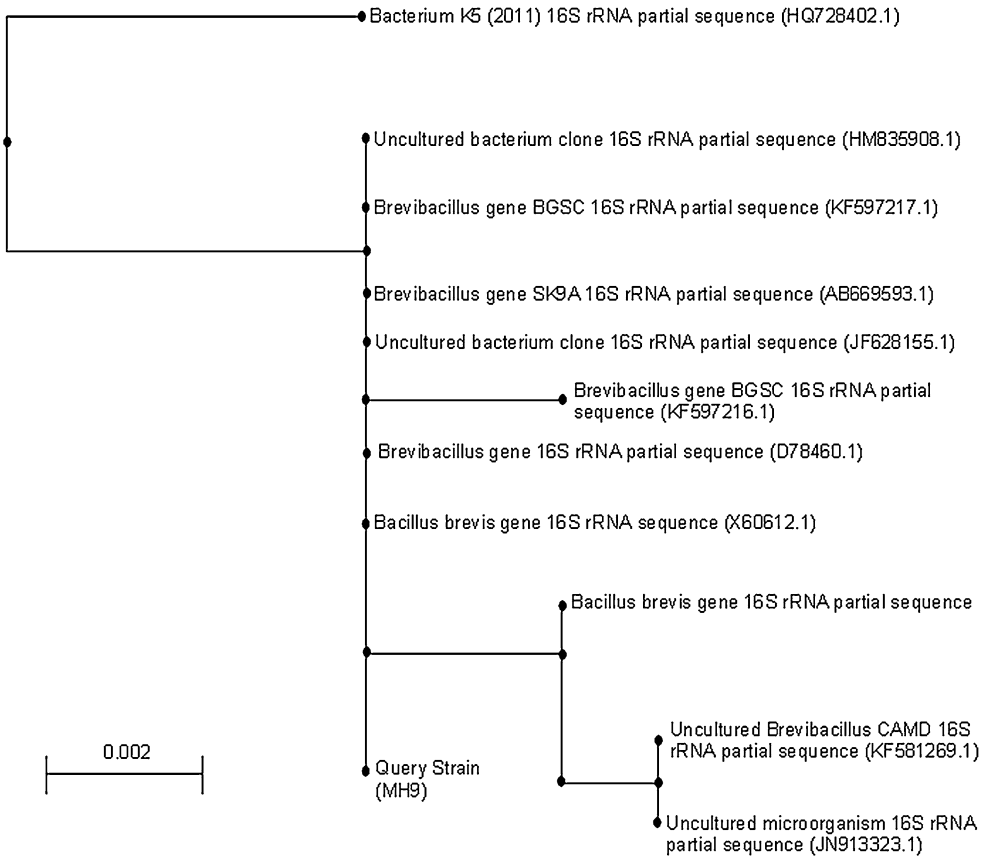

Fig. 1 Phylogenetic tree using the neighbor-joining method

3620 cm^{-1} (–OH) (Fig. 6). The ^{1}H NMR spectral signals demonstrated that the peptide is having both the aliphatic and aromatic groups: H-NMR (300 MHZ, 5 ppm): 7.25–7.37 (m, 4H, Ar–H), 5.37 (s, 2H, NH_2), 10.3 (bS, 1H, OH), and 13.9 (S, 1H).

Discussion

Bacillus species are known to produce secondary metabolites that are the object of natural product chemistry studies. Their antimicrobial activities have motivated the

pharmaceutical industry to search for lead structures in microbial extracts. Bacterial secondary metabolites including peptides antibiotics (Zuber and Marahiel 1997) are getting importance because of their increasing clinical usages (Schallmey et al. 2004). These polypeptides are having several advantages such as greater efficacy against broad spectrum multi-drug resistant bacterial pathogens among other uses (Augustin et al. 2009). The genus *Bacillus* is known for its antimicrobial peptides for decades against various microbial infections and industrial uses (Zhang and Falla 2004; Abriouel et al. 2011) and *Bacillus subtilis* reported to produce antimicrobial lipopeptides (Touré et al. 2004; Chen et al. 2008).

We have isolated *Bacillus* strains from Margalla Hills which is also found in some other geographical locations (Hill et al. 2004; Priest et al. 2004). Like the other *Bacillus* (Yasawong et al. 2011), our selected strain MH9 is morphologically Gram-positive, rod-shaped, aerobic, motile, and spore forming.

