

Annual Research & Review in Biology

32(4): 1-10, 2019; Article no.ARRB.50001 ISSN: 2347-565X, NLM ID: 101632869

## Novel Cathelicidin Antimicrobial Peptides from Paa robertingeri

Qinghua Luo<sup>1#</sup>, Huaiqing Deng<sup>1#</sup>, Mengguang Yin<sup>1</sup>, Chen Chen<sup>1</sup> and Jiang Zhou<sup>1\*</sup>

<sup>1</sup>School of Life Sciences, Guizhou Normal University, Guiyang, Guizhou, 550001, China.

#### Authors' contributions

This work was carried out in collaboration among all authors. Author JZ designed the study, performed the statistical analysis and wrote the protocol. Author CC wrote the first draft of the manuscript. Authors HD and MY managed the analyses of the study. Author QL managed the literature searches. All authors read and approved the final manuscript.

#### Article Information

DOI: 10.9734/ARRB/2019/v32i430093 <u>Editor(s):</u> (1) Dr. David E. Martin, Martin Pharma Consulting, LLC, Shawnee, OK, USA. <u>Reviewers:</u> (1) Mohini Chetan Kuchekar, Pune University, India. (2) Oshim, Ifeanyi Onyema, Nnamdi Azikiwe University, Nigeria. Complete Peer review History: <u>http://www.sdiarticle3.com/review-history/50001</u>

**Original Research Article** 

Received 02 May 2019 Accepted 10 July 2019 Published 02 August 2019

## ABSTRACT

This study aimed to describe two cathelicidins (cathelicidin-PR1 and cathelicidin-PR2) from the skin of *Paa robertingeri* (*Anura: Ranidae*). The deduced mature peptides cathelicidin-PR1 and cathelicidin-PR2 were composed of 29 and 25 residues, respectively. Cathelicidin - PR1 has higher antimicrobial activity it could kill Gram-positive and Gram-negative bacteria and even some fungal species. Cathelicidin-PR1 exhibited more effective than AMP in antimicrobial activity against *Pseydomonas maltophilia* clinical strain. On the contrary, cathelicidin-PR2 had very weak antimicrobial activity. Furthermore, cathelicidin-PR1 and cathelicidin-PR2 exhibited very low hemolytic activity against human erythrocytes and little hemagglutinating activity. The results suggested that the cathelicidin-PR1 might serve as a template for developing novel antibiotics.

Keywords: Antimicrobial activity; antimicrobial peptide; hemagglutinating activity; hemolytic activity; Paa robertingeri.

\*Corresponding author: E-mail: zhoujiang@ioz.ac.cn, 201402005@gznu.edu.cn;

\*These authors contributed to the work equally and are regarded as co-first authors.

#### **1. INTRODUCTION**

Antimicrobial peptide (AMP) is a kind of smallmolecule peptides characterized by strong and broad-spectrum bactericidal activity. In the last 30 years, the widespread distribution of AMPs has been discovered, providing insights into the innate defensive systems that permit multicellular organisms to live in harmony with microbes [1]. Cathelicidins and defensins are the two major AMP families in mammals [2,3]. They can defence against а variety of harmful microorganisms. The presence of cathelicidins in hagfish, the oldest jawless craniates, indicates that cathelicidin genes appeared early in phylogenesis [4-6], which illustrates their important role. Almost 100 kinds of cathelicidins were searched from the Antimicrobial Peptide (http://aps.unmc.edu/AP/main.php), Database and the data are constantly updated.

Since the discovery of the first cathelicidin (Bac5) from the cDNA of the bovine neutrophils [7], a variety of new cathelicidins have been found in most of the vertebrates, ranging from mammalians, birds, reptiles, amphibians to fishes [8,9]. Cathelicidins possess a conserved structure. Upon activation, most of the cathelicidin precursors are proteolytically cleaved to release the C-terminal mature peptide domain [5,6,10,11]. On the basis of the protein secondary structure, cathelicidins can be divided into four categories:  $\alpha$ -helix cathelicidins. extension-spiral cathelicidins, cyclic cathelicidins, and ß-sheet cathelicidins. Cathelicidins derived from mammals are mostly  $\alpha$ -helical, and the primary structure generally contains 23-40 amino acids [12,13]. Most of the cathelicidins antibacterial peptides are the  $\alpha$ -helical structure [14].  $\beta$ -sheet cathelicidins generally contain 16-18 amino acids, which are folded into a hairpin structure stabilized by disulfide bonds [15].

A known mechanism of action of amphibian antimicrobial peptides is that the positively charged polypeptides interact with the microbial cell membrane and induce changes in the membrane structure, resulting in the cytoplasm outflow and eventually causing microbial death [16]. However, evidence shows that the cell membrane is not the only target of antimicrobial peptides. They also act on other parts of microorganisms, such as intracellular DNA and RNA, thereby interfering with microbial metabolic pathways [17].

The amphibians face the challenge of adapting to moist environments. Their skins secrete a large

volume and variety of antimicrobial peptides. Also, amphibians lack lymphocytes. Hence, the secretion of mucous substances is particularly important in such an environment [18]. In this study, two cathelicidins were identified and characterized by Paa robertingeri.

#### 2 MATERIALS AND METHODS

#### 2.1 Tissue Preparation

An adult specimen of *Paa robertingeri* was captured from Fanjingshan in Guizhou province (108°45'55"–108°48'30" E; 27°49'50"–28°1'30" N). A 1- cm<sup>2</sup> piece of dorsal skin was removed from its back immediately and stored in liquid RNA protector (sample protector for RNA/DNA, TaKaRa, Japan) until use. After collection, this frog was sterilized with alcohol and then set free in its natural habitat.

## 2.2 cDNA Library Construction and Screening of the Skin cDNAs Encoding Cathelicidins

The stored skin was washed in water and then ground into powder in liquid nitrogen. The total RNA was extracted using TriZol reagent (Life Technologies, CA, USA). Then, the total RNA was used to construct the cDNA library using the Creator Smart cDNA Library Construction Kit (Clontech, CA, USA). First-strand cDNA synthesis was performed using SMARTScribe Reverse Transcriptase (Clontech) and SMARTer V Oligonucleotide and 3' IF SMARTer CDS Primer. Second-strand cDNA synthesis was performed by a long-distance polymerase chain reaction (PCR) method using Advantage 2 Polymerase Mix (Clontech) in the presence of 5' PCR Primer II A and 3' IF SMARTer PCR Primer. The synthesized cDNA was used as a template for the following PCR to screen the cDNAs encoding the cathelicidin peptides [18].

