American Journal of Biomedical Sciences
ISSN: 1937-9080
nwpii.com/ajbms

Antimicrobial Activity of Paneth Cells Derived Cryptdin-2 Against Selected Pathogens

Simran Preet, Praveen Rishi*

Department of Microbiology, Panjab University-160014, Chandigarh, India
***Corresponding author**
Dr (Mrs.) Praveen Rishi
Department of Microbiology
Basic Medical Sciences Building
Panjab University,
Chandigarh –160014.
INDIA.
TEL: +91-172-2524146
FAX: +91-172-2541770
Email address: rishipraveen @yahoo.com

Received: 6 April 2009; | Revised: 30 June 2009; | Accepted: 21 July 2009

Abstract

As cationic antimicrobial peptides are major players of the innate immune response with broad antimicrobial activities, we evaluated the activity of Paneth cells derived cryptdin-2 against selected bacterial pathogens i.e *Yersinia enterocolitica, Salmonella typhimurium* (Gram negative) and *Staphylococcus aureus* (Gram positive). Cryptdin(s) preparations were obtained from crypt homogenates as well as from Paneth cells and analyzed using SDS-PAGE. A peptide band corresponding to 3.5 KDa (appx.) was eluted and purity was confirmed using HPLC. It was identified as cryptdin-2 according to its amino acid sequence. The peptide was found to have antimicrobial activity against the selected pathogens as indicated by radial diffusion assay as well as standard colony count technique. However, the activity was found to be the highest for *S. aureus*. This study thus highlights an important role of cryptdin-2 during host-pathogen interactions and indicates its potential as an inhibitory agent against the enteric infections.

Keywords: Cryptdin-2, Paneth cells; pathogens, antimicrobial activity, sequential histology.

1.0 INTRODUCTION

The gastrointestinal tract is continuously exposed to a variety of foreign bacteria ingested through contaminated food and drinks. Despite the high bacterial exposure, infections are rare which suggests highly efficient mechanisms both to protect the host and to maintain a host-microbe balance [1]. Intestine possesses many arms of innate protection from microbial invasion

including peristalsis, mucus secretion, low pH, bile salts, digestive enzymes and endogenous antimicrobials [2] including cationic peptides to which acquisition of resistant mutants is less likely to occur [3, 4].

A variety of antimicrobial peptides termed cryptdins (for crypt defensins) released by Paneth cells contribute to mucosal barrier function and have been classified as cryptdin-1 to cryptdin-6 [5]. Paneth cells are epithelial granulocytes at the base of the crypts of Lieberkuhn in the small intestine of many mammalian species [6]. These cells, rich in secretory granules containing microbicidal peptides are thought to help maintain the relative paucity of bacteria within this environment [7] thereby providing protection from intestinal infections [8].

The most compelling evidence for a Paneth cell role in enteric resistance to infection is evident from studies of mice transgenic for a human Paneth cell alpha-defensin (HD-5), which are completely immune to infections caused by orally administered bacterial pathogens [9]. The sensitivity of *E. coli* as well as *Giardia lamblia* trophozoites to cryptdins has also been observed earlier [10].Studies using knock out mice have suggested that deficient expression of Paneth cell α-defensin may contribute to the pathophysiology of Crohn's Disease, a chronic inflammatory bowel disease [11].

Recently, there has been a lot of commercial interest in developing cationic peptides as potential antimicrobial agents. The present study was therefore, planned to assess the effect of Paneth cell derived cryptdin(s) on the intestinal pathogens, ranging from luminally restricted but epithelial adherent as well as epithelial invasive strains. Three bacterial strains i.e *Salmonella typhimurium* NCTC74, *Yersinia enterocolitica* ATCC 23715 as well as *Staphylococccus aureus* ATCC 9144 were selected to assess the antibacterial activity of cryptdin-2. *Salmonella* is responsible for a variety of infections in humans and domestic animals ranging from gastroenteritis caused by *S. typhimurium* to severe systemic disease caused by *S. typhi* [12, 13]. Although *Y. enterocolitica* commonly causes intestinal infection in the form of mesenteric lymphadenitis and enterocolitis, the clinical picture is nevertheless quite variable [14]. The membrane damaging α-toxin of *S. aureus* has been reported to circulate in the blood affecting the intestinal epithelia in such a way so as to render it permeable to Gram negative bacteria in the normal intestinal flora as well as their endotoxins (LPS) [15].This study demonstrates the antimicrobial activity of Paneth cell cryptdin-2 against *Yersinia enterocolitica* in addition to *Salmonella typhimurium and Staphylococcus aureus.*

2.0 MATERIALS AND METHODS

2.1 Animals

Balb/c mice (20-25g) used in this study were procured from the Central Animal House of Panjab University, Chandigarh (India).The animals were housed under standard conditions of light and dark cycle with free excess to food (Hindustan Lever Products, Kolkata, India) and water *ad- libitum*. The experimental protocols were approved by the Institutional Ethical Committee of Panjab University, Chandigarh, India.

