CHICKEN CATHELICIDINS: TISSUE EXPRESSION, DEVELOPMENTAL REGULATION, AND MASS SPECTROMETRIC QUANTIFICATION

By

MALLIKA ACHANTA

BACHELOR OF VETERINARY SCIENCE AND

ANIMAL HUSBANDRY

SRI VENKATESWARA VETERINARY UNIVERSITY

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Thesis Approved:

Dr. Guolong Zhang

Thesis Adviser

Dr. Steve Hartson

Dr. Lara Maxwell

Dr. Mark E. Payton

Dean of the Graduate College

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INTRODUCTION

Antimicrobial peptides (AMPs) are important effector molecules of the innate immune system. They are mostly cationic in nature and kill bacteria mainly by direct disruption of microbial cell membranes. In addition to antimicrobial properties, many AMPs stimulate the production of cytokines and chemokines. Cathelicidins constitute a major family of AMPs sharing a highly conserved precursor sequence at the amino-terminal region. Cathelicidins are generally synthesized from granules of leukocytes and macrophages as well as mucosal epithelial cells and skin keratinocytes. We have provided an extensive review of the literature on AMPs in Chapter 1. In chickens, four cathelicidins have been identified, known as fowlicidins 1-3 and cathelicidin B1. However, little is known about their tissue and developmental expression patterns. In Chapter 2, we quantitated the expression of four chicken cathelicidins in a large panel of tissues by real-time PCR. We also studied how the expressions of these four cathelicidins differ during the development of chickens from 2- to 28-days of age. We concluded that cathelicidins may function to protect chickens from infectious agents and environmental stress upon hatching until the immune system is fully established. Earlier work in this lab has revealed

that synthetic fowlicidins 1-3 possess potent antibacterial activities against a broad spectrum of microorganisms. Among all fowlicidin 9 analogs, carboxyl-terminal amidated fowl-1(6-26)-NH_2, capable of killing antimicrobial resistant bacteria, such as methicillin-resistant *Staphylococcus aureus,* both in vitro and in vivo, remains active at physiological salt concentrations and in the presence of serum.

Therefore, fow-1(6-26)-NH_2 has potential for further development as a novel antibacterial drug. However, the disposition of fow-1(6-26)-NH_2 with regard to its absorption, distribution, metabolism, and excretion following in vivo administration is presently unknown. As a first step to estimate its disposition, we have sought to develop and validate a liquid chromatography and tandem mass spectrometry (LS-MS/MS) approach to quantify fow-1(6-26)-NH_2 in Chapter 3.

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

The discovery of penicillin as an antibiotic by Alexander Fleming was considered a great advancement in science (1). Since then, hundreds of antimicrobial drugs have become commercially available and impact enormously on the public health. Mechanisms of action of antibiotics mainly include interference with the synthesis of bacterial cell walls, proteins and nucleic acids, inhibition of metabolic pathways, and disruption of bacterial membrane structure (2). However, the extensive use of antibiotics has also led to the rapid emergence of microorganisms that are resistant to antimicrobial agents (3). This resistance is primarily achieved through the acquisition of resistance genes from other microorganisms as in the case of *Staphylococcus aureus* (2). The resistance genes enable bacteria to produce antibiotic-inactivating enzymes, express drug-efflux systems, modify the drug target sites, or acquire new metabolic pathways that bypass the action of the drug (2).

Hence, the development of novel antibiotics with less likelihood of provoking bacterial resistance is critically important in ensuring public health. Antimicrobial peptides, a large

group of natural antibiotics produced by virtually all species of life, are being actively explored as a new generation of antibacterial drugs.

DISCOVERY OF ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are endogenous peptides of less than 100 amino acids that constitute an important component of the innate immune system. They are found in almost all species of life ranging from bacteria to yeasts and from plants to invertebrate and vertebrate animals (4). AMPs of animal origin are primarily produced from phagocytes as well as epithelial cells of the skin and gastrointestinal, respiratory, and uro-genital tracts (5). In general, AMPs are synthesized as precursor molecules, which have a signal and propeptide sequence. They are cleaved to form mature peptides (6). They have a broad spectrum of antimicrobial activity against bacteria, parasites, viruses, and fungi. Besides antimicrobial properties, they play an important immunomodulatory role in chemotaxis of immune cells, modulation of cell surface receptors and alteration in gene expression (4). AMPs also bridge the innate and adaptive immune systems. AMPs interact with dendritic cells, T cells and B cells to indirectly eradicate pathogens by either activating or inhibiting the production of chemokines and cytokines (7).

MECHANISM OF ACTION

Most AMPs kill microbes by disrupting cell membranes of microorganisms, similar to other innate immune components such as complement proteins, where killing occurs by membrane attack complex formation. Cationic AMPs interact with anionic cell membranes of bacteria, fungi, parasites and enveloped viruses (8). They penetrate into the cells and disrupt the microbial cell membranes either by pore formation or by

detergent-like solubilization (9). Three models have been proposed to explain membrane permeabilization: i. In the barrel stave model, peptide helices form pores into the bilayer, where the hydrophobic region of peptides align with lipid core of the bilayer and the hydrophilic region forms central region of the pore, ii. In the carpet model, peptides electrostatically bind to the membrane and spread over the bilayered membrane in a carpet like manner that eventually led to the formation of micelles and, iii. In toroidal pore model, the pore formation is similar to barrel stave model, but the antimicrobial peptide helices insert into the membrane in such a way that it induces the lipid bilayer to bend continuously through the pore so that the peptides are always associated with lipid head groups (10). In all these models, changes in membrane permeability lead to death of the microorganisms. However, non-membrane lytic mechanisms also exist for a few AMPs (11, 12). For example, PR-39 causes cell death by inhibiting bacterial protein synthesis (9), whereas human neutrophil defensins have been demonstrated to suppress DNA and protein synthesis (13) and mersacidin inhibits cell wall synthesis (14). Because of a difference in the membrane net charge, lipid composition, and transmembrane potential between bacterial and mammalian cell membranes, AMPs shows weak or no lytic activity toward mammalian cells (8).

CLASSIFICATION OF ANTIMICROBIAL PEPTIDES

AMPs are classified into four classes based on their charge, structure, sequence homology, and functional properties (7). They include: 1) linear cationic α-helical peptides, e.g., cercopins, chicken cathelcidins, LL-37, and SMAP-29; 2) peptides rich in certain amino acids, e.g., PR-39 rich in arginine and proline and indolicidin rich in

tryptophan; 3) β-sheet peptides consisting of one or multiple disulfide bonds, e.g., protegrins, human α- and β-defensins; and 4) anionic peptides, e.g., dermicidins (15).

In vertebrates, defensins and cathelicidins represent two major families of AMPs. Defensins are cationic peptides that contain 6-8 cysteine residues. Disulfide bridge formation is the characteristic feature of defensins, which are further grouped into three subfamilies, namely α-, β-, and θ-defensins, based on the cysteine spacing pattern and disulfide bond linkage (9). In humans, the α-defensins such as human neutrophil defensins (HNPs) 1-4 and human alpha defensin (HD) 5-6 are abundantly expressed in the granules of neutrophils and intestinal paneth cells respectively(16, 17). In addition to the antibacterial activity, α -defensins show an increased expression of TNF-α and IL-1 in activated macrophages (9) and also exhibit potent antiviral activity mainly against adenoviruses (18). There are over 35 distinct β-defensins reported in humans (9), whereas a total of 14 β-defensins exist in chickens (19, 20). Besides antibacterial activities, other immune related functions of β-defensins include promotion of histamine release and prostaglandin D2 production in mast cells (21).

Cathelicidins consist of a highly conserved prepropeptide sequence (cathelin) and an extremely variable carboxyl-terminal cationic, biologically active peptide. Cathelicidins are synthesized in bone marrow myeloid cells as prepropeptides. They are converted to proform by the removal of signal peptide, and then stored in the large granules of neutrophils (22). The conserved prosequence plays a role in targeting the antimicrobial peptides to the granules and also assists in correct proteolytic maturation. The prosequence is highly identical to the sequence of a protein called cathelin. Hence, the term cathelicidin is used, to indicate that this group contains cathelin-like proregion and a

C-terminal antimicrobial domain (23).The enzyme elastase cleaves off the N-terminal conserved region to produce matured antimicrobial peptide (24). LL-37 is the only cathelicidin found in humans (25), whereas four cathelicidins have been identified in chickens (26, 27). Chicken cathelicidins are termed as fowlicidins 1-3 and cathelicidin B1. Cathelicidin B1 is so called because of its selective expression in the bursa of fabricius (26).

THERAPEUTIC EFFECTS OF ANTIMICROBIAL PEPTIDES

AMPs have been shown to be effective in killing of various infectious agents like bacteria, virus, fungi and parasites. Production of endogenous AMPs is often positively correlated with infectious disease resistance. For example, a decrease in the expression of LL-37 and HBD-2 in human patients with atopic dermatitis has been linked to increased susceptibility to skin infections (28). A lack of cathelicidin expression in humans is considered to be a major cause in Kostamann's syndrome (9). Similarly, inhibition of cathelicidins in pigs enhanced bacterial colonization before the innate immune cells are activated (29). Similarly, Howell and their colleagues showed that an increased expression of AMPs in keratinocytes during inflammation resulted in an enhanced activity against *Staphylococcus aureus*, Herpes Simplex Virus, and Vaccinia Virus (30).

In addition to antimicrobial activity, AMPs play an important role in wound repair, angiogenesis, and immunomodulation. Inhibition of LL-37 by anti-LL-37 antibodies reduced wound healing (31) via suppression of the proliferation of keratinocytes (31, 32). Development of blood vessels was also noted in tissue cultures as well as in rabbit models by following addition of LL-37 (32). Because of pleiotropic effects beyond direct

antimicrobial actions, AMPs are being actively explored as a new class of antibiotics. Pharmacokinetic studies of several AMPs are currently being pursued (45, 46).

LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY (LC-MS/MS)

Pharmacokinetics of drugs is traditionally studied using enzyme linked Immunosorbent assay (ELISA), radio immunoassay, high performance liquid chromatography (HPLC), etc. However, immunoassay methods have certain limitations, such as time consuming steps in production of polyclonal or monoclonal antibodies against protein, cross reactions with precursors of target protein, and inability to identify the metabolized products of the protein (33). With the advent of new technologies, liquid chromatography and tandem mass spectrometry (LC-MS/MS) has gained much attention because of its sensitivity and specificity in detecting proteins/peptides as well as metabolic products. LC-MS/MS is a combination of high performance liquid chromatography and tandem mass spectrometry. Substances of interest are separated on a high performance liquid chromatography (HPLC) and then analyzed through a mass spectrometer. Acquisition of data involving scans of ion selection and its fragments is known as tandem mass spectrometry (MS/MS) (34). MS/MS increases the selectivity and specificity in the identification of drugs and also assists in analyzing the structural modifications that occur during metabolism of the drugs.

The principle of mass spectrometry is to generate ions from compounds and separate these ions based on their mass over charge ratios (m/z). These compounds are detected qualitatively and quantitatively based on their ions and abundance (48). The mass

spectrometer is comprised of an ion source, mass analyzer, and ion detector. Electrospray ionizes the sample at atmospheric pressure and then transfers it in to a mass spectrometer. A strong electric field is applied to a liquid, which assigns charge to these liquid droplets. The charged droplets are then heated up with inert gas like nitrogen to evaporate the solvent. The drop elongates under pressure and then attains taylor cone (48). There are many types of mass analyzers which include time-of-flight, quadrupole, ion trap, orbitrap, and fourier transform ion cyclotron resonance analyzer. Ions from mass analyzers are detected either by electron multipliers or Fourier transformation detectors. Recent advances in LC-MS/MS have allowed ionization on a nano scale. Nano-spray ionization (NSI) favors injection of a low volume of analytes, thereby reducing sample loss and system failure (35) with higher sensitivity and better signal to noise ratio (36). NSI is been applied to detect various pharmaceutical compounds, including proteins (37-39).

APPLICATIONS OF LC-MS/MS IN PHARMACOKINETIC STUDIES OF ANTIMICROBIAL PEPTIDES

LC-MS/MS has become perhaps the most widely applied technique in pharmacokinetic studies of drugs. A validated method should define specificity, recovery, lower limit of quantitation, accuracy, and precision of measurements (40). Sample pretreatment has become a crucial step for maximum recovery of the drug from biological matrices. Direct injection of plasma samples into LC-MS/MS interferes with ionization and also poses the danger of clogging the LC column. Plasma protein precipitation, solid-phase extraction, and liquid-liquid extraction are among commonly used techniques for sample pretreatment. Solvents used in protein precipitation often include ammonium sulfate,

aluminum chloride (5%, w/v), *n*-phosphoric acid (5%,w/v), trichloroacetic acid (TCA) (10%, w/v), zinc sulfate (10%, w/v), 0.5 N sodium hydroxide, ammonium acetate, acetonitrile, ethanol, or methanol (41).

All precipitants except ammonium sulfate and aluminum chloride were shown to be approximately 90% effective in precipitation of proteins at a volume ratio of 2:1 (precipitants: plasma). Although all precipitants proved to be effective, organic solvents are widely utilized for protein precipitation in drug analysis (41). Among ethanol, methanol, and acetonitrile, acetonitrile is considered a superior organic solvent when used at a ratio of <2:1 (precipitant: plasma). However, there are reports of direct injection of plasma samples without prior treatment. For example, plasma samples spiked with a peptide, AM336, were directly injected into a single polymer-coated mixed function (PCMF) column (42). Results are presented in single ion reaction monitoring, where a specific ion of interest is scanned.