On the basis of the conserved signal peptide domain of previously characterized host defence peptide (HDP) from ranid frogs, a sense oligonucleotide primer (5'-CCCCATGTTCACCTTGAAG-3') was designed and coupled with 3' antisense primer (5'-TACGCGACGCGATACGCGAAT-3') according to the sequence of 3' IF SMARTer CDS primer to screen the HDP encoded cDNAs. The PCR procedure was as follows: 5 min of denaturation at 94°C; 30 cycles: denaturation at 94°C for 30 s, primer annealing at °C for 30 s, and extension at 72°C for 1 min. The PCR product was purified by gel electrophoresis and cloned into pMD19-T vector (TaKaRa, Japan) for sequencing.

#### 2.3 Alignment of Amphibian Cathelicidins

Sequencing results used the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool to remove the carrier and identify the fragment. Then the fragment sequences were translated into amino acids by ExPASy (http://www.expasy.org/). The sequences were input into NCBI database, the complete gene sequence encoding cathelicidins of *P. robertingeri* was identified, and the amino acid sequence of the mature peptide was predicted according to the characterized cathelicidins.

## 2.4 Peptide Synthesis

Cathelicidin-PR1 and -PR2 were synthesized by GL Biochem Ltd. (Shanghai, China) and analyzed by high-performance liquid chromatography and mass spectrometry to ensure purity of more than 95%.

## 2.5 Antimicrobial Assay

Seven strains of standard and clinically isolated including microorganisms, Gram-positive bacteria, Gram-negative bacteria, and fungi, were used in the antimicrobial assay. Minimal inhibitory concentrations (MICs) of the peptides were determined by a standard twofold microdilution method in a 96-well microtiter plate, as described previously [19]. Briefly, the microorganisms were incubated in Mueller-Hinton broth (MH) at 37°C to exponential phase and diluted with fresh MH broth to 10<sup>6</sup> colony-forming unit (CFU)/mL. Then, 50 µL of serial dilutions of peptides in MH broth were prepared in 96-well microtiter plates and mixed with 50 µL of diluted bacterial inoculum. The plates were incubated at 37°C for 18 h. and the minimal concentration at which no visible growth occurred was recorded. The traditional antibiotic ampicillin was used as a positive control, and the assay was conducted in triplicate.

## 2.6 Bacterial Killing Kinetics Assay

The bacterial killing kinetics of cathelicidin-PR1 against *Bacillus cereus* clinical strain was determined by measuring the changes in the viable bacterial counts after peptide treatment. *B. cereus* clinical strain was incubated in the Luria-

Bertani (LB) liquid medium at 35°C and 200 rpm for 10-16 h and diluted to 10<sup>5</sup> CFU/mL in the fresh LB liquid medium. Cathelicidin-PR1 was added to the bacterial suspension to a final concentration of 5× MIC, and the bacterial suspension was incubated at 37°C for 0, 10, 20, 30, 45, 60, 90, and 120 min. At each time point, aliquots (10 µL) were removed and diluted with fresh LB broth 100 times. Next, 100 µL of the dilutions were coated on the LB solid medium and incubated for 10-16 h at 37°C. The viable colonies were counted. Ampicillin was used as a positive control, and sterile deionized water was used as a negative control, the assay was conducted in triplicate at least and took the average.

## 2.7 Hemolytic Assay

Fresh human erythrocytes were collected, mixed in 5 mL of mixing Alsever's solution (8.0 g sodium citrate, 0.55 g citric acid, 20.5 g glucose, and 4.2 g NaCl in 1 L deionized H<sub>2</sub>O, pH 6.1) at a volume ratio of 1:1, and centrifuged at 1000 rpm for 5 min. The supernatant was removed. washed with 0.9% saline three or four times, and resuspended to a final concentration of 2% (v/v). dilutions of cathelicidin-PR1 Serial and cathelicidin-PR2 were incubated with the erythrocyte solutions at 37°C for 30 min, and then the cells were centrifuged at 1500 rpm for 10 min. The supernatant was collected, and the absorbance at 540 nm was measured. The assay was conducted in triplicate. 1% Triton X-100 (v/v) was used as a positive control, and 0.9% saline was used as a negative control. The assay was conducted in triplicate at least. Percentage of hemolysis (1%) was calculated according to the following formula:

/%= (A<sub>sample</sub>-A<sub>negative control</sub>)/(A<sub>positive control</sub> - A<sub>negative control</sub>) × 100%

## 2.8 Anti-oxidant Assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable aliphatic nitrogen-centered radical. It can be used to detect the anti-oxidant activity of antimicrobial peptides by radical scavenging assay. DPPH (Sigma, USA) was dissolved in methanol to a final concentration of 6 × 10<sup>-5</sup>M. Next, 192 µL of DPPH solutions were mixed with 8 µL of serial concentrations of peptide solutions. The mixture was incubated in the dark at room temperature for 30 min, and the amount of reduced DPPH was quantified by measuring a absorbance decrease in at 517 nm. Deionized water was used as a negative control. Inhibition of free radicals by DPPH in percentage (*I*%) was calculated according to the formula:

 $I\% = (A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}} \times 100\%$ 

#### 2.9 Erythrocyte Hemagglutination Assay

Lectins are glycan-binding proteins that can specifically recognize glycan structures and have been identified from a wide variety of organisms [20]. Fresh human erythrocytes were collected and stored in Alsever's solution to prevent coagulation. The assay was performed in U-well microtiter plates (96 wells) according to the method described by Li et al. [21]. The erythrocytes were washed twice with Tris buffered saline (TBS) buffer (6.06 g Tris base and 5.84 g NaCl in 1 L of H<sub>2</sub>O, pH 7.5) and TBS + Ca<sup>2+</sup> buffer (6.06 g Tris base, 5.84 g NaCl, and 1.12 g CaCl<sub>2</sub> in 1 L of H<sub>2</sub>O, pH 7.5), centrifuged at 1000 rpm for 5 min, and resuspended in the same buffer to a final concentration of 2% (v/v). Then, 10 µL of peptide solutions (2 mg/mL) were mixed with 90 µL of erythrocyte solutions in a Uwell microtiter plate. The plate was incubated at room temperature for 45 min, and the result was observed. Deionized water was used as negative control, and the assay was conducted in triplicate.

