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Identification and Sequencing of a cDNA Clone Encoding Cathelicidin-like Antimicrobial Peptide from Chicken Heart Tissues

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Abstract

In the present scenario the antimicrobial peptides of animal sources are being considered as suitable alternatives to the classical antibiotics for preventing pathogenic microorganisms without allowing them to develop resistance. Cathelicidins are one of the major classes of antimicrobial peptides widely distributed in mammalian neutrophils and epithelial cells. They kill wide-range of bacteria (both gram positive and gram negative), viruses, fungi and protozoa. In the present work, a 215 bp cDNA transcript was identified from the heart tissues of white leghorn chicken (*Gallus gallus*) by reverse transcription. It was amplified by RT-PCR and partially sequenced to obtain a 175 bp sequence. The sequence homology study revealed that this gene encodes a cathelicidin-like pepttide. The deduced peptide was found to be 5.5 kDa in molecular weight with 59 amino acids in length. It showed sequence similarities with cathelicidin family proteins of mammals and birds and also phylogenetically resembled with human cathelicidin-like peptide. Thus, this study will provide an insight of using this antimicrobial peptide to treat infectious diseases of human beings.

Keywords: Antibiotics; Cathelicidins; Neutrophils; LL-37; cDNA clone; Evolution; Phylogeny.

1. Introduction

Endogenous cationic peptides play an important role in innate immunity. Unlike many natural antimicrobials synthesized through specialized metabolic pathways, these peptides are expressed as precursor molecules from individual gene and converted in to active peptides by proteolytic processing. Cathelicidins are one of the major classes of antimicrobial peptides (AMPs) found in all mammalian neutrophils and epithelial cells which are evolutionarily conserved at sequence as well as structural levels. Cathelicidin peptides share a highly conserved 12kDa *N*-terminus named 'cathelin'domain [1]. The *C*-terminals of the cathelicidin peptides express antibiotic activity after they have been cleaved from holoprotein [2].

A number of cathelicidins have been isolated and characterised from different animals. About 30 different cathelicidins like Bactenecins (Bac5 and Bac7) and PR-39 (proline-rich linear peptides) and Dodecapeptides and Protegrins (cyclic peptides) with their potential antimicrobial activities have been described in mammals [3-4]. Ambika et al., have also identified a cathelicidin cDNA (297 base pairs in length) from the Indian goat by RT-PCR. The human cathelicidin antimicrobial peptide (hCAP-18/LL-37) was the only cathelicidin peptide identified from human neutrophils [5]. The wound healing properties of hCAP-18 [6] and is *in vitro* antimicrobial activities of LL-37 from ocular surface epithelial cells [7] have been investigated in detail.

Apart from mammalian cathelicidins, some avian cathelicidin peptides have also been reported. Three cathelicidin genes encoding fowlicidin 1-3 were identified from the chicken genome. They showed high degree of similarity with all known mammalian cathelicidins [8-9] have also identified an expressed sequence tag (EST) clone which encoded a novel cationic antimicrobial peptide named 'chicken liver expressed antimicrobial peptide 2 (cLEAP-2) using *in silico* studies. This gene was also found to be expressed in a number of chicken epithelial tissues like small intestine, lung and kidney other than liver. Four other antimicrobial peptides, termed gallinacin 1 and 2 and turkey heterophil peptides 1 and 2 have been studied from avian heterophils [10-11]. Based on our very recent reports of antibacterial property of chicken heart tissues [12], we hypothesed that the chicken heart tissues might possess any human native antimicrobial peptides like human cathelicidin (hCAP 18/ LL 37). Hence the present study was aimed to clone and sequence the cDNA encoding human cathelicidin antimicrobial peptide from chicken heart tissues using the appropriate primers

(Primers for hCAP 18/ LL 37). This work also focused on the phylogenetic inference of deduced amino acid sequence of this clone with the related sequences of vertebrates.