Application of the OrbitrapTM (Thermo Fischer Scientific, MA, USA) mass spectrometer was reported for identifying the isotopic distributions of the parent ion and protonated fragment ions of vancomycin, a cyclic glycopeptide antibiotic (43). Multiple reaction monitoring (MRM) scan mode was applied in the analysis of protein phosphorylation sites on novel proteins within a signal network (44). Increased selectivity is obtained through SRM scan when compared to full and SIM scan (44). However, there are reports that MRM cannot be employed in quantifying the fragment ions of glucagon (45), due to the formation of many product ions with low intensities. Glucagon was found to ionize to form multiple charged states and shown that an alteration in capillary voltage led to the appearance of its most intense signal peak with +4 charged state (45), which

shows the importance of tuning of mass spectrometer settings for detection of the most intense peak from a multiply charged compounds. LC-MS/MS was applied to measure pharmacokinetic parameters of two cationic antimicrobial polymixin peptide analogs, NAB 7061 and NAB 739, following parenteral injection into rats (46). The limit of quantification (LOQ) for NAB 7061 was 0.125mg/L, while the LOQ of NAB 639 was 0.010mg/L and 0.050mg/L in acetonitrile-treated plasma and urine, respectively. A similar study was performed by using liquid chromatography – electrospray ionization-quadrupole time of flight (LC-ESI-QTOF) for plectasin, a peptide antibiotic obtained from saprophytic fungi (47).

CONCLUDING REMARKS

AMPs are being actively explored for their potential as novel antibiotics. The pharmacokinetic properties of these peptides need to be determined before large-scale clinical trials can occur. Therefore, it is essential to develop a reliable analytical approach to quantification of AMPs in biological samples and LC-MS/MS appears to be the method of choice.

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CHAPTER II

TISSUE EXPRESSION AND DEVELOPMENTAL REGULATION OF CHICKEN CATHELCIDINS

ABSTRACT

Cathelicidins represent a major family of vertebrate antimicrobial peptides that are an important component of innate immunity. Four cathelicidins, namely fowlicidins 1-3 and cathelicidin B1, have been found in chickens. However, little is known about their tissue and developmental expression patterns. In this study, we examined the expression of four cathelicidins in a large panel of tissues in 28-day-old chickens by real-time PCR. We also investigated the expression patterns of these four cathelicidins in chickens of different ages in order to understand the significance of cathelicidins during early development. We showed that all four cathelicidins were widely expressed in a variety of tissue types, with fowlicidins 1-3 showing a more similar pattern in four genes expression than cathelicidin B1. Fowlicidins 1-3 has the highest expression level in the lung, whereas cathelicidin B1 is expressed most abundantly in the bursa. We further revealed that four cathelicidins are differentially expressed during the early stage of development, largely with gradual increase in bursa and cecal tonsil, two important immune organs in intestinal host defense.

Collectively, our results suggested that chicken cathelicidins may play an important role in the protection of chicks from harmful agents during early development and before the immune system fully matures.

INTRODUCTION

Antimicrobial peptides (AMPs) are important effector molecules of the innate immune system with both bacterial killing and immunomodulatory activities (1). Cathelicidins, consisting of a highly conserved N-terminal pre-pro-sequence and a variable C-terminal mature sequence, are a major family of AMPs in vertebrate animals (2). In chickens, four cathelicidins, including fowlicidins 1-3 and cathelicidin B1 were recently identified (10, 11). Studies with limited tissue types revealed the expression of these chicken cathelicidins primarily in epithelial cells of mucosal tissues (20). A recent work also investigated the expressions of chicken cathelicidins and many other host defense genes during embryonic development (3). Fowlicidins 1-3 were expressed on day 3 and increased up to day 6 of development. Their expression levels were reduced on day 9 and subsequently increased in day 12 embryos. Cathelicidin B1 was not expressed until day 9. The expression pattern of fowlicidins 1-3 shows a similar profile, suggestive of their close phylogenetic relationship, whereas cathelicidin B1 expression was different indicating its distant evolutionary relationship from fowlicidins (12).

In this study, we investigated the tissue expression pattern of four chicken cathelicidins in a large panel of 23 different tissues of 28-day-old broiler chicks. Further, we examined the expression of four cathelicidins in bursa of Fabricius, lung, cecum, and cecal tonsils from 2- to 28-day-old chickens.

MATERIALS AND METHODS

Tissue collection and processing: One-day-old broiler chickens were purchased from a commercial hatchery and reared under standard care in the Lab Animal Resource Facility at Oklahoma State University. The chickens were given free access to commercial diets and water. A large panel of tissues was collected at various ages (day 2, 4, 7, 14 and 28), with 3-5 birds per age group. Cervical dislocation of chicken was performed and a total of 23 tissues were harvested, including crop, esophagus, proventriculus, gizzard, duodenum, jejunum, ileum, cecal tonsil, cecum, colon, lung, heart, trachea, liver, spleen, thymus, kidney, skin, breast muscle, brain, testis, ovary, and bursa of Fabricius. The tissue samples were snap frozen in liquid nitrogen and stored at -80°C until use.

Isolation and quantification of total RNA: Tissues were homogenized in Tri Reagent (Sigma Aldrich, MO), followed by total RNA extraction according to the manufacturer's instructions. Tri reagent contains guanidinium thiocyanate and phenol, which are involved in disruption of cells. Tri reagent maintains the integrity of RNA. The air-dried RNA pellet was suspended in nuclease-free water and mixed thoroughly until the pellet was completely dissolved in nuclease free water. RNA quantity and its purity were measured in duplicate using a Thermo Scientific NanoDrop™ Spectrophotometer (NanoDrop, Wilmington, De, USA). RNA concentration of all tissues was determined at 260nm. The purity of RNA was assessed by the ratio of absorbance at OD 260/280nm and OD 260/230nm.

Reverse transcription of total RNA: QuantiTect® Reverse Transcription Kit (Qiagen Inc.) was used to reverse transcribe total RNA into the first-strand cDNA following the

manufacturer's recommendations. Briefly, 0.3µg of total RNA was first freed from genomic DNA contamination in a genomic DNA wipeout buffer for 5 min at 42°C. Genomic DNA wipeout buffer contains DNase enzyme, which denatures DNA. Reverse transcription was performed in a total volume of 4 µL using Quantiscript® reverse transcriptase and a mixture of random hexamers and oligo(dT) primers for 30 min at 42°C, followed by 3 min at 95°C to inactivate the reverse transcriptase. The 1st strand cDNA concentration was then measured using the Thermo Scientific NanoDrop™ Spectrophotometer (NanoDrop, Wilmington, De, USA) following 10-fold dilution in nuclease-free water.

Real time PCR: QuantiTect® SYBR Green PCR Kit (Qiagen Inc.) was used for real-time amplification of the first-strand cDNA using MyiQ real-time PCR detection system (Bio-Rad Inc.) as previously described (4). Briefly, each PCR reaction was set up in a 96-well PCR plate in a total volume of 10 µL using 0.1 µg of the first strand cDNA. A volume of 10µL contains 9µL of SYBR green PCR master mix, sense and antisense primers (Table 1), and 1µL of cDNA. Real time PCR was programmed as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 15s, annealing at 55°C for 20s, and extension and data collection at 72°C for 30s. Comparative $\Delta\Delta C_T$ method was used for quantification of gene expression as mentioned by PE Applied Biosystems (Perkin Elmer, Forster city, CA) (5**).** Normalization was done by using GAPDH as the reference gene. Tissue with highest C_T value was used as a calibrator. Delta C_T value for all tissues were calculated by subtracting C_T values of GAPDH from C_T values of target tissues and then delta-delta C_T values were calculated by subtracting the highest ΔC_T

value obtained for one tissue. Relative fold difference was computed by using the formula $2^{-\Delta\Delta Ct}$ (Table 2) (5, 6).

Statistical Analysis: All data were analyzed with one-way ANOVA, followed by Tukey's multiple comparison test using Graph Pad Prism 5 (Graph pad software, San Diego, California). The results were considered significant, if $p < 0.05$.

RESULTS

RNA quality and statistical analysis: Our data showed that the quantity of RNA of all tissues obtained was more than 300ng/ml and the quality by OD_{260}/ OD_{280} nm, and OD_{260}/ OD_{230}nm absorption ratios were within the range of 1.8-2.0, which indicated that RNA was in good quality without any contamination from aromatic amino acid, proteins, guanidinium thiocyanate, and phenol. The data was normally distributed, verified by computing the value of (mean- 0.67 standard deviation) and compared with 25 percentile (18).

Tissue expression patterns of chicken cathelicidins: As shown in Fig. 1, all four cathelicidins were widely expressed in a variety of tissue types, with fowlicidins 1-3 showing a distinct and similar pattern in gene expression as compared to cathelicidin B1. Fowlicidins 1-3 were expressed throughout the digestive and respiratory tracts, with the highest expression in the lung (Fig. 1), consistent with the role of AMPs in mucosal host defense. On the other hand, although the cathelicidin B1 gene was also expressed in the digestive and respiratory tracts, the bursa of Fabricius was the dominant tissue where cathelicidin B1 is expressed (Fig. 1), in agreement with an earlier report (7). All four cathelicidins were also expressed abundantly in all four primary and secondary lymphoid

organs examined, including thymus, spleen, cecal tonsil, and bursa of Fabricius. The only tissue that showed no obvious expression of all four cathelicidins is breast muscle (Fig. 1).

Developmental expression of chicken cathelicidins*:* The variation in chicken cathelicidin expressions among different age groups of chickens was determined. As compared to that on day 2, an enhanced expression of all four cathelicidins was observed in the bursa of fabricius on day 4, and then the expressions were gradually decreased to the day 2 level on day 28 (Fig. 2). Interestingly, we observed an almost completely opposite trend with cecal tonsil, another immune organ unique in birds (Fig. 3).When compared to other tissues, cecal tonsils were only harvested from 7-, 14-, and 28-day-old chicks because of an inability to obtain sufficient sample mass at earlier ages. Relative to 7-day-old chickens, 14-day-old chickens showed an increased expression of cathelicidin B1, followed by a reduced expression in 28-day-old chickens, whereas a gradual increase of fowlicidins 1-3 expression was noted (Fig. 3).

In the cecum, the expressions of fowlicidins 1-3 remained largely unchanged during the first two weeks of age and then significantly increased on day 28 by as much as 25-fold in the case of fowlicidin-3 (Fig. 4). Cathelicidin B1 gene expression in the cecum showed a different pattern, with a significant decrease on day 7 as compared with day 2 or day4, followed by an age-dependent increase at day 28 (Fig. 4). On the other hand, there was no obvious difference during the first four weeks of development in the expression of four cathelicidin genes in the lung (Fig. 5), another mucosal tissue with the most abundant expression of three fowlicidins (Fig. 1). We also noted that cathelicidin B1

showed a relatively high, but statistically insignificant increase in the expression on day 14, relative to other age groups.

DISCUSSION

We determined both tissue and developmental expression patterns of four chicken cathelicidins in this study through RNA isolation, cDNA preparation, and quantifying fold difference in the gene expression. Comparative C_T method is the most widely used method for relative quantification in real-time RT PCR where inclusion of control levels standardize each reaction run with respect to RNA integrity, sample loading, and inter PCR reactions (5). Hence, in our study, RNA integrity was not verified through gel electrophoresis. GAPDH is used as housekeeping or reference gene for normalization, considering its mRNA synthesis to be constant in various tissues under experimental conditions (8). GAPDH normalizes the samples for differences in the amount of total RNA added to cDNA preparation and also normalizes the variations in reverse transcription efficiency among different cDNA reactions. Reports have demonstrated that the reference genes are regulated and vary under experimental conditions (9). But, in our study, reference gene values showed little or no variation among various tissues (Table 2). The fold difference in the expression of four cathelicidin genes revealed that they are expressed in a broad range of tissue types, consistent with earlier reports with limited tissue types (10). A comparative study of chicken genes in ileum, liver, cecal tonsil and spleen revealed that the antimicrobial peptides such as defensins 1, 2, 6 and 7 were highly expressed in spleen when compared to other tissues (11). Meade et al., studied the developmental expression pattern of all chicken cathelicidins in fertilized chicken eggs, which inferred that four cathelicidins are phylogenetically related (3). Fowlicidins 1-3

were most abundantly expressed in the lung, whereas cathelicidin B1 was highest in the bursa of Fabricius. It's noteworthy that bone marrow was also the place that actively synthesized many AMP transcripts including cathelicidins, but was not included in our study (7).

Interestingly, chicken cathelicidin expression levels were minimal in the skin of chickens, which is in contrast with many mammalian species. This difference could be attributed to species variation or the evolution of skin development in different species. The scenario is different in the case of humans, where human epidermal cells express abundant levels of LL-37 (7). Human skin can act either as a route of entry or as a site for replication for microorganisms. In order to prevent microbial invasion and their multiplication, skin has to synthesize host defense factors including cathelicidins for its immediate protection.

Majority of the infectious agents gain entry in to the body of chickens through feco-oral route (17). Hence, AMPs synthesis might not be required in the skin. However, a high level of fowlicidin-2/CMAP27 expression was reported in the uropygial (preen) gland tissue of chickens (12), which secretes wax and oil that spreads over plumage and feathers. Therefore, it seems possible that CMAP 27 provides protection against skin infections in chicken.

The expression of cathelicidins in bursa was inversely related to the age of the bird. Expression of cathelicidin genes gradually decreased as birds matured (Fig. 2). This expression pattern could be correlated with the growth of the bursa, which takes place rapidly up to 9 weeks of age and slows thereafter (17). The mucous membrane of the bursa has long folds termed as plicae which resemble villous projections (13). Each plica

has a large number of follicles separated by connective tissue. Inactive primary lymphoid follicles were observed in day-old chicks while development of secondary (active) lymphoid follicles was noticed from 7-day-old chicks (17). Ratcliffe demonstrated that bacterial antigens from the gastro-intestinal tract gain access to the bursa of Fabricius and may involve in the activation of innate and adaptive immunity (14). It is possible that bursa of Fabricius might have expressed effector molecules of innate immunity like, cathelicidins to protect from invading microorganisms. Cathelicidin expression has been reduced from 7th day onwards due to development of active T and B cells.