2.2 Bacterial strains

Y. enterocolitica ATCC 23715 and *Staphylococcus aureus* sub sp. *aureus* ATCC 9144 were procured from Institute of Microbial Technology, Chandigarh, India. *Salmonella typhimurium* NCTC74, procured from Central Research Institute, Kasauli, India was used in the present study. The strain has been maintained in our laboratory for the last several years and has also been used in recent studies [16, 17].

2.3 Extraction of cryptdins

Attempts were made to extract cryptdins from crypt preparation as well as from Paneth cell granules.

(i) *From crypt preparation*: Animals were subjected to fasting but were allowed to drink water *ad libitum* for 24h before being sacrificed. Crypts were isolated from the small intestine by the method as described by Bjernkes and Cheng [18].The intestinal segment under study was flushed with warm calcium magnesium free Hank's Balanced Buffered Salt Solution (CMF Buffer). After this, the lumen of the segment was

perfused with 1mM EDTA in CMF for 10 min and placed in cold CMF. It was then everted onto a glass rod, mounted on a stirrer assembly, and vibrated with ten short bursts into a tube of cold CMF to remove most of the villi (stage 1). Thereafter, the segment still mounted on the glass rod was incubated in a tube of cold 1mM EDTA in CMF for 10 minutes. At the end of this incubation, the segment was vibrated into cold CMF with five to ten short bursts to remove all residual villi (stage 2). Further, the segment was vibrated with three bursts into a fresh tube of CMF (stage 3). The loss of intestinal epithelium followed by the loss of villi and finally the crypts from the intestine into the EDTA buffer was confirmed by histological examination of the intestinal segment at various stages. The final fraction obtained thereby was homogenised and termed as P1. A small piece of the intestinal segment at various stages of the experiment was cut and fixed in 10% buffered formalin. Samples were processed, stained with heamotoxylin-eosin and examined microscopically. Microphotographs were taken with an Olympus binocular photomicroscope equipped with an automated digital camera (model DP12)**.**

(ii) *From Paneth cell granules*: Animals were subjected to fasting for 12-24h but were given water *ad libitum* before being sacrificed. Cells were isolated from the small intestine by the method as described by Eisenhauer et al. [19].The entire small intestine was removed and cut into four equal length segments, which were flushed with 0.9% NaCl containing 1mM dithiothreitol to remove luminal contents. Each segment was carefully everted on to a 0.1ml glass pipette, which was then placed in a tube containing phosphate buffered saline (PBS) (pH 7.4) with 27mM sodium citrate [20, 21]. After 30 and 60 minutes, the solution was removed and replaced with PBS that contained 0.5mM dithiothrietol and 1.5mM EDTA. The two exfoliated cell preparations obtained (rich in Paneth cells) between 30 and 60 minutes were washed twice with PBS containing 1mM EDTA. Both cell rich preparations were combined and centrifuged at 450 x g for 10 minutes at 4℃ to separate the supernatant from Paneth cells. The cells were suspended in 5ml of ice-cold 0.34M sucrose (pH 7.4) and homogenised. The homogenate was centrifuged at 450xg for 10min at 4℃, and the resulting pellet was centrifuged at 27,000 x g for 20 min to separate the supernatant from the granules rich pellet. This fraction was termed as P2. Total protein concentration in all the desired samples was calculated by the method of Lowry et al. [22].

2.4 Tricine SDS-polyacrylamide gel electrophoresis

Tricine sodium dodecyl sulphate – polyacrylamide gel electrophoresis (Tricine SDS-PAGE) for low molecular weight proteins was performed as described by Schagger and Jagow [23]. The SDS gel had a resolving gel of 16.5% acrylamide with a 10% acrylamide spacer gel and 4% stacking gel. The gel was subjected to electrophoresis for 6h at 80 V and stained by silver staining.The molecular weight of the peptide of interest was calculated according to the standard molecular weight markers. (Bangalore Genei, India).

2.5 Peptide elution and HPLC Analysis

The band corresponding to the peptide of interest (putative cryptdin) was selected and eluted out of the preparative gels as described by us earlier [24]. For further purification of the peptide, a10µl volume of the eluate obtained thereby was injected into a 4.6 X 250 mm sinochrom ODS-BP-5 column with a mobile phase system consisting of 0.1% triflouroacetic acid in 100% acetonitrile (solvent A) and 0.1% triflouroacetic acid in 100% water (solvent B). Gradient conditions were 20% (0.01min), 45% (25min), 100% (25.1min) for solvent A and 80%, 55% and 0% for solvent B for 30min at a flow rate of 1.0ml/min.The elution profile was monitored at 220nm.

2.6 Amino acid sequencing

The 3.5 KDa band (putative cryptdin) was transferred on to poly vinylidene diflouride (PVDF) membranes and sequenced using a PROCISE sequence Pro ™ 491 amino acid sequencer (Applied biosystems, USA) according to standard protocols of the manufacturer.