Most of the *Bacilli* are mesophilic that produce peptide antibiotics (gramicidins, tyrocidines, and bacitracins) (Egorov et al. 1987), while few of them are capable of growing at temperature above 40 °C. Such strains are *B. brevis* var. G-B that produces gramicidin C (Egorov 1999) and *Bacillus polymyxa* that manufactures gavaserin and saltavalin (Pichard et al. 1995). Gramicidins produced by *B. brevis* strains are heterogeneous mixture of polypeptide antibiotics (Bourinbaiar and Coleman 1997) that contains ~15 amino acid residues (Burkhart et al. 1999). Similarly, tyrocidine is the major constituent of tyrothricin, which also contains gramicidin. It is a mixture of cyclic-decapeptides obtained from *B. brevis* and contains 4-different amino acid sequences. Tyrocidine has been reported to be toxic to human blood and reproductive cells (Mootz and Marahiel 1997). While in our study, the isolated polypeptide antibiotic from the strain MH9 was found to contain 11 amino acid residues. Most of the antimicrobial peptides produced by *Bacillus* either acts against Gram-positive or Gram-negative pathogens (Anderson et al. 2001). Gramicidin, for example has been reported against Gram positive and selectively against Gram negative *Neisseria* species, while tyrocidine has been used only against localized Gram-

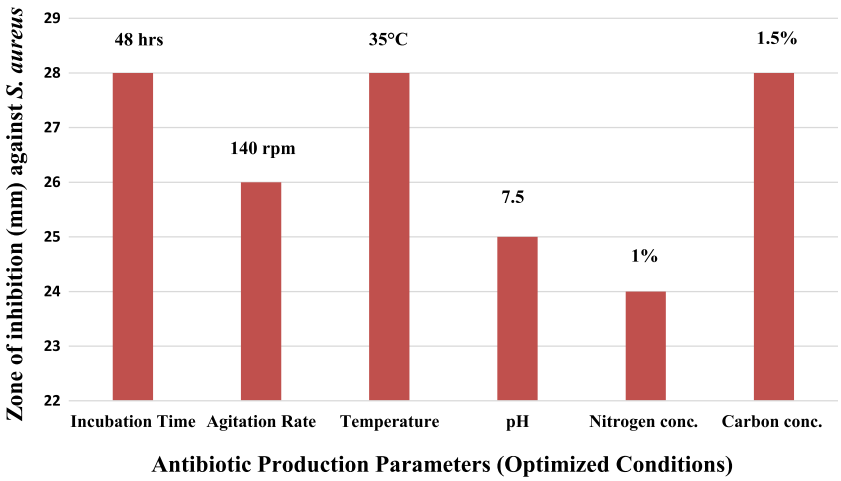

Fig. 2 Production of peptide antibacterial compound by *Bacillus brevis* MH9 at optimized conditions against *Staphylococcus aureus* as test strain

Table 2 Sequential steps of purification of peptide antibacterial compound produced by *Bacillus brevis* MH9 and estimation of total peptide contents

Aspects/Parameters	Sequential steps of purification		
	Culture supernatant[a]	NH_4SO_4 precipitation	Preparative HPLC
Volume (ml)	1000	30	1
Zone of Inhibition (mm)[b]	28	30	34
Zone of Inhibition (mm)[c]	22	24	28
Protein (mg\ml)[d]	6.2	3.0	0.12
Compound Recovery (%)[e]	100	48	2.0

[a] Supernatant obtained after centrifugation and membrane filtration

[b] Zone of Inhibition using *Staphylococcus aureus* as test strain by agar well diffusion assay

[c] Zone of Inhibition using *E. coli* as test strain by agar well diffusion assay

[d] Protein conc. was estimated by the Lowry method

[e] Recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration (initial concentration-final concentration/initial concentration × 100)

positive bacterial infections (Bourinbaiar and Coleman 1997; Robertson and Maibach 1998). But, we observed that our isolated peptide from MH9 strain is equally active against both the Gram-positive and Gram-negative test bacteria. The significant antibacterial activity of MH9 sample with a maximum zone of inhibition of 28 mm was observed against *E. coli* as compared to gramicidin which indicated 2 mm zone of inhibition (Robertson and Maibach 1998) against the same bacterial species.

Antibiotic production by bacteria varies quantitatively and qualitatively depending on the culture conditions (Chen et al. 1996; Fang et al. 1997) and strain (Berditsch et al. 2007). Important factors in culture conditions include-the time of incubation, pH, temperature, aeration rate, and chemical components including nitrogen, and carbon concentrations (Awais et al. 2008; Webster et al. 2002). *B. subtilis* strain SK.DU.4 is reported to produce antimicrobial peptides under 14 % NaCl after 24 h of growth (Baindara et al. 2013). In our case, the *B. brevis* MH9 exhibited optimum level of peptide antibiotic production at 35 °C after 48 h of incubation. The optimum pH, glutamic acid, and glucose concentration were 7.5, 1, and 1.5 %, respectively.