The results observed in cecum and cecal tonsil suggested that the expression of cathelicidin was enhanced during development, likely due to exposure to normal microflora. Shapiro et al., reported that commensal bacteria colonizes in the gastro-intestinal tract of chicken within 24 hours of hatching (15) and the density of these microflora is greater in the distal region of the intestine. Evidence showed that a diverse group of bacteria, such as genera of Bacteriodes, Bifidobacterium, Clostridium, Enterococcus, Escherichia, Fusobacterium, Lactobacillus, Streptococcus, and Campylobacter resides in the cecum (15). The commensal microflora is beneficial in preventing the colonization of pathogenic microorganisms by forming biofilms on intestinal epithelium and blocking the pathogens from entry (16). However, excessive proliferation of these commensal bacteria may become pathogenic to chickens. Therefore, production of chicken cathelicidins is essential to maintain intestinal homeostasis. In addition to the gene expression of cathelicidins in healthy chickens, studies have to be performed to differentiate the fold change from healthy and infected chickens.

Our study of tissue and developmental expression patterns of four chicken cathelicidins has led to a better understanding of the chicken innate immunity as well as the development of the innate immune system. The results from this study suggest that the differential expression of cathelicidins in mucosal and immune tissues might play an important role in the protection of chicks from harmful agents during early development and before the immune system is fully functional.

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Table 1: Forward and reverse primers of genes used in quantitative RT-PCR.

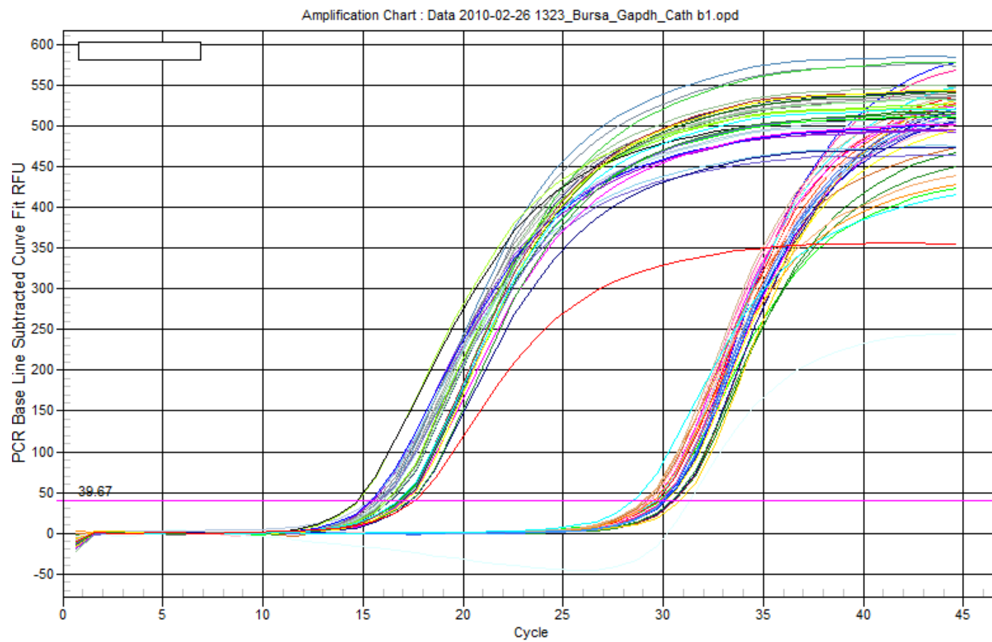
Name	Forward primer (5'-3')	Reverse primer (5'-3')
gGAPDH	GCACGCCATCACTATCTTCC	CATCCACCGTCTTCTGTGTG
g CATH B1	CCGTGTCCATAGAGCAGCAG	AGTGCTGGTGACGTTCAGATG
Fowlicidin-1	GCTGTGGACTCCTACAACCAAC	GGAGTCCACGCAGGTGACATC
Fowlicidin-2	CAAGGAGAATGGGGTCATCAG	CGTGGCCCCATTTATTCATTCA
Fowlicidin-3	Same as fowlicidin 1 forward primer	CCACCGTGTTGATGGCCACTG

Table 2: An example of calculation of fold change of cathelicidin B1 in bursa of Fabricius using delta-delta C_T method.

	Cath B1 ct	Gapdh ct	dct	avg dct	ddct	fold change
D2#1	16.11	29.75	-13.64		-0.81	**1.753211**
	16.33	30.53	-14.2		-1.37	**2.584706**
D2#2	15.76	29.61	-13.85		-1.02	**2.027919**
	15.93	29.96	-14.03		-1.2	**2.297397**
D2#3	15.98	30.08	-14.1		-1.27	**2.411616**
	15.94	30.59	-14.65		-1.82	**3.530812**
D4#1	15.43	30.51	-15.08		-2.25	**4.756828**
	15.5	29.9	-14.4		-1.57	**2.969047**
D4#2	15.83	30.09	-14.26		-1.43	**2.694467**
	15.31	30.4	-15.09		-2.26	**4.789915**
D4#3	15.65	29.64	-13.99		-1.16	**2.234574**
	15.81	29.67	-13.86		-1.03	**2.042024**
D7#1	16.92	29.91	-12.99		-0.16	**1.117287**
	16.83	29.86	-13.03		-0.2	**1.148698**
D7#2	16.29	29.45	-13.16		-0.33	**1.257013**
	16.14	29.29	-13.15		-0.32	**1.248331**
D7#3	14.76	29.24	-14.48		-1.65	**3.138336**
	14.72	29.49	-14.77		-1.94	**3.837056**
D14#1	16.8	29.13	-12.33		0.5	**0.707107**
	16.74	29.41	-12.67		0.16	**0.895025**
D14#2	15.38	28.97	-13.59		-0.76	**1.693491**
	15.36	29.04	-13.68		-0.85	**1.802501**
D14#3	16.98	30.05	-13.07		-0.24	**1.180993**
	16.68	30.82	-14.14		-1.31	**2.479415**
D28#1	**17.35**	**30.09**	**-12.74**	-12.83	0.09	**0.939523**
	16.88	**30**	**-13.12**		-0.29	**1.22264**
D28#2	**17.05**	**29.92**	**-12.87**		-0.04	**1.028114**
	17.26	**30**	**-12.74**		0.09	**0.939523**
D28#3	**16.71**	**28.44**	**-11.73**		1.1	**0.466516**
	17.55	**31.33**	**-13.78**		-0.95	**1.931873**

Note: dct = Cath B1 C_T - GAPDH C_T; avg dct is average of control;

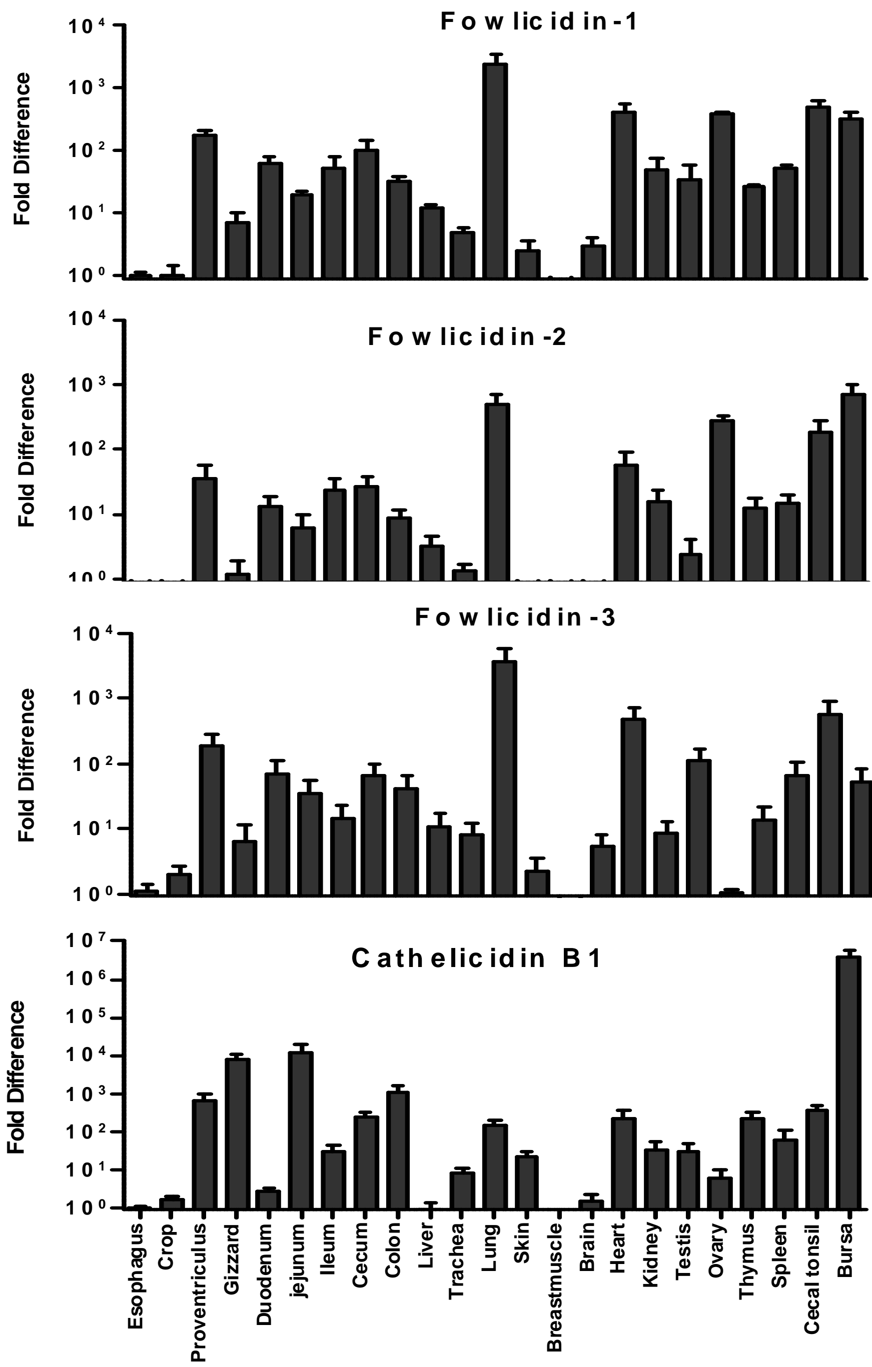
ddct = dct of target - avg dct of control; fold change = $2^{-\wedge ddct}$.



Note: Chart showing amplification curves of both GAPDH (right) and Cathelicidin B1 (left) genes in the bursa of Fabricius.

FIGURE LEGENDS

Figure 1. Tissue expression patterns of four chicken cathelicidin genes. The expression patterns were obtained by real-time RT-PCR of total RNA isolated from 28-day-old broiler chickens. Each bar represents mean ± SEM of three chickens.



Fowlicidin-1
Fowlicidin-2
Fowlicidin-3
Cathelicidin B1
Fold Difference
10⁰
10¹
10²
10³
10⁴
10⁵
10⁶
10⁷
Esophagus
Crop
Proventriculus
Gizzard
Duodenum
jejunum
Ileum
Cecum
Colon
Liver
Trachea
Lung
Skin
Breastmuscle
Brain
Heart
Kidney
Testis
Ovary
Thymus
Spleen
Cecal tonsil
Bursa

Figure 2. Developmental expression of four chicken cathelicidins in the bursa of Fabricius. Bursas were harvested from chickens of 2, 4, 7, 14, and 28 days of age and subjected for RNA isolation and real-time PCR analysis of individual chicken cathelicidins. Each bar represents mean ± SEM of three chickens. Means with different letters are significantly different ($p < 0.05$).