2.7 Determination of the antibacterial activity by radial diffusion assay

The antibacterial activity of the peptide was evaluated by the radial diffusion assay, a modification of the sensitive assay for defensins by Lehrer et al. [25]. A single colony of each of the test strain was inoculated in 50ml of full strength (3%w/v) trypticase soy both (TSB) for 18h at 37^0C separately. 100µl of this culture was inoculated into 50ml of fresh trypticase soy broth to obtain mid log phase cells and incubated for six h at 37^0C. The cells were pelleted, washed twice with 10mM sodium phosphate buffer, pH 7.4 (NAPB) and resuspended in 1ml 0f cold NAPB. A 10^{-3} dilution of this suspension containing appx. 10^6 bacterial cells were added to 10ml of preautoclaved, warm (42^0C) 10mM NAPB that contained 3mg of powdered TSB medium, 1% (w/v) low electroendosmosis agarose. After a rapid dispersion of bacteria, the agar was poured into agar plate to form a uniform layer. When the agar was solidified, it was punched to make evenly spaced wells. 10µl of the eluted peptide fraction containing 2.5 µg (as per the standardization in the laboratory) of cryptdin-2 or 0/01% acetic acid (as control) was added to wells. The plates were incubated for 3h at 37^0C and then overlayed with 10ml sterile overlay agar (maintained in fluid phase).0.01% acetic acid served as control. The overlay agar consisted of double strength (6% w/v) tryptic soy broth (TSB) and 1%w/v agarose. After overnight incubation at 37℃, the size of the clear zone surrounding each well was measured. The antibacterial activity was represented by measuring the size of the clear zone of inhibition of bacterial growth around the wells.

2.8 Standard colony count assay for antibacterial activity

Standard colony count assays were performed to assess the bactericidal activity of the extracted cryptdin-2 against selected strains. In these assays, 3µl of the cell suspension containing 55 x 10^5 cfu/ml of *S. typhimurium* and 98 x10^5 cfu/ml of *S. aureus* was added to 3ml of nutrient broth medium in specially designed flat bottom flasks. Similarly 3µl of the cell suspension containing 110 ×10^6 cfu/ml of *Y. enterocolitica* was added to 3ml of tryptose broth with thiamine HCl. 2.5µg of the peptide was added to all the test flasks and which were then incubated at 37^o C for 24h. Serial dilutions were prepared at 0, 4, 8, 12 and 24h and spread plated on nutrient agar plates for *S. typhimurium and S. aureus* while for *Y. enterocoltica* , the dilutions were spread plated on tryptose agar containing 0.025% thiamine HCl.

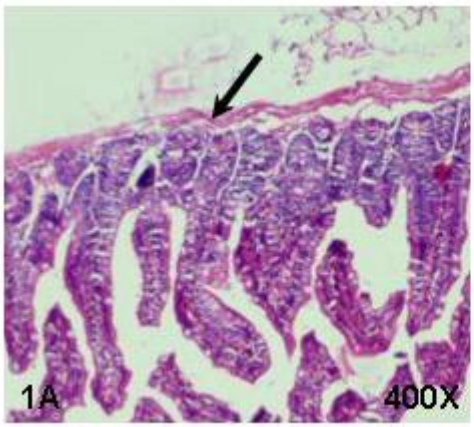

Figure 1A

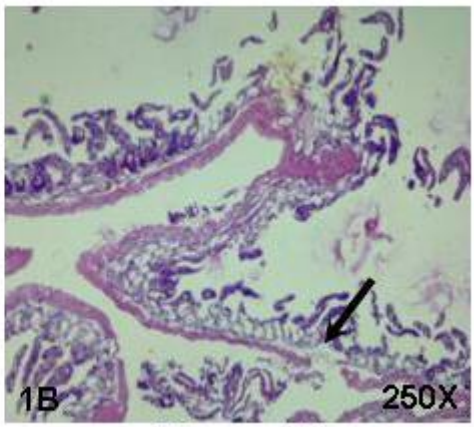

Figure 1B

Figure 1A-B: (1A) Photomicrographs of the control small intestinal ileum of Balb/c mice showing normal intestine. The ileum is within normal limits, mucosa shows prominent villi (cut in various planes) and the crypts look normal [400 x]. **(1B):** Photomicrograph of the mouse small intestine after vibration in cold Calcium magnesium free HBBS solution leading to the thinning out of villi with sporadic loss of lining cells (Stage 1). The entire row of crypts has only the basement membrane and the glands are disrupted. Villi have lost their integrity [250 x] **(Stage-1).**

3.0 RESULTS AND DISCUSSION

Recent investigations in murine models provide compelling support for the hypothesis that enteric cryptdins play a pivotal role in defence against food and water borne pathogens in the intestinal lumen [11]. Since cryptdins reportedly have potent activity in the micromolar range, these provide effective protection to the crypts. To evaluate the effect of various Paneth cell cryptdin isoforms on the pathogens, isolation of relatively pure epithelial preparations has long been a matter of interest. The present study demonstrates the extraction of antimicrobial peptide from the intestinal epithelium following sequential histology. Sequential loss of intestinal epithelium, followed by the loss of villi and finally crypts containing Paneth cells was observed by histological examinations of the small intestinal segment at various stages. (Stage I – Figure 1 A, B; Stage II – Figure 2 A-D; Stage III – Fig.3 A, B) These stages indicate the complete extraction of cryptdins from Paneth cell granules into the buffer leaving behind empty crypts. Thus, the evidence for the loss of granules containing cryptdins from Paneth cells has been provided in this study for the first time. Complete loss of crypts was observed indicating the migration of the crypts into the EDTA buffer. The protein content of P1 and P2 fractions as estimated by Lowry method was found to be 6 µg/ml and 7 µg/ml respectively.