2. Methods

2.1 Preparation of cDNA by RT-PCR and DNA sequencing

The heart of white leghorn chicken (*Gallus gallus*) used in the experiments was procured from a slaughter house, Tiruchirappalli. Total RNA from the tissue sample was extracted using an RNeasy kit (Qiagen). Two hundred and fifty ng of total RNA was used for reverse transcription reaction to produce cDNA (First-Strand cDNA synthesis kit; Fermentas). Reverse transcription was performed at 54^{o}C for 60 minutes. After denaturation of reverse transcriptase at 94^{o}C for 5 minutes, amplification of cDNA was performed by polymerase chain reactions. Two µl of the reaction product was used for PCR amplification. The PCR reactions were performed in Eppendorf Master Cycler as per the manufacturer's instruction (Quagen hot stat taq Master Mix kit). Total PCR volume was 16µl with 2 µl of primers per assay. The sequences of the primers for hCAP-18/LL-37 such as 5'-ATCATTGCCCAG-GTCCTCAG-3' for forward reaction and 5'-GTCCCCATACAC-CGCTTCAC-3' 251bp for reverse reaction were synthesised according to [7] and used to clone the human cathelicidin encoded gene. The concentration of each primer (both forward and reverse) used was 3 pmol per assay. The PCR products were visualized in the gel after the capillary electrophoresis using an Alpha Imager gel documentation system (Alpha Innotec, San Leandro, CA, USA) along with the DNA ladders between 15 to 1500 base pair lengths. The identified DNA band of 215 bp was eluted after agarose gel electrophoresis. This eluted DNA sample was further sequenced using automated DNA sequencing (ABI DNA sequence, USA) and then verified.

2.2 Sequence and structural analysis

The sequence of cDNA product was annotated for coding regions, conservd domains, motifs, structure and its biological function. The cDNA sequence was translated to corresponding amino acid sequence and then subjected to domain and motif predictions using MyHits server (http://hits.isb-sib.ch/cgi-bin/PFSCAN) from motif databases. Conserved domain classification of this sequence was searched from NCBI conserved domain architecture [13]. Pairwise similarity of this sequence with some related sequences were analyzed with LALIGN tool (http://www.ch.embnet.org/software/LALIGN_form.html). A primary and secondary structural feature of translated peptide sequence was computed by ProtParam (http://expasy.org/tools/protparam.html) and SOPMA server [14] respectively. ModWeb is an automatic comparative protein modeling server (http://salilab.org/modweb), where the same sequences were uploaded to build three dimensional structures based a template using ModPipe Version SVN.r665. A quality of modeled protein structure was further validated by SAVS (Structure Analysis and Verification) server (http://nihserver.mbi.ucla.edu/SAVS/) using Prove and ProCheck algorithms. The function of modeled protein was annotated by ProFunc server [15] by uploading PDB file as input.

2.3 Phylogenetic analysis

The highly identical sequences for cathelicidin antimicrobial peptide (CAMP) were retrieved from NCBI database using BLASTn and PSI-BLAST with default parameters [16]. Sequences with more significant identities were clustered by multiple sequence alignment program in ClustalX software [17] using Smith-Waterman substitution matrix and trimmed to consensus. Neighbor-Joining (NJ) and Minimum Evolution (ME) algorithms were used to construct phylogenic trees using MEGA 4.0 [18] with 1000 bootstraps at uniform divergence rates using Jukes and Cantor evolutionary model and 0.25 gamma distribution factors.

2.4 GenBank accession

The sequence used in this study has been deposited in GenBank of NCBI with accession number FJ755847.

3. Results

RT-PCR analysis was performed to detect the hCAP-18/LL-37 (human cathelicidin) mRNA from the chicken heart tissues. An isomer of 215 base pairs transcript was identified along with a co-amplified 50 bp cDNA clone as shown in Figure 1. Among two bands identified, the band at 215 bp transcript was distinct and dense and which was purified by agarose gel electophoresis and subjected to sequencing analysis. This was resulting into only partial sequencing that is 175bp in length instead of expected length (215bp).