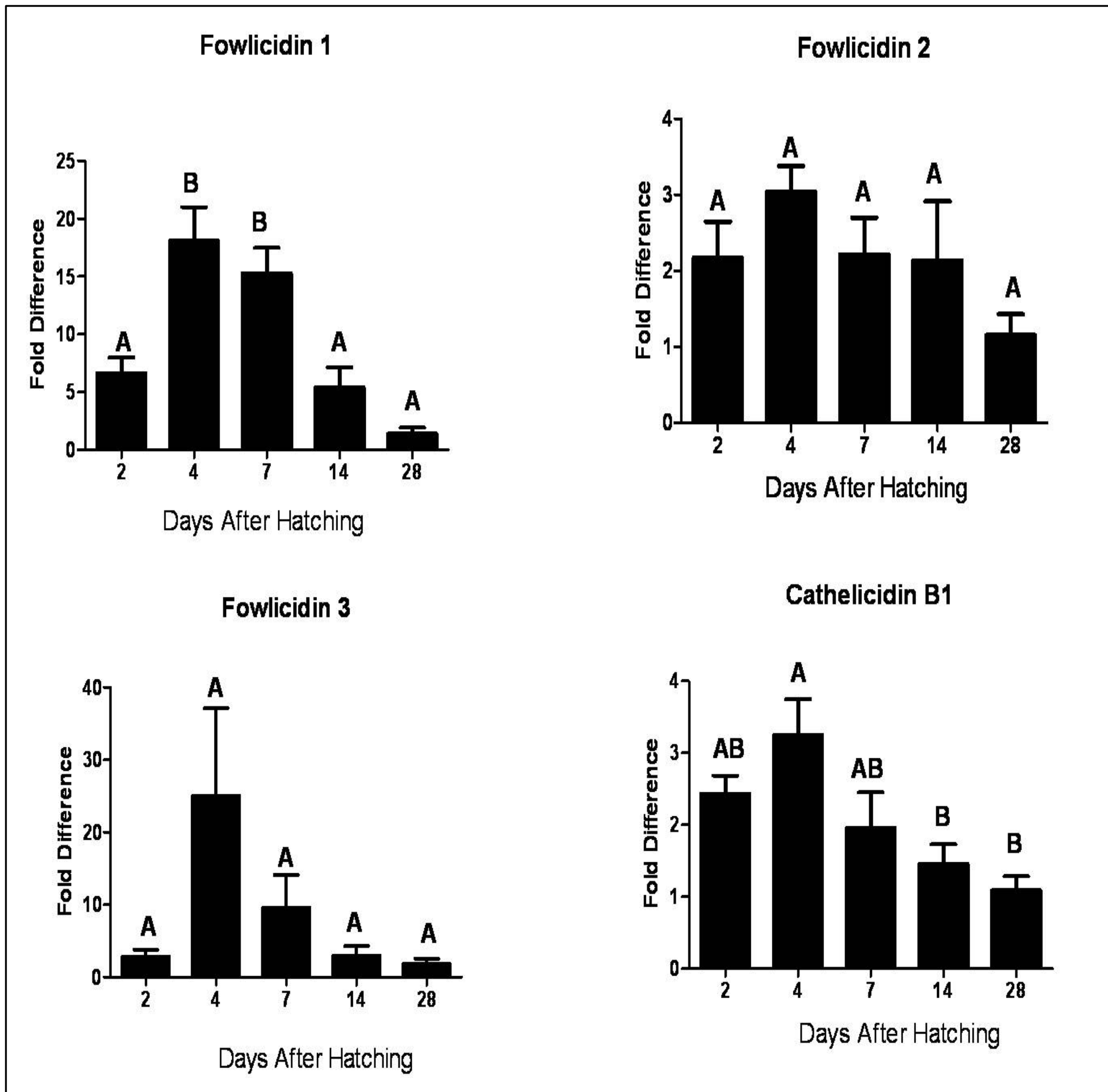


Figure 3. Developmental expression of four chicken cathelicidins in the cecal tonsil. Cecal tonsils were harvested from chickens of 7, 14, and 28 days of age and subjected for RNA isolation and real-time PCR analysis of individual chicken cathelicidins. Each bar represents mean ± SEM of three chickens. Means with different letters are significantly different ($p < 0.05$).

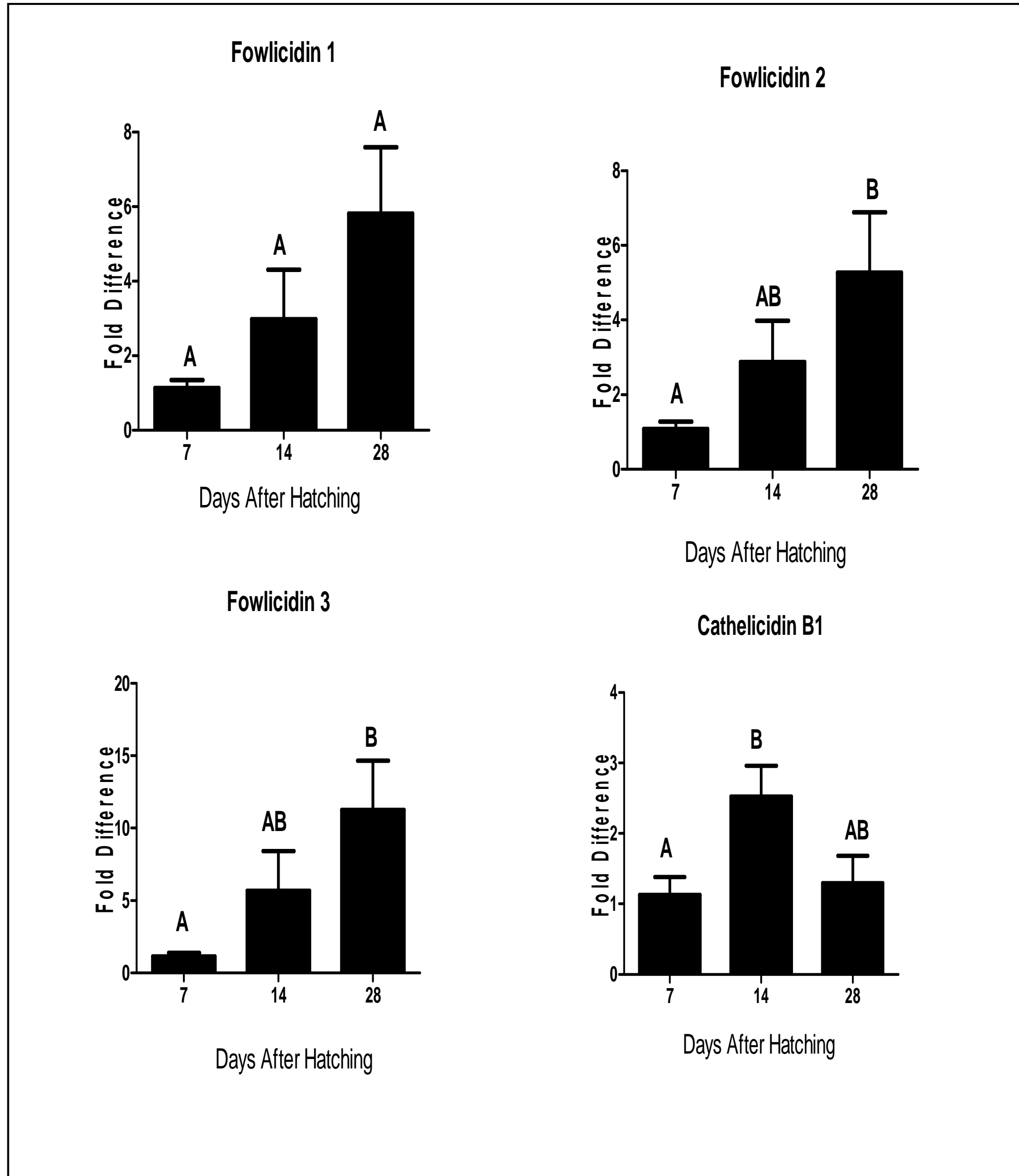


Figure 4. Developmental expression of four chicken cathelicidins in the cecum. Cecum was harvested from chickens of 2, 4, 7, 14, and 28 days of age and subjected for RNA isolation and real-time PCR analysis of individual chicken cathelicidins. Each bar represents mean ± SEM of three chickens. Means with different letters are significantly different ($p < 0.05$).

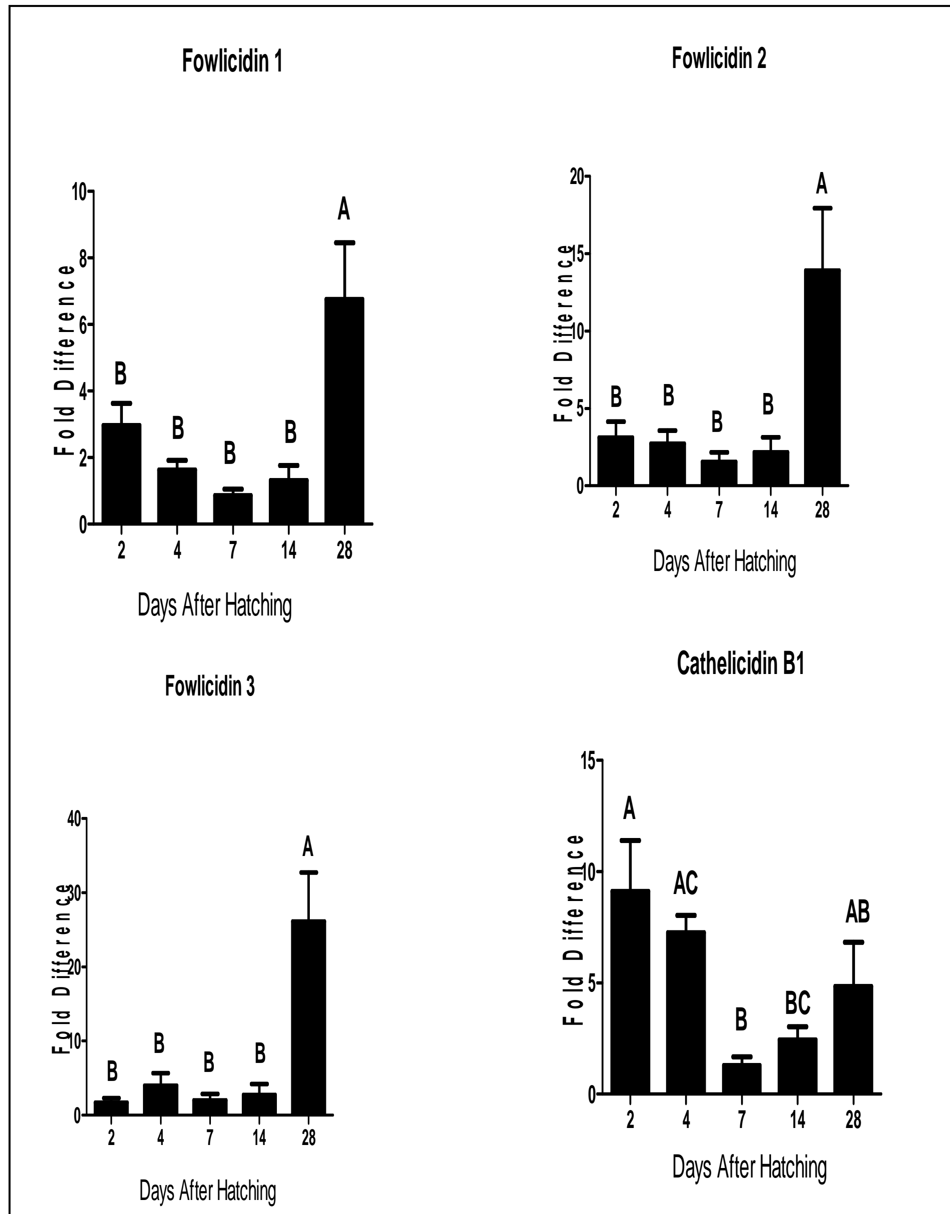
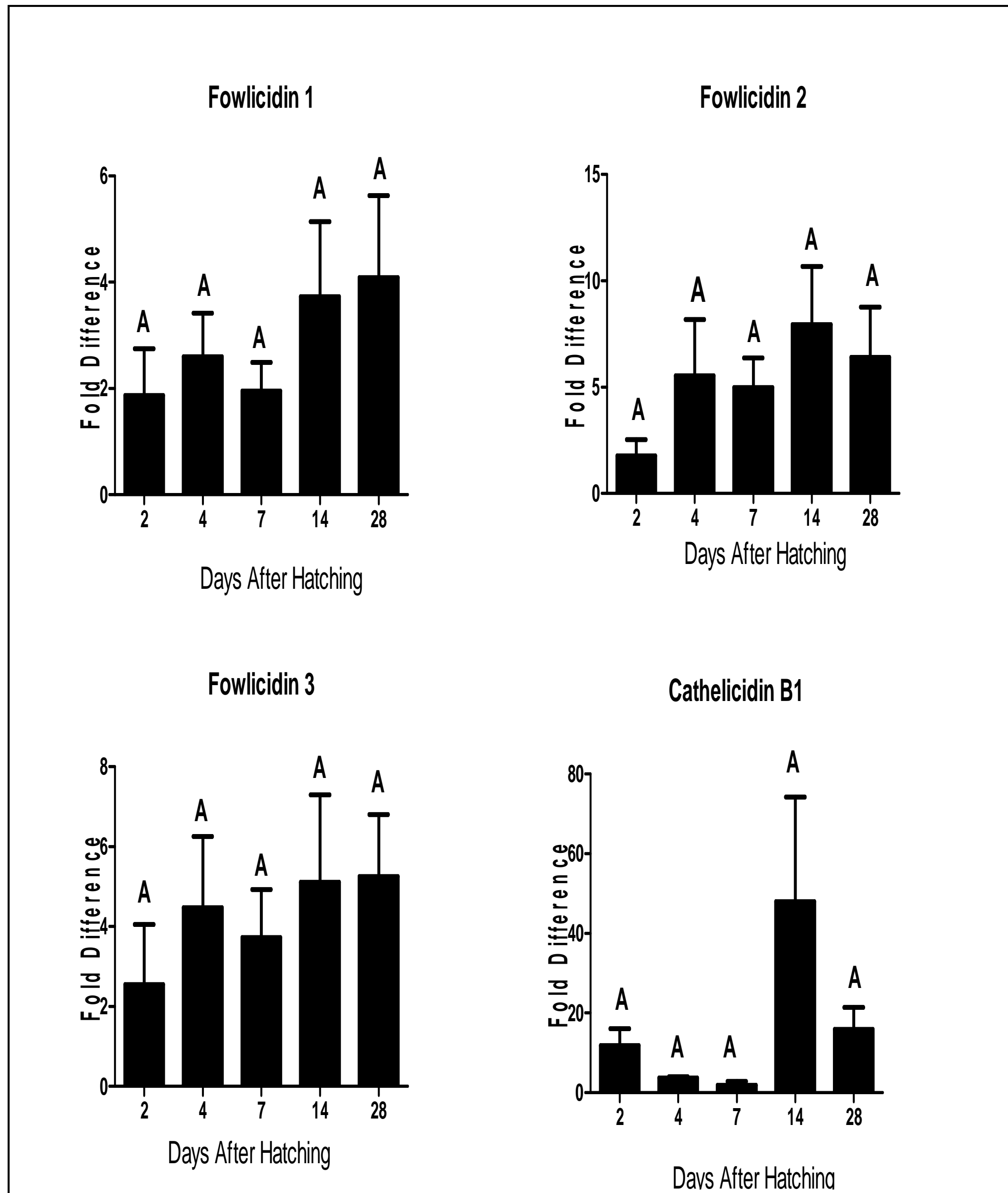


Figure 5.Developmental expression of four chicken cathelicidins in the lung. Lungs were harvested from chickens of 2, 4, 7, 14, and 28 days of age and subjected for RNA isolation and real-time PCR analysis of individual chicken cathelicidins. Each bar represents mean ± SEM of three chickens. Means with different letters are significantly different ($p < 0.05$).