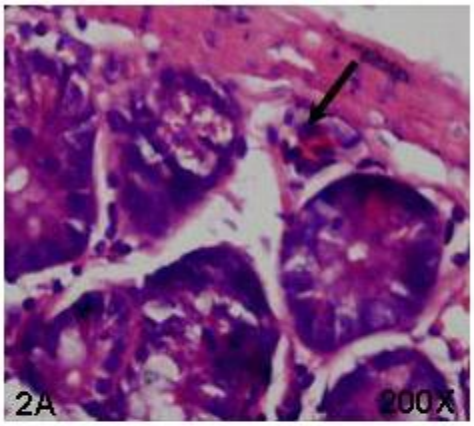

Figure 2A

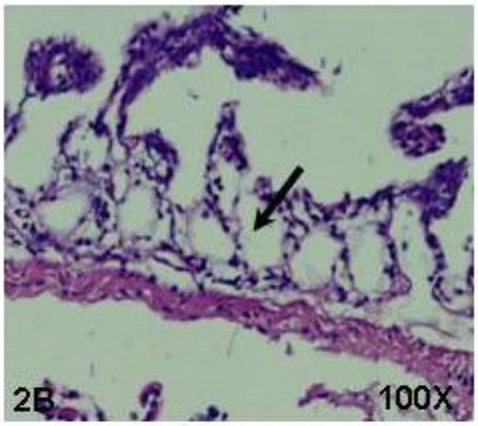

Figure 2B

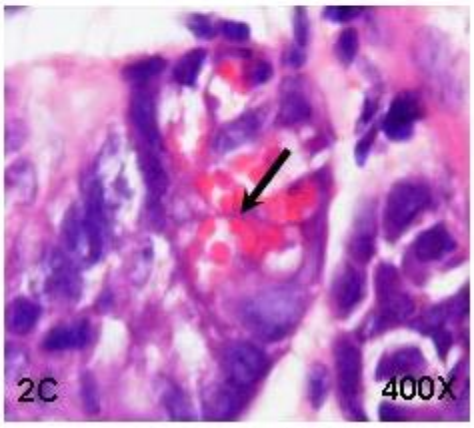

Figure 2C

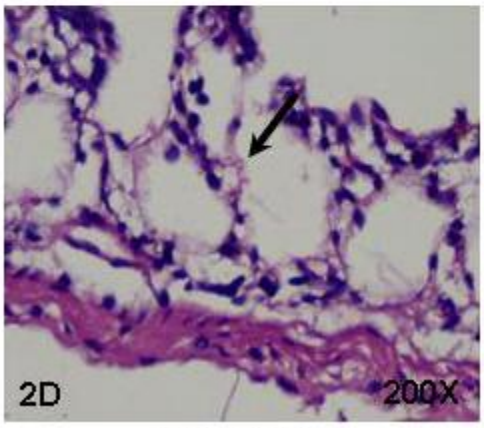

Figure 2D

Figure 2A-D: (2A) Photomicrograph of small intestinal crypts showing Paneth cells at their base (200 x). **(2B)** Photomicrograph of the small intestine after vibration in cold CMF followed by incubation under non shaking conditions in CMF with 1mM EDTA for ten minutes. Empty crypts are seen with basement membrane left behind [100 x]. **(2C)** Photomicrograph of the small intestine showing pink coloured Paneth cell granules containing cryptdins [400 x]. **(2D)** Epithelium of the villi is lost further and crypts ghosts are visible [200 x]. **(Stage II)**

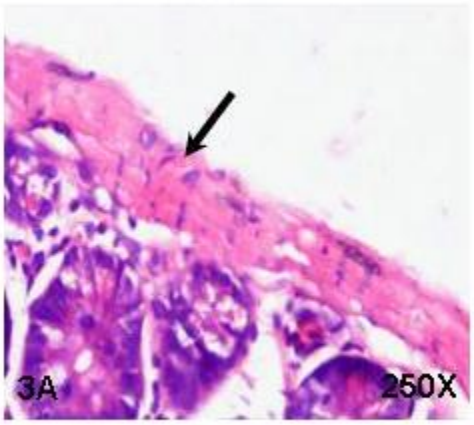

Figure 3A

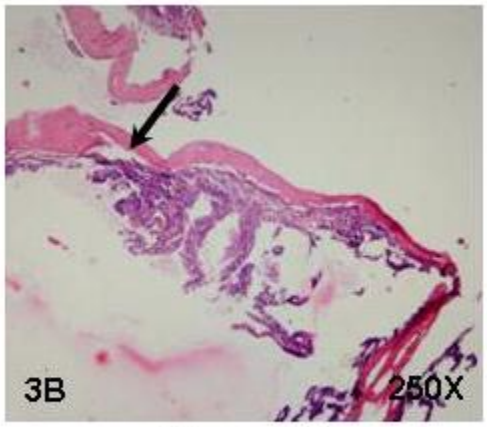

Figure 3B

Figure 3 A-B: (3A) Photomicrograph of the mouse small intestine showing a thicker normal ileum [250 x].**(3B)** Small intestine after vibration in cold CMF, incubation in EDTA buffer for ten minutes followed by vibration in cold CMF and bursts in fresh CMF solution .Villi ghosts are seen. A relatively thinner ileum can be observed **(Stage III)** [250 x].

