Characterization of transcription profile and structural properties of avian NK-lysin

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ABSTRACT This study aimed to determine the transcription profile of NK-lysin gene in native chickens. Moreover, it was targeted toward determining the primary, three-dimensional, and molecular dynamic structures of NK-lysin and granulysin peptides to understand their mode of action and intracellular transduction pathways using in silico analysis. The results revealed that NK-lysin gene in native chickens and *Gallus gallus* were closely related to those of other avian species. However, there was a low sequence homology when aligned with the mammalian peptides. The coding region of NK-lysin peptide in native chickens encoded 140 amino acids as found in *G. gallus* with a homology of 98% that declined to 20%, particularly in mammalian species. The results revealed that the NK-lysin in native chickens was closely related to that in avian species at a range of 71–76%. However, it was different from that of other mammalians in terms of nucleotide and amino acid identities. The mRNA transcripts of NK-lysin had high and moderate expression levels in the testis and pancreas, respectively. Nonetheless, the small intestine, kidney, spleen, and liver had a low expression level. The NK-lysin peptides contained more than 50% of the total AA with a nonpolar feature, whereas polar AA constituted up to 30% of AA. The results also indicated that the hydrophilic regions and positively charged amino acids were predominant on the surface of the investigated peptides. The NK-lysin was folded in 4–5 helical units and 3–4 loop structures in their saposin domain. The third helical peptide was long in both avian and bovine species (104–123 residues). However, the fourth helical peptide was short in humans, pigs, and chimpanzees (101–123, 104–123, and 102–124 residues, respectively), with the helical unit residues of 95–97, 96–99, and 96–98, respectively. The obtained results can be helpful in developing novel approaches that could be used as alternatives or adjuncts to the existing means of control.

Key words: NK-lysin gene, prediction, secondary structure, tertiary structure, transcription profile

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INTRODUCTION

One of the most potent milestones of the immune system is antimicrobial peptide (**AMP**), which represents the first line of innate defense against a large number of pathogens. These peptides are widely found in animal and plant kingdoms and play fundamental roles against different kinds of pathogens (Boman, 1994). Recently, they have been found to have significant immune-modulatory functions in the activation of acquired or adaptive immunity by releasing cytokines and chemotaxis.

In addition, AMP stimulate wound healing, immune-cell proliferation, and angiogenesis (Bals and Wilson, 2003; Beisswenger and Bals, 2005). These peptides are cysteine-rich molecules, which form conserved disulfide bridges with a low molecular weight of around 40 kDa (Van der Sluis, 2000; Van Immerseel et al., 2004, 2009; Cooper and Songer, 2009). These immune-stimulated peptides exhibit a great efficacy against plenty of fungi, gram-positive and gram-negative bacteria, viruses, and protozoa (Yount et al., 2006).

As per to some investigations, variations in bacterial membrane contents and the interaction of peptides with mammalian membrane would lead to an abundance of AMP functions (Hallock et al., 2002; Epand et al., 2006; Lee et al., 2014; Sudheendra et al., 2015). Some

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reports have concluded that the negative charge of lipids increases the binding affinity of the bacterial membranes with AMP (Sudheendra et al., 2015). In contrast, the presence of cholesterol inhibits the performance of AMP in membrane disruption by increasing the selectivity of AMP (Ramamoorthy et al., 2010). The positively charged arginine and lysine residues at the peptide C-terminal were revealed to interact with the phosphate groups of the lipid that are negatively charged. This interaction is important as it enforces membrane disruption with their dynamics (Sudheendra et al., 2015).

The differences in the net charge of antimicrobial molecules may influence their antimicrobial capacity; therefore, a decline in net charge may decrease antimicrobial efficacy (Cuperus et al., 2013). On the other hand, an increase in the positive charge of some of these molecules from 0.7 to 2.7 may improve their antimicrobial impact against gram-negative bacteria (*Listeria monocytogenes*); however, this has no significant effect against *Staphylococcus aureus* (Derache et al., 2009). Avian NK-lysin has a similarity of less than 20% with that of mammalians. Nonetheless, it can significantly penetrate the cell membrane of the infected cells or pathogens with a high capacity owing to its helical secondary structure (Jin et al., 2000; Koczulla and Bals, 2003; Mader and Hoskin, 2006; Iwasaki et al., 2009; Gross and Andra, 2012; Lin et al., 2013; Park et al., 2013; Lee, 2014).

One of the ways to address these issues may be the development of novel approaches that could be used as alternatives or adjuncts to the existing means of control. The stimulation of innate host responses by AMP and NK-lysin or granulysin in the modulation of signaling pathway is a novel and effective strategy to protect chickens against different emerging diseases. However, this approach has not been investigated adequately yet. With this background in mind, the present study was conducted to investigate the transcription profile of NK-lysin gene in native chickens. In addition, this research was targeted toward determining the primary, three-dimensional (3D), and molecular dynamic structures of NK-lysin and granulysin peptides to better understand their mode of action and intracellular transduction pathways using in silico analysis.

MATERIALS AND METHODS

Sample Collection

A total of 50 native chickens were obtained from the farm of King Abdulaziz University, Jeddah, Saudi Arabia. The birds were kept in cages and provided with water and food ad libitum. The study protocol was approved by the Ethics Committee of King Abdulaziz University. This study was conducted based on the health guidelines for completing animal experiments.

Tissue Collection

The tissue specimens were collected from 17 organs, including the bone marrow, spleen, liver, oviduct, ovum, large and small intestines, pancreas, skin, egg yolk, muscles, heart, testis, duodenum, gizzard, uterus, and kidney. These tissues were dissected and frozen in liquid nitrogen until use.

RNA Isolation and Complementary DNA Synthesis

Total RNA was isolated from 30–60 mg of the bone marrow, spleen, liver, oviduct, ovum, large and small intestines, pancreas, skin, egg yolk, muscles, heart, testis, duodenum, gizzard, uterus, and kidney tissues using the EZ RNA Clean Up Plus DNase Kit (EZ BioResearch, St. Louis, MO). Moreover, the RNA concentrations were measured using the NanoDrop spectrophotometer (Jenway, UK). Furthermore, reverse transcription was performed using the oligo(dT) primers (Bioneer, Inc., Daejeon, Republic of Korea) in a 20-μL reaction, including 5 μL of RNA.

The complementary DNA were amplified using a PCR master mix (Bioneer Inc., Daejeon, Republic of Korea) with a set of primers (i.e., forward 3′-ATG GCC GCT GCT CTC ATC GTG-5′ and reserve 3′-CAG CCC TTG CAC AGC CCC AG-5′) designed based on the open reading frame of the NK-lysin sequence deposited in the NCBI database (GeneBank accession number KT962967). The PCR amplification reactions were performed at a volume of 50 μL. The obtained PCR products were cloned using the CloneEZ PCR Cloning Kit (www.genscript.com). Subsequently, the amplified gene was sent to the Bioneer Incorporation (Daejeon, Republic of Korea) for sequencing.