As shown in Table 1, pairwise sequence similarity analysis reported that the identified new nucleotide sequence had 100% similarities with the sequences of CAP-18 of *H. sapiens* at the positions of 170-193 and 367-390 where as CAMP of *Macaca mulatta* at the positions of 170-193 and 357-380. However, it had only 70.2% similarity with GAL2 of *Gallus gallus* at the positions of 48-90 and 274-318 with the score value of 67. Motif and conserved domain similarity of this sequence showed its resemblance with collagen alpha type 4 and then with hCAP of *Hamo sapiens*.

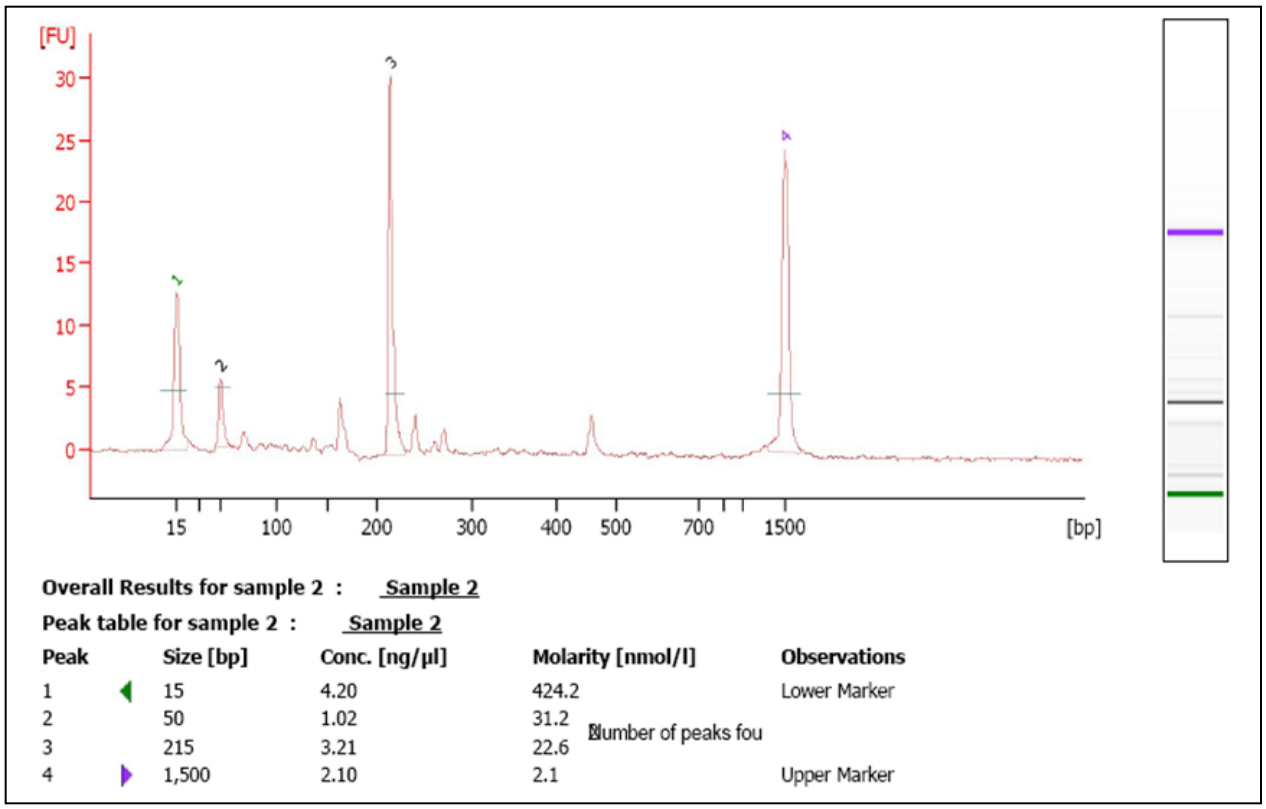

Figure 1. Electropherogram of cDNA clones from chicken heart tissues.