The first step in antibiotic purification is separation of crude antibiotic from the microbial growth followed by precipitation of proteins by 70 % ammonium sulfate (Shimogki et al. 1991). Subsequently, HPLC can be used for the purification and collection of active fractions containing bacitracin and other antibacterial polypeptides (Sunaryanto et al. 2010). Using a strong isocratic-HPLC technique Oka and co-workers (Oka et al. 1989) had purified 22 bacitracin constituents in isocratic conditions with 25 min run time while in current study, the peptide antibiotic from *B. brevis* strain MH9 was precipitated by

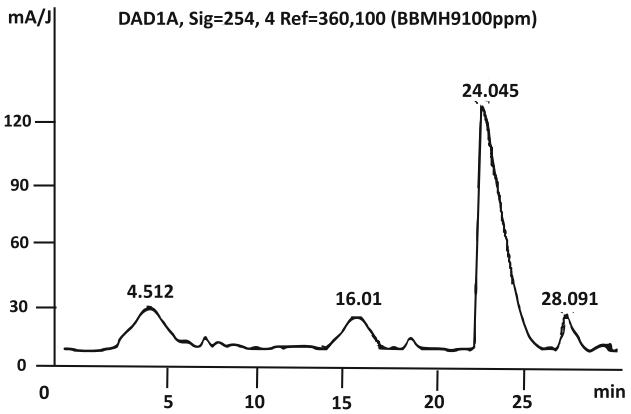

Fig. 3 Purification of peptide antibiotic produced by *Bacillus brevis* MH9 using preparative HPLC

Table 3 The amino acid analysis of the peptide antibacterial compound produced by *Bacillus brevis* MH9

Serial #	Amino acids (aa) residues	Abbreviations	Percentage*
1	Glycine	Gly	0.521
2	Leucine	Leu	ND*
3	Tryptophan	Trp	0.230
4	Tyrosine	Tyr	0.146
5	Lysine	Lys	0.222
6	Isoleucine	Ile	0.456
7	Alanine	Ala	0.712
8	Phenylalanine	Phe	0.201
9	Arginine	Arg	0.102
10	Aspartic Acid	Asp	0.431
11	Proline	Pro	ND*

* ND: Not determined

* Mole percent represents the amount of each amino acid present as a percentage of the total amino acids recovered in the sample that was analyzed using Amino acid analyzer LC3000. Mole percent can be useful for samples in which there is no known composition or molecular weight

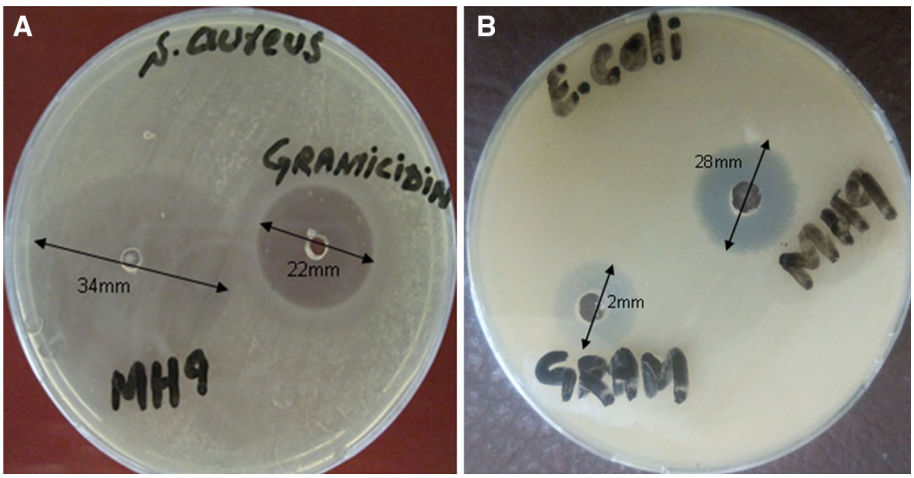

Fig. 4 Comparative antibacterial activity of purified peptide sample (MH9) and gramicidin antibiotic against test strains **a** *Staphylococcus aureus* **b** *Escherichia coli*

50 % saturated solution of ammonium sulphate and further purified by reverse phase column HPLC. The purified antibiotic peptide is ~1.4 kDa which is in agreement with a previous study where gramicidin from some *B. brevis* strain is reported to have molecular weight less than 2 kDa (Woolley and Wallace 1994).

