

Antibiotic Resistance and Strategies to Develop New Antibiotics

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Introduction

- **Wide spread and indiscriminate use of antibiotics lead to the emergence of microorganism that are resistant to these agents**
- **Antibiotic resistant have been posing increasingly serious concern to the public, health specialist and animal food producers**
- **To overcome antibiotics resistance health specialist and animal food producers need alternative means of preventing and treating emerging and re-emerging diseases**
- **New approaches to the problem of antimicrobial resistance and development of novel classes of antimicrobial agents with less likelihood to gain resistance are needed**

- ❖ African clawed frog *Xenopus laevis* can thrive in water filled with microbes and infection free wound healing was observed in responds to incisions
- ❖ The active principle was isolated and characterized (Zasloff *et al.*, 1987)
- ❖ Active principle was found to be two peptides of 23 amino acids and were named as magainin-1 and 2
- ❖ These two peptides were exactly identical except at position 10 and 22 and inhibited the growth of many organisms
- ❖ Following the isolation of these two peptide molecules, the amphibian skin secretions were studied in further details and large number of peptide with broad spectrum activity have been isolated

Ubiquitous Expression of Host Defense Peptides

- Host Defense peptides are prevalent throughout the nature as a part of the intrinsic defenses of most organisms
- Represents an ancient host defense effectors molecules
- Present in organisms across the evolutionary spectrum
- Fundamental in successful evolution of complex multicellular organism
- Played important role in innate immunity

Merits of Host Defense Peptides

- Traditional antibiotics usually have single or limited types of target molecules
- No specific receptors are involved in the action of Host Defense Peptide
- Host Defense peptide have dual potential as it can be used as template for drug synthesis or gene of choice for production of transgenic animals

Major Host Defense Peptides

- Two broad classes of Host Defense peptides : Defensins and Cathelicidins
- Epithelial cell lining and myeloid cells bone marrow are the crucial site of expression
- Defensins are polycationic 3-5 kDa characterized by the presence of six to eight conserved cysteine residues
- Defensins are divided into three classes : α -defensin , β -defensin and θ -defensin
- α -defensins are 29-35 residues long , containing three disulfide bridges at 1-6 , 2-4 and 3-5

Contd.

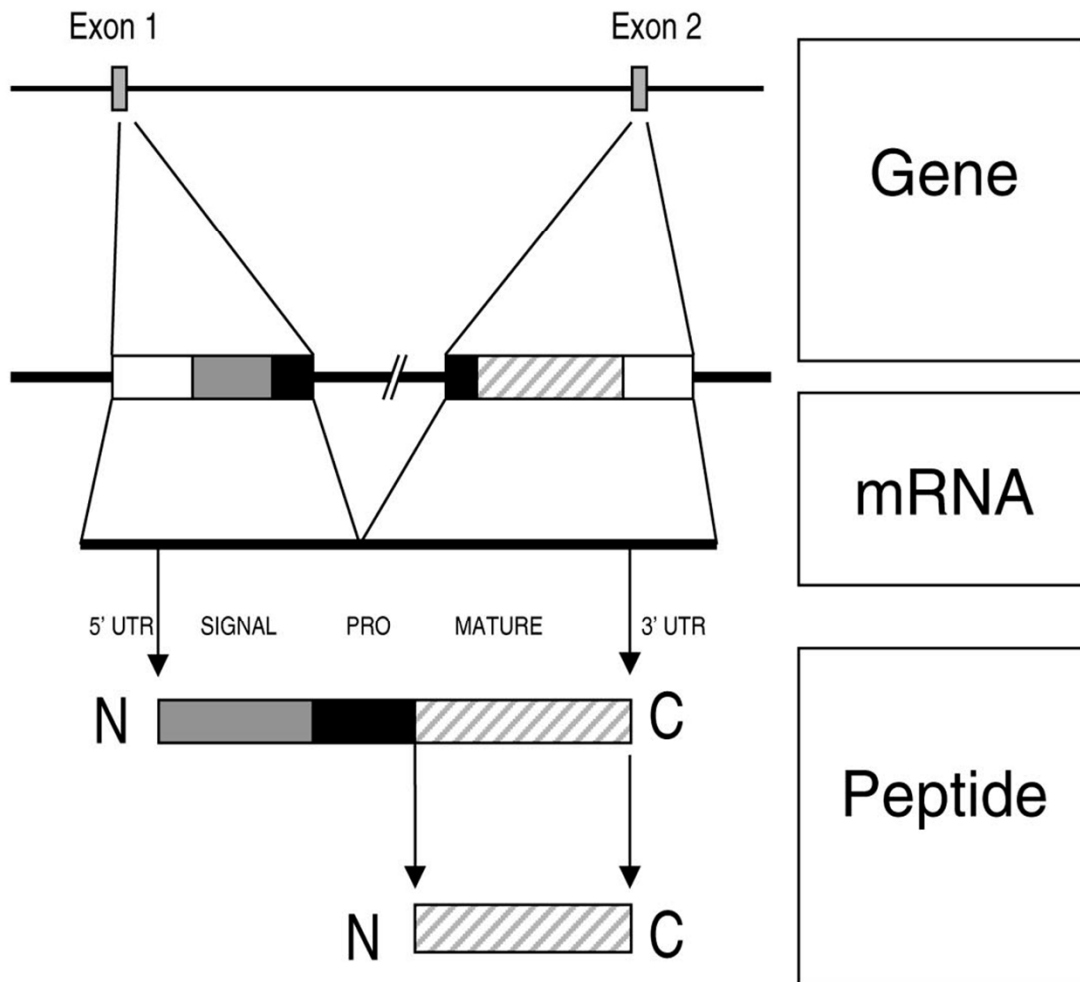
- β -defensin possesses three disulfide alignment at 1-5, 2-4 and 3-6 position
- \emptyset -defensin , novel class of defensin named for their circular structure and disulfide bridges at 1-6, 2-5 and 3-4
- Both α and β -defensins have similar tertiary structures and have triple stranded β sheets