Table 1. Pairwise sequence similarity analysis of cDNA clone sequence from *Gallus gallus* with cathelicidine gene sequences of other vertebrates.

Accession	Gene	Organism	Score	Position	Identity (%)
NM_204992	*GAL2 (G)*	*Gallus gallus*	67	48-90:274-318	70.2
			66	65-134:38-106	57.1
NM_001033509	*CAMP (M)*	*Macaca mulatta*	111	170-193:358-381	95.8
			107	102-183:92-165	64.6
			99	109-173:68-127	66.2
X89658	*CAP-18 (H)*	*Homo sapiens*	120	170-193:367-390	100
			98	102-183:101-174	63.4
			88	130-179:81-130	64
AF288284	*CAMP (H)*	*Homo sapiens*	120	170-193:357-380	100
			98	102-183:91-164	63.4
			64	130-179:71-120	88

The translated peptide was found to be 5.5 kDa in molecular weight with 59 Amino acids length. It has 69.5% random coil, 13.5% alpha helix, 10.2% extended strand and 6.8% beta turn at secondary structure level. It was structurally aligned with the position 12-58 of template (2PNE) wherein it showed 43% identity and 0.0012 e-values. Homology modeling of this peptide showed that it has structurally homologous to collagen alpha chain type 4 (MPQS 1.34 and z-DOPE -1.17). The more conserved amino acid residues in this peptide were Gly57, Asp58 and Gly59 (Table 2). While the homology model was superimposed on hCAP-18 peptide structure, it showed the significant structural similarity and conservation of this peptide.

Table 2. Annotation of functional features of modelled protein using ProFunc server.

Search	Feature	Quality
InterPro Hit	Collagen α-chain, type 4	PTHR10499
PDB Hit	TY0F (B): Collagen α-chain, type I	Identity: 64.3%
UniProt Hit	Collagen α-chain, type I	Identity: 97.6%
Active site	Gly57, Asp58, Gly59	Score: 1.667
Reverse template	3BOG: Anti freeze protein	Score: 246.8

In the phylogenetic tree (Figure 2), there were 5 major clades in which human cathelicidin-like peptides from *Gallus gallus* was closely related with collagen alpha type 1 and 2 peptides and then clustered with gallicidin 2 of *Gallus gallus* and CAMP of *Macaca mulatta*.