Real-Time Polymerase Chain Reaction

The constitutive expression of NK-lysin mRNA in the different tissues of native chickens was analyzed using the real-time PCR (**RT-PCR**) via the SYBR Green qPCR Master Mix, with ROX being applied as a reference dye (Biotool LLC, Houston, TX). The total RNA was isolated from different tissues. Subsequently, RT-PCR was conducted using oligo(dT) primers (BioneerInc, Daejeon, Republic of Korea) in a 20-μL reaction, including 5 μL RNA. The RT-PCR was performed using the Strata gene Mx3005P QPCR System (Agilent Technologies, Germany).

The cycling conditions included a denaturing step at 95°C for 15 s and an annealing/extension step at 62°C for 30 s for 40 cycles. The melting curve was determined at a range of 60°C–95°C with one read every 0.2°C and a 5-s interval between the reads. All of the amplified fragments were obtained in 3 independent replicates, and the results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase as the reference gene using the comparative Ct method.

Sequencing and Statistical Analysis

The obtained sequences were subjected to sequence alignment to investigate their similarities with related

avian species using the nucleotide–nucleotide BLAST software at http://www.ncbi.nlm.nih.gov/blast/ and the CLUSTALW program (version 2.0.12). The phylogenetic tree was constructed using the MEGA software, version 6.1 (Tamura and Nei, 1993; Tamura et al., 2013), by using maximum likelihood method based on Jones–Taylor–Thornton model (Jones et al., 1992). The pairwise distance was measured using this Jones model through using the neighbour-joining method. The reliability of the branching of the tree was checked by 1,000 bootstrap samplings (Felsenstein, 1985). The branches corresponding to the partitions were collapsed when they were reproduced in less than 50% of the bootstrap replicates. The gaps and missing data at all positions were eliminated (Onteru et al., 2020). The analysis of expression level was performed in triplicate, and the obtained data were presented as mean and SE. The RT-PCR data were analyzed by the comparative CT method. Moreover, a two-tailed test was performed on the data using Microsoft Excel 2013.

Data Collection

The primary sequences of NK-lysin were retrieved from UniProt (http://www.uniprot.org) and NCBI databases for different kinds of species as depicted in Table 1.

Distribution and Composition of AA

The ProtParam tool of ExPASy proteomic server was used to determine and predict the distribution of positively and negatively charged AA residues, as well as polar and nonpolar side chains, in the given NK-lysin peptides.

Prediction of NK-Lysin Secondary Structure

The YASPIN secondary structure predictor (http://www.ibi.vu.nl/programs/yaspinwww/; Lin et al. 2005; Bioinformatics 21:152-9) was used to emphasize the secondary structure. Moreover, the UCSF ChimeraX software (Goddard et al., 2018) was used to determine the number and length of helices for each given peptide. Furthermore, the evaluation of the percentage of each Nk-lysin protein structure was accomplished using the Chou and Fasman Secondary Structure Prediction Server.

Table 1. The profile of saposin B-type domain of Nk-lysin peptide.

				Glycosylation	
Species	Domain	Disulfide bridges	AA	Type	Position
Gallus gallus Q2I811	62-140	66-138 69-132 97-107	140	N-linked GlcNAc	Asn 83
Coturnix japonica T2HFY1	62-137	63-135 66-129 94-104	137	N-linked GlcNAc	Asn 80
Meleagris gallopavo *XM_010712309.1*	58-130	58-130 61-124 89-99	132	-	-
Ovis aries *A6YT17*	62-142	66-138 69-132 97-107	146	N-linked GlcNAc	Asn 83
Capra hircus *A0A452FYB7*	62-138	66-138 69-132 97-107	146	N-linked GlcNAc	Asn 83
Camelus dromedarius *A0A4U5RLE4*	62-142	66-138 69-132 97-107	145	N-linked GlcNAc	Asn 83
Bos taurus *Q29RG7*	62-142	66-138 69-132 97-107	145	N-linked GlcNAc	Asn 83
Equus caballus *F6VY56*	62-142	66-138 69-132 97-107	145	N-linked GlcNAc	Asn 83
Danio rerio *Q7T3Q1*	43-121	47-119 50-113 78-88	121	N-linked GlcNAc	Asn 64
Homo sapiens *P22749*	62-142	66-138 (absent) 69-132 97-107	145	N-linked GlcNAc	Asn 83
Sas scrofa M9TM62	62-142	66-138 69-132 97-107	145	N-linked GlcNAc	Asn 83
Pan troglodytes *H9YYG7*	62-143	66-139 (absent) 69-133 97-108	146	N-linked GlcNAc	Asn 83

The domain structure of Nk-lysin peptide was determined used Swiss-modeling server and Uniprot databases (http://www.uniprot.org).

Modeling of 3D Structure of Nk-Lysin

The 3D structure of Nk-lysin peptides was generated through the homomodeling severs of the Swiss Institute of Bioinformatics (https://swissmodel.expasy.org/). Based on the Research Collaboratory for Structural Bioinformatics Protein Data Bank information, the pig file of Nk-lysin was used as a model template to figure out sequence identity, score, and quality.

Electrostatic Potential and Hydrophobicity Profiling

The UCSF ChimeraX software was applied to predict the distribution of positively and negatively charged AA (electrostatic potential), as well as the hydrophobic and hydrophilic regions on the surface of Nk-lysin.

RESULTS AND DISSCUSSION

Sequence Similarity

The open reading frames of NK-lysin gene in the local chickens in Saudi Arabia were subjected to amplification and sequencing. Sequence alignment was conducted to find identity with other deposited sequences in different avian species and mammalian counterparts using the nucleotide–nucleotide BLAST software and CLUSTALW program (version 2.12). The results revealed a homology of up to 98% between native NK-lysin and *Gallus gallus* as shown in Figure 1. This homology with other species was found to range from 20 to 76%.

Phylogenetic Construction

The phylogenetic tree indicated that NK-lysin genes in the native chickens and *G. gallus* were closely related to those in other avian species, such as quails and turkeys. However, granulysin showed a low sequence homology when aligned with that in mammalians. Based on the alignment results, the NK-lysin peptides in *Coturnix japonica* and *Meleagris gallopavo* were closely related to each other. The same view was monitored concerning *Bos taurus, Equus caballus*, and *Camelus dromedarius*, as well as *Ovis aries* and *Capra hircus*, which share the same clusters. The tree revealed that both *Homo sapiens* and *Pan troglodytes* had one cluster, and *Danio rerio* was out of the group as depicted in Figure 1.