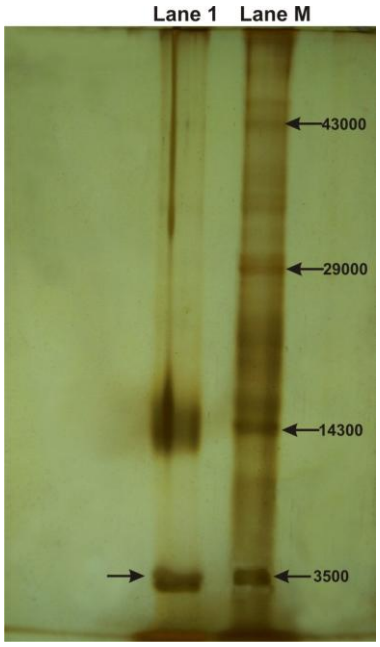

Figure 4: Silver stained Tricine SDS-PAGE gel. Lane 1 showing a 3.5KDa (arrow) band of the pooled fraction (P1 and P2) resolved on a 16.5%T, 6%C resolving gel. Lane M showing the standard protein marker.

As the two fractions (P1 and P2) could not be resolved individually on SDS-PAGE, the exfoliated Paneth cell preparation was pooled with the crypt homogenates. The peptides ranging from 3 to 10 KDa [26] have been reported to be best resolved using 16.5% resolving gels with urea [23]. In the present study also, both the fractions were pooled together and analyzed using 16.5%T and 4%C gels. Silver staining of the gels revealed two bands corresponding to 14KDa and 3.5 KDa (putative cryptdin band) (lane1) (Fig.4). The additional granule proteins such as phospholipase IIa, lysozyme and RNAse7 might have been released earlier during the experimental procedure with vibration bursts and therefore not retrieved in the final step. Since the molecular weight of the distinguishably visible 3.5KDa band corresponded to that of one of the enteric defensins, this band was eluted from the preparative gels and subjected to HPLC. On analyzing the HPLC chromatogram, a single peak was observed (Fig.5) which confirmed the purity of the extracted peptide. The amino acid sequence (PROCISE SequencePro^{TM}, 491) of the eluted peptide band consisting of 35 amino acid residues was found to be LRDLVCYCRTRGCKRRERMNGTCRKGHLM YTLCCR [**PDB Accession No. AAB22838.1**]. Interestingly, the amino acid sequence determined in the present study was found to be identical to that of native cryptdin-2 which has already been reported [27].

Earlier, it has been reported that *Salmonella* may decrease the expression of cryptdins thereby evading potent host immune defense mechanisms [28]. However, antimicrobial activity of cryptdin isoforms has also been demonstrated against *S. aureus* [29] as well as *S. typhimurium* carrying

PhoP mutations [30]. In the present study also, activity of cryptdin-2 has been demonstrated qualitatively as well as quantitatively against *Salmonella typhimurium* NCTC74 and *Staphylococcus aureus*. Although there are reports suggesting the sensitivity of *Yersinia enterocolitica* to the action of polycationic peptides (poly-L-lysine, poly-L-ornithine, melittin) [31], activity of cryptdin-2 against *Yersinia enterocolotica* has been demonstrated in the present study for the first time to the best of our knowledge.

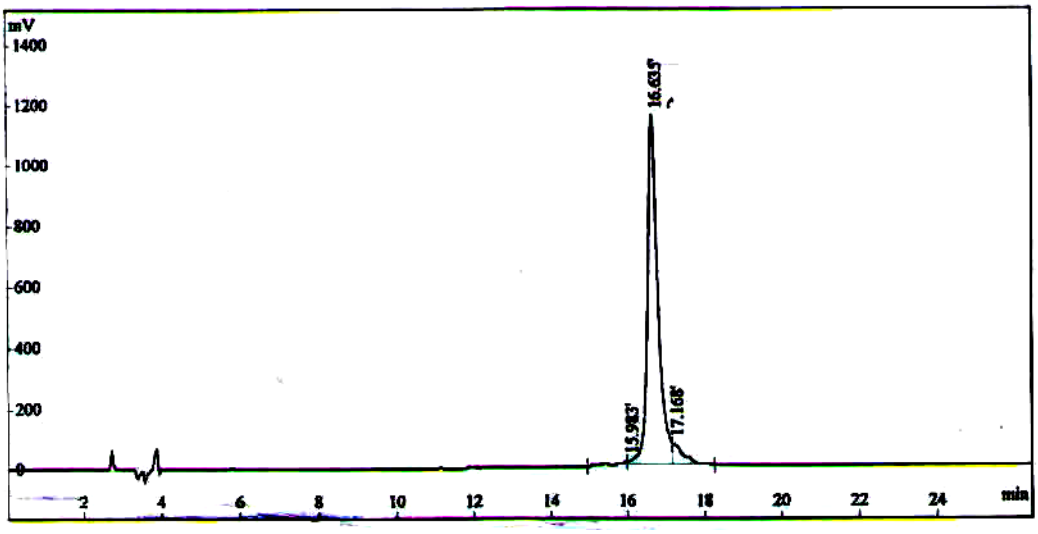

Figure 5: Autoscaled HPLC chromatogram of the eluted peptide showing a single peak