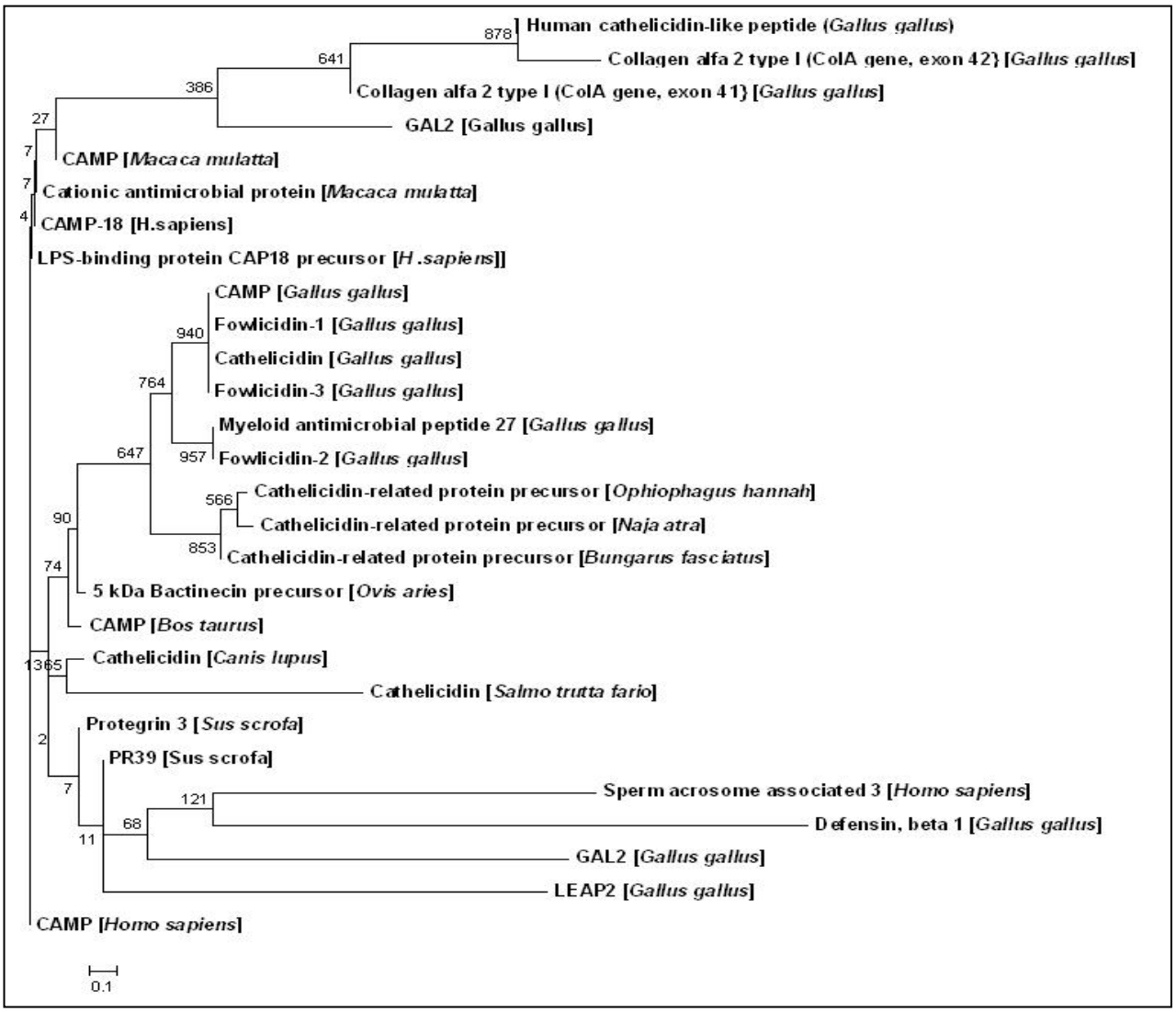

Figure 2. Phylogenetic analysis of mRNA sequence (human cathelicidin-like) isolated from *Gallus gallus*. The tree was constructed with 1000 bootstraps value with NJ algorithm.

This clade has phylogenetic proximity with CAP-18 and LPS-binding protein of CAP-18 of *Homo sapiens*, suggesting that the functional and evolutionary divergence of human cathelicidin-like peptides may be occurred. Other antimicrobial peptides, fowlicidin from *Gallus gallus*, bactinecin from *Ovis aeries*, protegrin from *Sus scrofa* have also shown a close phylogenetic resemblance with this peptide sequence.

4. Discussion

It is a well known fact that cathelicidins are proantibacterial proteins of neutrophils from a variety of mammalian species. The antibacterial activity of vathelicidins is confined to the *C*-terminal parts that differs greatly among the different cathelicidins, but share the characteristic features of being highly positively charged (cationic) and containing a high proportion of hydrophobic amino acids [19]. These features are essential for the antimicrobial activity of cathelicidins. The occurrence of such cationic amino acids and high proportion of hydrophobic regions in this peptide revealed to support the preserving antimicrobial activity against some pathogens of vertebrates.