CHAPTER III

DEVELOPMENT OF A MASS SPECTROMETRIC ASSAY FOR CHICKEN FOWLICIDINS

ABSTRACT

Fowlicidin-1 is a recently identified chicken antimicrobial peptide with potent antibacterial activity. Fowl-1(6-26)-NH_2, an amino-terminal five-amino-acid truncation analog of fowlicidin-1 with carboxyl-terminal amidation, is capable of killing drug resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* both in vitro and in vivo, remains active at physiological salt concentrations and in the presence of serum. Therefore, fowl-1(6-26)-NH_2 has potential for further development as a novel class of antibacterial drugs. However, it remains unknown about fowl-1(6-26)-NH_2 with regard to its kinetics of absorption, distribution, metabolism, and excretion following in vivo administration. As a first step to estimate its pharmacokinetic parameters, we have sought to develop and validate a liquid chromatography and tandem mass spectrometry (LC-MS/MS) approach to quantify fowl-1(6-26)-NH_2. Extensive optimizations on mass spectrometer settings including capillary voltage, tube lens, scan time, and injection control settings were carried out to gain sensitive and accurate detection.

Our results showed that, after optimization, five different charge states of fowl-1(6-26)-NH_2 ranging from +2 to +6 were readily detected, with the most intense ion being 501.31($[M+5H]^{5+}$), followed by 626.39 ($[M+4H]^{4+}$), 418.09 ($[M+6H]^{6+}$), and 835.19 ($[M+3H]^{3+}$). A concentration-response curve was also established fowl-1(6-26)-NH_2 in the range of 0.05 - 5 µg/ml, with a R^2 of 0.988. We further revealed that the peptide can be reliably detected when acetonitrile-treated plasma samples were diluted up to 5 percent plasma before injection into LC-MS/MS. However, acetonitrile precipitation caused a loss of 70% of the peptide from plasma, suggesting that an alternative protein precipitation procedure or use of unprecipitated samples is needed for quantification of fowl-1(6-26)-NH_2 in plasma. Collectively, these preliminary data have established a solid foundation on the quantification of the plasma concentrations of fowl-1(6-26)-NH_2 peptide, which will be essential in calculating pharmacokinetic parameters.

INTRODUCTION

Cathelicidins represent a large family of antimicrobial peptides with a highly conserved N-terminal pre-pro-sequence and a variable *C*-terminal mature peptide sequence (1). They have been found in various vertebrate species including mammals, birds, reptiles, and fishes (2-4). Fowlicidins 1-3 are chicken cathelicidins identified recently through a combination of genomic sequence screening and molecular cloning (5). The peptide sequences of the three fowlicidins indicate that they are homologous to each other, with fowlicidins 1 and 3 sharing 90% identity (5). Presence of cationic residues like arginine and lysine at the *C*-terminal region are suggestive of the positively charged nature of fowlicidins. Putatively mature fowlicidins 1-3 are devoid of cysteines and composed of 26, 32 and 29 amino acid residues with a net charge of +8, +10 and +7 respectively (5).

Among the three fowlicidins, fowlicidin-1 adopts primarily an alpha helical structure with a kink at the center and a short flexible unstructured region at the N-terminal region (6). Studies with a series of synthetic fowlicidin-1 analogs with either amino- or carboxyl-terminal deletion demonstrated that fowl-1(6-26) with a deletion of the first five amino acids retained antibacterial activity (6, 7). The *C*-terminal amidation of fowl-1(6-26) enhanced its stability in the presence of serum (8). Furthermore, fowl-1(6-26)-NH_2 reduced bacterial titers in the spleen of mice and protected mice from MRSA-induced lethal infection (8). Thus, fowl-1(6-26)-NH_2 has potential for further development as a possible drug candidate for antimicrobial therapy.

Pharmacokinetics describes the time course of absorption, distribution, metabolism, and excretion (ADME) of a drug in the body, which is essential information required in drug development. Pharmacokinetic parameters of a drug can be traditionally studied by employing techniques such as enzyme linked immunosorbent assay (ELISA). However, because of relatively low sensitivity, low specificity, and high costs associated with many of the traditional techniques (9, 10), liquid chromatography and tandem mass spectrometry (LC-MS/MS) has proven useful in pharmacokinetic studies of many drugs (11, 12). Such an approach has also been employed to study cationic AMPs such as NAB 7061 and NAB 739 (13) as well as plectasin (14). In the case of plectasin, its linear response range was 4-1440 ng/mL by liquid chromatography-electrospray ionization-quadrapole time of flight (LC-ESI-QTOF) (14).The primary objective of the current study was to develop and optimize the LC-MS/MS settings for sensitive and accurate quantitation of fowl-1(6-26)-NH_2 peptide with and without serum as a prelude for future pharmacokinetic studies.

MATERIALS AND METHODS

Fowl-1(6-26)-NH$_2$ peptide preparation: Fowl-1(6-26)-NH$_2$ peptide was commercially synthesized at 95% purity and dissolved in 0.1% formic acid and acetonitrile (50:50) for electrospray infusion. In the case of nanospray LC-MS/MS, the peptide was suspended in buffer A (0.1% formic acid), and serial 2-fold dilutions were prepared to derive a concentration-response curve.

Precipitation of plasma proteins: Acetonitrile treatment was employed in the precipitation of plasma proteins as previously described (15). Mouse plasma (50 µL) was mixed with 150 µL of chilled acetonitrile, thoroughly vortexed, and incubated on ice for 2 h to precipitate proteins. The precipitated proteins were then pelleted by centrifugation at 10,000 x *g* for 10 min at 4°C. The supernatant was transferred, aliquoted, and stored at -80°C until further use.

HPLC separation of fowl-1(6-26)-NH$_2$: The peptide samples were separated on a NanoLC HPLC system (Eksigent), prior to mass spectrometry. The NanoLC system was equipped with a C18 column, a microwell plate autosampler and reservoirs containing mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A total 10 µL of the sample was injected via the autosampler for each 20-min run. A linear 30-90% acetonitrile gradient was applied at a flow rate of 300 nL/min for 15 min, followed by a reduction in the acetonitrile gradient from 90% to 30% in 1 min, which was then held constant for another 4 min (Table 1). Scan time and AGC settings were then optimized to achieve at least twenty points on the chromatographic peak. Each concentration of the peptide was subjected to at least 2-3 independent runs to obtain

replicate ion counts, which were expressed as mean ± SEM. For each run, a full MS scan was performed with a mass range of m/z 300-1500 in fourier transformation detector, followed by a MS/MS scan for the parent ion 626 ($[M+4H]^{4+}$) → 772 (fragment *m/z*) in ion trap.

Optimization of mass spectrometer settings: An LTQ Orbitrap XL™ Mass Spectrometer (Thermo Scientific MA, USA) connected to the NanoLC system was used to analyze the mass and concentration of the fowl1-1(6-26)-NH_2 peptide. The instrument was tuned with electrospray infusion of 400 µg/ml of fowl-1(6-26)-NH_2 in acetonitrile/0.1% formic acid (50:50) at a nominal flow rate of 80 nL/ min. The capillary voltage and tube lens were adjusted until the most intense ion of the peptide with ion counts of $>10^6$ were observed. In order to achieve optimal spray voltage, different voltages were applied and the spray was observed for 10 min until an appropriate taylor cone was noted.

RESULTS

Optimization of mass spectrometer settings and Liquid Chromatography conditions: To increase the accuracy and sensitivity in detection of ions of fowl-1(6-26)-NH_2 peptide, various settings of the mass spectrometer were optimized following an electrospray infusion of the peptide at 400 µg/ml. For all experiments, the capillary voltage, capillary temperature, and tube lens, and spray voltage were finally set at 30 V, 199°C, 156 V, and 1.9 KV, respectively, to maintain a perfect taylor cone. The scan time and injection control settings were further adjusted. Under the factory default settings (Table 2), a 10µL infusion of 400 µg/ml fowl-1(6-26)-NH_2 only yielded two weak charge states, namely ($[M+3H]^{3+}$) of 835 and ($[M+4H]^{4+}$) of 626 (Fig. 1A). Under tuned settings (Table 2), several charge states of the peptide were readily revealed, with the most

intense ion being m/z 501 ($[M+5H]^{5+}$), followed by 626 ($[M+4H]^{4+}$) and 835 ($[M+3H]^{3+}$) (Fig. 1B).

The highest ion count was noted for *m/z* 501, followed by quadruple charged *m/z* 626. Other ions such as *m/z* 418 and *m/z* 835 were found at low ion intensity (Fig. 1B). Ion 1225 ($[M+2H]^{2+}$) charge state was observed only at high concentrations (Table 3). In full scan, these charge states were calculated based on the distance between the isotopes formed by these masses. The charge state and the isotopic distribution of the respective ions were also demonstrated (see the inserts of Fig. 2A-D). Overall, results in all experiments showed a similar pattern of ion distribution in the following sequence: $[M+5H]^{5+}$ (56% ± 5), $[M+4H]^{4+}$ (28% ± 6), $[M+6H]^{6+}$ (14% ± 4) and, $[M+3H]^{3+}$ (2% ± 2). Therefore, we concluded that fowl-1(6-26)-NH_2 is multiply charged, ranging from +2 to +6, consistent with the calculated peptide mass of 2502Da.

Each precursor ion has produced characteristic product ions (Table 3). The SRM scan for all precursor ions was performed and fragmented with 35 percent collision energy. The product *m/z* 772 of precursor ion *m/z* 626 ($[M+4H]^{4+}$) was shown to be the most abundant and detectable among the other product ions. This product ion was considered as the best transition to monitor the peptide. Execution of more number of scans in our experiment has reduced the number of chromatographic points and also the ion counts (data not shown). Hence, a single SRM scan was performed in ion trap for precursor ion, 626 and its product ion, 772 (626 → 772). Both parent ion and its fragment showed an identical retention time of 8.2 min (Fig. 3).

Quantification of fowlicidin peptide: To ensure that the optimized mass spectrometer settings can be used for quantitative measurement of fowl-1(6-26)-NH_2, we first developed a 20-min HPLC protocol using a linear gradient of 30-90% acetonitrile. Under this protocol, the peptide showed a retention time between 7.8 to 8.2 min and was eluted at 56% acetonitrile. Serial 2-fold dilutions of the peptide were then prepared in 0.1% formic acid, ranging from 1.25 to 40 μg/ml, and subjected to LC-MS/MS under the optimized instrument settings. As shown in Fig. 4, total ion counts of all charge states showed a concentration-dependent increase with increasing concentrations of the peptide, with saturation of detection occurring at 10 μg/ml.

To determine the lower limit of detection, serial 2-fold dilutions from 3.2 to 0.05 μg/ml were prepared. Response was estimated from the area of the product ion 772 of *m/z* 626 ($[M+4H]^{4+}$). The power trend line clearly demonstrated a good fit for response over concentration plot with an R^2 value of 0.994 (Fig. 5). It is noteworthy that the ion counts have become unreproducible when the peptide concentration is below 25 ng/ml. The limit of detection was approximately 50 ng/ml. Collectively, our results indicated that the response curve for fowl-1(6-26)-NH_2 is between 0.05 to 5.0 μg/ml using the LC-MS/MS approach that we developed.

To determine the best scan between full and SRM modes for the quantification of fowl-1(6-26)-NH_2 peptide, serial two-fold dilutions from 3.2 to 0.2 μg/mL were prepared. The resulting data were double log (10) transformed and fitted simple linear regressions in Microsoft excel (Microsoft, Redmond, Washington). The resulting regression equation was used to back calculate the concentrations and percent deviation of the calibrants as compared to their nominal concentrations (Table 4 & Fig. 6). Based on R- squared value

and percent deviation from nominal concentrations, full scan was chosen as the most accurate method of constructing a calibration curve for fowl-1(6-26)-NH_2 peptide. Further, to choose between single ion and combined total ion count in full scan mode for the quantification of the fowl-1(6-26)-NH_2 peptide, similar approach was adopted. The single ion *m/z* 501 was considered as the best ion for comparison with the summated approach due to its high abundance. Based on R- squared values and percent deviation from nominal concentrations (Table 4 & Fig. 6), there was no difference between single ion and combined total ion counts. Therefore, we adopted full scan, combined total ion count for estimation of the amount of the fowl-1(6-26)-NH_2 peptide in buffer and plasma.

Influence of plasma on quantification of fowl-1(6-26)-NH_2: In order to determine the impact of plasma on measurement of the peptide, mouse plasma proteins were first precipitated in acetonitrile in the ratio of 1:3. Clarified plasma was then diluted to 1, 2.5, and 5% plasma in 0.1% formic acid, followed by fortifying with fowl-1(6-26)-NH2 peptide to a final concentration of 5 μg/ml. The same concentration of the peptide in 0.1% formic acid was used as a positive control. Plasma fortified with peptide sample each was freshly prepared before each duplicate run on LC-MS/MS under the optimized instrument settings. The results indicated that inclusion of clarified plasma had a minimum impact on the quantification of the peptide, as the ion counts of the peptide were at least 70% in 5% plasma, relative to the peptide in 0.1% formic acid alone (Fig. 7). Therefore, it suggests that the plasma samples could be diluted up to 5% with no impact on peptide detection before loading on to the LC-MS/MS.

