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Complete List of Authors:	Yacoub, Haitham ; King Abdulaziz University El-Hamidy, Salem ; King Abdulaziz University Mahmoud, Maged ; King Abdulaziz University, King Fahd Medical Research Center Baeshen, Mohamed ; King Abdulaziz University Almehdar, Hussein ; King Abdulaziz University Uversky, Vladimir; University of South Florida, Molecular Medicine Redwan, Elrashdy ; King Abdulaziz University Elazzazy , Ahmed ; University of Jeddah, Biological Sciences Department
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Biocidal activity of chicken defensin-9 against microbial pathogens

Haitham A. Yacoub,[1,2,*] Salem M. El-Hamidy,[3] Maged M. Mahmoud,[4,5] Mohamed Nabih Baeshen[1,3], Hussein A. Almehdar,[3] Vladimir N. Uversky,[3,6,7,*] Elrashdy M. Redwan,[3,8,*] , Omar.A.Al-Maghrabi[1] , and Ahmed M. Elazzazy[1,9]

[1]*Biological Sciences Department, Faculty of Science, University of Jeddah, Jeddah, Saudi Arabia & Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, P.O. Box 80203, Jeddah, Saudi Arabia.*

[2]*Cell Biology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Dokki, Gizza, P.O. Box 12622, Egypt*

[3]*Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, P.O. Box 80203, Jeddah, Saudi Arabia*

[4]*King Fahd Medical Research Center, King Abdulaziz University, P.O. Box 80216, Jeddah 21589, Saudi Arabia*

[5]*Department of Molecular Genetics and Enzymology, Davison of Human Genetics and Genome Research, National Research Centre, Dokki, Gizza, P.O. Box 12622, Egypt;*

[6]*Institute for Biological Instrumentation, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia;*

[7]*Therapeutic and Protective Proteins Laboratory, Protein Research Department, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, New Borg EL-Arab 21934, Alexandria, Egypt;*

[8]*Department of Molecular Medicine and USF Health Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, FL, USA;*

[9]*Department of Chemistry of Natural and Microbial Products, Division of Pharmaceutical and Drug Industries, National Research Centre, Dokki, Gizza, P.O. Box 12622, Egypt.*

***Corresponding authors:** Yacoub, H.A. (Haithamyacoub46@gmail.com), Redwan, E.M. (redwan1961@yahoo.com), Uversky, V.N. (vuversky@health.usf.edu).

Abstract

In this study we identified the expression patterns of β-defensin-9 in local chicken from Saudi Arabia, evaluated antimicrobial activities of the synthetic chicken β-defensin-9 (sAvBD-9) against pathogenic bacteria and fungi, and investigated the mode of sAvBD-9 action on the bacterial cells. The *AvBD-9* gene of the local Saudi chicken encodes a polypeptide of 67 amino acids, which is highly similar to the duck, quail, and goose polypeptides (97%, 86%, and 87%, respectively) and shares a low sequence similarity with the mammalian defensins. AvBD-9 is expressed in various organs and tissues of the local chicken and is able to inhibit the growth of both Gram-negative and Gram-positive bacteria, as well as is active against unicellular and multicellular fungi, *Aspergillus flavus*, *Aspergillus niger*, and *Candida albicans*. The sAvBD-9 completely inhibited the growth of both Gram-positive and Gram-negative bacterial strains as well as *Candida albicans*. The haemolytic effects of sAvBD-9 were limited. The morphological analysis by TEM revealed that sAvBD-9 induces shortening and swelling of *Staphylococcus aureus* and *Shigella sonni* cells, opens holes and deep craters in their envelopes, and leads to the release of their cytoplasmic content. Our data shed light on the potential applications of sAvBD-9 in the pharmaceutical industry.

Keywords: defensin; antibacterial peptide; antifungal peptide; synthetic defensin

Introduction

Many representatives of the family of antimicrobial peptides are frequently found in various living organisms including mammalian, avian, plant, and insect species. These peptides display pronounced antimicrobial potentials against broad spectrum of microorganism, such as bacteria, fungi, parasites, and viruses. Such peptides are known now as host defense peptides (HDPs). The name reflects the involvement of these peptides in the immunomodulation, which is a relatively recent addition to their longer known functions related to the control and inhibition of the microbial growth (Cuperus et al. 2013; Sugiarto and Yu 2004). These peptides have a short amino acid chain that is typically less than 100 residues. They are cationic molecules rich in arginine, lysine, and histidine. HDPs are amphipathic entities containing hydrophobic and hydrophilic regions. They serve as ancient defense pathways against various kinds of pathogenic agents. Currently, full sequences and phylogenies of defensin and cathelicidin genes have been established for avian species. In addition, there are several studies aiming to figure out the potential use of these peptides as templates for the novel anti-microbial agents (Cuperus et al. 2013).