However, the AA chain length of NK-lysin varied with respect to other avian species even in mammalian granulysin peptides (Figure 2). The coding region of NK-lysin peptide in native chickens encoded 140 AA as found in *G. gallus* with a homology of 98%. Nevertheless, the AA residues were 137 and 132 aa in *C. japonica* and *M. gallopavo* with the homology values of 71.9 and 76%, respectively. The coding region of mammalian granulysin varied from 145 to 146 aa; in addition, 121 AA were noted in zebrafish.

Tissue Distribution and Expression

The constitutive expression patterns of NK-lysin were addressed in different tissues based on RT-PCR as depicted in Figure 3. The results revealed that NK-lysin gene had high and moderate expression levels in the testis and pancreas, respectively. However, a low level of expression was observed in the small intestine, liver, kidney, and spleen. The results also revealed very low or no expression in the other investigated organs.

AA Composition of NK-Lysin Peptides

The side chain of the AA residues of NK-lysin peptides was determined using the ProtParam tool of ExPASy proteomic server. Based on the results, the proportion of positively charge residues had a range of 12–15%, whereas the negatively charged amino acids ranged from 8.2% in buffalos to 19.2% in Japanese quails (Figure 4). The highest percentage of polar aminoacyl residues was recorded in the sheep, whereas a low rate was found in turkeys. The analysis revealed a moderate percentage of polar residues in mammalian species. In addition, nonpolar AA were high in Arabian camels (51.1%), and the sheep was found to have a low rate in this regard (24.7%). This value ranged from 45.3 to 49.7% in other investigated species.

Saposin β-type Domain Profiling in NK-Lysin Peptides

The domain structure of NK-lysin peptide was determined using the Swiss-Model server and UniProt

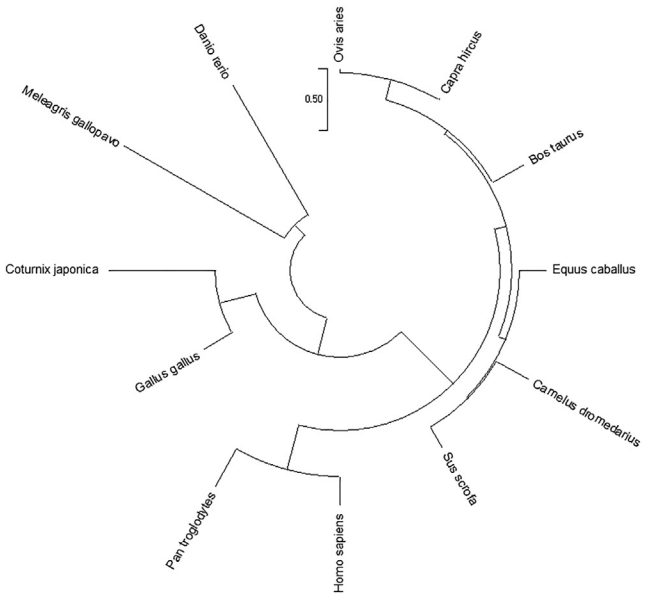

Figure 1. Phylogenetic relationship among Nk-lysin genes in *Gallus gallus* (*Q2I811*) and other species (*Meleagris gallopavo* [XM_010712309.1], *Coturnix coturnix* [*T2HFY1*], *Bos taurus* [*Q29RG7*], *Ovis aries* [*A6YT17*], *Capra hircus* [*A0A4J52FYB7*], *Equus caballus* [*F6VY56*], *Danio rerio* [*Q7T3Q1*], *Homo sapiens* [*P22749*], *Coturnix coturnix* [GQ985499], *Sas scrofa* [M9TM62], *Camelus dromedarius* [A0A4U5RLE4], *Pan troglodytes* [*H9YYG7*]). The phylogeny was inferred by using the neighbor-joining and maximum likelihood methods based on the Tamura-Nei model.

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Danio.rerio                 MLRGIIL-LT----------------------LLISSVCAVQWEMHKEQHSGI-ELEGS        35
Coturnix.japonica           MAAAIIVMMA---MG-AVLQVVVTEPPHDDQRDVAAGSPWEQQWQLLQDGSAVWD--EGD        54
Gallus.gallus               MAAALIVLLA---LG-AAVQVAVTEPPRDDHRDLDAGSHWEQQWHLLQDGSAAWDADEGD        56
Meleagris.gallopavo         --------HP---AP-PAVQVAVTELPRDEHQDVAAGSHWEQQWHLLGDGSAAWDAAEGD        48
Homo.sapiens                MATWALLLLAAMLLGNPGLVFSRL---SPEYYDLARAHLRDEE-KSCPCL--AQEGPQGD        54
Pan.troglodytes             MATWALLLLAATLLGNPGLVFSRL---SPEYYDLARAHLRDEE-KSCPCL--AQEGPQGD        54
Bos.taurus                  MTSWAVLLVTSVLLVAPGLAFSGL---TPECHDQETAHLWDGD-KLLQGL--APEDPQGD        54
Camelus.dromedarius         MTSRVLLVLVSVLLGAPGLAFSGL---TPEHSDGATAHLCDGE-HLFQGL--APQDPQGD        54
Ovis.aries                  MTSWAVLLIASVLLVAPGLAFSGL---APERHYQATAHLCNGD-ELCQGL--SQEYTQGG        54
Capra.hircus                MTSWAVLLIASVLLVAPGLAFSGL---SPERHDQAMAHLCNGD-ELCQGL--AQEYPQGG        54
Equus.caballus              mtsrallllasallgtpgltfsgl---npesydlatahlsdge-qfcqgl--tqedlqgd        54
Sus.scrofa                  MTSWAVLLLTLVLLGTPGLAFSGL---TPEHSALARAHPCDGE-QFCQNL--APEDPQGD        54

Danio.rerio                 GEIPTEQLPGMCWACKWALGKVKRKISNGATQDEIKVQLSQVCDQI-GFLKSLCRGFVNK        94
Coturnix.japonica           A--MGPGK-MKCSACIKLVKKLQKIVGDDPDEEAIGTALDQVCGTK-RILKGICRQLGKK       110
Gallus.gallus               A--MGPGKGIKCRFCVSLVKKVQKIVGDDPDEDAINNALNKVCSTG-RRQRSICKQLLKK       113
Meleagris.gallopavo         A--MGPGKGIKCRACVGLVKKLQKIVGDDPDEEAINTALDKVCGTG-KRQKSICRQLVKK       105
Homo.sapiens                LLTKTQELGRDYRTCLTIVQKLKKMV-DKPTQRSVSNAATRVCRTGRSRWRDVCRNFMRR       113
Pan.troglodytes             LLTKTQELGRNYRTCLMIVQRLKDVVEDQPTQRSVSNAATRMCRTGRSQWRDVCRNFMRR       114
Bos.taurus                  LVLQREELNQFCGPCQKIMQMLKYMVGNRPSKNVIIHVTSNVCSKM-GLWSILCNQMMKK       113
Camelus.dromedarius         LQPRGEGLGFICHSCQMLIQKVEDLVGQQPTQDTIAQAASQVCRKF-KRLRGLCKMITKL       113
Ovis.aries                  LLLQGEELGLLCGPCRKIIKSLEDMVGDQPNEDTIREAASKVCSKM-KLLKRPCQSIMKK       113
Capra.hircus                LLLQGEELGLLCGPCRKIIKSLEDMVGDQPNEDTIREAASKVCSKM-KLLKRPCQSIMKK       113
Equus.caballus              lltererqgiacwscrkilqkledlvgeqpneatineaasrvcrnl-gllrgackkimrt       113
Sus.scrofa                  QLLQREELGLICESCRKIIQKLEDMVGPQPNEDTVTQAASRVCDKM-KILRGVCKKIMRT       113

