Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6715 - 6721
Received 15 December 2020; Accepted 05 January 2021.

Activity and Toxicity Assessment of Partially Purified Bacteriocin from Lactic Acid Bacteria against *Mycobacterium Kansasii*

Revathy Kalyanasundaram[1], Manikkam Radhakrishnan*[2] and Sivaraj Anbarasu[3]

[1]Research Scholar, Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu

[2]Scientist E, Centre for Drug Discovery and Development, Col. Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu

[3]Scientist B, Centre for Drug Discovery and Development, Col. Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu

ABSTRACT

Non Tuberculous Mycobacteria (NTM) comprises of more than 170 different species. Their resistance and lack of new therapeutic agents poses the challenge to the effective treatment of diseases caused by NTM pathogens. Mycobacterium kansasii is one of the most frequently isolated NTM which cause pulmonary diseases in humans. The treatment of M. kansasii disease includes multiple antimicrobials for the period of more than 12 months. However, their adverse effects and uncertain effectiveness remains challenging. Lactic Acid Bacteria (LAB) is a diverse group of bacteria who inhabits in a wide range of environment such as dairy, vegetables, gastrointestinal tract of humans, etc., They produces an antimicrobial peptide called Bacteriocins which are active against closely related species as well as others. In this study, we have screened the partially purified bacteriocins (PPB) extracted from LAB of cow milk samples against M. kansasii (MTCC3058). PPB of LAB strain BLN34 showed more than 50% of reduction in the growth of M. kansasii by Colony Forming Unit (CFU) estimation method and exhibits less in vitro toxicity in Vero cell lines. Also 50% of zebrafish larvae were survived upto 144 hpf in the presence of BLN34 PPB. The LAB strain BLN34 was identified as Enterococcus italicus by 16s rRNA analysis.

Keywords: Bacteriocins, Lactic Acid Bacteria, Mycobacterium kansasii, Non Tuberculous Mycobacteria, Zebrafish larvae

INTRODUCTION

Non Tuberculous Mycobacteria (NTM) comprises of ever growing list of 170 different species (Forbes, 2017; Ahmed et al., 2020). The importance and interest towards NTM are recently increasing due to their intrinsic resistance to antibacterial agents, lack of new therapeutic agents, poor therapeutic strategies, etc., (Huang et al., 2020a). NTM infections are rising in the immunocompromised and vulnerable individuals. Mostly NTM infections are not considered as a problem till the end of the treatment course of disease (Ahmed et al., 2020). Frequent exposure to the NTM inhabitants accepts the transmission mode (Honda et al., 2018).Usually, they transmits by aerosol, ingestion, inoculation, inhalation, etc., and also by human to human transmission (Bryant et al., 2016).

Mycobacterium kansasii is one among the six frequently isolated NTM species worldwide. It is highly virulent NTM strains causing pulmonary disease in humans and rarely reported in wildlife. *M. kansasii* was first described by Buhler and Pollak in 1953 from the respiratory sample of patients with TB like disease. The treatment of *M. kansasii* diseases includes multiple antimicrobials for more than 12 months however the challenges in the effective treatment remains like intolerable adverse effects, uncertain effectiveness, etc., (Buhler and Pollak, 1953; Ford et al., 2020; Huang et al., 2020b).

Lactic Acid Bacteria (LAB) are diverse group of organisms phylogenetically which includes gram positive, cocci or bacilli, non sporulating, non motile, aerophilic bacteria. They produces lactic acid as

major fermentation product. Some of the important genera of LAB includes *Aerococcus, Enterococcus, Lactobacillus, Streptococcus, Pediococcus,* etc., It inhabits a wide range of environment such as dairy, vegetables, meat, gastrointestinal and vaginal tract of humans, etc., (Roberts et al., 2020). Many health benefits have been seen with LAB like lactose intolerance prevention, anticarcinogenic, anti obesity, anti diabetic, antimicrobial activity, etc., (de Melo Pereira et al., 2018).

LAB produces potential antimicrobial peptides called bacteriocins that are active against closely related species as well as others. Bacteriocins from LAB are effective in lower concentration when compared to the bacteriocins from eukaryotic origin (Nissen-Meyer et al., 2001). Non-toxic and Generally Recognised As Safe (GRAS) status of LAB bacteriocins made them a most extensively studied subject in biotechnology (Riaz et al., 2020).