## 2.10 Bioinformatics Analysis and Structure Prediction

The physical and chemical parameters of cathelicidin-PR1 and cathelicidin-PR2 were determined by the Prot Param tool (http://web.expasy.org/protparam/) through ExPASy Bioinformatics Resource. The secondary structure was predicted using the PSIPRED protein structure prediction server provided by Bioinformatics Group of UCL Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/).

## 2.11 Circular Dichroism Analysis/ Spectroscopy

The samples were prepared by dissolving the peptide powder in 60mM sodium dodecyl sulfate  $(SDS)/H_2O$  solutions to a concentration of 0.5 mg/mL. The spectra were measured at 298 K (25°C) between 192 and 250 nm using a 0.1-cm path length cell with 1-nm bandwidth, 1-s response time, and a scan speed of 100 nm/min. Three consecutive scans per sample were performed and averaged, followed by subtraction of the solvent signal.

#### 3. RESULTS AND DISCUSSION

## 3.1 Identification and Characterization of *P. robertingeri* Cathelicidins

Total RNA was extracted from the skin of P. robertingeri, and cDNA library was constructed using a cDNA library construction kit. Two cDNAs encoding two different cathelicidins were obtained from the cDNA library by the PCRbased cDNA cloning method. The complete nucleotide sequences and translated amino acid sequences of the two cathelicidin precursors are shown in Fig. 1. The cDNAs encoding cathelicidin-PR1 and cathelicidin-PR2 precursors were composed of 587 bp and 607 bp, respectively. The translated protein precursors comprised of 147 and 145 amino acid residues. respectively. Consistent with other cathelicidins, precursors of cathelicidin-PR1 and cathelicidin-PR2 possessed a typical signal peptide sequence, a highly conserved cathelin domain, and a cationic C-terminal mature peptide sequence.

The mature peptides of cathelicidin-PRs were predicted in this study. Cathelicidin-PR1 was composed of 29 amino acid residues, and the amino acid sequence was RKCNLFCKAKQKLKSLSSVIGTVVHPPRG. In contrast, cathelicidin-PR2 was composed of 25 amino acid residues, and the amino acid sequence was KECKDYLCKLLMKLG SSSHIESIDP.

#### 3.2 Antimicrobial Activity of cathelicidin-PRs

Cathelicidin-PR1 and cassthelicidin-PR2 were chemically synthesized and their purity was confirmed to be 95%. The minimal inhibitory concentrations (MICs) of the two peptides against seven microorganisms, including Grampositive bacteria, Gram-negative bacteria, and fungi, were determined. As listed in Table 1, except for *Acinetobacter baumannii*, cathelicidin-PR1 exhibited potent and broad-spectrum antimicrobial activity against most in the tested clinical strain. Cathelicidin-PR1 is more effective than AMP in antimicrobial activity against *Pseydomonas maltophilia* clinical strain. Unlike cathelicidin-PR1, cathelicidin-PR2 exhibited very weak antimicrobial activity.

#### 3.3 Bacterial Killing Kinetics of cathelicidin-PR1

Using ampicillin as a positive control, the killing kinetics of cathelicidin - PR1 against

Microorganisms	Minimal inhibitory concentrations (µg/ml)						
	Cathelicidin-PR1	Cathelicidin-PR2	Amp				
Acinetobacter baumannii clinical strain	>100	>100	4.69				
Pseydomonas maltophilia clinical strain	75	>100	>100				
Staphylococcus aureus clinical strain	37.5	>100	<0.10				
Bacillus cereus clinical strain	37.5	>100	4.69				
Bacillus subtilis clinical strain	37.5	>100	4.69				
Candida albicans clinical strain	37.5	>100	4.69				
Candida glabrata clinical strain	37.5	>100	4.69				