To study the influence of acetonitrile precipitation of plasma proteins on the recovery of the peptide, we next fortified 100 percent mouse plasma with fowl-1(6-26)-NH_2 peptide

to 1 μg/ml, then proceeded with acetonitrile treatment in the ratio of 1:3 as described above. Clarified plasma fortified with peptide sample was then subjected to LC-MS/MS. As compared with peptide alone, only 29 percent of the fowl-1(6-26)-NH_2 peptide was recovered following acetonitrile precipitation procedure (Fig. 8), suggesting a large proportion of the peptide was precipitated.

DISCUSSION

In this study, we have developed an LC-MS/MS approach for detection of fowl-1(6-26)-NH_2, a short antimicrobial peptide analog with commercial potential in antimicrobial therapy. The developed method provides a preliminary idea in detection of fowl-1(6-26)-NH_2 in biological matrix, which will be useful in estimating pharmacokinetic parameters during pharmacokinetic study.

We developed a preliminary method for the detection of fowl-1(6-26)-NH_2 peptide in full and SRM scan modes. The method development varies from peptide to peptide. Wilbert et al., demonstrated the quantitative analysis of multiple charged compound NR58-3.14.3 (A), a panchemokine inhibitor in biological fluids choosing selected ion monitoring. Selected ion monitoring (SIM) was applied to quantify the peptide by combining ion currents of all three charges to generate a total area under curve (16). They also reported that choosing a single ion for quantitative analysis have produced data with relative standard deviation (RSD) of more than 20 percent. But, in our study, percent deviation of back calculated value from the observed values for both single ion and combined ion count were in acceptable limits (Table 4 and fig. 6). However, we have chosen combined total ion counts for our analysis. Therefore, our results have proven to produce a

reproducible LC-MS/MS method for detection of fowl-1(6-26)-NH_2 peptide using full scan, where this method can be a useful tool in further analyzing other fowlicidin analogs.

Limited preliminary data regarding the detection and concentration-response curve of fowl-1(6-26)-NH_2 peptide were obtained from the present study. A follow up study would be required to estimate the limit of detection (LOD) and lower limit of quantification (LLOQ) of fowl-1(6-26)-NH_2 peptide in plasma. The lower limit of quantification can be defined as the lowest concentration of an analyte that can be quantified with a precision and accuracy equal to or better than 20 percent (17), while, the limit of detection describes the analyte concentration yielding a signal that significantly differs from that of the blank samples (18). However, our preliminary data provides an idea of fowl-1(6-26)-NH_2 response in buffer and plasma, which will be useful in additional experiments. Further work is expected to include biological samples and inclusion of other fowlicidin analogs.

A low recovery rate was observed for fowl-1(6-26)-NH_2 following plasma precipitation by 75 percent acetonitrile. In fact, more than 70 % of the peptide was lost following precipitation (Fig. 8), which suggested that the peptide might have precipitated or bound to certain plasma proteins. However, precipitation of plasma proteins before chromatographic separation is necessary to increase the availability of free drugs and to remove extraneous proteins and other interfering agents that may bind to the column which decreases the life of the column, suppress ionization, shifts the retention time, and alters peak shape. Therefore, a further study has to be performed to prevent loss of the peptide either by employing internal standards, lower acetonitrile concentrations, and or

use of methanol or ammonium acetate as an alternate plasma precipitants (15). Internal standards such as isotope analogs, stereoisomers including atropisomers and diastereomers can be used to correct for analyte loss during sample preparation, matrix effect and, drifting ion currents of mass spectrometers (16). Wolf et al., reported the addition of protease or other inhibitors to prevent the degradation of peptides in the biological matrix (19). Lower acetonitrile concentrations (50% and 67%) were found to give a better recovery for two other antimicrobial cationic peptides, NAB 7061 and NAB 739 (13).

Alternatively, peptide-containing plasma samples may be directly subjected to LC-MS/MS analysis in order to minimize peptide loss during precipitation. An increased sensitivity in detection of a peptide in biological fluids without any pretreatment was reported (20). The utility of direct injection of plasma could be examined by injection of un-manipulated plasma samples with determination of the minimum dilutions required without interfering with the sensitivity and accuracy in fowl-1(6-26)-NH_2 detection.

In summary, we demonstrated the feasibility of quantitation of fowl-1(6-26)-NH_2 by LC-MS/MS and revealed that it can be reliably detected in the range of 0.05- 5 μg/mL. This preliminary study will be helpful in determining the optimal MS/MS conditions for analysis of fowl-1(6-26)-NH_2, which will lead to a method suitable for use in mouse plasma.

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Table 1: Gradient used for separation of fowl-1(6-26) NH_2 peptide.

Time (min)	%A	%B
0	70	30
15	10	90
16	70	30
20	70	30

Note: %A: 0.1% formic acid in water; % B: 0.1% formic acid in acetonitrile.

Table 2: Optimization of the scan time and injection control settings in ion trap and FT detector.

	Scan Time Setting (ms)		AGC[a] Target Setting (ion counts)	
	Before optimization	After optimization	Before optimization	After optimization
Ion trap				
Full scan	10	100	3.00e + 04	3.00e + 04
SIM[b]	50	50	1.00e + 04	1.00e + 04
MSn[c]	100	100	1.00e + 04	1.00e + 04
Zoom	50	50	3000	3000
FT[d]				
Full scan	10	500	2.00e + 05	3.00e + 05
SIM [b]	50	500	1.00e + 05	1.00e + 05
MSn[c]	100	500	1.00e + 05	3000

Note: [a] AGC- Automatic gain control, [b]SIM- Selective ion monitoring, [c]MSn- Multiple reaction monitoring, [d]FT- Fourier transform.

Table 3: Mass over charge ratios (m/z) of precursor and product ions of fowl-1(6-26)-NH_2.

Charge State	Full Scan	SRM[a] Scan
+6	418.09	475.00
+5	501.51	580.11
+4	626.64	772.83
+3	835.19	786.00,1114.00
+2	1252.52	-------

Note: [a] SRM- Selective reaction monitoring.

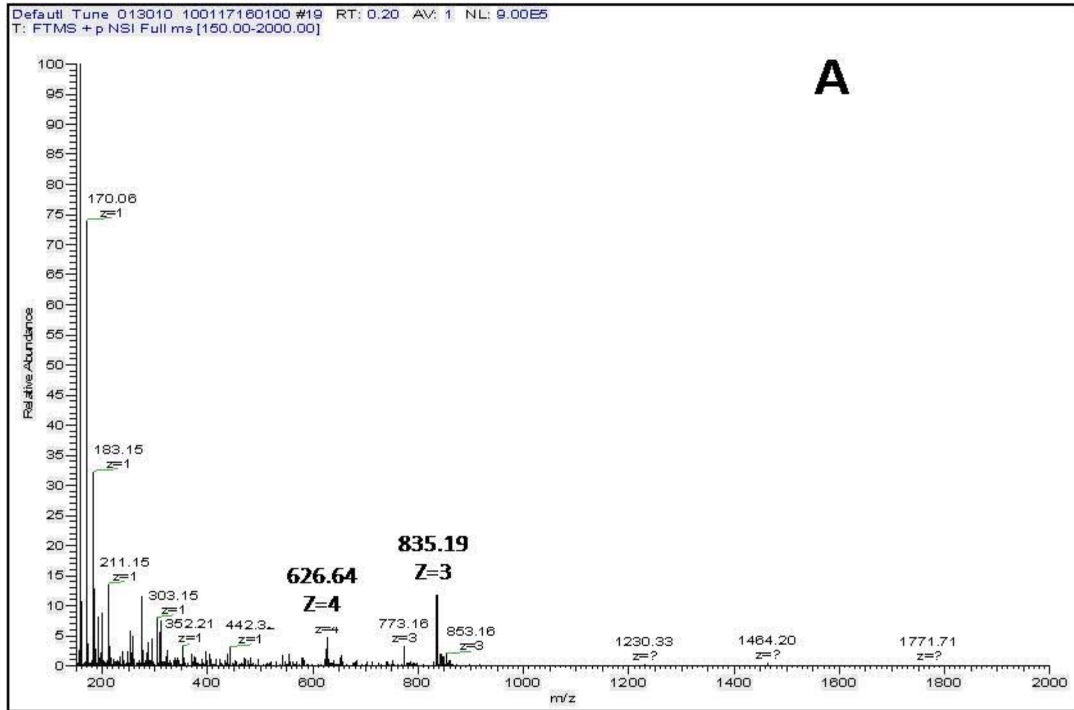
Table 4: Percent deviation values estimated by back calculations using regression equations.

Actual concentration (µg/ml)	Full Scan		SRM Scan[a]
	Single ion count (m/z 501) Percent Deviation [b]	Combined total ion count Percent Deviation [b]	Percent Deviation [b]
0.2	8	8	10
0.4	13	14	32
0.8	7	8	20
1.6	11	11	1
3.2	4	4	27

Note: [a] Selected Reaction monitoring;

[b] Percent deviation = $\frac{|\text{Actual concentration} - \text{Estimated concentration}|}{\text{Actual concentration}} \times 100$

Figure 1. Mass spectra of fowl-1(6-26)-NH_2 peptide before (A) and after (B) optimization of instrument setting. The spectra were obtained by direct electrospray infusion of 10 µl of the peptide at 400 µg/mlin 50% acetonitrile at 80 nL/min.