In this study, we have screened four bacteriocins extracted from Lactic Acid Bacteria (LAB) isolated from cow milk samples against *M. kansasii* using Colony Forming Unit (CFU) estimation method. The toxicity analysis of potential bacteriocin was assessed under both *in vitro* and *in vivo* conditions and their taxonomy was also defined.

MATERIALS AND METHODS

Bacteriocins of LAB

Four partially purified bacteriocins (PPB) were obtained from LAB of cow milk samples and the method was published in Revathy et al., (2019 & 2020)

Anti *M. kansasii* activity of PPB

PPB were dissolved in PBS and the stock was made in the concentration of 10mg/ml (w/v). The stock was filtered using membrane filter with a pore size of 0.45 micron. Subsequently, suspension of *M. kansasii* (MTCC3058) was prepared by inoculating a loopful of culture into 0.3ml of Middlebrook 7H9 broth and vortexed for 30 seconds to homogenize. Then the volume was made upto the required volume using 7H9 broth. In a sterile cryovial, 400µl of 7H9 broth was taken as growth control, 350µl and 50µl of PPB was taken as test. 100µl of *M. kansasii* suspension was added to both the vials and incubated at 37˚C for 72 hours. Following incubation, 100µl of aliquot from both the vials was spreaded onto Middlebrook 7H11 agar plate and incubated at 37˚C for 15 days. After incubation, the colonies were counted and the percentage of inhibition by PPB was evaluated in test by compared to control.

Taxonomy of potential LAB

The genomic DNA of LAB strain BLN34 was isolated using solute ready genomic DNA kit. The isolated DNA was analysed by gel electrophoresis and quantified using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Gloucester, UK). The 16S rRNA gene sequence of the strain was amplified using the primers: 27F 5´AGAGTTTGATCMTGGCTCAG3´ (forward) and 1492R 5´TACGGYTACCTTGTTACGACTT3´ (reverse) (Kumar Gothwal et al., 2007). The PCR amplified product of the strain was sequenced and analyzed at National Chemical Laboratory (NCL), Pune, India. The sequence obtained was aligned with similar sequences available in GenBank using MEGA 7 program (Saitou and Nei, 1987). The aligned sequences of the strain BLN48 was used to construct the phylogenetic tree by following neighbor joining algorithm (Saitou and Nei, 1987) in MEGA 7 program. The bootstrap estimation (Felsenstein, 1985) was used to determine the confidence of the branches of the phylogenetic tree. The partial 16S rRNA nucleotide sequence of all the four strains has been deposited in GenBank database.

***In vitro* toxicity analysis**

Toxicity of potential PPB BLN34 was evaluated under in vitro conditions by MTT (3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay using Vero cell lines. In the assay, 100µl of desired concentration of PPB was added to 100µl of RPMI 1640 medium and mixed well. Then 100µl of the aliquot was serially diluted to the next well. The cultured Vero cell lines were harvested by trypsinization and pooled in 50ml vial. Then the plating of cells were done were done at 1x100 cells/ml. 200µl of Vero cells alone were used as control. The culture plate was incubated for 24 hours at 37˚C in incubator with 5% CO2. After incubation, 20µl of MTT solution was added to all the wells and incubated 37˚C for 4 hours. The media and MTT was mixed well and the absorbance was measured at 450nm and the percentage of viability was calculated manually (Vijayarathna and Sasidharan, 2012; Revathy et al., 2020).

***In vivo* toxicity analysis**

Toxicity analysis of PPB under in vivo condition was done using zebrafish model (Sisman et al., 2008). Freshly laid zebrafish eggs were obtained from zebrafish aquarium in Kanchipuram district, Tamil Nadu, India. Twenty healthy post hatched eggs were inoculated to the 24 well plate containing 1ml of embryo water (60mg of sea salt per litre of ultra pure water). Different concentrations of PPB were added to them and incubated upto 144h at 28.5˚C. Mortality of the zebrafish was noted at 24, 48, 96 and 144h. The embryos appeared opaque and white in colour. The dead embryos were degraded whereas the intact embryo structures were more visible by 48 hours post fertilization (hpf) which showed a clear difference between dead and alive embryos. The mortality rate was calculated. The embryos were photographed using light microscope at 10X magnification.

RESULTS AND DISCUSSION

Anti *M. kansasii* activity of PPB

Among the four PPB tested against *M. kansasii* (MTCC3058), BLN34 showed 0.37 log reduction viz., 57.34% of reduction in the growth of *M. kansasii* followed by BLN48 showed 0.15 log reduction viz., 28.44% of growth reduction whereas BLN36 and BLN39 did not show any inhibitory activity against *M. kansasii* (Table 1).