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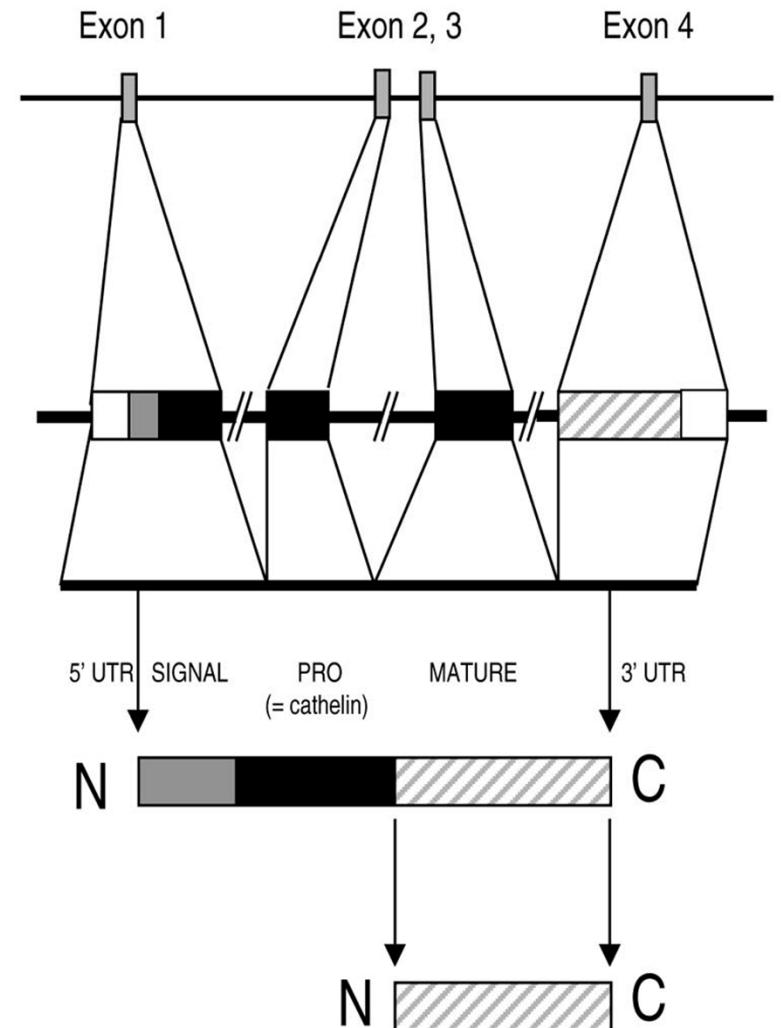
- **Cathelicidins are linear peptides of 16-26 kDa and have three different domain**
- **N-terminal signal peptide (30 aa) , a highly conserved cathelin like domain in the middle (94-112 aa) and less conserved C-terminal (12-100 aa)**
- **C-terminal - there is substantial heterogeneity which act as mature peptides**