Danio.rerio                 YMDVLIEELSTTDNARTICANISVCKK------ 121
Coturnix.japonica           LRQQLSDALQDDSDPRSVCTTLGLCKG------ 137
Gallus.gallus               LRQQLSDALQNNDDPRDVCTTLGLCKG------ 140
Meleagris.gallopavo         LRQQLSDALQNDDDPRSVCTTLGMCKG------ 132
Homo.sapiens                YQSRVTQGLVAGETAQQICEDLRLCIPSTGPL- 145
Pan.troglodytes             YQSRVTQGLVAGETAQQICEDLRLCIPSTGPL- 146
Bos.taurus                  YLNRISKDIMARKTPQAICVDIKLCKRKAGLI- 145
Camelus.dromedarius         ALNRISQDIIAGKKPREICVDLKKCKPKAGLI- 145
Ovis.aries                  FLRRITEDIKAGKKPQAICVDIKVCKSKAVGFI 146
Capra.hircus                FLRRITEDIKAGKQPQAICVDIKVCKSKAVGFI 146
Equus.caballus              qlrlisrdilagkkpqevcvdiklckhkagli- 145
Sus.scrofa                  FLRRISKDILTGKKPQAICVDIKICKEKTGLI- 145
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Figure 2. AA sequence alignment of Nk-lysin protein in *Gallus gallus* (Q2I811) and other species (*Meleagris gallopavo* [XM_010712309.1], *Coturnix coturnix* [T2HFY1], *Bos taurus* [*Q29RG7*], *Ovis aries* [A6YT17], *Capra hircus* [*A0A452FYB7*], *Equus caballus* [*F6VY56*], *Danio rerio* [*Q7T3Q1*], *Homo sapiens* [*P22749*], *Coturnix coturnix* [GQ985499], *Sas scrofa* [M9TM62], *Camelus dromedarius* [A0A4U5RLE4], *Pan troglodytes* [*H9YYG7*]). The mature peptides of cathelicidin genes are underlined in bold and the 6 conserved cysteines residues (C) are underlined in bold. The differences among avian species are highlighted in yellow.

database. As shown in Table 1, the NK-lysin peptides in different species belong to the saposin β-type domain. The domain position varied in avian species, with the ranges of 62–140, 62–137, and 58–130 in domesticated chickens, Japanese quails, and turkeys, respectively. The same trend was observed in mammalian species, and a unique domain structure was observed in zebrafish (43–121).

In the same line, the results demonstrated a significant variation in the number of AAin the investigated species.

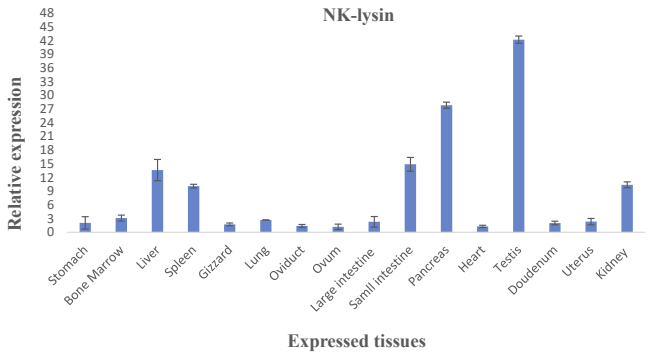


Figure 3. The expression patterns of Nk-lysin gene in native chicken in different tissues. The expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase as a reference gene. Three independent assays and each point is the mean ± SE.

The numbers of AA were 140, 137, and 132 in chickens, Japanese quails, and turkeys, respectively. An identical trend was found in mammalian species, with zebrafish having a unique NK-lysin with 121 AA. The results showed that all NK-lysin peptides had N-glycosidic bonds in asparagine residues at the position of 83 in all species, except for Japanese quails (at the position of 80). The NK-lysin peptides were stabilized with 6 conserved cysteine residues through 3 disulfide bridges as depicted in Table 1.

The results demonstrated that sulfide bonds had different positions in avian species, whereas all NK-lysin peptides in bovine species had the same positions of sulfide bonds (i.e., cys.66-cys.138, cys.69-cys.132, and cys.97-cys.107). Controversially, human and chimpanzee granulysin peptides had only two disulfide bridges at the positions of cys.69-cys.133/cys.97-cys.132 and cys.69-cys.133/cys.97-cys.108, respectively. Furthermore, zebrafish possessed 3 bonds in terms of cysteine residues at the positions of cys.47-cys.119, cys.50-cys.113, and cys.78-cys.88.

Prediction of Secondary Structure of NK-Lysin Peptides

Table 2 tabulates the secondary structure units of NK-lysin peptides. As per this table, helices constitute

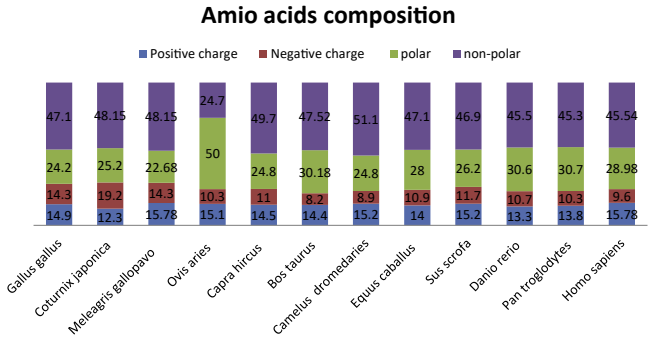


Figure 4. Prediction of posively, negatively, polar, non-polar AA residues distribution in Nk-Ysin peptides in different animal species.

70–84.7% and 80–87.8% of the secondary structure of NK-lysin peptides in avian and bovine species, respectively. However, this rate was lower in humans (51%) and chimpanzees (51.4%). The zebrafish represented the highest proportion of helical units with 91.7%. Concerning strand units, the recorded units ranged from 38.6 to 46.7% and 39 to 59% in avian and bovine species, respectively. However, this value was obtained as 54.4 and 59.6% in humans and chimpanzees, respectively. Regarding the zebrafish, this value was estimated at 61.2%. The lowest proportion of secondary structure belonged to turn units.