|--|

#### cathelicidin-PR1

_	-	var	99a	cee	~~~				100	-									1.0			
V	1 1	L	W	I	S	A	L	Т	L	. 1	Q	A	A	R	S	Q	S	P	D	Ģ	) E	2
gaat	gg	gtc	aga	gag	gcc	ttg	gga	tct	ct	ac	aac	cag	agg	gaa	igat	gga	agai	gtte	ctt	ct	ttt	12
E	A	A	R	E	A	L	D	L	•	Y	N	Q	R	Ε	D	G	E	F	F		F	4
aagt	tc	ctg	tct	gat	cto	ccg	gga	cgo	co	tc	ctg	gag	gag	gag	ggag	gga	aga	ctc	tcc	ag	şcc	18
K	F	L	S	D	L	Ρ	D	A	1	L	L	Ε	Ε	Ε	E	G	D	S	P	8	A	6
atcg	gc	ttc	cta	atc	aag	gag	gac	gga	at	gc	ccc	aaa	tcc	gaa	agad	tge	cga	ctt	gga	ga	aa	24
I	G	F	L	Ι	K	E	Т	E		С	Ρ	K	S	E	D	С	D	L	E		K	8
tgcg	ac	tac	agg	aag	gac	ggs	gga	ggt	ga	ag	gto	tgo	gct	ctg	stad	cg	gga	ggai	aga	gg	gac	30
C	D	Y	R	K	D	G	E	V	1	K	V	С	A	L	Y	R	E	E	E		D	10
gtga	ag	tgc	gtc	agt	ctg	tco	cga	gaa	tt	ca	cgo	gcc	cgg	cgg	ggco	ago	caa	caa	gcg	ga	ag	36
V	K	С	V	S	L	S	E	N	I	S	R	A	R	R	A	S	N	K	R	t	K	12
tgta	ac	ttg	ttc	tgc	aaa	gcg	gaa	gca	iga	ag	ctg	aaa	tct	ctg	gago	tco	gt	cat	cgg	ga	acg	42
c	N	L	F	С	K	A	K	G	)	K	L	K	S	L	S	S	V	I	G		T	14
																						40
steg	stt	cat	cca	cct	cga	gga	itg	aac	gg	sca	itt	cgo	rgo	tgo	gg	gg	gea	aaa	aag	aa	icg	48.
	17	н	P	P	R	G	-															14
V cggc atcc cath	gg ag neli	cag aat cid	cgc aat in-l	cga caa PR2	ccg taa	cca	ac	gct tca	to ta	etc 1aa	gca tcc	ccg tto	ggc gta	aaa tat	icta tgat	itca ;	acti	gcg	ctt	co	caa	54 58
V atcc cath gtg1	v cag cag neli tgc V	cag aat cid tat L	cgc aat in-l gga W	cga caa PR2 atc1 I	ccg taa ! tccs	cca aad got A	acto cto L	gct tca aca T	to ita at	etc aaa tgo L	gca tcc cag: Q	iccg itto gogi A	ggc gta gct A	aaa tat cgc R	tct tct	itca ; cag Q	tct S	gog cog P	ctt gga D	tc	aa agg Q	54 58 aa E
V cggc atcc cath gtg1 y gaat	v cag neli tgc V tgg	cag aat cid tat L	cgc aat in-l gga ¥	cga caa PR2 atc1 I agag	ccg taa ! tcc; S	got A ctt	ac ct L gga	gct tca ac: T	tc	tcaa tgo L tao	gca tcc cag Q caa	iccg itto gogi A cca	ggc gta gct A gag	aaa tat cgc R gga	tct tct S aga	itca cag Q tgg	tct S	sce cce P igte	gga D gct	tc tc	agg Q Lttt	54 58 aa E
V atco cath gtg1 V gaat E	v cag neli tgc V tgg ¥	cag aat cid tat L gto V	cgc aat in-l gga ¥ aga R	cga caa PR2 itci I igag E	ccg taa ! tcci S sgco A	got A ctt L	act ctc L gga	gct tca ac: T itc ]	tc at tc L	tc aaa tgo L tao Y	gca tcc cag Q caa N	iccg itto gcgi A cca Q	gct gct gag R	aaa tat cgc R gga E	tct S aga D	cag Q tgg G	tct S aga	sccs P igtg	gga D gct	tc tc	agg Q ttt F	54 58 aa E
v atcc cath gtg1 y gaat E aaat	v cgg cag tgc tgc V tgg V ttgg V ttgg	cag aat cid tat L gtc V	cgc aat in-l gga ¥ aga R tct	cga caa PR2 atc1 I I gag E tga1	ccg taa ! tccg S gco A tcto	scca aad got A ott L ccc	acto cto L gga gga	gct tca aci T itc I lcg	tc at tc L cc	tgo tgo tao Y	gca tcc cag Q caa N	iccg itto gcg A cca gga	gct gct A gag R gga	aaa tat cgc R gga E gga	tct S aga D aaa	cag Q tgg Cga	tct S aga tcc	sce P igtg cac	ga ga gct caa	tc tc tc tc	agg Q ttt F acg	54 58 aa E
V cggc atcc cath gtgt gaat E aaat K	v cag; cag; tgc V tgg V tgg V ttc F	cag aat cid tat L gtc V cctg L	cgc aat in-l gga ¥ aga R (tct S	cga caa PR2 atc1 I igas E tga1 D	ccg taa ! tcc; S sgc A tct L	got A ctt Coc P	aaci oot L ggaa D ggaa	gct tca T itc I icg	tc at L cc	tgo tgo tao Y cto L	gca tec Q caa N cet L	iccg itto gcg A cca gga E	ggct gct gag gag gga E	aaa tat cgc R gga E gga E	tct s aga D aaa N	cag Q tgg cga D	tct S aga tcc	sce P stg ceg gate f gate f gate	gga D Sct Caa	tc tc tc tc tc	agg Q ttt F acg T	54 58 aa E
V cggc atcc cath gtg1 gaa1 E aaa1 K ttc1	v cgg cag; neli tgc V tgg ¥ ttc F tta	cag aat cid tat L sgtc V cctg L ata	cgc aat in-l gga W caga R tct S aag	cga caa PR2 itci I igaş E tgat D ggaş	ccg taa tccq S ggcc A tctc L tctc L gacq	get A Cott D Sga	acto ctc L gga D gga L atg	gct tca T itc i cg i cg	tc ita ita itc itc itc itc itc	tgo tao tao tao tao L cto L aas	gca tcc Q caa N cct: L ct:	cccg gcg: A ccca: Q gga: E tga	ggta gct A gag gag gga E aga	aaa tat cgc R gga E gga E tat	tcta tgat S aga D aaa N caa	cag Q tgg G cga D ctt	tct S aga E tcc F gga	scs P igts ccs gac J I iggs	gga D got Caa	tc tc tc tc tc tc	agg Q ttt F acg T gac	54 58 aa E
V cggc atco cath gtgt V gaat E aaaat K ttct F	v cgg cag; tgc V tgc V tgc V ttc F tta L	cag aat cid tat L sgtc V cctg L atz	cgc aat in-l gga W aga R tct S aag K	cga caa PR2 atci I igag E gag E	cccg taa ! tccs S ggco A tcto L tcto T	gota aac A ctt E gga E	acto ctc gga gga D atg C	get tea T ite i i i i i i i i i i i i i i i i i i	tc at tc L cc A tg L	tgo tgo tao Y cto L aas K	gca tcc Q caa N ccti L stc S	cceg ttc gcg: A cca: gga: E tga. E	ggta gct A gaga B gga E aga D	cgc R gga E gga E tat I	acta tgat S agaa D aaaa N caaa N	cagg Q tgg Cga D ctt L	tct S aga tcc F gga . E	cccg P igtg cccg cccg c cccg c c c c c c c c c c	gga D ggt Caa I iat	tc tc tc tc tc tc tc tc tc tc tc	agg Q ttt F acg T gac D	54 58 aa E