Defensins are cysteine-rich antimicrobial peptides with six conserved cysteine residues that form three pairs of disulfide bridges. In vertebrates (including avian species), there are several types of defensins, which are classified into three sub-clusters, α-defensins, β-defensins, and θ-defensins, based on their length and distribution of the cysteine residues that form the disulfide bridges (Sugiarto and Yu 2004; Yacoub et al. 2015; Yacoub et al. 2011). α-Defensins are found in mammals (Lehrer and Ganz 2002), θ-defensins are present in all vertebrates (Klotman and Chang 2006; Lehrer and Ganz 2002), whereas avian species are characterized by the presence of β-defensins (Cuperus et al. 2013). Most defensins, and particularly avian β-defensins, are known to exhibit an effective antimicrobial activity against a broad spectrum of pathogens including bacteria and fungi.

The actual mechanisms defining the ability of these β-defensins to inactive and kill pathogenic agents are not fully understood. However, several studies supported the hypothesis that the action of these peptides is depended on two major features, their cationic and amphipathic properties (Evans and Harmon 1995; Powers and Hancock 2003). Many studies concluded that the action of β-defensins begins with the attachment of this peptide to bacterial membrane through the electrostatic interaction between the negatively charged bacterial membrane and the exposed cationic sites of the β-defensins. These defensin-membrane interactions result in the formation of hollow defensin-based channels penetrating the phospholipid bilayer that eventually leads to the membrane disruption (Brogden 2005; Ganz 2004; Powers and Hancock 2003; Sugiarto and Yu 2004; Zasloff 2002). The aims of the study were to identify the expression patterns of the β-defensin-9 in local Saudi chicken, to evaluate the antimicrobial activities of the synthetic avian β-defensin-9 (sAvBD-9) against pathogenic bacteria and fungi, and to investigate the mode of the sAvBD-9 action on the bacterial cells using transmission electron microscopy (TEM).

Materials and methods

Animals

Fifty healthy local Saudi chicken used in this study were obtained from the King Abdulaziz University farm, Jeddah, Saudi Arabia. The birds were kept in cages and provided with water and food ad libitum.

Tissue collection

Tissue specimens were collected from nineteen organs of the local Saudi chicken including

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 used.

RNA isolation and cDNA synthesis

Total RNAs were isolated from 30-60 mg of bone marrow, spleen, liver, oviduct, ovum, large and small intestines, pancreas, skin, egg yolk, muscles, heart, testis, duodenum, gizzard, uterus, and kidney tissues using EZ RNA Clean Up Plus DNase Kit (EZ BioResearch, St Louis, MO, USA). RNA concentrations were measured using NanoDrop Spectrophotometer (Jenway, UK). Reverse transcriptions (RT) was performed using oligo-dT primers (Bioneer Inc, Daejeon, Republic of Korea) in a 20 µL reaction including 5 µL RNA. The cDNAs obtained were amplified by using PCR Master Mix (Bioneer Inc, Daejeon, Korea) with primers designed by (Ma et al. 2012c) as shown in Table 1. PCR amplification reactions were performed in a 50 µL volume (i.e., 50 ng of template DNA, 10 pmol of each primer, 0.25 U of Taq DNA polymerase, 250 µM of dNTPs mix, 10 µM of Tris-HCl (pH 9.0), 30 µM of KCl, 1.5 µM of MgCl2, and sterile nuclease-free water). PCR amplification was conducted in a thermocycler (Labnet International Inc.). The following cycling conditions were used: pre-denaturation at 94 ºC for 5 minutes, denaturing at 94 ºC for 30 seconds, annealing at 58 ºC for 30 seconds, extension at 72 ºC for 30 seconds for 35 cycles, and a final extension at 72 ºC for 10 minutes. In addition, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. The amplified fragments were analyzed by gel electrophoresis using a DNA ladder in order to assess the size of the amplicons products. The images were obtained using a gel documentation system (Ultra-Violet Products Ltd.). The

size of the amplicons was determined using software available with the gel documentation system.

Cloning

The obtained PCR products were cloned using GenScript's CloneEZ PCR cloning kit (www.genscript.com). After that, the amplified gene was sequencing at (Bioneer Inc, Daejeon, Republic of Korea). The sequencing alignment was done using nucleotide-nucleotide BLAST (BLASTN) software in http://www.ncbi.nlm.nih.gov/blast/ and CLASTALW 2.0.12 to compare the similarities with related avian species. The phylogenetic tree was constructed using MEGA version 6.1 software (Tamura and Nei 1993; Tamura et al. 2013).

Sequence analyses

The order/disorder propensities of the β-defensins-9 from the local chicken (identical to β-defensin-9 or gallinacin-9 from *Gallus gallus*, UniProt ID: Q6QLR1), duck (*Anas platyrhynchos*, UniProt ID: A3FPG7), quail (*Coturnix coturnix*, UniProt ID: D0F1S0), goose (*Anser cygnoid*, UniProt ID: G0Z9C4), rat (*Rattus norvegicus*, UniProt ID: Q32ZI2), mouse (*Mus musculus*, UniProt ID: Q8R2I6), cow (*Bos taurus*, UniProt ID: P46167), and buffalo (*Bubalus bubalis*, UniProt ID: A3RJ39) were evaluated using the PONDR® (Predictor Of Natural Disordered Regions) VSL2 algorithm (Peng et al. 2005), which is one of the more accurate stand-alone disorder predictors (Fan and Kurgan 2014; Peng et al. 2005; Peng and Kurgan 2012). The outputs of the evaluation of disorder propensity are represented by the real numbers between 1 (ideal prediction of disorder) and 0 (ideal prediction of order). An arbitrary threshold of ≥0.5 was used to identify disorder.