Molecular Modeling of NK-Lysin Peptides

The 3D structure of NK-lysin and granulysin peptides was built using the Chimera package based on the Protein Data Bank database and presented in Table 3. The analysis was performed based on the saposin domain sequence for each investigated species. The 3D structures exhibited a sequence similarity range of 18–28% depending on the availability of nuclear magnetic resonance and X-ray crystal structures. As shown in Table 4, the NK-lysin peptides are folded in 4–5 helical units and 3–4 loop structures in their saposin domain. The third helical peptide was long in both avian and bovine species (residues 104–123). On the other hand, the fourth helix was found to be short in humans, pigs, and chimpanzees (residues 101–123, 104–123, and 102–124), with the residues of 95–97, 96–99, and 96–98, respectively.

Hydrophobicity Analysis and Electrostatic Potential

The surface hydrophobicity profile and electrostatic potential of NK-lysin and granulysin peptides were analyzed by means of the Chimera software (version 1.14) as shown in Figure 5. The hydrophobicity distribution on the surface of NK-lysin peptides demonstrated that the surface contained more hydrophilic regions rather than hydrophobic and neutral areas. Both chickens and Japanese quails had the same hydrophobicity patterns, and the same issue was observed in bovine species.

Table 2. Prediction of secondary structures units of monomeric Nk-lysin.

Species	Domain	Helix %	B-sheet %	Turn %
Gallus gallus Q2I811	62-140	70.7	39.3	15
Coturnix japonica T2HFY1	62-137	84.7	46.7	14.6
Meleagris gallopavo XM_010712309.1	58-130	70.7	38.6	15
Ovis aries A6YT17	62-142	87.8	39	13.7
Capra hircus A0A452FYB7	62-138	86.3	36.3	13.7
Camelus dromedarius A0A4U5RLE4	66-142	82	49	10.3
Bos taurus Q29RG7	62-142	83.4	59.3	11
Equus caballus F6VY56	62-142	80	38.6	14.5
Danio rerio Q7T3Q1	43-121	91.7	61.2	6.6
Homo sapiens P22749	62-142	51	54.4	13.1
Sas scrofa M9TM62	62-142	84	46	11.7
Pan troglodytes H9YYG7	62-143	51.4	59.6	13.7

The secondary structure using YASPIN secondary structures predictor (http://www.ibi.vu.nl/programs/yaspinwww/). The UCSF ChimeraX software (Goddard et al., 2018) was used to determine the number and length of helices for each given peptide.

However, in turkeys, NK-lysin peptides possessed more predominant hydrophilic regions on their surface. Controversially, humans and chimpanzees had the same surface areas based on the hydrophobicity analysis. Concerning the distribution of electrostatic potential on the surface of the investigated peptides, it was found that these peptides had predominantly more positively charged regions, compared with negatively charged and neutral regions, especially in cattle. Nonetheless, both negatively and positively charged regions were distributed with the same ratio on the surface area of the zebrafish peptides.

A way to address disease control may be the development of novel approaches that could be used as alternatives or adjunct to the existing means of control. The stimulation of innate host responses by AMP in the modulation of signaling pathway is a novel and effective way to protect chickens against different emerging diseases. However, this approach has not been investigated adequately yet.

Regarding the native chickens, some studies reported that these chickens play an important socioeconomic role in rural and desert communities. According to Al-Yousef (2007), native chickens are widely varied in terms of body shape and feather colors. In addition, they have various combinations of plumage colors, comb types, and body size. Moreover, local birds are generally smaller in size, with the mean mature body weights of 1.26 ± 0.23 and 1.8 ± 0.26 kg in females and males, respectively.

Classical approaches to the genetic analysis of the structure and origin of native chicken breeds are based on cytogenetics and morphologic studies (Ma et al.,

Table 3. Prediction of the tertiary structure of Nk-lysin peptides and saposin domain identity.

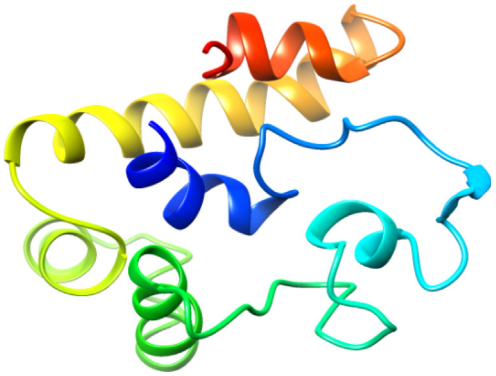
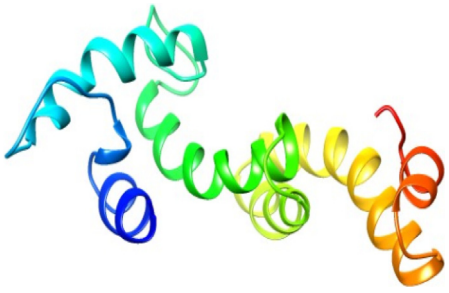
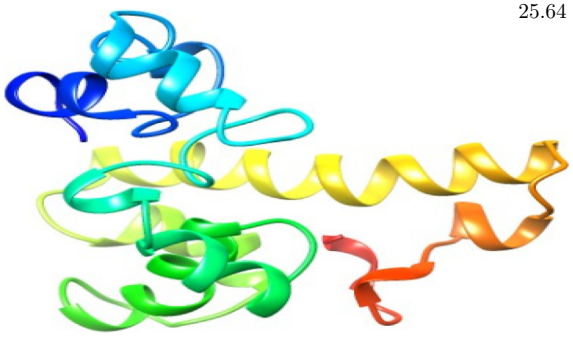
Species	Three-dimensional structure	Sequence identity %
Gallus gallus Q2I811		28.21
Coturnix japonica T2HFY1		25.97
Meleagris gallopavo XM_010712309.1		26.58
Ovis aries A6YT17		25.64

Table 3. (*continued*)