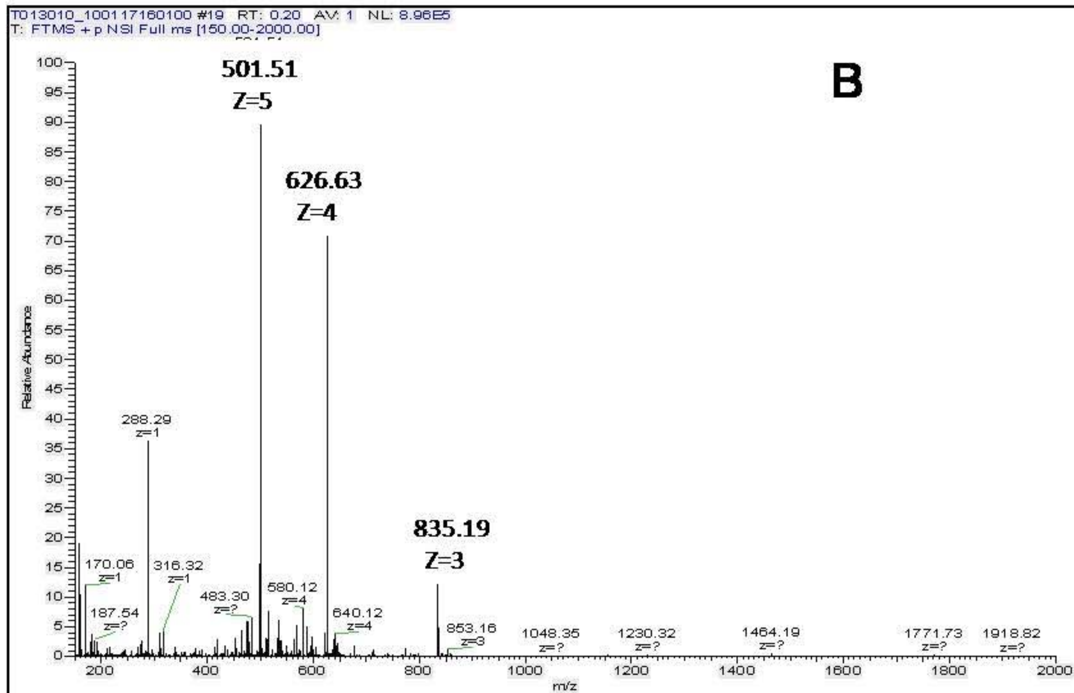
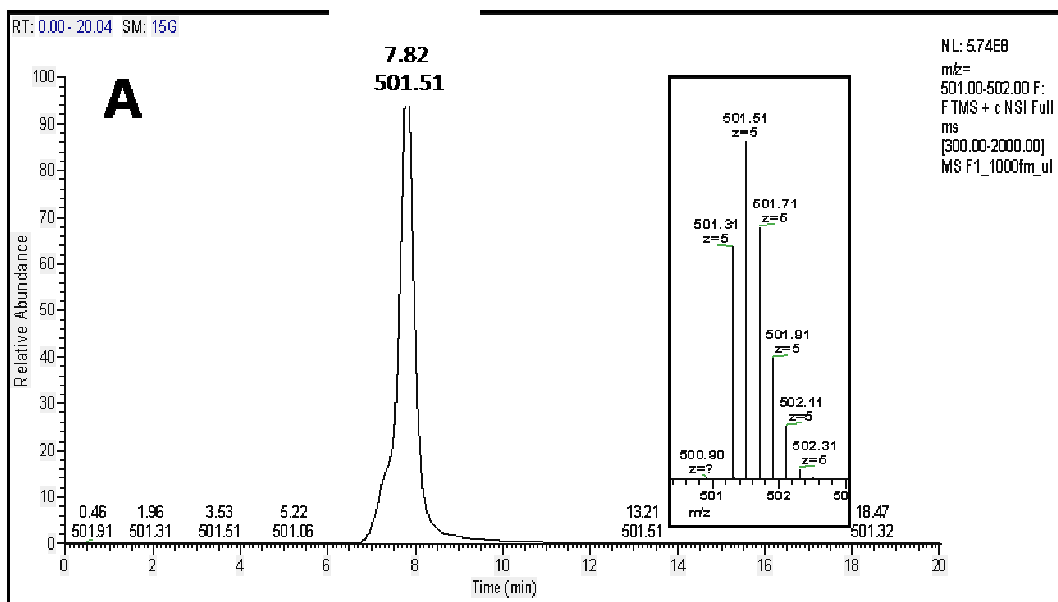
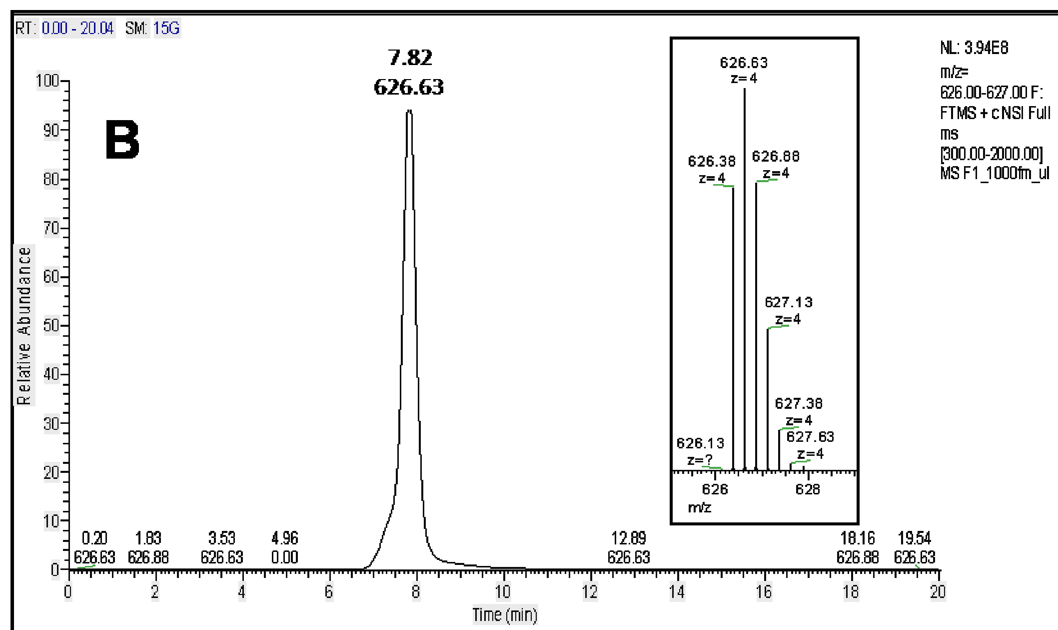
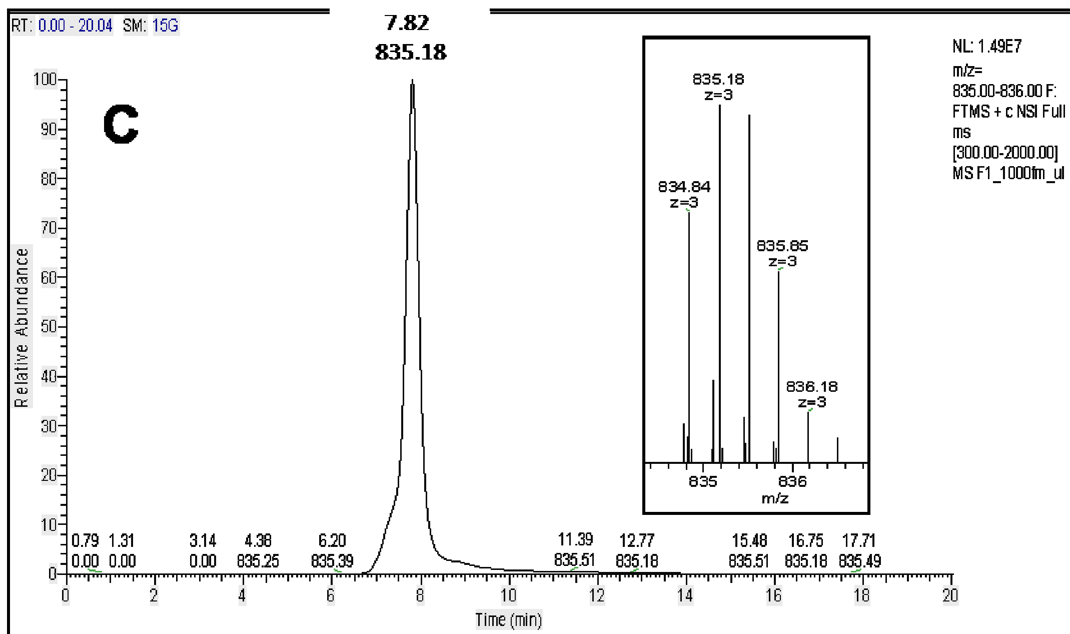


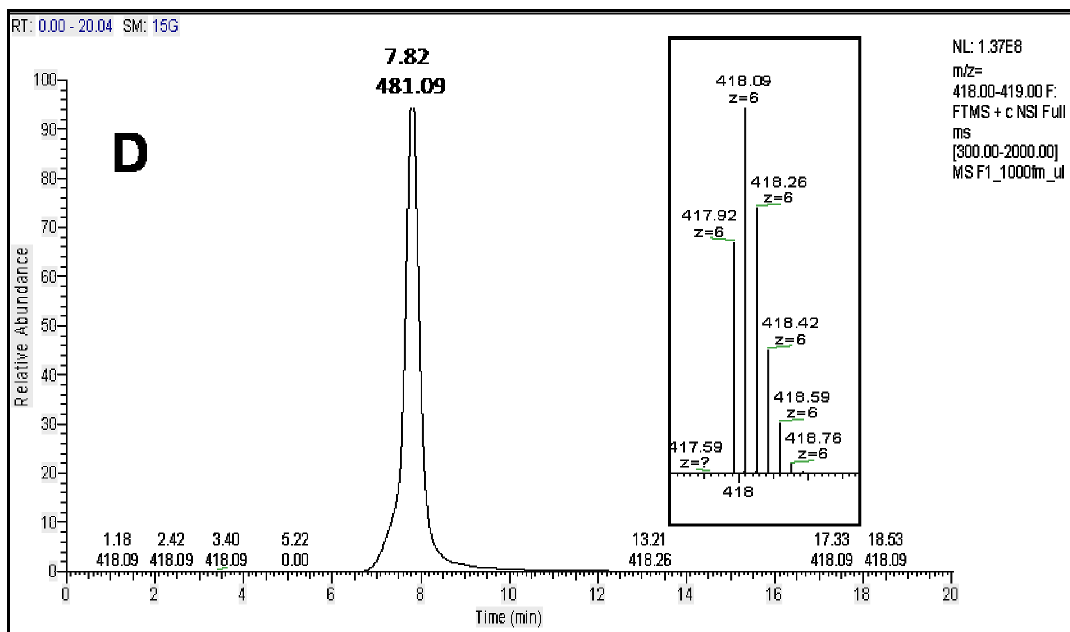
Figure 2. Nanospray mass spectrum of each charge state of fowl-1(6-26)-NH_2. The Gaussian peaks of four major charge states including *m/z* 501.31 ($[M+5H]^{5+}$) (A), 626.64 ($[M+4H]^{4+}$) (B), 835.19 ($[M+3H]^{3+}$) (C), and 418.09 ($[M+6H]^{6+}$) (D) were shown. The isotopic distributions for each charge state were also indicated in the insert. of all ions, 501 ($[M+5H]^{5+}$) was abundantly produced.







RT: 0.00 - 20.04 SM: 15G
7.82
835.18
C
NL: 1.49E7
m/z=
835.00-836.00 F:
FTMS + c NSI Full
ms
[300.00-2000.00]
MS F1_1000fm_ul
835.18
z=3
834.84
z=3
835.85
z=3
836.18
z=3
835
836
m/z
Relative Abundance
0.79 0.00
1.31 0.00
3.14 0.00
4.38 835.25
6.20 835.39
11.39 835.51
12.77 835.18
15.48 835.51
16.75 835.18
17.71 835.49
Time (min)



RT: 0.00 - 20.04 SM: 15G
7.82
481.09
D
NL: 1.37E8
m/z=
418.00-419.00 F:
FTMS + c NSI Full
ms
[300.00-2000.00]
MS F1_1000fm_ul
418.09
z=6
418.26
z=6
417.92
z=6
418.42
z=6
418.59
z=6
418.76
z=6
417.59
z=?
418
Relative Abundance
1.18 418.09
2.42 418.09
3.40 418.09
5.22 0.00
13.21 418.26
17.33 418.09
18.53 418.09
Time (min)

Figure 3. LC-MS/MS full and SRM chromatograms of fowl-1(6-26)-NH_2. The ions of the precursor (A) and product (B) of the 626.64 ($[M+4H]^{4+}$) charge state were shown.

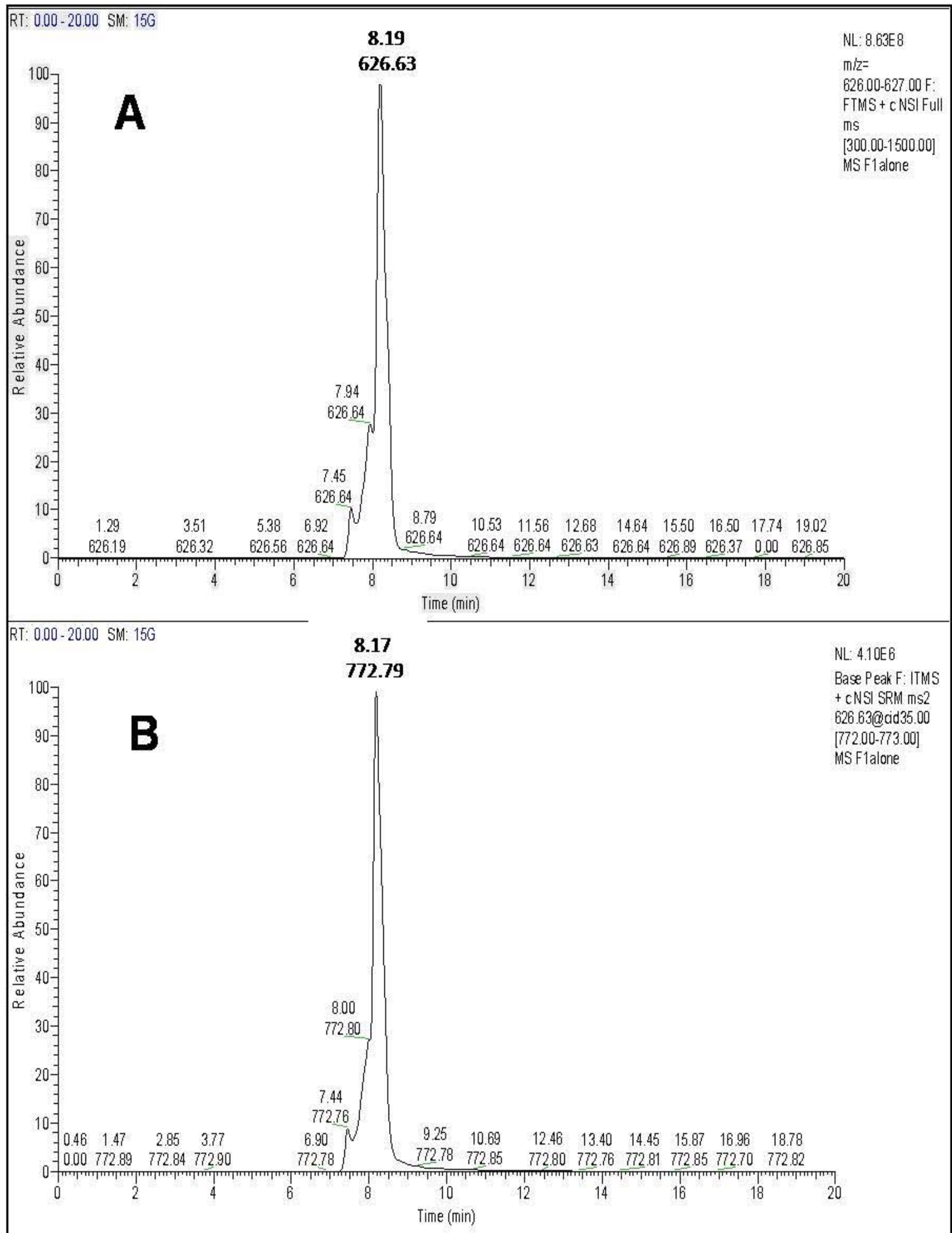


Figure 4. Concentration-response curve of fowl-1(6-26)-NH_2 by LC-MS/MS. Serial 2-fold dilutions of the peptide in 0.1% formic acid were subjected to LC-MS/MS analysis, with duplicate runs for each dilution. The total ion counts of all charge states were recorded for each concentration and saturation of the fowl-1(6-26)-NH_2 peptide was observed at 10μg/mL.