In addition to the per-residue disorder distribution in proteins, their overall disorder status was evaluated by the so-called binary disorder classifier, the charge-hydropathy (CH) plot (Oldfield et al. 2005; Uversky et al. 2000), which is the computational tool that evaluate the predisposition of a given protein to be ordered or disordered as a whole. In this approach, the absolute mean charge <R> for each of the β-defensin-9 proteins was calculated as the absolute value of the difference between the numbers of positively charged and negatively charged amino acid residues divided by the total number of amino acids. The hydrophathy of each amino acid residue was computed by the Kyte and Doolittle approximation (Kyte and Doolittle 1982) with a window size of 5 and normalization on the scale from 0 to 1. The mean normalized hydrophathy <H> was defined as the sum of the normalized hydrophobicities of all residues divided by the total number of residues minus 4 (Oldfield et al. 2005; Uversky et al. 2000). The results were presented in a charge-hydropathy plot.

Real time PCR

The constitutive expression patterns of β-defensin-9 in different tissues of local Saudi chicken were performed through the real-time RT-PCR method using SYBR Green qPCR Master mix containing ROX as a reference dye (Biotool LLC, Houston, USA). Total RNAs were isolated from different tissues, then RT was conducted using oligo-dT primers (Bioneer Inc, Daejeon, Republic of Korea) in a 20 μL reaction including 5 μL RNA. The real time PCR was achieved with (Stratagene Mx3005P QPCR Systems, Agilent Technologies, Germany), the cycling conditions used as follows: activation at 95˚C for 10 minutes, denaturing at 95˚C for 15 seconds, annealing/extension at 58˚C for 30 seconds for 40 cycles. The melting was conducted from 95 to 60˚C with read every 0.2˚C and 5 s hold between reads. All amplified fragments were achieved in three independent replicates; in addition, the results were

normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene using comparative C_t method.

Bacterial species

Bacterial species used in the biocidal analysis included both Gram-negative and Gram-positive strains. The five Gram-positive bacterial strains used were: Methicillin-Resistance *Staphylococcus aureus* ATCC 43330 (MRSA), Staphylococcus epidermidis ATCC 12228 (*S. epidermidis*), *Enterococcus faecalis* ATCC 29212 (*E. faecalis*), *Streptococcus bovis* ATCC 49147 (*S. bovis*), and *Micrococcus luteus* ATCC 49732 (*M. Luteus*). Five strains of Gram-negative bacteria involved: *Shigella sonnei* ATCC 25931 (*S. sonnei*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Salmonella typhimurium* ATCC 14028 (*S. typhimurium*), *Klebsiella pneumonia* ATCC 700603 (*K. pneumonia*), and *Escherichia coli* ATCC 25922 (*E. coli*). The bacterial strains were cultured on tryptone soy agar and incubated for 24 h at 37˚C and stored in slants at 4˚C.

Fungal species

As in previous study, three fungal species were used; *Candida albicans* ATCC 10231 (*C. albicans*), *Aspergillus flavus* (*A. flavus*) and *Aspergillus niger* (*A. niger*) isolates (obtained from the Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia. Filamentous fungi and Candida were cultured in Sabouraud dextrose agar and incubated at 25- 28 ºC for 72 h for multicellular fungi and h at 30 ºC for 24 in the case of Candida and stored in slants at 4 ºC.

Chicken β-defensin peptide synthesis

The full amino acid sequence of chicken β-defensin-9 peptide was synthesized and purified to 80 % level using high-performance liquid chromatography (HPLC) by GL Biochem Ltd. (Shanghai, China) (http://www.glschina.com/en/profile) (Table 1). The peptide was checked for endotoxin content according to (Redwan 2012).

Antimicrobial activity assay

Minimal inhibitory concentration (MIC) assays for the peptide were performed by the broth dilution method with Mueller Hinton II broth according to the procedures suggested by the CLSI (Clinical and Laboratory Standards Institute) (CLSI 2009, 2012). In summary, as in previous study, bacteria and candida were grown to reach the exponential phase, which took around three hours. Cells were then centrifuged at 2000×g for 15 min. The resulting pellets were washed and resuspended in 10 mM PBS (pH 7.0). Two-fold serial dilutions of the sAvBD-9 were prepared in appropriate culture medium in 96-microwell plates. Inoculum (100 μL) from the culture with a bacterial density of 106 CFU/ml was added to each individual well containing 100 μL of either M-H alone, or M-H containing two fold diluted peptide to give a final concentration of the peptide ranging from 0-200 μM. Tetracycline was used for bacteria, while and Ketoconazole was used in the case of both yeasts and fungi as a reference. The MIC values were recorded as the minimum concentration that showed no visible growth after overnight incubation at 37˚C.