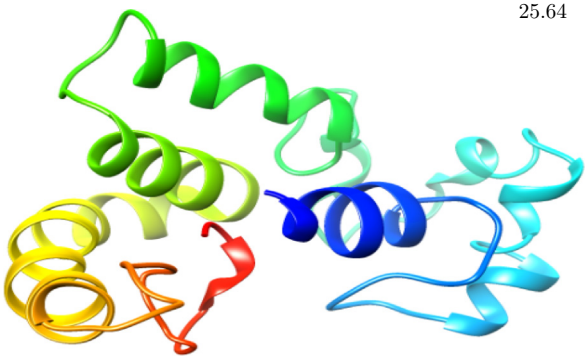
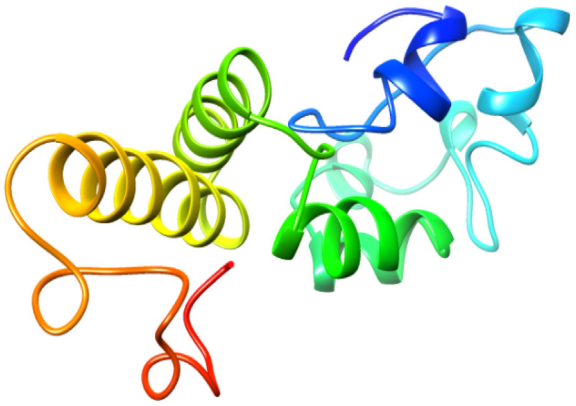
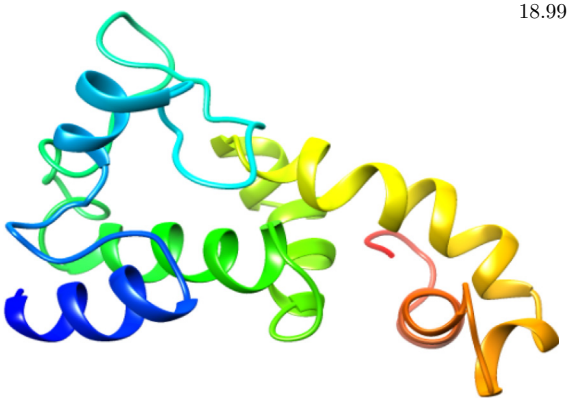
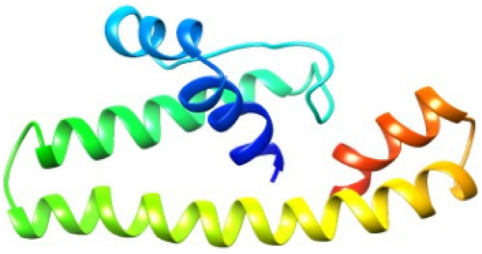
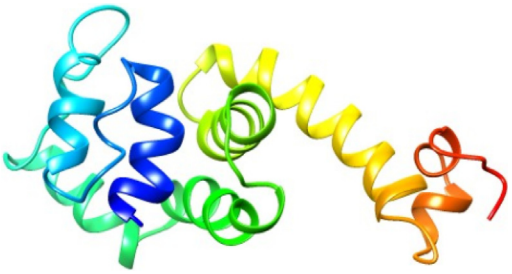
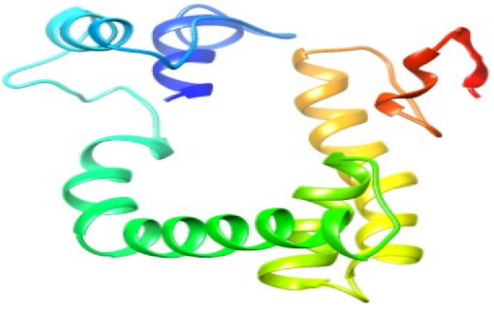
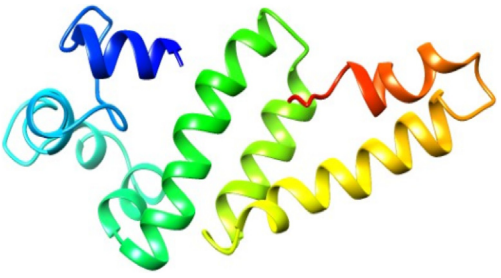
Species	Three-dimensional structure	Sequence identity %
Capra hircus A0A452FYB7		25.64
Camelus dromedarius A0A4U5RLE4		21.79
Bos taurus Q29RG7		18.99
Equus caballus *F6VY56*		23.08

Table 3. (*continued*)

Species	Three-dimensional structure	Sequence identity %
Danio rerio *Q7T3Q1*		25.64
Homo sapiens P22749		17.95
Sas scrofa M9TM62		91.03
Pan troglodytes H9YYG7		17.95

The three-dimensional structure of Nk-lysin peptides was generated through homomodeling severs of the Swiss Institute of Bioinformatics (https://swissmodel.expasy.org/). The (N-termini) is colored with blue and the (C-termini) are colored with orange using Scratch Protein Predictor and ChimeraX software.

1993; Cheng et al., 1996; Li et al., 1996; Liu et al., 1997). Many reports revealed that native chicken strains were closely related to the cluster of *G.gallus* and subspecies of *Gallus*, suggesting that they may be separated from the same origin. According to these studies, it was concluded that the native chicken strains were genetically closer to *G. gallus*, and they could be successfully distinguished from the other wild types of *Gallus* chickens based on Cytochrome b gene information (Yacoub and Fathi, 2013; Yacoub et al., 2014).

Antimicrobial molecules are cysteine-rich and cationic in nature; moreover, they are composed of 3 conserved disulfide bridges and β-sheet structure. These peptide structures are widely found and located in all living creatures (Lehrer and Ganz, 2002; Selsted and Ouellette, 2005; Klotman and Chang, 2006), whereas avian species are known to possess unique peptide structure and characteristics (Cuperus et al., 2013).

All avian NK-lysin and granulysin peptides were examined for their antimicrobial activities against both bacteria and fungi. The killing mechanism of these

Table 4. Profile of saposin B-type domain of Nk-lysin peptide.

Species	Domain	H-number	H- length	L- number
Gallus gallus Q2I811	62-140	α-Helices 1	65-80	3
		α-Helices 2	86-99	
		α-Helices 3	104-123	
		α-Helices 4	128-134	
Coturnix japonica T2HFY1	62-137	α-Helices 1	62-77	3
		α-Helices 2	83-96	
		α-Helices 3	101-120	
		α-Helices 4	125-131	
Meleagris gallopavo XM_ 010712309.1	58-130	α-Helices 1	57-74	3
		α-Helices 2	78-90	
		α-Helices 3	96-115	
		α-Helices 4	120-126	
Ovis aries A6YT17	62-142	α-Helices 1	66-80	3
		α-Helices 2	86-99	
		α-Helices 3	104-123	
		α-Helices 4	128-134	
Capra hircus A0A452FYB7	62-138	α-Helices 1	66-80	3
		α-Helices 2	86-99	
		α-Helices 3	104-123	
		α-Helices 4	128-134	
Camelus dromedarius A0A4 U5RLE4	62-142	α-Helices 1	65-80	3
		α-Helices 2	86-99	
		α-Helices 3	104-123	
		α-Helices 4	128-134	
Bos taurus Q29RG7	62-142	α-Helices 1	66-80	3
		α-Helices 2	86-99	
		α-Helices 3	104-123	
		α-Helices 4	128-134	
Equus caballus F6VY56	62-142	α-Helices 1	65-80	3
		α-Helices 2	86-99	
		α-Helices 3	104-123	
		α-Helices 4	128-134	
Danio rerio Q7T3Q1	43-121	α-Helices 1	46-61	3
		α-Helices 2	67-80	
		α-Helices 3	85-104	
		α-Helices 4	109-115	
Homo sapiens P22749	62-142	α-Helices 1	64-79	4
		α-Helices 2	85-92	
		α-Helices 3	95-97	
		α-Helices 4	101-123	
		α-Helices 5	128-135	
Sas scrofa M9TM62	62-142	α-Helices 1	65-80	4
		α-Helices 2	86-94	
		α-Helices 3	96-99	
		α-Helices 4	104-123	
		α-Helices 5	128-134	
Pan troglodytes H9YYG7	62-143	α-Helices 1	64-79	4
		α-Helices 2	86-93	
		α-Helices 3	96-98	
		α-Helices 4	102-124	
		α-Helices 5	129-136	