Basic Structure of Defensin and Cathelicidin

β -defensin



Cathelicidin



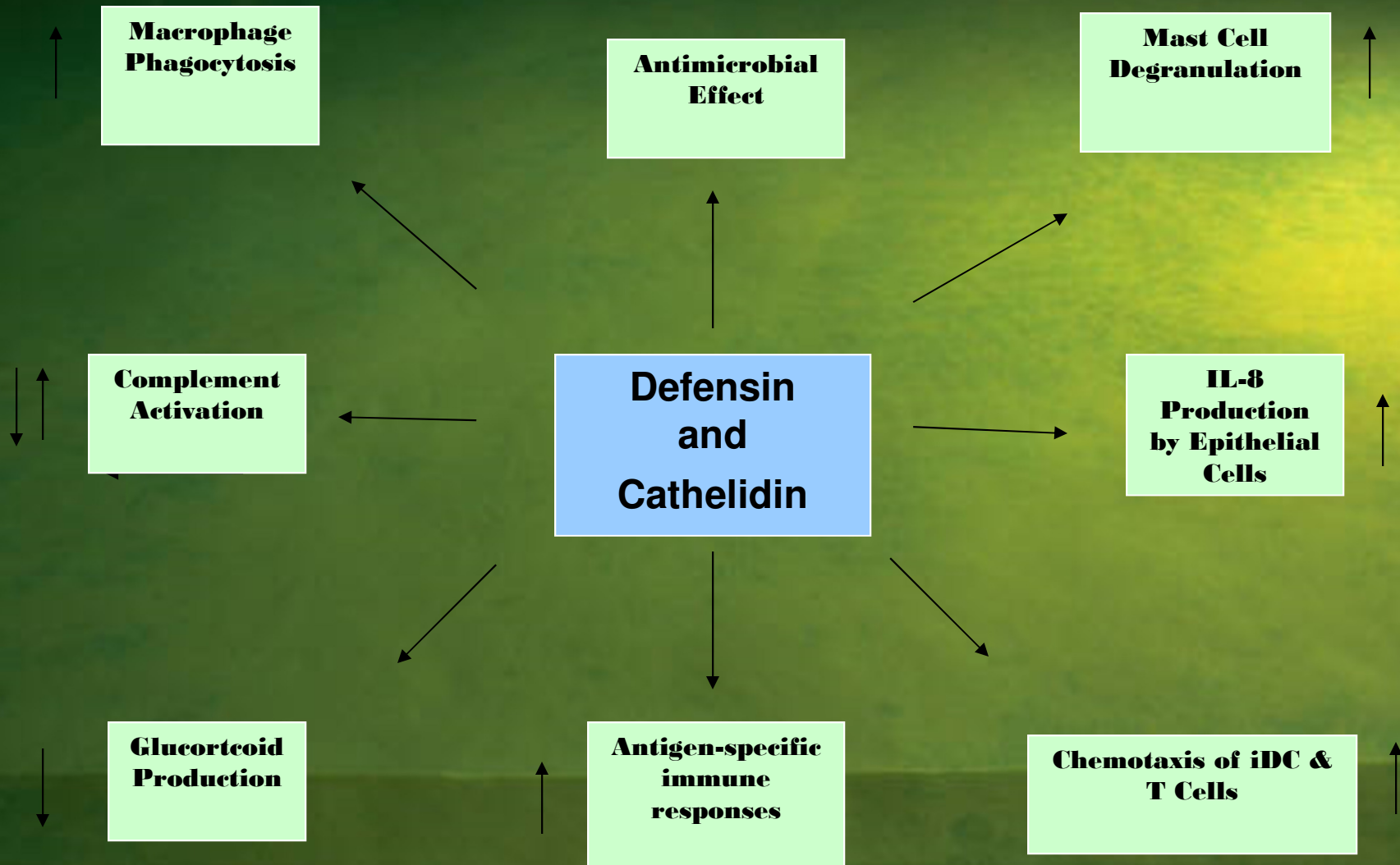
Mechanism of Action

- **Host Defense peptides are cationic molecules with spatially separated hydrophobic and charged residues**
- **Mammalian cells are enriched in PC, PE and SM where as microbial cell membrane comprise of PG and CL**
- **Presence of cholesterol in EC causes stabilization of lipid bilayers**
- **Fundamental differences of microbial and mammalian cell membrane exert selective toxicity of HDP against microorganisms**

Contd.