The minimum inhibitory concentration for the bacteria (MBC) or minimum inhibitory fungicidal concentration (MFC) values were determined by sub-culturing 10 μL from each MIC positive well onto minimal M-H agar. The lowest peptide concentration yielding more than 99% of either bacterial or fungal growth inhibition was noted as the MBC or MFC,

respectively. In case of multicellular fungi *A. niger* and *A. flavus* which used to evaluate the anti-fungal activity of β-defensin-9, overnight cultures of the fungi were prepared by inoculating 100 ml of Sabouraud dextrose broth with a 105 spores/ml concentration.

Kinetics of the microbe inactivation by sAvBd-9

Three organisms, MRSA, and *E. coli* (1×108 CFU/ml) and *C. albicans* (1×105 CFU/ml), were used to study the killing kinetics of sAvBD-9. The sAvBD-9 concentration used in these experiments was 2 times of that of the MIC. Overnight bacterial cultures were prepared. The cultures were spun down and resuspended in fresh Müller-Hinton agar (MH medium) at a concentration of 1×106 CFU/ml. sAvBD-9 was then added to the bacterial suspension, at a concentration equivalent to 2 times that of the MIC. The mixture was incubated at 37 ºC. The colony forming units (CFUs) were determined at 0, 15, 60, 120 and 180 min (Ma et al. 2011). At each time point, 1 μL of the suspension was inoculated into 1 ml of MH medium. Diluted bacterial suspension (50 μL) was plated out and incubated at 37 ºC for 12 h before counting.

Effect of ionic stress on the peptide antimicrobial activity

In order to evaluate the effects of NaCl concentrations on the antibacterial activity of sAvBd-9, E. coli was used. The tested bacteria was subcultured at 37°C to the mid-log phase, and suspended to 106 CFU/ml in MH medium. A suspension of *E. coli* (1ml) was incubated with the different concentrations of peptide (0-200 μg/mL) and the different NaCl concentrations (0, 20, 50, 150 mM) in 10 mM sodium phosphate buffer, pH 7.4. The tested bacteria were cultured at 37°C for 2h before diluting 1000 times followed by plating. Survived bacteria were counted (Baricelli et al. 2015; Ma et al. 2011; Wang et al. 2011; Yacoub et al. 2015).

Hemolysis test

The hemolytic activity of the synthetic defensin was measured according to (Ma et al. 2013; Shin et al. 2001; Yacoub et al. 2015; Yu et al. 2001). Briefly, fresh chicken blood was collected from King Abdulaziz University farm, Jeddah, KSA. The blood was spun down for erythrocytes harvesting by centrifugation (3000 rpm, 10 min, at 20°C). The resulting erythrocytes were then washed twice with sterile PBS at a concentration of 0.5% vol/vol and were used for the assay, by dispensing 90 µL into each well of the 96-well plates. Then, 10 µL of solution containing different peptide concentrations were added to the cells and incubated at 37°C for 2 h. After incubation, the microtiter was spun down at 800×g for 10 min. The supernatants were withdrawn and transferred to a new 96-well plate and checked for released hemoglobin as measured spectrophotometerically at 405 nm. For (0 hemolysis) as well as (100% hemolysis) controls, cells were resuspended in PBS only and in 1% Triton X-100, respectively.

Morphology of the sAvBd-9 nanoparticles

For the transmission electron microscopy (TEM) assay, the β-defensin-9 nanoparticles were suspended in (0.01 %) acetic acid, then treated in an ultrasonic bath (BRANSON, 1510) for 30 min. A small drop of this suspension was placed on carbon-coated copper grid which was dried at room temperature. The specimens were examined with a transmission electron microscope JEM-1011 (JEOL, Tokyo, Japan).

Effects of chicken β-defensin-9 on the bacterial morphology

Cultures of *S. aureus*, MRSA ATCC (43330) and *S. sonnei* ATCC (25931) were grown in MH broth to early exponential phase then harvested by centrifugation. The bacterial pellets

were washed in PBS and resuspended to an OD600 of 0.2. The cell suspension was treated with β-defensin-9 (at 2.5× and 5×MIC). After one-hour of incubation, the cells were centrifuged and washed with PBS, then fixed with 2.5% glutaraldehyde. After fixation, the sample were dropped on carbon coated copper grid and waiting until dry. The grid then was stained with the Phosphotingestic Acid (PTA) stain. Finally, the grid was dried at room temperature and become ready to examine with the transmission electron microscopy (TEM).

Statistical analysis

Data were entered using IBM SPSS Statistics 20, and was analyzed by Kaplan-Meier analysis. A P level of < 0.05 was considered to be significant.