Taxonomy of BLN34

Amplification of 16S rRNA gene from BLN34 strain resulted in 894 bp sequences. BLAST analysis and phylogenetic tree also showed that LAB strain BLN34 is closely related to *Enterococcus italicus* (Figure 2). The nucleotide sequence of strain BLN34 was submitted to Genbank with accession number MT322789.

***In vitro* toxicity analysis**

In MTT assay, BLN34 showed 75% of viability in 10mM concentration and 64% of viability in 100mM concentration. The result shows that BLN34 is less cytotoxic to Vero cell lines (Table 2).

***In vivo* toxicity analysis**

In the toxicity study done using zebrafish larvae, around 50% of zebrafish were survived in the different concentration of PPB upto 144 hpf with healthy morphology under microscopic observation. The features like fin movement, swimming nature, tail development, etc., are active and normal with the viable embryos (Table 3) (Figure 1).

Antimicrobial peptides like bacteriocins are considered as promising candidates for drug development due to their various functions such as direct killing, immune modulators in infectious diseases, etc., Bacteriocins from LAB are extensively studied against *M. tuberculosis* whereas very few studies focused on their screening against non tuberculous mycobacteria (Teng et al., 2015; Abedinzadeh et al., 2015). Carroll et al (2010) have screened the bacteriocin produced by *Lactococcus lactis*, named

Lacticin 3147 against *M. kansasii* along with other NTM, M. avium. In this study, Lacticin 3147 showed 90% of reduction in *M. kansasii* growth. da Silva et al (2020) have screened various antimicrobial peptides against *M. abscessus*.

CONCLUSION

The importance and incidence of NTM diseases growing worldwide. *Mycobacterium kansasii* is one of the clinically significant slow growing NTM causing severe pulmonary diseases in humans and rarely wildlife infections. The complex treatment, their adverse effects and ineffectiveness necessitates the development of new therapeutic agents against *M. kansasii*. This study showed that partially purified bacteriocin of *Enterococcus italicus* BLN34 could be able to inhibit the growth of *M. kansasii* and also exhibits less toxicity under both in vitro and in vivo analysis. Therefore, further purification and characterisation of bacteriocin of *E. italicus* BLN34 can develop an effective candidate against *M. kansasii* as well as other slow growing NTM.

REFERENCES

1. Abedinzadeh, M., Gaeini, M., & Sardari, S. (2015). Natural antimicrobial peptides against *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy*, *70*(5), 1285-1289.
2. Ahmed, I., Tiberi, S., Farooqi, J., Jabeen, K., Yeboah-Manu, D., Migliori, G. B., & Hasan, R. (2020). Non-tuberculous mycobacterial infections—A neglected and emerging problem. *International Journal of Infectious Diseases*, *92*, S46-S50.
3. Bryant JM, Grogono DM, Rodriguez-Rincon D, et al. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. Science. 2016;354:751–7.
4. Buhler, V. B., and Pollak, A. (1953). Human infection with atypical acid-fast organisms; report of two cases with pathologic findings. Am. J. Clin. Pathol. 23, 363–374.
5. Carroll J, Draper LA, O'Connor PM, Coffey A, Hill C, Ross RP, Cotter PD, O'Mahony J: Comparison of the activities of the lantibiotics nisin and lacticin 3147 against clinically significant mycobacteria. Int J Antimicrob Agents 2010;36:132-136.
6. da Silva, J. L., Gupta, S., Olivier, K. N., & Zelazny, A. M. (2020). Antimicrobial peptides against drug resistant Mycobacterium abscessus. *Research in microbiology*, *171*(5-6), 211-214.
7. de Melo Pereira GV, de Oliveira CB, Júnior AIM, Thomaz-Soccol V, Soccol CR (2018) How to select a probiotic? A review and update of methods and criteria. Biotechnol Adv 36:2060–2076.
8. Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(4), 783-791.
9. Forbes BA. Mycobacterial taxonomy. J Clin Microbiol 2017;55(2):380–3.
10. Ford, A. K., Niedringhaus, K. D., Anderson, A. N., LaCour, J. M., & Nemeth, N. M. (2020). Disseminated *Mycobacterium kansasii* infection in a white-tailed deer and implications for public and livestock health. *Journal of Veterinary Diagnostic Investigation*, *32*(1), 147-151.
11. Huang, H. L., Lu, P. L., Lee, C. H., & Chong, I. W. (2020b). Treatment of pulmonary disease caused by *Mycobacterium kansasii*. *Journal of the Formosan Medical Association*.
12. Huang, W. C., Yu, M. C., & Huang, Y. W. (2020a). Identification and drug susceptibility testing for nontuberculous mycobacteria. *Journal of the Formosan Medical Association*.