The presence of mRNA identified for human cathelicidin (hCAP-18/LL-37) gene in the chicken heart tissues may be due to the up-regulation of this gene when the respective antibacterial peptide is depleted by utilization during infections. The up-regulation of cLEAP-2 gene in small intestine and liver of chicken after Salmonella infection has also been studied [9]. Further they have reported that birds are universally recognized as a major reservoir of human enteropathogens but are themselves often asymptomatic. Though both the nucleotide and

deduced amino acid sequences of the identified cDNA clone were primarily matched with collagen type proteins, they have also significant similarities with different cathelicidin peptides including human cathelicidin. The molecular weight of the translated peptide (5.5 kDa) was approximately equal to the molecular weight of 4 to 5 kDa LL-37 functional region of human cathelicidin [20]. In addition, it was also similar to the molecular weight (5-7 kDa) of cathelicidin peptides such as Bactenecin 5 and Bactenecin 7 [21]. Hence, this cDNA clone coding peptide obtained from chicken heart tissues has suggested as a functional region of cathelicidin-like peptide (Figure 3). Moreover, it was also phylogenetically resembled with different cathelicidin family proteins of mammals and birds, revealing that molecular function of this peptide could be acquired from a common ancestor of birds by slow evolutionary process.

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1      GAG CAT CTT GTG TCT GCC TGG CTC TCG GGG AGA ATT GGT CTT CCA    45
1       E   H   L   V   S   A   W   L   S   G   R   I   G   L   P    15

46     GGC ATT GCT GGA GCA ACA GGT GAA CCT GGT CCT CTG GGT GTT TCT    90
16      G   I   A   G   A   T   G   E   P   G   P   L   G   V   S    30

91     GGT CCT CCT GGT GCT CGT GGT CCC TCT GGT CCT GAG GGT TCT CCT    135
31      G   P   P   G   A   R   G   P   S   G   P   E   G   S   P    45

136    GGT CCT AAT GGT GCT CCT GGT GAA GCG GTG TAT GGG GAC    174
46      G   P   N   G   A   P   G   E   A   V   Y   G   D


2      AGC ATC TTG TGT CTG CCT GGC TCT CGG GGA GAA TTG GTC TTC CAG    46
1       S   I   L   C   L   P   G   S   R   G   E   L   V   F   Q    15

47     GCA TTG CTG GAG CAA CAG GTG AAC CTG GTC CTC TGG GTG TTT CTG    91
16      A   L   L   E   Q   Q   V   N   L   V   L   W   V   F   L    30

92     GTC CTC CTG GTG CTC GTG GTC CCT CTG GTC CTG AGG GTT CTC CTG    136
31      V   L   L   V   L   V   V   P   L   V   L   R   V   L   L    45

137    GTC CTA ATG GTG CTC CTG GTG AAG CGG TGT ATG GGG ACA    175
46      V   L   M   V   L   L   V   K   R   C   M   G   T


3      GCA TCT TGT GTC TGC CTG GCT CTC GGG GAG AAT TGG TCT TCC AGG    47
3       A   S   C   V   C   L   A   L   G   E   N   W   S   S   R    14

48     CAT TGC TGG AGC AAC AGG TGA ACC TGG TCC TCT GGG TGT TTC TGG    92
15      H   C   W   S   N   R   *   T   W   S   S   G   C   F   W    29

93     TCC TCC TGG TGC TCG TGG TCC CTC TGG TCC TGA GGG TTC TCC TGG    137
30      S   S   W   C   S   W   S   L   W   S   *   G   F   S   W    44

138    TCC TAA TGG TGC TCC TGG TGA AGC GGT GTA TGG GGA    173
45      S   *   W   C   S   W   *   S   G   V   W   G    56
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Figure 3. Codon usages of identified genes in three frames.

5. Conclusions

Even though the identified nucleotide sequence could not be confirmed a human cathelicidin peptide, due to all the above mentioned facts, it can be considered as a cathelicidin-like gene. Overall, we concluded that this gene isolated from chicken heart may provide a new insight to develop a potential antimicrobial peptide against pathogenic microorganisms withstanding resistance to classical antibiotics after the successful completion of full-length cDNA cloning of this gene using the appropriate cloning vectors and its expression

studies. This study also reveals that it is very convenient to prepare such natural antibacterial peptides economically at industrial level.

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