peptides is analogous to those of other cationic AMP. The underlying mechanism through which AMP exert their effect is their interaction with the negatively charged phospholipids bilayer of the cell, which leads to the disorganization of the cell membrane under hydrophobic conditions, thereby resulting in the disruption and elimination of pathogens (Ganz, 2004). The present research aimed to determine the primary, 3D, and molecular dynamic structures of NK-lysin and granulysin peptides to better understand their mode of action and intracellular transduction pathways using in silico analysis.

This study illustrated that native chickens were closely related to avian species (71–76%). However, they were significantly different from other mammalian species in terms of the nucleotides and amino acid identities of NK-lysin. These findings are consistent with the results reported by Lee et al. (2014).

They demonstrated that NK-lysin in chickens possessed less than 20% similarity to that in mammalians and showed significant efficacy in penetrating the cell membrane with full capacity owing to its helical secondary structure. The present study was the first attempt targeted toward characterizing the constitutive transcription patterns of NK-lysin peptides in native chickens. The mRNA transcripts of NK-lysin had high and moderate expression levels in the testis and pancreas, respectively. On the other hand, the small intestine, kidney, spleen, and liver had a low expression level. Our findings were in good agreement with those of other studies, which illustrated that NK-lysin expression was high in the intestinal intraepithelial lymphocytes, moderate in the spleen, and low in the lymphoid organs (Lillehoj and Choi, 1998; Hong et al., 2006).

Furthermore, the results demonstrated that NK-lysin peptides contained more than 50% of all AA with a nonpolar feature, whereas polar AA constituted up to 30% of AA. The distribution of positively and negatively charged AA shaped the rest of the proportion. In this regard, these structural conformations might enhance the folding process of NK-lysin after emerging from ribosomes to form secondary structure units with the strong constitution of nonpolar AA, which might force its segregation into the interior of NK-lysin peptide.

As the results of the present study indicated, NK-lysin peptides belong to saposin domain with a slight variation in the domain length. This domain was folded in 4–5 helical bundles as per species with 3 disulfide bonds and 6 cysteine residues that stabilized the peptide conformational structure. These results are consistent with those of other studies concluding that the peptides had 5 helical folded structures in a single globular chain and stabilized disulfide bridges with 6 cysteine residues in pigs. However, human NK-lysin domain is composed of 4 cysteine residues to form two disulfide bonds (Liepinsh et al., 1997; Zhai and Saier, 2000; Olmeda et al., 2013).

Based on their structural content, it seems that they have a fundamental advantage over the antimicrobial effects of NK-lysin and granulysin peptides. This is owing to the fact that they share the same pathways by which AMP inactivate bacteria. This process is interposed by the physical interactions of positively charged peptides with negatively charged cell membrane bilayer phospholipids, thereby leading to membrane disruption (Zasloff, 2002). Owing to this mode of action, these peptides have bactericidal effectiveness with little probability of strong bacterial resistance (Zasloff, 2002).

The results of the present study revealed that the hydrophilic regions and positively charged AA were predominant on the surface area of the peptides and may determine the mode of action of most of the AMP. These results are in agreement with those of other studies, reporting that the intramolecular structures of NK-lysin were bound by 6 cysteine residues through bridge formation to join the units of helical structures 1/4/5 and 2/3.