13. Kumar Gothwal, R., Kumar Nigam, V., Mohan, M. K., Sasmal, D., & Ghosh, P. (2007). Extraction of bulk DNA from Thar Desert soils for optimization of PCR-DGGEbased microbial community analysis. Electronic Journal of Biotechnology, 10(3), 400-408.

14. Nissen-Meyer, J., H.H. Hauge, G. Fimland, V.G.H. Eijsink and I.F. Nes (2001). Ribosomally synthesized antimicrobial peptides by lactic acid bacteria: their function, structure, biogenesis and their mechanism of action. *Recent Res. Dev. Microbiol.,* **1:** 141–154.

15. Revathy K, Manikkam R and Sivaraj. Evaluation of inhibitory activity of bacteriocins from *Enterococcus italicus* BLN48 against *Mycobacterium fortuitum* and its toxicity profiling. Bioscience Biotechnology Research Communications. Vol 13 (3) July-Aug-Sep 2020 Pp-1156-1161.

16. Revathy K, Radhakrishnan M, Sivaraj A. Isolation, characterization of lactic acid bacteria from cow and buffalo milk and evaluation for antibacterial and antimycobacterial activity *in vitro.* Asian Journal of Microbiology Biotechnology and Environmental Sciences. Vol. 21, No. (4): 2019: 1041-1046

17. Riaz, N., Faheem, S. M., & Ahmad, M. U. A thematic review on potential antimicrobial potential applications of bacteriocins from lactic acid bacteria.

18. Roberts, A., & Barrangou, R. (2020). Applications of CRISPR-Cas systems in lactic acid bacteria. *FEMS Microbiology Reviews*, *44*(5), 523-537.

19. Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution, 4(4), 406-425.

20. Şişman, T., İncekara, Ü. and Yıldız, Y.Ş., 2008. Determination of acute and early life stage toxicity of fat- plant effluent using zebrafish (*Danio rerio*). *Environmental Toxicology: An International Journal*, *23*(4), pp.480-486.

21. Teng, T., Liu, J., & Wei, H. (2015). Anti-mycobacterial peptides: from human to phage. *Cellular Physiology and Biochemistry*, *35*(2), 452-466.

22. Vijayarathna, S., & Sasidharan, S. (2012). Cytotoxicity of methanol extracts of Elaeis guineensis on MCF-7 and Vero cell lines. Asian Pacific Journal of Tropical Biomedicine, 2(10), 826-829.

Table 1: Anti *M. kansasii* activity of PPB

PPB	Log Reduction	Percentage Reduction
BLN34	0.37	57.34
BLN36	0.04	9.4
BLN39	0.02	3.9
BLN48	0.15	28.44

Table 2: In vitro toxicity analysis by MTT Assay

PPB	Test concentration	Percentage of Viability
BLN34	100mM	64.18
	10mM	75.23

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6715 - 6721
Received 15 December 2020; Accepted 05 January 2021.

Table 3: In vivo toxicity analysis using zebrafish larvae

Concentration HPF	Control	10 µg/ml	50 µg/ml	100 µg/ml
0	20	20	20	20
24	20	18	18	17
48	18	14	13	12
96	16	11	9	9
144	13	8	7	6