V cggcc atcc cath gggat E aaaat K ttct F tacs	v cgg cag tgc tgc V tgg ttc F tta L aag	cag aat cid tat L stat V cctg L ata I saag	cgc aat in-l gga R tct S aag tct S aag K gao	cga caa PR2 itci I igai E ggag E cggg	ccg taa ! tccf Sgco A tcto L tcto T gacf T	get A Ctt E gga E ggt	aac ctc L ggaa D ggaa C gaa	gct tca T tc itc i tc i c g c c i t g g g g g g	tc ita ita ita ita ita ita ita ita ita	tgo L tao Y cto L aas K tgo	gca tco Q caa N ccti L ccti S cgg	ecce ttc gcg: A ggga: E tga E atg	ggct gct A ggga B agga D gta	cgc R gga E gga I tat I cccc	acta tgat S aga D aaaa N caaa N gga	cag Q tgg Cga D ctt L gga	tct S aga E tcc F gga gga gga	scs P ssts cccs P ssts cccs P ssts cccs P ssts cccs ccc	gga D Sct Liat	tc tc tc tc tc tc tc tc tc tc tc tc tc	agg Q 1 ttt F acg T gac D atg	54 58 aa E 1 2
V cggc atcc cath gggat E aaaat K ttct F tacs Y	v sgg ag; tgc V tgg V ttc F tta L aag K	cag aat cid tat L sgtc V cctg L aats I saag K	cgc aat in-l gga k caga K caaa K cgac D	cga caa PR2 itcf I igas E gas E cgas G	ccg taa S sgco A tcto L sacs T sgas E	got A bott B gga E ggt V	acto cto L ggaa D atg C gaa K	gct tca T itc itc icc icc icc icc icc	tc at tc L cc A tg L tc V	tgo tao tao tao tao L tao L aas K tgo C	gca tco Q caas Q caas N ccts L tcc S G G	cca gcg: A cca Q gga E tga E atga ¥	ggct gct A gaga B aga D gga Y	cgc R gga E gga E tat I cccc P	acta tgat S agaa D aaaa N caaa N gga E	tca Q tgg Cga Cga L gga E	tct S aga tcc F gga . E gga . E	scs P sts cccs cccs c sgac F sgac c F sga c f F	gga D get Lat Liga	tc tc tc tc tc tc tc tc tc tc tc tc	caa agg Q l tttt F acg T gacc D atg M	54 58 2 1 1 1 1
V cggccatcc cath gtgt E aaaat K ttct F tacs Y aaaga	v cgg cag; tgc tgc V tgg V ttg F tta L aag K act	cag aat cid tat L sgtc V ctg ata I saag K	cgc aat in-J gga R saga K sgac D saas	cga caa PR2 itci I igag E gag E G G itgi	cccg taa ! tccs S ggco A tcto L gacs T ggas E E tgto	scca aac get A ctt E gga E ggt V cag	acto ctc gga gga tg gaa K cct	gct tca T itc i i c c c c c c c c c c c c c c c c	tc ita tc L cc A tg L tc V cc	tgo tao tao tao tao tao tao tao tao tao ta	gca tcc Q caa N ccti L tcc S cgg G gaa	CCC gCC A CCC B CCC CCC CCC CCC CCC	ggct gct A ggga R ggga E aga D gta Y tcg	cgc R gga E gga E tat I cccc P cgc	acta tgat S agaa D aaaa N caaa N ggaa E caa	tca cag Q tgg Cga Cga Ctt L gga E gcg	tct S aga E tcc F gga E gga G gga G ago	gog P ggtg C gga E ggga E ggga E ccac	ctt gga D sct Caa Caa Caa Caa Caa Caa Caa Caa Caa Ca	tc tc tc tc tc tc tc tc tc tc tc tc tc t	agg Q l tttt F acg T gac D atg M aaa	54 58 8 1 1 2
V cggccatco cath gtgt gaat E aaaat K ttct F ttcs Y aaaga K	v cgg cag tgc tgg V ttgg V ttc F tta L aag K act	cag aat cid tat L sgto V ctg L atsa E K L ctg L	cgc aat in-l gga R stct S aaag K gaa D saaag K	cga caa PR2 itci I igas E igas E gas G G itgi C	cccg taa tccs S sgco L tcto L sgacg E tgto V	got A ctt B gga E ggt V cag S	acto ctc L ggaa D atg gaa K cct L	gct tca T tc itc itc icc c c c c c c c c c c c c	at at tc L cc A tg L tc T	tc tgo L tao Y cto L aaaa tgo C aaaa K	gca tcc Q caa N ccti L ccti C S c gga X N	ecce gcg: A ggga E tga E atga ¥ ttt F	ggct ggct A ggga B gga B gga D gta Y tcg. R	cgcc R gga E tat I cccc P cgc A	acta tgat S agaa D aaaa N caaa N gga E caaa K	tca cagg Q tggg cga C tt L ggga E gcg R	tct S aga tcc F ggs S S S S S S S S S S S S S S S S S S	sce P sgtg co gga E sgga E sgga E sca A T	ctt gga D gct Caa Caa Caa Caa Caa Caa Caa Caa Caa Ca	tc tc tc tc tc tc tc tc tc tc tc tc tc t	caa agg Q I tttt F accg T gacc D catg M aaaa K	542 58 58 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