- **Induction of hydrolases**
- **Damaging of the critical intracellular structure after internalization of the peptides**
- **Natural Host Defense Peptides are Lacking unique epitopes to bind by protease**
- **Synthesis of multiple peptide of different structural classes**

Host Defense Peptides are Unique and quite complex host defense tool, having many blades with overlapping functions



HDP act as Template for Synthesis of New Antimicrobial Agent

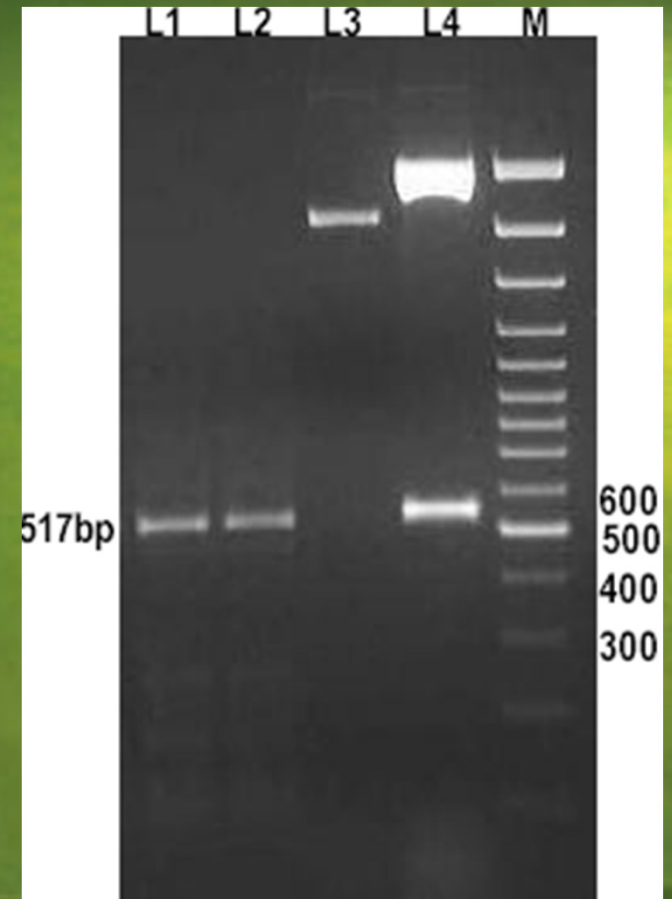
- ❖ Host Defense peptide can be use as blueprint for the design of novel antimicrobial agents
- ❖ Complete genome sequences and development of Bioinformatics provide opportunity for peptide based drug design
- ❖ In order to design the synthetic Host Defense Peptides, the most common approach is to have genomic sequences of HDP
- ❖ This can either be achieved by cloning a particular gene or by retrieving the required genomic sequences from NCBI gene data bank

Cloning of Host Defense Peptide Gene

- Isolation of Total RNA
- RT-PCR of Isolated RNA
- Electrophoresis for Confirmation of PCR Products
- Purification of PCR product
- Ligation of purified PCR product in cloning vector
- Transformation of ligated product in Competent cells
(Chung *et al.*, 1989)
- Isolation of plasmid (Sambrook and Russel (2001)
- Screening of Isolated Recombinant Plasmid
- Sequencing of Recombinant plasmid
- Sequence Analysis

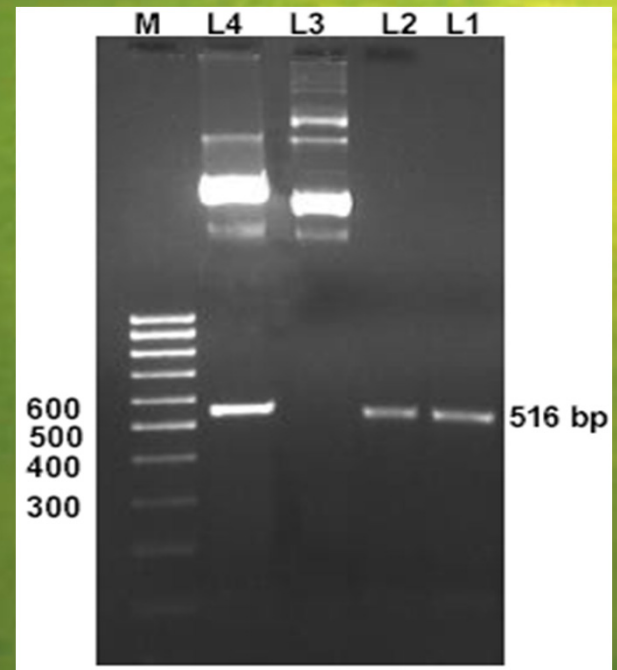
Cathelicidin Antimicrobial Peptide Gene (Testis) Accession No DQ 832665

**Fig. : Lane M : 100 bp DNA ladder
Lane L1 : PCR product
Lane L2 : Purified PCR product
Lane L3 : Undigested Plasmid
Lane L4 : Digested Plasmid**