Figure 1: Phylogeny of BLN34

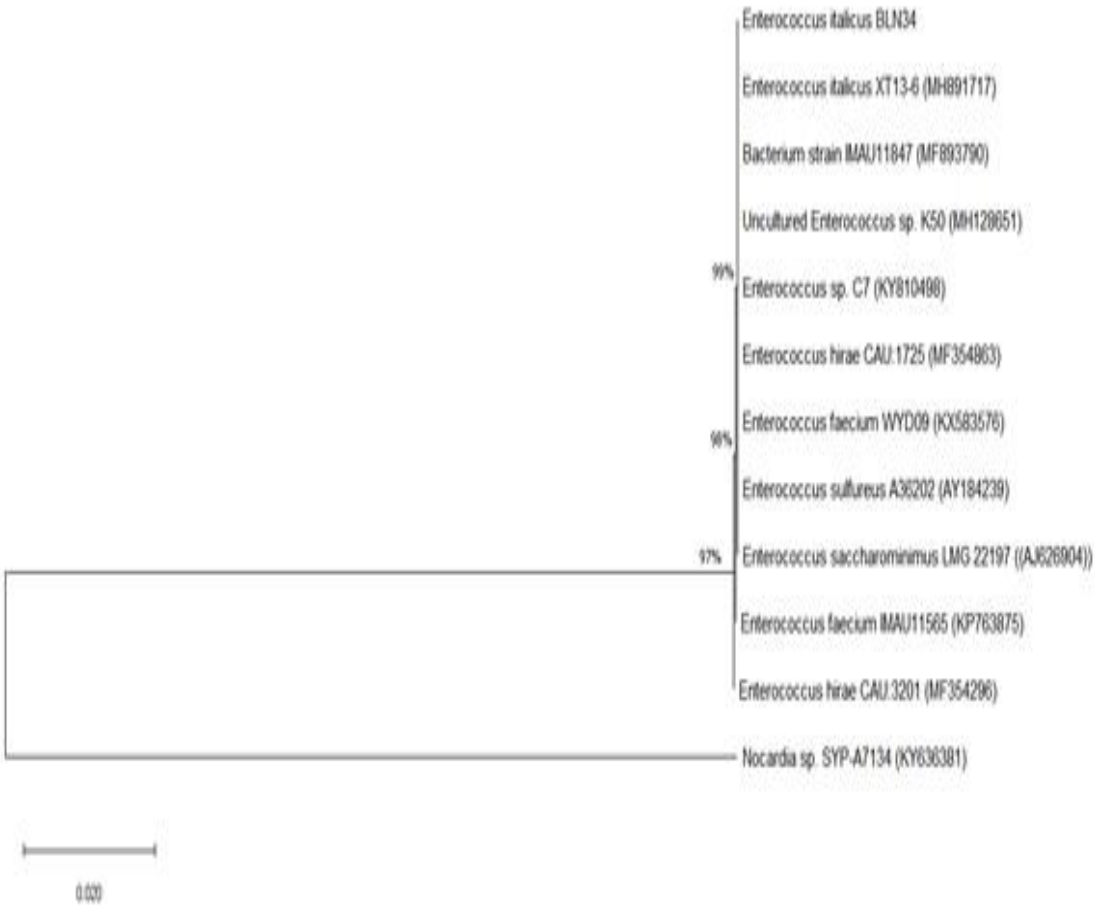
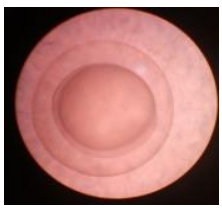
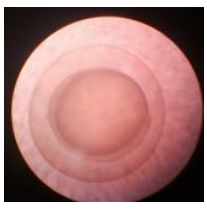
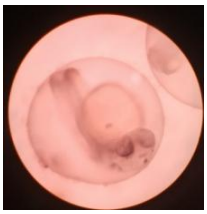
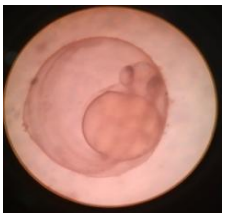
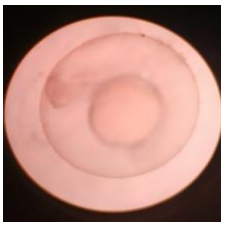
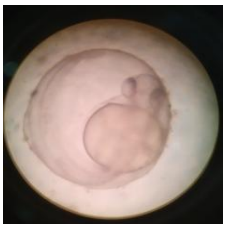
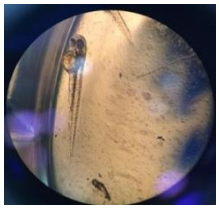
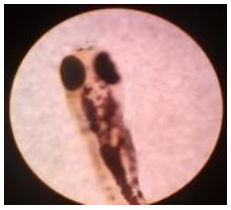


Figure 2: Microscopic Observation of Zebrafish Larvae

CONC HPF	Control	10 µg/ml	50 µg/ml	100 µg/ml
24 hpf				

Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6715 - 6721
Received 15 December 2020; Accepted 05 January 2021.

48 hpf				
96 hpf				
144 hpf