V cggccatcc cath gggat E aaaat K ttct F taca Y aaaga K aaaag	v cgg; cag; tgc V tgg V ttc F tta L aag K aact T gag	cag aat cid tat L sgtc V ctg L atz K ctg L ctg L	cgc aat in-l gga R stct S aaa S gaa D gaaa K saaa K	cga caa PR2 itci I igas E igas G G G G itgi C igas	cccg taa tccs S ggco A tcto L gacs T ggas E tgto V	goti A cott B gga B ggt V cag S ttt	acto ctc L gga D ggaa C gaa K cct L gtg	gct tca itca itc itc icc icc icc icc icc icc icc icc	at tc L cc A tg L tc V cc T aa	tc tac tac tac tac tac tac tac tac tac t	gca tco Q caas N ccti L ccti S gg G gaa N sct	atga E ttto gcg; A cca Q gga: E tga E tga E ttga F tttt F tat;	ggct gct A ggga B agga D gta T tcg R agaa	cgcc R ggga E tat I cccc P cgcc A act	acta tgat S aga D aaaa N caaa N gga E caa K gga	tca cag Q tgg Cga Cga C C C C C C C C C C C C C C C	acti S aga tcc F ggs S ggs G ggs G ggs C agc	cccg P igtg cccg C igtg cgac F iggga c E iggga c E iggga c i E icac cag c i cag c i cag c i cag c i c c c i c c i c c c i c c c i c c c i c c c i c c c i c c c i c c c i c c c i c c c i c	ctt gga D gct Caa I iga Cca Scc	tc tc tc tc tc tc tc tc tc tc tc tc tc t	caa agg Q tttt F acg D atg A aaa K catc	542 58 8 1 1 2 2 2 1 1 2 2 1 2 2 2 2 2 2 2 2
V cggccatco cath gtgt K gaat E aaaat K ttct F tacca Y aaaga K	v cag; cag; tgc tgc V tgg ttc F tta aag K aact T gag	cag aat cid tat L sgtc V ccts L aasg K ccts L tgc	cgc aat in-l gga R tct Saag K gaa K aag K aag K aag K	cga caa PR2 atc1 I igag E gag G G itg1 C igat	cccg taa S Sgco A tcto Sgacg T Sgag E tgto V tta	get A Cett B gga E ggt V cag S ttt	acto ctc ggaa D ggaa C gaa K cctt L gtg C	gct tca T tc l l gcc l ggg ta ta	at tc L cc A tg L cc T aa K	tgo L tao Y cto L aass K tgo C aass K cts L	gca tco cag: Q caa N cct: L cgg: G gaa N gct L	ccca gcgi A ccca gga E tga E tga E tga F tttt F	gggct ggta ggga gga gga gga gga tcg gaa K	cgcc R ggga E ggga E tat I cccc P cgcc A act	acta tgai S agaa D aaaa N caaa N ggaa E caaa C a a gga C a a a a a a a a a a a a	atca cag Q tggg cga C gga gga gga R atc	tct S aga E tcc F ggs G ggs G ago C tct S	cccg P igtg cccg ccgac F igga E igga F igga C F igga C F igga C F igga C F igga C F igtg C C C C C C C C C C C C C C C C C C	sga D sct Lat Liga Scc Scc	tc tc tc tc tc tc tc tc tc tc tc tc tc t	caa agg Q tttt F acg acg D aatg aatg M aaaa K catc	54: 58 8 1 1 1 1 1 1 1
V cggccatcc cath gtg1 K taaaat K ttc1 F tacs X aaags K gaaa	v cgg cag tgc tgg V tgg V ttc F tta L aag K act T gag	cag aat cid tat L sgtc V cctg L ats K cctg L tgc C	cgc aat in-l gga R stct S aag C C D saas K saas K saas K	cga caa PR2 atcri I I I I I I I I I I I I I I I I I I I	cccg taa tcccg S sgcc A tctc S sgcc T sgacg E tgtc V ttat Y	scca aac aac A Ctt L Cccc B gga E ggt V Cag S ttt L	acto L ggaa D ggaa C gaa K cctt L gtg gtg C Q atco C	gct tca T tc itc i gcc i ggg i ggg i ta i ggc i ta	at tc tc tc tc tc tc tc tc tc tc tc tc tc	tgo L tao Y cto L aass K ctgo C aagg C aagg L ggo	gca tco Q caa N ccti L tco S gaa N gaa N gct L ccc	cca gcg A cca gga E tga E atg W ttt F tat; M	ggct: gct: A ggga B aga D gta T C gaa K gaa	cgc R gga E tat I cccc P cgc A act L	acta tgat S aga D aaa N caaa N gga E caa K tgg C ace	cag Q tgg Cga Cga Ctt L gga E gcg R ato S	tct S aga E tcc F ggss G ggs C C c tcc S ggs C C c tcc S ggs C C C C C C C C C C C C C C C C C C	cccg P igtg C gac I I gga E E gga E E gga C I I E ccac C C C C C C C C C C C C C C C C C	ctt gga D sct Lat Lat Scca Scc	tc tc tc tc tc tc tc tc tc tc tc tc tc t	caa agg Q ttt F acg acg T gac T aaa K catc I I ggca	542 58 8 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
V cggccatco cath gtg1 K taca X aaaga K gaaaa K gaaaa	v cgg cag tgg tgg V tgg V ttc tta a ag t gag E agc	cag aat cid tat L sgtc V ctg L aag K ctg C catc	cgc aat in-l gga R saas K saas K saas K saas K saas K saas K	cga caa PR2 Itci I gas E gas E gas C S C S C S C S C C S C C S C C S C C S C C S C	ccg ttaa tccc S ggco A tcto L gac gac E tgto V ttat Y	get A cett C gga B ggt V cag S ttt L acc	aac ctc L ggaa D atg gaa K cctt L ggaa K cct L gtg ct	gct tca T tc i tc i c c c c c c c c c c c c c c c	tc at cc tc tc tc tc tc tc tc tc tc tc tc tc	tgo L tao Y cto L aas K tgo C aas L ggo	gca tco Q caa N ccti L cgg gaa N gca N ccti L cgg c gaa N ccti L cgg c gaa S ccti C C c gg c c c c c c c c c c c c c c c	ttca:	gga gga gga gga gga gga gga tcg gaa K gga K	cgc R gga E tat Cccc P cccc A act L gta	acta tgat S aga D aaa N caa N ggaa Caa K tgg G acg	cag Q tgg G cga C t L ggga E gcg R atc S cac	acti S aga E ggs G ggs C ago C ago C ggt	gog P P ggg ggg F gggg F Gggg F C cac C C C C C C C C C C C C C C C C C	gga D Sct Lat Liga Scc Scc Sag	tc tc tc tc tc tc tc tc tc tc tc tc tc t	caa agg Q ttt F acg T gac D gac D M aaaa K catc I I gca	542 58 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1