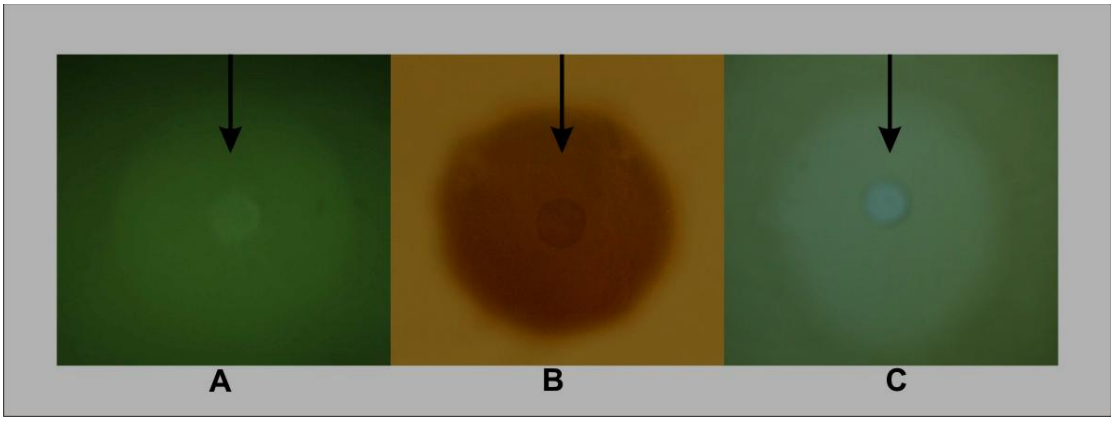

Figure 6A-6C: Radial Diffusion Assay. [6A] Growth inhibition zone of *Yersinia enterocolitica* ATCC 23715 (zone diameter- 3mm appx.) **[6B]** Growth inhibition zone of *Salmonella typhimurium* NCTC74 (zone diameter -4mm appx.) **[6C]** Growth inhibition zone of *Staphylococcus aureus* ATCC 9144 (zone diameter-4.1 mm appx).

In the present study, cryptdin-2 inhibited the growth of all these pathogenic strains which is evident from the respective zones of inhibition of growth around the wells filled with the peptide. The zone diameters were found to be 3.5mm (Figure 6A), 4mm (Figure 6B), and 4.2mm (Fig.6C) for *Y. enterocolitica*, *Salmonella typhimurium* and *Staphylococcus aureus* respectively. The peptide might have inhibited the growth either by permeabilizing and/or disrupting

the bacterial cell membranes or by translocating through the cell membrane and interacting with a cytosolic target [32].

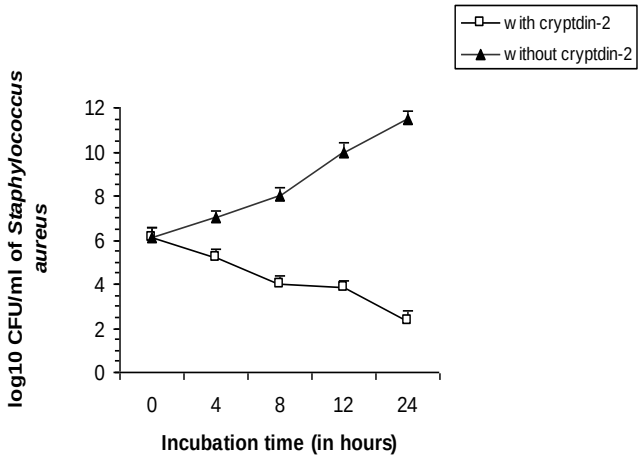

Figure 7: Log_{10} CFU/ml of *Staphylococcus aureus* ATCC 9144 at different time intervals in presence and absence of cryptdin-2. Values are expressed as mean ± standard deviation.

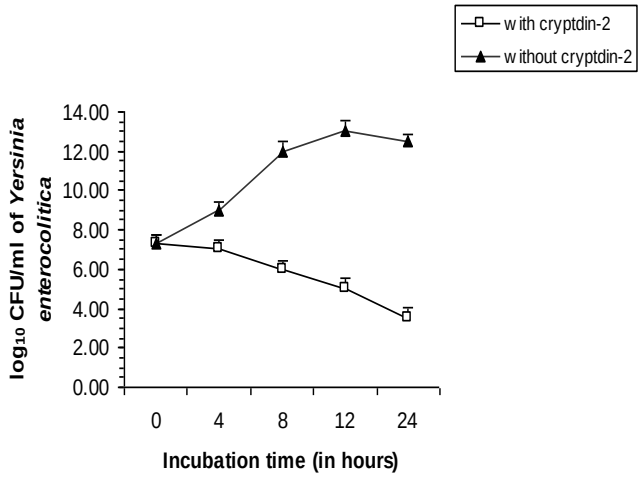

Figure 8: Log_{10} CFU/ml of *Yersinia enterocolitica ATCC* 23715 at different time intervals in presence and absence of cryptdin-2. Values are expressed as mean ± standard deviation.