Results

In this study, the transcriptional profile of β-defensin-9 in local chicken from Saudi Arabia and the antimicrobial activity of the synthetic β-defensin-derived peptide-9 (sAvBD-9) against the most frequent human pathogens were characterized. Furthermore, to investigate the effect of this peptide on the morphology of bacterial cells, the electron transmission microscopy (TEM) was used.

Identification and phylogenetic analysis of β-defensin-9

We were able to amplify a DNA fragment of 204 bp that corresponded to a complete nucleotide sequence of the cDNA of β-defensin-9 in local chicken from Saudi Arabia. The amplified fragment was sequenced and deposited in GenBank (accession number, KR136304). The analysis of sequence alignments was used to find the similarities of this β-

defensin-9 from local Saudi chicken with the nucleotide sequences of related avian species using nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast) and CLASTALW 2.12 software. This analysis revealed that the nucleotide sequence of a newly identified β-defensin-9 has a high similarity (99%, 97% and 91%) with the β-defensin-9 sequences of duck, quail, and goose, respectively (Figure 1). Next, the phylogenetic tree was constructed based on the nucleotide sequences of the β-defensin-9 from the local Saudi chicken, the other related avian species, and some mammalian β-defensins-9.

This analysis revealed that the local chicken β-defensin-9 is closely related to the β-defensins-9 from *Gallus gallus* and duck, with all three sequences being clustered in same group as shown in Figure 2. However, β-defensins-9 of two other avian species, quail and goose, were grouped together in a single sub-cluster. The same trend observed in case of mammalian defensins (Figure 2). The multiple polypeptide sequence alignment of the complete amino acid sequence of the β-defensin-9 from the local chicken revealed a highly similarity with β-defensins-9 from duck, quail, and goose (97%, 87%, and 86%, respectively) (see Figure 3).

Intrinsic disorder propensity of β-defensins

The intrinsic disorder propensities of avian and mammalian β-defensins-9 were evaluated by PONDR® VSL2 (Peng et al. 2005) (see Figure 4A). Here, the scores above 0.5 are considered to correspond to the disordered residues/regions. This analysis revealed that β-defensins-9 are expected to be rather ordered, a conclusion illustrated by Figure 4A representing the aligned PONDR® VSL2-based disorder profiles for various β-defensins-9. It is also seen that although these proteins are predicted to be mostly disordered, some of them possess disordered or flexible regions. Figure 4A also illustrates that based on the peculiarities of

their per-residue disorder propensities, β-defensins-9 analyzed in this study can be grouped into three sets. Here, β-defensins-9 from chicken, duck, quail, and goose have rather similar disorder profiles and form one group, β-defensins-9 from rat and mouse are also characterized by comparable disorder profiles and form another group, with the third group being formed by β-defensins-9 from cow and buffalo. Close similarities of disorder profiles within these groups suggest that the group-conserved disorder-related sequence features can have functional implications. The most ordered N-terminal parts of the avian β-defensins-9 and defensins from mouse and rat correspond to the signal peptide (residues 1-19).

One of alternative approaches for the evaluation of the disorder propensity of a protein is based on classifying an entire protein as mostly ordered or disordered based only two parameters of a particular amino acid sequence – absolute mean net charge and mean hydrophobicity (Oldfield et al. 2005; Uversky et al. 2000). The resulting plot of the absolute mean net charges of proteins <R> against their mean hydropathy <H> represents a charge-hydropathy plot (CH-plot) which shows that ordered and disordered proteins tend to occupy two different areas within the charge-hydrophobicity phase space, separated by an estimated boundary line, $<R> = 2.785<H> - 1.151$ (Uversky et al. 2000). It was also pointed out that the CH-plot is able to discriminate proteins with substantial amounts of extended disorder (random coils and pre-molten globules, which are located above the boundary) from proteins with globular conformations (molten globule-like and ordered globular proteins, which are positioned below the boundary) based on charge and hydropathy of their sequences (Oldfield et al. 2005). Figure 4B shows that all β-defensins-9 are located below the boundary separating compact proteins and extended disordered proteins and are, therefore, predicted to have compact structures. In addition, all the β-defensins-9 are positioned deep within the area corresponding to compact/ordered proteins and is characterized by high mean hydropathy. Furthermore, Figure 4B shows that the hydropathy is unequally distributed within the β-

defensin-9 sequence, with the N-terminally located signal peptide being the most hydrophobic part of this protein.

Tissue distribution and expression of β-defensin-9

To characterize the expression patterns of β-defensin-9 within the local chicken, a wide range of its tissues were collected. The real time RT-PCR was used to determine the expression levels of β-defensin-9 in local chicken (Figure 5). In our study, we used the baseline 1 or -1, therefore the range of changes in the expression level was defined to be between +X and +1 when the gene was up-regulated and between -X and -1 when it was down-regulated. Figure 5 shows that the β-defensin-9 gene is highly expressed in sexual organs, especially in testis and ovum. Furthermore, the β-defensin-9 transcripts were found in the gastrointestinal tract. The highest levels of β-defensin-9 expression were found in several tissues, such as liver, large intestine, muscle, and gizzard, whereas a moderate expression was recorded in stomach, ovum, duodenum, lungs, uterus, testis and kidney. Finally, no expression β-defensin-9 was found in spleen, oviduct, bone marrow, skin and heart.