## Fig. 1. cDNA sequences encoding cathelicidin-PR1 and the predicted prepropeptide sequences. The putative mature peptides of cathelicidin-PRs are boxed and shaded

*Bacillus cereus* clinical strain was investigated by a colony counting method. As illustrated in Table 2, at a concentration of 5× MIC, cathelicidin-PR1

rapidly exerted its antimicrobial function. It just took 45 min for cathelicidin-PR1 to kill all the *B. cereus* clinical strain cells. More importantly, the

colony forming units (CFUs) remained zero when the incubation time was to 120 min, implying that the extended antimicrobial property of cathelicidin-PR1 at the was lethal. In contrast, same concentration of 5× MIC, it took at least 90 min for the positive control ampicillin to completely kill the B. cereus clinical strain cells.

It indicated that cathelicidin-PR1 could rapidly and efficiently kill *B. cereus* clinical strain cells. Therefore, cathelicidin-PR1 might be used as a potential antibiotic.

#### 3.4 Hemolytic and Anti-oxidant Activity

Cathelicidin-PR1 did not show any hemolytic activity. At a concentration of 200  $\mu$ g/mL, the rate of hemolysis of cathelicidin-PR1 and cathelicidin-PR2 was 1.78% and 2.01%, respectively. However, at a concentration of 100  $\mu$ g/mL, the rate of hemolysis of cathelicidin - PRs was 3.87% and 1.12%, respectively (Table 3).

At a concentration of 80 µg/mL, cathelicidin-PR1 and cathelicidin-PR2 showed slight DPPH radical

Luo et al.; ARRB, 32(4): 1-10, 2019; Article no.ARRB.50001

scavenging activity, with *I*% values of 2.92% and 2.30%, respectively.

#### 3.5 Erythrocyte Hemagglutinating Activity

In this study, cathelicidin-PR1 did not show any hemagglutinating activity irrespective of the presence of  $Ca^{2+}$ . However, cathelicidin-PR2 showed a weak hemagglutinating activity in the presence of  $Ca^{2+}$ , but it did not show any hemagglutinating activity in the absence of  $Ca^{2+}$  (Fig. 2).

#### 3.6 Physical Properties Analysis and Secondary Structure Prediction

The physical and chemical parameters of the two cathelicidin-PRs were computed by ProtParam (http://web.expasy.org/protparam/); they are listed in Table 4. Besides, the secondary structures of the two cathelicidin-PRs were also predicted by the online prediction software from the University College London (UCL) Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/). Cathelicidin-PR1 was mainly composed of a helix and random coil (Fig. 3). Cathelicidin-PR2 was similar to cathelicidin-PR1.





Pred: Pred: AA:	CCHHHHHHHHHHHHHHHHHHHHHCCCCCCCCC RKCNLFCKAKQKLKSLSSVIGTVVHPPRG 10 20	cathelicidin-PR1
Conf: Pred: Pred: AA:	CHHHHHHHHHHHHHHCCCCCCCCCC KHCKDYLCKLLMKLGSSSHIESIDP 10 20	cathelicidin-PR2
Leger	d: - helix Conf:	fidence of prediction dary structure

Fig. 3. Secondary structure prediction of cathelicidin-PRs

Time	Colony Forming Units (CFUs)									
	0min	10min	20min	30min	45min	1h	1.5h	2h		
cathelicidin-PR1(5xMIC)	49.33±12.06	51±8.19	41±3.00	14.33±7.02	0±0.00	0±0.00	0±0.00	0±0.00		
Ampicillin(5xMIC)	37.67±6.11	31.33±11.15	26.67±11.02	15.67±2.52	7.00±2.65	1.33±2.31	0.33±0.58	0±0.00		
Blank control (sterile water)	39.67±10.07	32.33±7.57	26.33±3.06	55±9.64	95.67±20.21	98.33±14.57	132.33±15.37	219.67±11.15		

## Table 2. Bacterial killing kinetics of cathelicidin PR1 against Bacillus cereus clinical strain

Note: 5xMIC is 5 times of the minimum inhibitory concentration; cathelicidin-PR1 concentration is 187.5ug/ml; ampicillin concentration is 23.45ug/ml; the results are the average value of three independent repeated experiments (M±SD)

Peptide	Number of amino acids	Molecular weight (Da)	Net charge	Theoretical pl	Grand Average of Hydropathicity (GRAVY)
Cathelicidin-PR1	29	3195.88	+7	10.59	-0.226
Cathelicidin-PR2	25	2838.34	0	6.74	-0.328

Table 3. Physical and chemical parameters of cathelicind-PR1 and cathelicind-PR2

Table 4. The hemolysis ratios of cathelicidin-PR1 and cathelicidin-PR2

	cathelicidin-PR1	cathelicidin-PR2
100ug/ml	3.87%	1.12%
200ug/ml	1.78%	2.01%



# Fig. 4. Circular dichroism analysis of cathelicidin-PRs in ultrapure water (a) and SDS (60mM, b) solvent

Circular dichroism analysis is shown in Fig. 4. Both cathelicidin-PR1 and cathelicidin-PR2 had random coil configuration in sterile deionized water, while in 60mM sodium dodecyl sulfate (SDS) solvent, they had helix configuration, as predicted.

## 4. CONCLUSIONS

Recent studies have shown that cathelicidins act by interacting with the cell membrane of pathogenic microorganisms, leading to the formation of holes in the cell membrane, leakage of cell contents, and hence killing of pathogens [22]. Not every cathelicidin has antimicrobial activity [15,23]. This study showed that the charge of cathelicidin-PR1 was +7, while the cathelicidin-PR2 net charge was 0, and the cathelicidin-PR2 no antibacterial activity. It suggested that the antibacterial activity of cathelicidins was related to not only it's  $\alpha$ -helix structure, but also its charge number.

Luo et al.; ARRB, 32(4): 1-10, 2019; Article no.ARRB.50001

Cathelicidins have potential clinical and agricultural value. At present, Cathelicidin PR1 and cathelicidin PR2 genes were tandem ligated and successfully expressed in *E. coli* BL21 by prokaryotic expression [24].