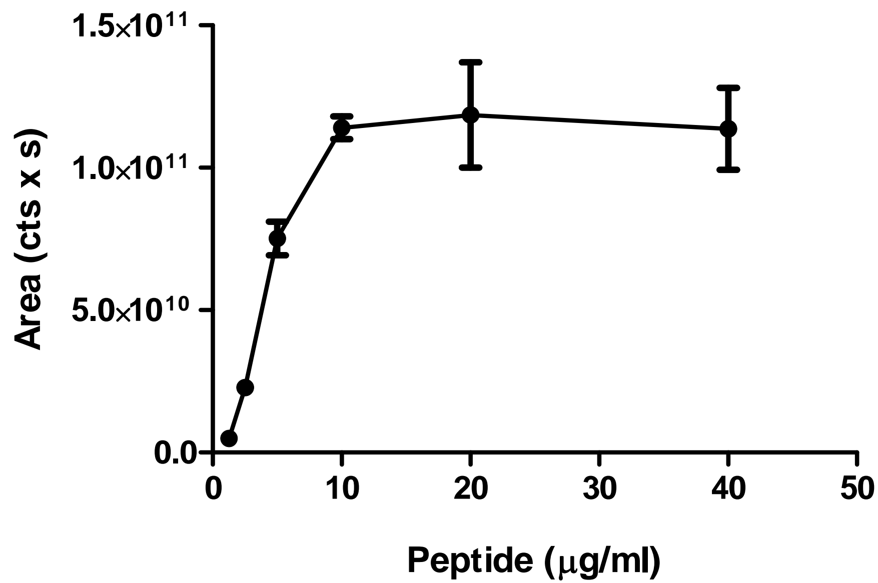


Figure 5. Concentration-response curve of fowl-1(6-26)-NH_2 in linear scale. Serial 2-fold dilutions of the peptide in 0.1% formic acid were subjected to LC-MS/MS analysis, with duplicate runs for each dilution. Response was estimated from the area of product ion (772) of the precursor ion 626 ($[M+4H]^{4+}$). The concentrations used were 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μg/ml. A good fit of concentration over response plot was noted in power trend line with R^2 value of 0.994.

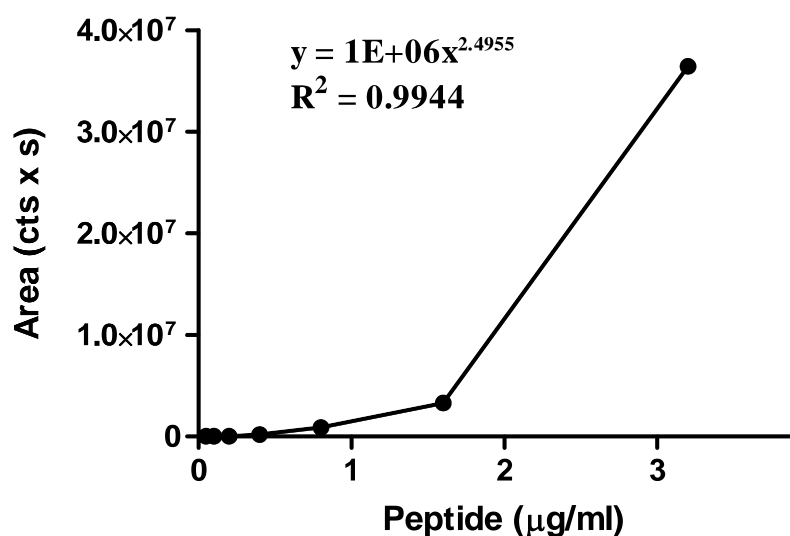


Figure 6: Concentration response curves of fowl-1(6-26)-NH_2 in SRM and Full (combined total ion count and single ion count) scans. Based on R^2 values and percent deviation, full scan mode was adopted. A SRM Scan; B Full scan (combined total ion count); C full scan (single ion m/z 501)

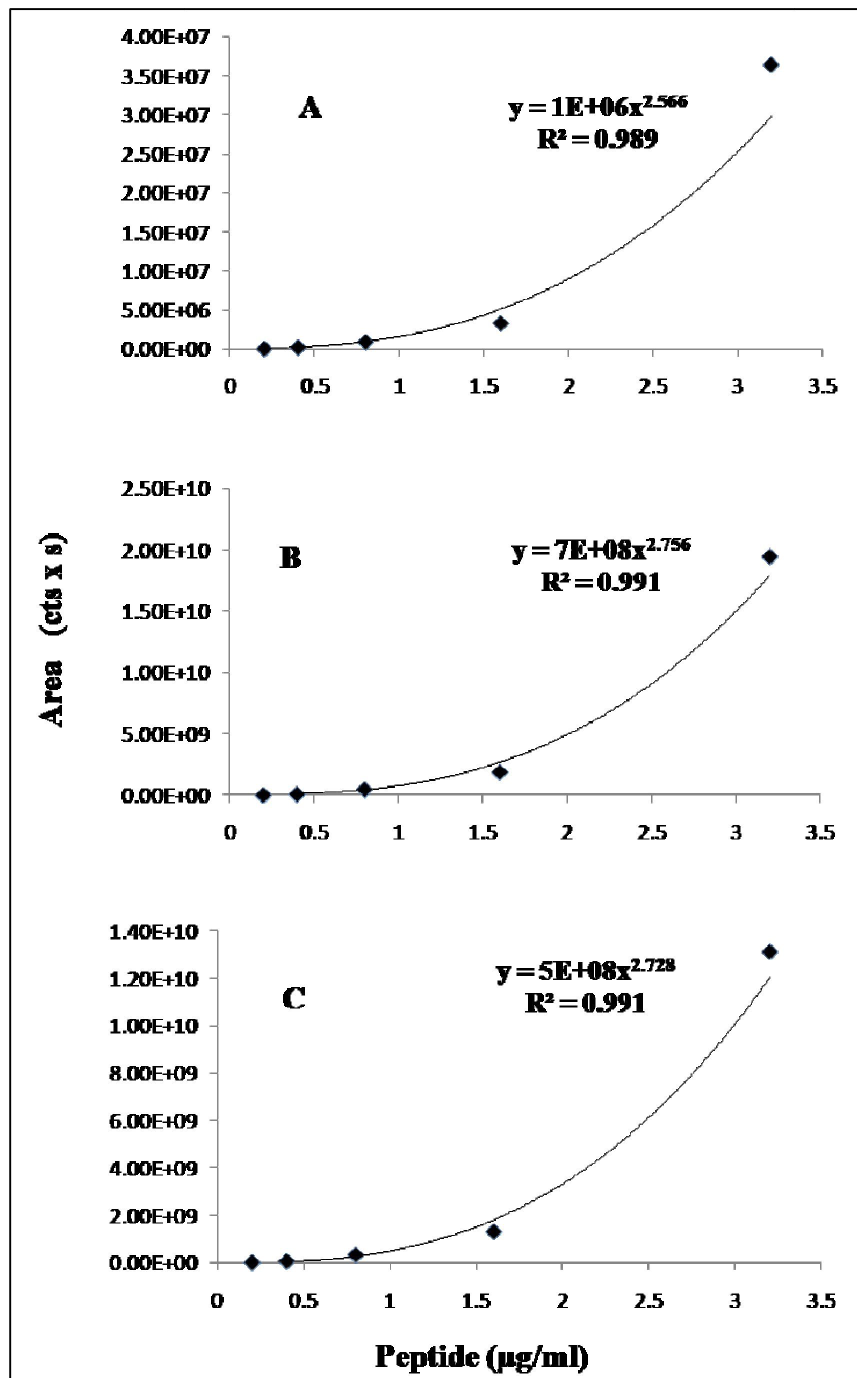


Figure 7. Influence of plasma on LC-MS/MS quantification of fow1-1(6-26)-NH_2. Mouse plasma samples were precipitated with acetonitrile and then diluted to 1, 2.5, and 5% plasma in 0.1% formic acid, followed by addition of fowl-1(6-26)-NH_2 to 5μg/ml.10μl of the samples were injected into LC-MS/MS at a flow rate of 300nl/min. Total ion counts of all charge states were recorded.

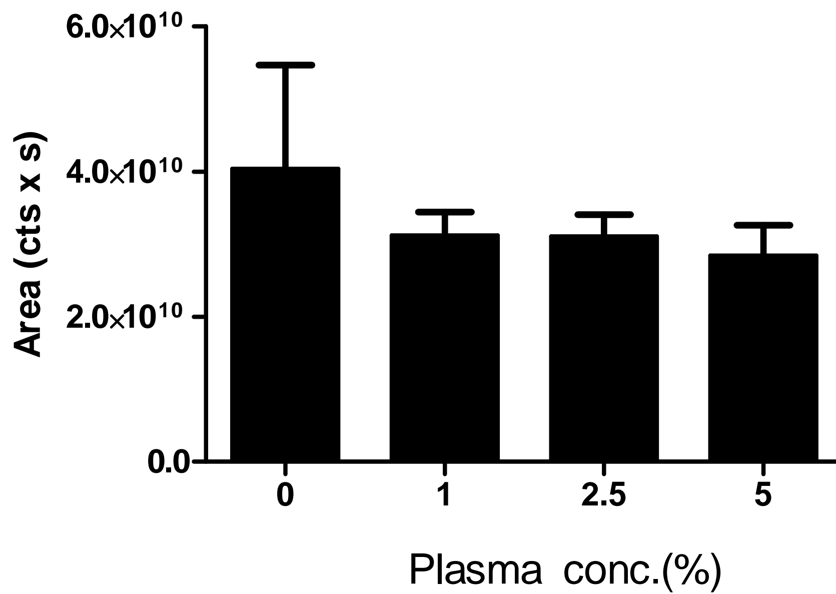
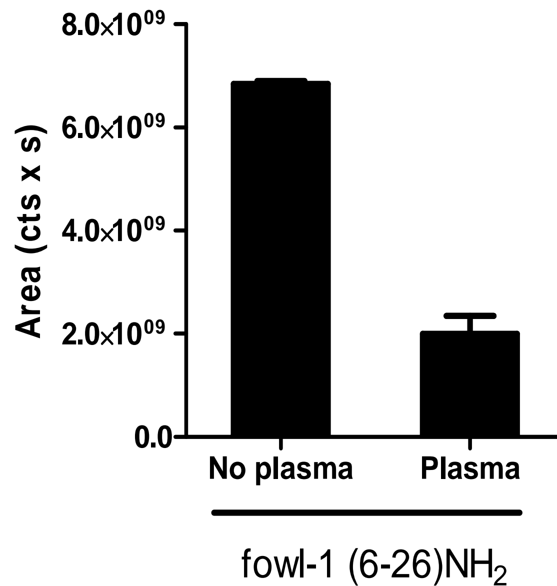


Figure 8. Effect of acetonitrile precipitation on the recovery of fow1-1(6-26)-NH_2. Peptide was added in plasma which was then precipitated with 75% acetonitrile. Spiked plasma (10µL) was injected into LC-MS/MS and the amount of fowl-1(6-26)-NH_2 peptide recovered was compared with that of positive control (peptide alone). It was noted that 29 % fowl-1 (6-26)-NH_2 was recovered.



VITA

MALLIKA ACHANTA

Candidate for the Degree of

Master of Science

Thesis: CHICKEN CATHELICIDINS: TISSUE EXPRESSION, DEVELOPMENTAL REGULATION, AND MASS SPECTROMETRIC QUANTIFICATION

Major Field: Animal Science

Biographical:

Education:

Master of Science, Oklahoma State University, Stillwater, Oklahoma,July, 2010

Bachelor of Veterinary Science and Animal Husbandry, Sri Venkateswara Veterinary University, Tirupati, India, 2007

Experience:

Worked as a graduate teaching asistant for Animal Genetics course in spring semesters of 2009 and 2010
Graduate research assistant in Animal Molecular Biology and Immunology lab, fall 2008 – summer 2010

Professional Memberships:

The American Society for Mass Spectrometry
American Association for Advancement of Science
Veterinary Council of India

Awards:

George R Waller Mass spectrometry fellowship
Gold medal in Animal Genetics and Breeding, 2007
Silver medal in table tennis at Inter University competitions, India, 2004

Name: MALLIKA ACHANTA Date of Degree: July, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CHICKEN CATHELICIDINS: TISSUE EXPRESSION, DEVELOPMENTAL REGULATION, AND MASS SPECTROMETRIC QUANTIFICATION

Pages in Study: 64 Candidate for the Degree of Master of Science

Major Field: ANIMAL SCIENCE

Scope and Method of Study: The purposes of the study are to determine the tissue and developmental expressions of chicken cathelicidins and to develop a mass spectrometric assay for quantification of chicken cathelicidins. RNA was isolated from a panel of chicken tissues of different ages and real-time PCR was performed to measure the gene expression levels of four chicken cathelicidins. A mass spectrometric method was also developed by employing LC-MS/MS for quantification of fowl-1(6-26)-NH_2, a chicken cathelicidin peptide analog.

Findings and Conclusions: Four chicken cathelicidins were widely expressed in a variety of tissue types, with fowlicidins 1-3 showing a more similar pattern than cathelicidin B1. Lung showed the highest expression of fowlicidins 1-3, while cathelicidin B1 was expressed most abundantly in bursa of Fabricius. Four cathelicidins were differentially expressed during the early stages of development, largely with a gradual increase in bursa and cecal tonsil, two important immune organs in intestinal host defense. Collectively, our study of tissue and developmental expression patterns of four chicken cathelicidins has led to a better understanding of the chicken innate immunity as well as the development of the innate immune system. In the LC-MS/MS assay, five different charge states of fowl-1(6-26)-NH_2 ranging from +2 to +6 were detected, with the most intense ion being 501.31 ($[M+5H]^{5+}$), followed by 626.39 ($[M+4H]^{4+}$), 418.09 ($[M+6H]^{6+}$), and 835.19 ($[M+3H]^{3+}$). Results also showed the response curve for fowl-1(6-26)-NH_2 is between 0.05 to 10.0 µg/ml with saturation of detection occurring above 10.0 µg/ml using the LC-MS/MS approach that we developed. Taken together, these preliminary data have established a solid foundation on the quantification of the plasma concentrations of fowl-1(6-26)-NH_2 peptide, which will be essential in calculating pharmacokinetic parameters.

ADVISER'S APPROVAL: Dr. Guolong Zhang