The antimicrobial activity of sAvBD-9

A significant antimicrobial activity of the synthetic β-defensin-derived peptide (sAvBD-9) was observed against all bacterial pathogens tested in this study (Table 2). This analysis revealed that the sAvBD-9 had an ability to inhibit both Gram-negative and Gram-positive bacteria, being present in the medium at low concentrations. Specifically, the efficiency of sAvBD-9 against *S. sonnei* was characterized by the MIC of 3.2 μg/ml and the MBC of 6.25 μg/ml, whereas for *K. pneumonia, E. faecalis*, and *S. aureus* (MRSA) the MIC and MBC were of 6.25 μg/ml and 6.25 μg/ml, respectively.

Furthermore, it is worthy to mention that sAvBD-9 exerted effective toxicity against the three fungal species analyzed in this study (Table 2), *A. flavus* (MIC 3.2 µg/ml and MFC 3.2 µg/ml) than *A. niger* (MIC 3.2 µg/ml and MFC 6.25 µg/ml) and *C. albicans* at (MIC 6.25 µg/ml and MFC 6.25 µg/ml).

Kinetic characterization of the sAvBD-9 biocidal activity

The kinetics of the sAvBD-9 killing efficacy was determined for the representative organisms from both Gram-positive and Gram-negative bacterial strains, as well as the fungal *C. albicans* strain (Figure 6). These analyses revealed that the sAvBD-9, being present at the concentration of 25 µg/L, was a fast and efficient killer of fungi, Gram-negative and Gram-positive bacteria, reducing cell survival within the first hour to below the detection limit of 100 cells/ml. At the second hour, the peptide exhibited higher antimicrobial efficacy against of MRSA strain than against other tested organisms (P<0.001). Finally, sAvBD-9 was more effective in reduction of the survival levels of the Gram-positive bacteria than of the Gram-negative bacteria and fungi.

Effect of ionic stress on the sAvBD-9 antimicrobial activity

The antibacterial effect of the sAvBD-9 against tested bacteria was evaluated in the presence of 0, 12.5, 25, 50, 100, and 150 mM NaCl at different concentrations of tested peptide (0, 12.5, 25, 50, 100, 150, and 200 µg/mL) as depicted in (Figure 7). This analysis revealed that the sAvBD-9 had similar bactericidal effect against tested bacteria at concentrations ranged from 0 to 50 mM NaCl. In contrast, the antimicrobial activity of this synthetic peptide was significantly reduced in the presence of 100 and 150 mM of NaCl (P < 0.001).

The haemolytic activity of sAvBD-9

The haemolytic activity of the synthetic peptide was evaluated using the freshly isolated chicken erythrocytes that were incubated with the sAvBD-9 peptide. The hemolysis was observed by measuring the absorbance of the released hemoglobin at 400 nm as noticed in Figure 8. The sAvBD-9 peptide displayed a limited haemolytic effect against chicken erythrocytes at different peptide concentrations.

Effect of chicken β-defensin-9 on bacterial cell morphology

Figure 9 shows that according to the TEM analysis, the size of the avian β-defensin-9 peptide particles does not exceed 10 nm as pointed by arrows. Then we monitored the ultrastructural changes of *S.aureus* (MRSA) and *S. sonnei* cells upon exposure to β-defensin-9 at 2.5× and 5×MIC. Untreated individual *S.aureus* and *S. sonnei* cells appeared spherical and rod shaped with smooth and intact cell membranes, and no cell damage was obvious in the control samples (see Figures 10a, 11a). The abilities of the synthetic peptide to induce damage of the bacterial cell membrane and to promote cell lysis were observed through the TEM analysis (Figures 10 and 11). At the concentration of 2.5×MIC, the defensin was able to induce significant deterioration of the microbial cell membrane ultrastructure (Figures 10b and 11b). The increase of the sAvBD-9 concentration to 5×MIC promoted extensive damage to the cell wall, loss of cell walls, decrease in the cell volume, and other alterations in the cellular morphology (Figures 10c and 11c). At concentrations of 5×MIC, the evanescence of cytoplasm constituents was very clear for both Gram-positive and Gram-negative bacteria.

Discussion

The excessive and uncontrolled use of antibiotics in the treatment of infectious disease has resulted in the emergence of highly multi-drug resistant bacteria insensitive to antibiotics, thus creating a major problem to the public health at large. For this reason, finding suitable alternatives to antibiotics has been sought for a long time.

One of the possible natural substitutions includes the use of the host defense peptides (HDPs) that have a fundamental role in the innate immunity in several vertebrates and invertebrates. They have also shown to have immunomodulatory functions. One thousands of HDPs have been discovered so far. Three of those HDPs are related to chickens, β-defensins, cathelicidens, and liver antimicrobial peptide-2.