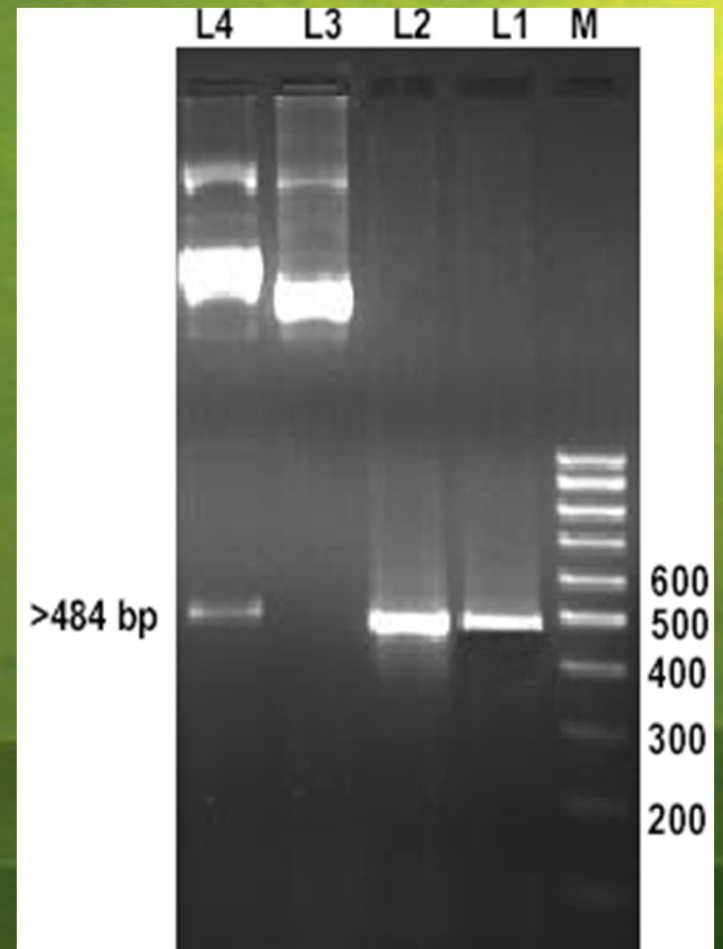
Cathelicidin Antimicrobial Peptide Gene (Uterus) Accession No EF 050433

- **Fig. : Lane M : 100 bp DNA ladder Lane L1 : PCR product Lane L2 : Purified PCR product Lane L3 : Undigested Plasmid Lane L4 : Digested Plasmid**



Cathelicidin Antimicrobial Peptide Gene (Myeloid Cell)
Accession No DQ 832666

- **Fig. : Lane M : 100 bp DNA ladder**
Lane L1 : PCR product
Lane L2 : Purified PCR product
Lane L3 : Undigested Plasmid
Lane L4 : Digested Plasmid



Prediction of Peptide from cDNA Sequence (Testis)

- atg cag agc cag agg gcc atc ctc gtg ctg ggg cgg tgg tca ccg tgg ctt ctg ctg ctg ggg ctt gtg 69
- M Q S Q R A I L V L G R W S P W L L L L G L V 23
- gtg tcc tgc acc agc gcc cag gac ctc agc tac agg gaa gcc gtg ctt cgt gct gtg gat cag ctc aat 138
- V S S T S A ↑ Q D L S Y R E A V L R A V D Q L N 46
- gag cgg tct tca gaa gct aat ctc tac cgc ctc ctg gag cca gaa cca cct ccc aag gat gat gaa gat 207
- E R S S E A N L Y R L L E P E P P P K D D E D 69
- ctg ggc act cga aag cct gtg agc ttc acg gtg aag gag act gtg tgc ccc agg acg act cag cag cct 276
- L G T R K P V S F T V K E T V C P R T T Q Q P 92
- gcg gag cag tgt gac ttc aag gag gaa ggg cgg gtg aag cag tgt gtg ggg aca gtc acc ctg gac ccg 345
- A E Q C D F K E E G R V K Q C V G T V T L D P 115
- tcc aat gac cag ttt gac cta aac tgt aat gcg ctc cag agt gtc agg ata cgc ttt cca tgg cc a tgg 414
- S N D Q F D L N C N A L Q S V ↓ R I R F P W P W 138
- cga tgg cca tgg tgg cgc aga gtc cga ggt tga 447
- R W P W W R R V R G * 148