Bacillus cereus can cause human food poisoning, causing symptoms such as nausea, vomiting and abdominal pain. Cathelicidin PR1 has higher antimicrobial activity than ampicillin on the kill *B. cereus*, and also cathelicidin-PR1 has broadspectrum antimicrobial activity. This indicates that Cathelicidin-PR1 is an important resource for the development of new anti-infection drugs, especially some strains that are resistant to traditional antibiotics. Cathelicidin-PR1 and cathelicidin-PR2 exhibited very low hemolytic activity against human erythrocytes and little hemagglutinating activity. The results suggested that the cathelicidin-PR1 might serve as a template for developing novel antibiotics.

## ETHICAL APPROVAL

All animal experimental protocols were approved by the Animal Care and Use Ethics Committee of Guizhou Normal University.

## ACKNOWLEDGEMENT

This study was supported by Deng's Doctor Research Fund of Guizhou Normal University. We are thankful for this support.

## **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

## REFERENCES

- 1. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;415:389-395.
- 2. Zanetti M, Gennaro R, Romeo D. Cathelicidins: a novel protein family with a common proregion and a variable Cterminalantimicrobial domain. FEBS Letters. 1995;374:1-5.
- Zaiou M, Gallo RL. Cathelicidins, essential gene-encoded mammalian antibiotics. J Mol Med. 2002;80: 549-561.
- Uzzell T, Stolzenberg ED, Shinnar AE, Zasloff M. Hagfish intestinal antimicrobial peptides are ancient cathelicidins. Peptides. 2003;24:1655-1667.

- 5. Du"rr UHN, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochim Biophys Acta. 2006;1758:1408-1425.
- Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, Wang A, Lin D, Lai R. Snake Cathelicidin from Bungarus fasciatus is a Potent Peptide Antibiotics. PLoS ONE. 2008;3:e3217.
- Zanetti M, Del SG, Storici P, Schneider C, Romeo D. The cDNA of the neutrophil antibiotic Bac5 predicts a pro-sequence homologous to a cysteine proteinase inhibitor that is common to other neutrophil antibiotic. J Biol Chem. 1993;268:522-526.
- Hao X, Yang H, Wei L, Lai R. Amphibian cathelicindin fills the evolutionary gap of cathelicidin in vertebrate. Amino Acids. 2012;43:677-685.
- Wei L, Yang JJ, He XQ, Mo GX, Hong J, Yan XW, Lin DH, Lai R. Structure and function of a potent lipopolysaccharidebinding antimicrobial and anti-infiammatory peptide. Journal of Medicinal Chemistry. 2013;56:3546-3556.
- Kopitar M, Ritonja A, Popovic T, Gabrijelcic D, Krizaj I, Turk V. Primary structure of a new cysteine proteinase inhibitor from pig leukocytes, FEBS Lett. 1989;255:211-214.
- Zanetti M, Gennaro R, Scocchi M, Skerlavaj B. Structure and biology of cathelicidins. The Biology and Pathology of Innate Immunity Mechanisms. 2000;479:203-218.
- 12. Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI. Activities of LL-37, a cathelinassociated antimicrobial peptide of human neutrophils. Antimicrob Agents Chemother. 1998;42:2206-2214.
- Tack BF, Sawai MV, Kearney WR, Robertson AD, Sherman MA, Wang W, Hong T, Boo LM, Wu HY, Waring AJ, Lehrer RI. SMAP - 29 has two LPS - binding sites and a central hinge. Eur J Biochem. 2002;269:1181-1189.
- Ling GY, Gao JX, Zhang SM, Xie ZP, Wei L, Yu HY, Wang YP. Cathelicidins from the Bullfrog Rana catesbeiana provides novel template for peptide antibiotic design. PLoS ONE. 2014;9(3): e93216.
- Guang H, Li Z, Wang Y, Lai R, Yu H. Progress in cathelicidins antimicrobial peptides research, zoological research 33:523-526 Hancock REW, Scott MG. 2000. The role of antimicrobial peptides in animal defense. Proc. Natl. Acad. Sci.

Luo et al.; ARRB, 32(4): 1-10, 2019; Article no.ARRB.50001

2012;97(16):8856-8861.

- 16. Hancock REW. Cationic peptides: Effectors in innate immunity and novel antimicrobials. The Lancet Infectious Diseases. 2001;I:156-164.
- 17. Lai R, Liang J, Zhang Y. Antimicrobial peptides in amphibian skins and their application. Zoological Research. 2004;25:456-468
- Lu Z, Zhai L, Wang H, Che Q, Wang D, Feng F, Zhao Z, Yu H. Novel families of antimicrobial peptides with multiple functions from skin of Xizang plateau frog, Nanorana parkeri, Biochimie. 2010;92:475-481.
- 20. Fujii Y, Dohmae N, Takio K, Kawsar SMA, Matsumoto R, et al. A lectin from the mussel Mytilus galloprovincialis has a highly novel primary structure and induces glycan-mediated cytotoxicity of globotriaosylceramide expressing lymphoma cells. J Biol Chem. 2012;287:44772-44783.

- 21. Li J, Wu H, Hong J, Xu X, Yang H, et al. Odorranalectin is a small peptide lectin with potential for drug delivery and targeting, PLoS ONE. 2008;3:e2381.
- 22. Wei L, Gao J, Zhang S, Wu S, Xie Z, Ling G, Kuang Y, Yang Y, Yu H, Wang Y. 2015. Identification and characterization of the first Cathelicidin from sea snakes with potent antimicrobal and anti-inflammatory activity, and special mechanism. Journal of Biological Chemistry. 2015;290:1633-1652.
- 23. Ma QQ, Jiao WJ, Wang ZY, Wu CX, Shan AS, Wang YB, Cai J. Tissue specificity and species superiority of cathelicidin gene expression in Chinese Indigenous Min pigs. Livestock Science. 2013;161:36-40.
- 24. Deng HQ, Chen C, Xiao N, Zhou J. Prokaryotic expression of antimicrobial peptide CATH PR1–2 from the skin of *Paa robertingeri* in *Escherichia coli*. Asian Herpetological Research. 2017;8(4):275-283.

© 2019 Luo et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history: The peer review history for this paper can be accessed here: http://www.sdiarticle3.com/review-history/50001