We conducted this study to identify the transcriptional profile of the β-defensin-9 in local chicken from Saudi Arabia, to determine the antimicrobial activities of the derived synthetic (sAvBD-9) peptide against pathogenic bacteria and fungi, and to investigate the mode of action of sAvBD-9 on bacterial cells using transmission electron microscopy.

We report here the results of the transcriptional profiling of the β-defensin-9 in local chicken from Saudi Arabia. The results of the multiple nucleotide sequence alignments using BLAST (http://www.ncbi.nlm.nih.gov/blast) and CLASTALW 2.12 indicated that the β-defensin-9 sequence of the local chicken is highly homologous to other avian defensins, such as duck, quail, and goose. At the protein level, the β-defensin-9 polypetide from the local chicken is identical to the duck β-defensin-9 and has high similarity to the quail peptide followed by the goose counterpart. These findings are in agreement with the results of earlier studies on duck, goose, and quail β-defensins-9 (Lu et al. 2014; Ma et al. 2012a; Ma et al. 2011; Ma et al. 2008; Ma et al. 2012b; Ma et al. 2012c; Zhang and Sunkara 2014).

Our analysis also revealed that the β-defensin-9 from local chicken has four exons region and the corresponding transcipt contains six highly conserved cysteins, starts with the highly hydrophobic signal peptide at N-terminus (residues 1-19) and terminates as a C-terminally-located mature peptide. This peptide structure could be related to its antimicrobial activity as reported in (Zasloff 2002). The phylogenetic analysis of the local chicken β-defensin-9 indicated that this peptide is grouped with the duck protein and forms the sub-cluster with quail and goose counterparts. This analysis also demonstrated the presence of very close ancestor and evolutionary relationships between the avian β-defensins-9. In contrast, this peptide shared a very low homology with mammalian defensins as was previously reported for the most of the avian β-defensins-9 (Cormican et al. 2008; Cuperus et al. 2013; Lu et al. 2014; Xiao et al. 2004).

Analysis of the intrinsic disorder predisposition of various defensins revealed that although all of them are expected to be mostly ordered, the disorder propensity and the sequence charge/hydropathy are unequally distributed within the β-defensin-9 sequence, with the N-terminally located signal peptide being the most ordered and highly hydrophobic part of this protein.

The high overall hydropathy of the full-length avian β-defensins-9 is expected to make them insoluble. It is also expected that the unequal distribution of the disorder propensity, hydropathy, and charge within these proteins would force them to form micelle-like structures, with the N-terminal signal peptides being located within the core of the resulting particles, and with the less hydrophobic and more charged/polar C-terminal regions being solvent-exposed and located on the surface of these particles.

Analysis of the tissue distribution of the β-defensin-9 of local chicken revealed that peptide was expressed in different organs and tissues, and these results were comparable with the

results of earlier studies (Higgs et al. 2005; Lynn et al. 2004; Ma et al. 2008; van Dijk et al. 2007; Zhao et al. 2001). The strong expression the β-defensin-9 was found in liver, large intestine, muscle, gizzard, pancreas, and kidney (Lynn et al. 2004; van Dijk et al. 2007; Xiao et al. 2004). Female and male reproductive tracts were characterized by a moderate expression of this protein, whereas the lymphoid organs possessed low to moderate levels of the β-defensin-9 (Cuperus et al. 2013; Lynn et al. 2004; van Dijk et al. 2007; Xiao et al. 2004; Zhao et al. 2001). Such tissue/organ distribution of defensins has biological meaning since it is well-known that these antimicrobial peptides might be expressed constitutively or be inducible as a response to the invading pathogen, and the site of their production is controlled by their secretions (Ma et al. 2011; Michailidis et al. 2012; Milona et al. 2007; Zhao et al. 2001).

We also revealed that the synthetic avian the β-defensin-9 (sAvBD-9) displayed broad-spectrum antimicrobial activities against both Gram-positive and Gram-negative strains in comparison with the conventional antibiotics. Our results are consistent with other reports in respect with the antimicrobial activity of various avian β-defensins (Evans et al. 1995; Evans et al. 1994; Harwig et al. 1994; Higgs et al. 2005; Lynn et al. 2004; Ma et al. 2012a; Ma et al. 2011; Ma et al. 2012b; Ma et al. 2012c; Soman et al. 2010; Thouzeau et al. 2003; van Dijk et al. 2008; Wang et al. 2010; Zhang et al. 2011; Zhang et al. 2012). For example, the β-defensins-2 and 7 from ostrich were shown to exhibit strong antibacterial activities against both Gram-positive and Gram-negative bacteria (Lu et al. 2014).

Similarly, the duck-derived recombinant β-defensin-2 showed a potent bactericidal potential against *P. multocid* and *S. aureus*. However, this peptide exhibited weak potential against *S. choleraesuis* and *E. coli*, and similar trends were reported for other defensins, which were either chemically synthesized or produced recombinantly (Evans et al. 1994;

Harwig et al. 1994; Higgs et al. 2005; Ma et al. 2008; Milona et al. 2007; Sugiarto and Yu 2004; Thouzeau et al. 2003; van Dijk et al. 2007; Yu et al. 2001).