Alignment of Predicted Cathelicidin peptide

	M Q T Q R A S L S L G R W S P W L L L L G L V U S S T S A Q	Majority
	10 20 30	
1	M Q S Q R A I L V L G R W S P W L L L L G L V U S S T S A Q	Buffalo Testis CATML.PRO
1	M Q T Q R A S L S L G R W S P W L L L L G L V U S S T S A Q	Buffalo Uterine CATML.PRO
1	M Q T Q R A S L S L G R W S L W L L L L G L V U P S A S A Q	B taurus CATML4.PRO
	D L S Y R E A V L R A V D Q L N E R S S S E A N L Y R L L E L	Majority
	40 50 60	
01	D L S Y R E A V L R A V D Q L N E R S S S E A N L Y R L L E P	Buffalo Testis CATML.PRO
01	D L S Y R E A V L R A V D Q L N E R S S S E A N L Y R L L E L	Buffalo Uterine CATML.PRO
01	A L S Y R E A V L R A V D Q L N E L S S E A N L Y R L L E L	B taurus CATML4.PRO
	D P P P K D D E D L G T R K P V S F T V K E T U C P R T T Q	Majority
	70 80 90	
61	E P P P K D D E D L G T R K P V S F T V K E T U C P R T T Q	Buffalo Testis CATML.PRO
61	D P P P K D D A D L G T R K P V S F T V K E T U C P R T T Q	Buffalo Uterine CATML.PRO
61	D P P P K D N E D L G T R K P V S F T V K E T U C P R T I Q	B taurus CATML4.PRO
	Q P A E Q C D F K E E G R V K Q C V G T U T L D P S N D Q F	Majority
	100 110 120	
91	Q P A E Q C D F K E E G R V K Q C V G T U T L D P S N D Q F	Buffalo Testis CATML.PRO
91	Q P T E R C D F K E E G R V K Q C V G T U T L D P S N D Q F	Buffalo Uterine CATML.PRO
91	Q P A E Q C D F K E K G R V K Q C V G T U T L D P S N D Q F	B taurus CATML4.PRO
	D L N C H E L Q S V R I R F P W P W X W P U W R R X R G -	Majority
	130 140	
121	D L N C H A L Q S V R I R F P W P W R W P U W R R V R G .	Buffalo Testis CATML.PRO
121	D L N C H E L Q S V R I R F P W P W P W P U W R R F R G .	Buffalo Uterine CATML.PRO
121	D L N C H E L Q S V I L - - - P W K W P U W P W R R G .	B taurus CATML4.PRO

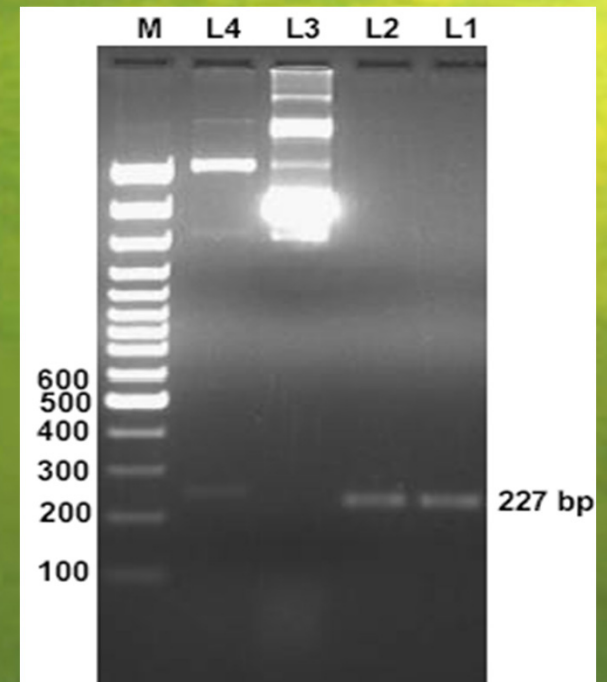
Alignment of Active/Mature Cathelicidin peptide for Synthesis

	<u>R L R F P W P W X W P W W R R</u>	Majority
		10
1	<u>I L - - - - P W</u> K W P W W P W	B taurus CATHL4 Active.PRO
1	R I R F P W P W P W P W W R R	Buffalo Uterine CATHL Active.PRO
1	R I R F P W P W R W P W W R R	Buffalo Testis CATHL Active.PRO
1	G L - - - - P W I L L R W L F	Buffalo Indolicidin Mature Peptide.PRO
	<u>F R G -</u>	Majority
12	<u>R R G .</u>	B taurus CATHL4 Active.PRO
16	<u>F R G .</u>	Buffalo Uterine CATHL Active.PRO
16	<u>V R G .</u>	Buffalo Testis CATHL Active.PRO
12	<u>F R G .</u>	Buffalo Indolicidin Mature Peptide.PRO