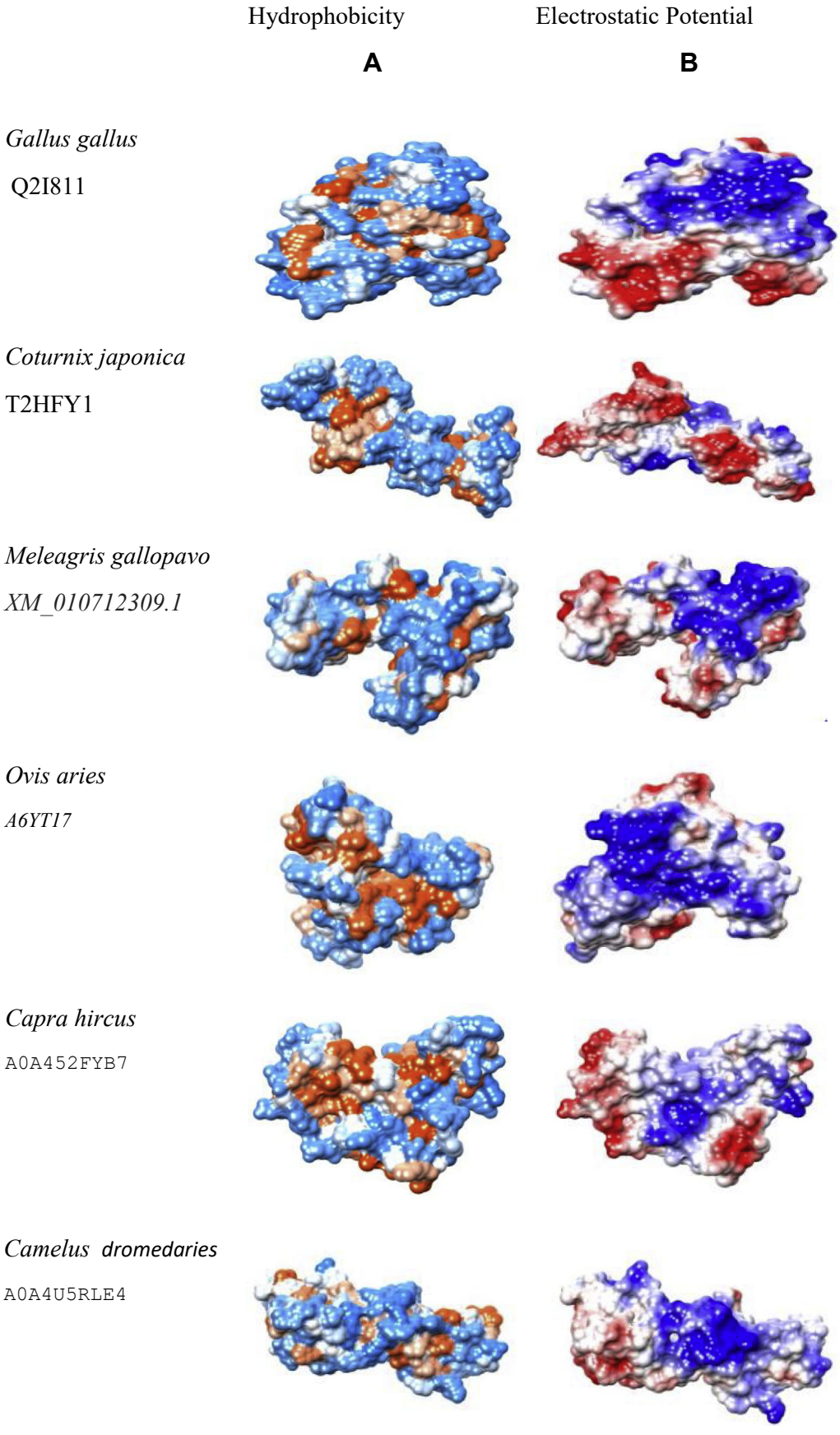


Figure 5. The distribution of hydrophilic, hydrophobic regions, positively, negatively charged AA, neutral residues on the surface of Nk-lysin peptides in different species based on ChimeraX software. (A) represented the hydrophobicity profiling, where blue colors are hydrophilic region, orange are hydrophobic regions, white are neutral. (B) represented electrostatic potentials, where dark blue colors are positively charged residues, orange colors are negatively charged AA, whereas white colors represented neutral regions.

Bos taurus

Q29RG7

Equus caballus

F6VY56

Danio rerio

Q7T3Q1

Homo sapiens

P22749

Sas scrofa

M9TM62

Pan troglodytes

H9YYG7

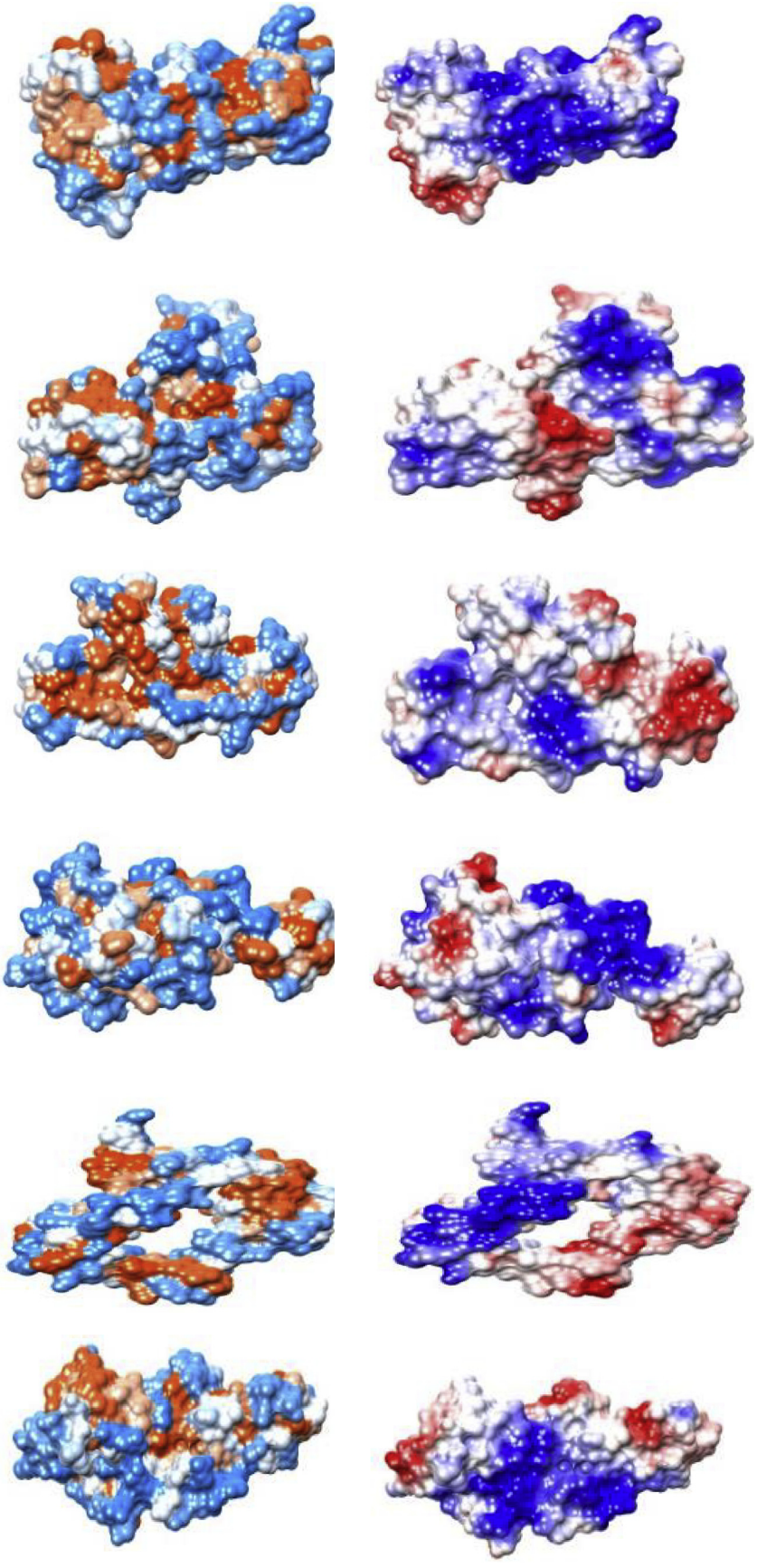


Figure 5. Continued

They also proposed that the mode of NK-lysin peptide action relied on the electrostatic interactions of positively charged AA, especially lysine and arginine, on the surface area with negatively charged lipoproteins in the phospholipid bilayer of the pathogens. This interaction may lead to the formation of pores on the membrane, followed by membrane disruption, in bilayer lipids, thereby resulting in morphologic changes and finally cytoplasmic leakage and cell death (Bruhn, 2005; Olmeda et al. 2013).

In summary, this study determined the mRNA transcription patterns of NK-lysin gene in native chickens. Based on the findings, the mRNA transcripts encoded 140, 137, and 132 aa in chickens, Japanese quails, and turkeys, respectively. However, it varied in other mammalian species. These peptides contained predominant hydrophilic regions and positively charged AA, revealing their biological functions based on the primary structure, domain constitution, 3D structure, and molecular dynamic modeling analysis. Based on the findings, these peptides may be a possible alternative to antibiotics and serve as natural antimicrobial agents that will hopefully produce no resistant bacteria because of their naturally occurring properties.

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