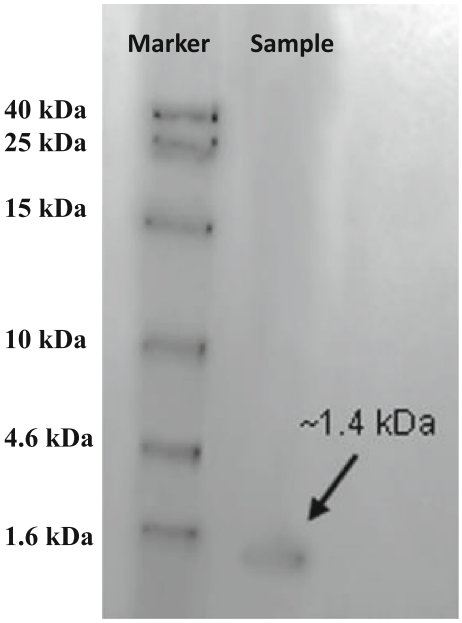

Fig. 5 SDS-PAGE analysis of purified peptide antibiotic produced by *Bacillus brevis* MH9, *arrow* indicates the protein band in the sample compared with the Marker

FT-IR spectra of gramicidin components were recorded in the region of 1000–3750 cm^{-1} (Zuber and Marahiel 1997) while the spectra of peptide antibiotic produced by *B. brevis* MH9 was recorded in the region of 794–3620 cm^{-1} supporting the finding of Rijs et al. (2011). These findings showed that peptide antibiotic may fall under gramicidin class. ^{1}H NMR spectra also suggested that the peptide sample contains alkyl groups, amide linkage, and carbonyl groups and is composed of amino acids: Gly, Leu, Trp, Tyr, Lys, Ile, Ala, Phe, Arg, Asp, and Pro. We studied a new peptide antibiotic produced by *B. brevis* strain MH9 with molecular weight of ~1.4 kDa. It is effective against Gram-positive and Gram-negative bacteria as compared to previously reported gramicidin and tyrocidine antibiotics. The peptide contains 11 amino acids residues however the exact sequence of the polypeptide chain is yet to be determined and also the gene that is translated into this peptide.

Conclusion

With increased emergence of bacterial resistance depicts the need of antimicrobial drug development. Results of this study suggested that the peptide molecule is a novel antibacterial agent that is effective against both Gram-positive and Gram-negative bacteria. This study may have important implications for new peptide antibiotic that could be a new addition to treat infections. It will be helpful in projecting low production cost, less toxicity and promising

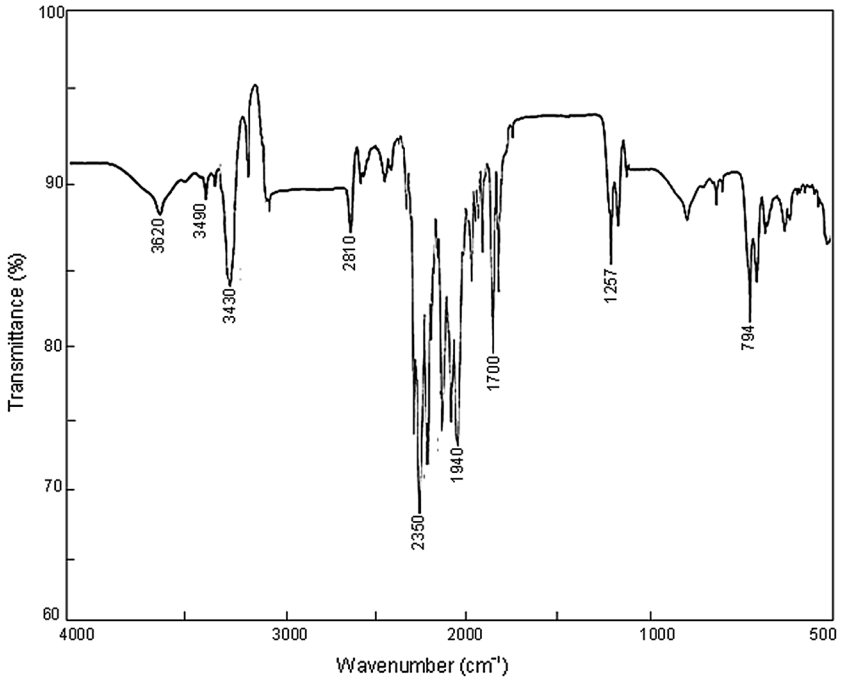

Fig. 6 FT-IR spectrum of peptide antibiotic produced by the *Bacillus brevis* MH9

pharmacokinetics to develop highly effective antimicrobial peptides with high biological activities.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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