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

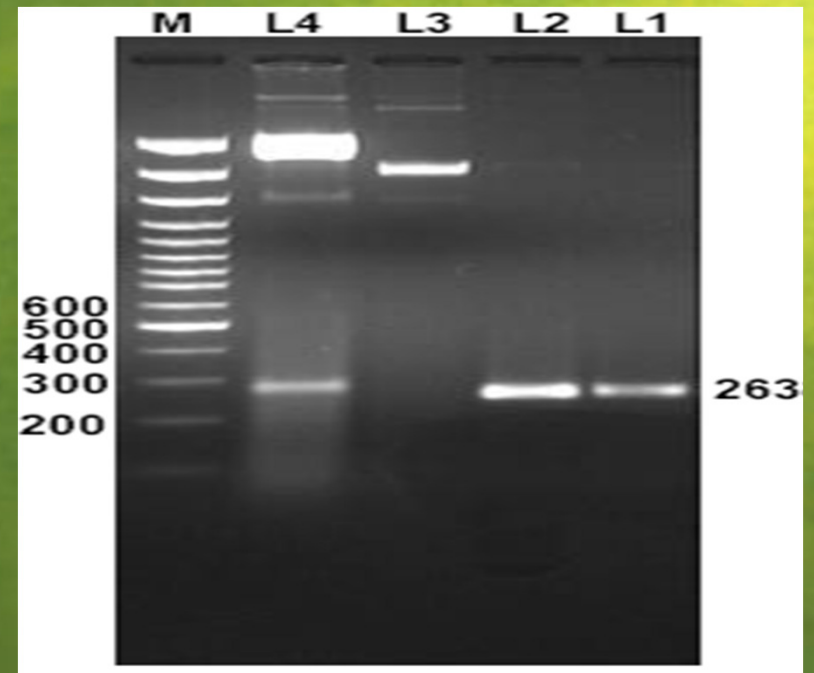
Defensin Antimicrobial Peptide Gene (Tongue) Accession No DQ 458768.

- Fig. : Lane M : 100 bp DNA ladder Lane L1 : PCR product Lane L2 : Purified PCR product Lane L3 : Undigested Plasmid Lane L4 : Digested Plasmid



Defensin Antimicrobial Peptide Gene (Mammary Gland) Accession No DQ 886701

- **Fig. :** Lane M : 100 bp DNA ladder
Lane L1 : PCR product
Lane L2 : Purified PCR product
Lane L3 : Undigested Plasmid
Lane L4 : Digested Plasmid