Cryptdin-2 decreased the colony forming units of the test strains in a time dependent manner also. Approximately, 60% reduction in colony forming units could be observed in *S. aureus* (Figure 7) whereas in case of *Y. enterocolitica* (Figure 8) and *S. typhimurium (*Figure 9), 50% (appx.) reduction in the number of colony forming units was observed. Therefore, cryptdin-2 was found to be more effective against *S. aureus* than the Gram negative pathogens. Earlier also, this variation in potency against various bacterial strains has been observed for murine cryptdins [27]. It has been suggested that most likely, these differences may be due to the variations in the nature of interactions between antimicrobial peptides and microbial membrane components present in Gram positive and Gram negative bacteria. The study thus highlights the activity of cryptdin-2 against the three bacterial pathogens which might play an important role during host-pathogen interactions.

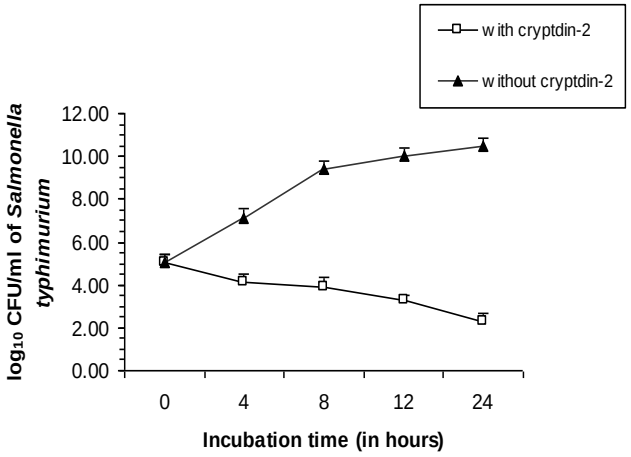

Figure 9: Log_{10} CFU/ml of *Salmonella typhimurium* NCTC 74 at different time intervals in presence and absence of cryptdin-2. Values are expressed as mean ± standard deviation.

ACKNOWLEDGEMENTS

The authors are grateful to the Indian Council of Medical Research (ICMR), New Delhi, India for providing assistance to carry out the research work. We also appreciate the help of Dr. B. N Datta, Ex-Professor of Pathology, Post Graduate Institute of Medical Education and Research, Chandigarh for providing help to carry out the histological studies.