Alignment of Predicted Defensin

	M	R	L	H	L	L	L	L	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	V	R	N	P	Q	S	C	R	R	N	K	G	V	C	V	P	Majority			
	10										20										30										40											
1	M	R	L	H	H	L	L	L	L	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	V	G	N	P	V	S	C	A	R	N	K	G	I	C	V	P	Buffalo LAP.PRO		
1	M	R	L	H	H	L	L	L	L	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	V	R	N	P	Q	S	C	H	R	N	K	G	I	C	V	P	Buffalo EBD.PRO		
1	M	R	L	H	H	L	L	L	L	V	L	F	V	L	S	S	V	S	G	F	T	Q	G	I	R	S	P	L	S	C	R	G	N	R	G	V	C	L	P	Buffalo NBD4.PRO		
1	M	R	L	H	H	L	L	L	L	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	V	R	N	S	Q	S	C	H	R	N	K	G	I	C	V	P	Cattle LAP.PRO		
1	M	R	L	H	H	L	L	L	L	L	F	L	V	L	S	A	M	S	G	F	T	Q	G	V	G	N	P	V	S	C	V	R	N	K	G	I	C	V	P	Cattle TAP.PRO		
1	M	R	L	H	H	L	L	L	L	T	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	I	S	N	P	L	S	C	R	L	N	R	G	I	C	V	P	Cattle EBD.PRO	
1	M	R	L	H	H	L	L	L	L	A	V	L	F	L	V	L	S	A	G	S	G	F	T	Q	R	V	R	N	P	Q	S	C	R	M	N	M	G	V	C	I	P	Cattle NBD4.PRO
1	M	R	L	H	H	L	L	L	L	V	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	I	R	S	R	S	C	H	R	N	K	G	V	C	A	L	Goat BD1.PRO		
1	M	R	L	H	H	L	L	L	L	A	L	F	L	V	L	S	A	G	S	G	F	T	Q	G	I	I	N	H	R	S	C	Y	R	N	K	G	V	C	A	P	Goat BD2.PRO	
1	M	R	L	H	H	L	L	L	L	L	F	V	L	S	A	A	S	G	F	T	Q	G	V	X	T	P	Q	S	C	H	R	N	K	G	V	C	V	P	Reindeer BD1.PRO			
	Y R C P G N M R Q I G T C L G P P V K C C R R K -																																								Majority	
	50																				60																					
41	S	R	C	P	G	N	M	R	Q	I	G	T	C	L	G	P	P	V	K	C	C	R	R	K	.	Buffalo LAP.PRO																
41	I	R	C	P	G	N	M	R	Q	I	G	T	C	L	G	P	P	V	K	C	C	R	R	K	.	Buffalo EBD.PRO																
41	I	R	C	P	G	R	L	R	Q	I	G	T	C	F	G	P	R	V	P	C	C	R	-	R	.	Buffalo NBD4.PRO																
41	I	R	C	P	G	S	M	R	Q	I	G	T	C	L	G	A	Q	V	K	C	C	R	R	K	.	Cattle LAP.PRO																
41	I	R	C	P	G	S	M	K	Q	I	G	T	C	V	G	R	A	V	K	C	C	R	K	K	.	Cattle TAP.PRO																
41	I	R	C	P	G	N	L	R	Q	I	G	T	C	F	T	P	S	V	K	C	C	R	W	R	.	Cattle EBD.PRO																
41	F	L	C	R	V	G	M	R	Q	I	G	T	C	F	G	P	R	V	P	C	C	R	R	.	Cattle NBD4.PRO																	
41	T	R	C	P	R	N	M	R	Q	I	G	T	C	F	G	P	P	V	K	C	C	R	K	K	.	Goat BD1.PRO																
41	A	R	C	P	R	N	M	R	Q	I	G	T	C	H	G	P	P	V	K	C	C	R	K	K	.	Goat BD2.PRO																
41	I	R	C	P	R	S	M	R	Q	I	G	T	C	L	G	A	P	V	K	C	C	R	R	K	.	Reindeer BD1.PRO																

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Alignment of Active/Mature Defensin peptide for Synthesis

	V	G	N	P	V	S	C	X	R	N	K	G	I	C	V	P	I	R	C	P	Majority
1	V	G	N	P	V	S	C	V	R	N	K	G	I	C	V	P	I	R	C	P	Cattle TAP Mature.PRO
1	V	R	N	S	Q	S	C	R	R	N	K	G	I	C	V	P	I	R	C	P	Cattle LAP Mature.PRO
1	V	R	N	P	Q	S	C	H	R	N	K	G	I	C	V	P	I	R	C	P	Buffalo EBD Mature.PRO
1	V	G	N	P	V	S	C	A	R	N	K	G	I	C	V	P	S	R	C	P	Buffalo LAP Mature.PRO
	G	S	M	R	Q	I	G	T	C	L	G	P	P	V	K	C	C	R	R	K	Majority
21	G	S	M	K	Q	I	G	T	C	V	G	R	A	V	K	C	C	R	K	K	Cattle TAP Mature.PRO
21	G	S	M	R	Q	I	G	T	C	L	G	A	Q	V	K	C	C	R	R	K	Cattle LAP Mature.PRO
21	G	N	M	R	Q	I	G	T	C	L	G	P	P	V	K	C	C	R	R	K	Buffalo EBD Mature.PRO
21	G	N	M	R	Q	I	G	T	C	L	G	P	P	V	K	C	C	R	R	K	Buffalo LAP Mature.PRO
	-																				Majority
41	.																				Cattle TAP Mature.PRO
41	.																				Cattle LAP Mature.PRO
41	.																				Buffalo EBD Mature.PRO
41	.																				Buffalo LAP Mature.PRO

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Synthesis and Evaluation

- Solid phase methodology (devised by Bruce Merrifield for which he got Nobel Prize in 1984) can be used for its synthesis
- Screening of synthetic peptide or its analogue can be done by anti microbial sensitivity test
- It has also to be tested for its toxicity on normal host cells by estimating haemolytic activity of the peptide as well as by studying the permeability of the cell to propidium iodide (PI) by Fluorescence Activated Cell Sorter (FACS)
- Secondary structure of the peptide can be quantified by analyzing the Circular Dichroism (CD) spectroscopy

Conclusions

- **Designing and synthesis of peptides represents a promising strategy for the development of a new class of antimicrobial agents to prevent and treat systemic and topical infections**

THANKS