REFERENCES

1. Karlsson, J.; Putsep, K.; Chu, H.; Kays, RJ.; Bevins, CL.; Mats A. Regional variations in Paneth cell antimicrobial peptide expression along the mouse intestinal tract, *BMC Immunol*, 2008, 9**,** 37.
2. Didierlaurent, A.; Simonet, M.; Sirard, JC. Innate and acquired plasticity of the intestinal immune system, *Cell Mol Life Sci*, 2006, 62(12)**,** 1285–1287.
3. Lupetti, A.; Dissel, JT.; Brouwer, CPJM.; Nibbering, PH. Human antimicrobial peptides' antifungal activity against *Aspergillus fumigatus*, *Eur J Clin Microbiol Infect Dis*, 2008, 27, 1125-1129.
4. Hancock, REW. Cationic antimicrobial peptides: towards clinical applications, *Exp Opin Invest Drug*, 2000, 9**,** 1723-1729.
5. Inoue, R.; Tsuruta, T.; Nojima, I.; Nakayama, K.; Tsukahara, T.; Yajima, T. Postnatal changes in the expression of genes for cryptdins1-6 and the role of luminal bacteria in cryptdin gene expression in mouse small intestine. *FEMS Immunol Med Microbiol*, 2008, 52, 407–416.
6. Ouellette, AJ.; Darmoul, D.; Tran. D et al. Peptide localization and gene structure of cryptdin-4, a differentially expressed mouse Paneth cell α-defensin, *Infect Immun*, 1999, 67(12)**,** 6643-6651.
7. Bevins, CL.; Porter, E M.; Ganz, T. Defensins and innate host defence of the gastrointestinal tract, *Gut*, 1999, 45, 911-915.
8. Menard, S.; Forster, V.; Lotz, M. et al. Developmental switch of intestinal antimicrobial peptide expression**,** *J Exp Med*, 2008, 205(1)**,** 183-193.
9. Ouellette, AJ. Paneth cell alpha-defensins: peptide mediators of innate immunity in the small intestine, *Springer Semin Immunopathol*, 2005, 27(2)**,** 133-146.
10. Aley, SB.; Zimmerman, M.; Hetsko, M.; Gillin, FD. Killing of *Giardia lambia* by cryptdins and cationic neutrophil peptides, *Infect Immun*, 1994, 62, 5397-5403.
11. Bevins, CL. Events at the host-microbial interface of the gastrointestinal tract. Paneth cell defensins in intestinal host defence, *Am J Physiol Gastr-L*, 2005, 289, 173–176.
12. Gianella, RA: Salmonella. In: Baron S, ed. Medical. Microbiology, 2nd edn. California**:** Addison Wesley Publishing Company 1998, 452-458.
13. Sood, S.; Rishi, P.; Vohra, H.; Sharma, S.; Ganguly, NK. Cellular immune response induced at peripheral and mucosal level by *Salmonella enterica* serotype Typhi iron regulated outermembrane proteins, *J Med Micro*, 2005, 54, 815-821.
14. Salem, AHA.; Elbashier, AM.; Nazer, MA. *Yersinia enterocolitica* colitis with peritonitis in a child with beta-thalassemia major, *Ann Saudi Med*, 2002, 22, 339-340.
15. Arbuthnottd, JP; Coleman, DC; Azavedo, JSD. Staphylococcal toxins in human disease, *J Appl Bacteriol* (symposium supplement), 1990, 101S-107S.
16. Rishi, P.; Kaur, S.; Bhalla, MPS; Preet, S.; Tiwari, RP. Selection of probiotic *Lactobacillus acidophilus* and its prophylactic activity against murine Salmonellosis, *Int J Probiotic Prebiotic*, 2008, 3 (2)**,** 89-98.
17. Rishi, P.; Kaur, P.; Virdi, JS.; Shukla, G.; Koul, A. Amelioratory effects of zinc supplementation on Salmonella-induced hepatic damage in the murine model. *Dig Dis Sci*, 2008, 53(4), 1063-70.
18. Bjernkes, M.; Cheng, H. Methods for isolation of intact epithelium from the mouse small intestine**,** *Anat Rec*, 1981, 199, 565-574.
19. Eisenhauer, PB.; Harwig, SSSL.; Lehrer, RI. Cryptdins: Antimicrobial defensins of the murine small intestine, *Infect Immun*, 1992, 60(9), 3556-3565.
20. Lawson, AJ. ; Smit, RA.; Jeffers, NA.; Osborne, JW. Isolation of rat intestinal crypt cells, *Cell Tissue Kinet*, 1982, 15**,** 69-80.
21. Lee, JY.; Boman, A.; Chuanxin, S. et al. Antibacterial peptides form the pig intestine: isolation of a mammalian cecropin**,** *Proc Natl Acad Sci USA*, 1989, 86, 9159-9162.
22. Lowry, OH.; Rosebrough, NJ.; Farr, AL.; Randall, RJ. Protein measurement with Folin's reagent, *J Biol Chem*, 1951, 193, 265-275.
23. Schagger, H.; Jagow, GV. Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel

Electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal Biochem*, 1987, 166, 368-379.
24. Chanana, V.; Majumdar, S.; Ray, P.; Sharma, M.; Rishi, P. Coordinated expression and immunogenicity of an outer membrane protein form *Salmonella enterica* serovar Typhi under iron limitation, oxidative stress and anaerobic conditions, *J Biomed Sci*, 2006, 13(3), 303-312.
25. Lehrer, RI.; Roseman, M.; Harvig, SSSL.; Jackson, R.; Eisenhauer, P. Ultrasensitive assays for endogenous antiicrobial polypeptides, *J Immunol Meth*, 1991, 137, 167-173.
26. Swank, RT.; Munkres, KD. Molecular weight analysis of oligopepetides by electrophoresis in PAGE with SDS, *Anal Biochem*, 1971, 39**,** 462-477.
27. Selsted, ME.; Miller, SI; Henschen, AH; Ouellette, AJ. Enteric defensins: antibiotic peptide components of intestinal host defence, *J Cell Biol*, 1992, 118, 929–936.
28. Salzman, N.H.; Chou, M. M.; de Jong, H.; Liu, L. ; Porter, E.M. ; Paterson,Y. Enteric *Salmonella* infection inhibits Paneth cell antimicrobial peptide expression, *Infect. Immun*, 2003, 71(3), 1109-1115.
29. Walton, E.; Gladstone, GP. Factors affecting the susceptibility of staphylococci to killing by the cationic proteins from rabbit polymorphonuclear leukocytes: the effects of alteration of cellular energetics and of various iron compounds, *Br J Exp Pathol*, 1976, **57,** 560-570.
30. Miller, SJ.; Kukral, AM.; Mekalanos, JJ. A two component regulatory system (PhoP and PhoQ) controls *Salmonella typhimurium* virulence, *Pro Natl Acad Sci*, 1989, 86, 5054-5058.
31. Bengoechea, JA.; Lindner, B.; Seydel, U.; Diaz, R.; Moriyon, I. *Yersinia pseudotuberculosis* and *Yersinia pestis* are more resistant to bactericidal cationic peptides than *Yersinia enterocolitica*, *Microbiology*, 1998, 144,1509-1515.
32. Shi, Y.; Song, W.; Feng, ZH; Zhao, YT; Li, F.; Tian, Y.; Zhao, YM. Disinfection of maxillofacial silicone elastomer using a novel antimicrobial agent: recombinant human beta - defensin-3, *Eur J Clin Microbiol Infect Dis*, 2008, Doi 10.1007/s 10096-008-0634.