Interestingly, besides its antibacterial effects, the sAvBD-9 analyzed in our study exhibited a prominent fungicidal activity against both unicellular and multicellular fungi. The high levels of the fungicidal activity reported in our study are comparable with the previous reports showing that the sAvBD-4 and 10 had significant anti-fungal potentials against several fungi species. However, the sAvBD-9 fungicidal effects have not been studied before. Overall, our results of the analysis of the biocidal potential of the sAvBD are consistent with the earlier reports which demonstrated that the defensin-like proteins where able to display a strong inhibitory potential against human and plant infectious agents (Lacadena et al. 1995; Marx 2004; Meyer 2008; Meyer and Stahl 2002).

The kinetic analysis of the killing potential of the sAvBD-9 peptide against MRSA, *E. coli* and *C. albicans* revealed that this defensins was noticeably more efficient against MRSA than against two other pathogens, *E. coli* and *C. albicans*. The sAvBD-9 activity was confirmed to be lethal for MRSA, which could not be revived after the 3-hour-exposure to the peptide, suggesting its sufficient antibacterial potency. The variations in the kinetics describing the peptide antibacterial activity may be determined by its high cationicity (pI 9.75) and distinguished hydrophobicity (0.38), as well as by the types of bacteria used. The aforementioned intrinsic structural characteristics of the β-defensin-9, such as high hydrophobicity, high cationicity, high pI values (>9), and noticeable helical propensity are known to be important determinants of the antimicrobial potency of protein and peptides (Redwan et al. 2014). Similar data were reported for pheasant cathelicidin-1 (Pc-CATH1) that inhibited *E. coli* growth at the first hour and the bacterial growth was not resumed after 6 hours (Wang et al. 2011).

Although the sAvBD-9 was resistant to salt up to 50 mM NaCl, it lost its bactericidal and fungicidal potentials in the presence of higher NaCl concentrations. These effects of salinity on the sAvBD-9 activity are in agreement with other reports on most tested defensins and other antimicrobial peptides (Bals et al. 1998; Porter et al. 1997; Veldhuizen et al. 2008; Zucht et al. 1998). Results reported earlier for three goose defensins revealed that their antimicrobial activity against both Gram-negative and Gram-positive bacteria was efficiently inhibited by sodium chloride (Ma et al. 2011).

Furthermore, beside the significant antimicrobial potential of sAvBD-9, this peptide displayed low haemolytic activity and low toxicity against animal cells. These findings are in agreement with the results of earlier investigations of other avian β-defensins and their mammals counterparts (Ma et al. 2011; Ma et al. 2012b; Ma et al. 2012c; Milona et al. 2007; Veldhuizen et al. 2008). In fact, other reports indicated that the avian defensins and their derivatives/counterparts typically possessed low cytotoxic activity toward the animal cells. This potentiality was attributed to the higher levels of cholesterol in the membranes of these cells and to the lack of negatively charged molecules incorporated into the phospholipid bilayer of animal membranes (Ishitsuka et al. 2006; Lu et al. 2014; Ma et al. 2013; Matsuzaki et al. 1995).

Nowadays, the bioactive peptides that are derived from the natural compound have received essential attention especially in their relation to the public health, due to the safety and low level of side effects of these natural biocides. Several reports indicated that the electron microscopy can provide some relevant information on the peptide-induced damage in the bacterial cell membranes (Cellini et al. 2008; Hillier and Baker 1946, 1947; Mcfarlane 1949). The modes of the antibacterial action of such biocidal peptides are based on the type of the bacterial organisms, being especially sensitive to the structure of the cell wall and the outer membrane arrangement. Some peptides may be able to penetrate the bacterial cell

membrane and successfully led to the release of the cytoplasmic contents into the surrounding environment. We observed this mode of the sAvBD-9 action for both Gram-positive and Gram-negative bacteria.

The fact that sAvBD-9 and other synthetic or recombinant defensins have strong biological activity in the aggregated state is of special importance. In fact, in the protein literature, there is a strong believe that the non-specific protein or peptide aggregation are processes with strong negative connotation typically related to dysfunction. Data reported in our work and in earlier studies suggest that such non-specific aggregation of defensins might be beneficial. Díaz-Visurraga *et al*. reported that the nanoparticles are expected to generate higher local concentrations of charged particles at the bacterial membrane, thus enhancing the antimicrobial activity (Díaz-Visurraga et al. 2010).

Conclusions

In local chicken from Saudi Arabia, the coding region of β-defensin-9 contained 204 bp and encoded a 67 amino acid-long polypeptide. The amino acid sequence of this β-defensin-9 possessed a highly similarity to the analogous peptides from duck, quail, and goose (97%, 86%, and 87%, respectively). In contrast, this peptide shared a low homology with mammalian defensins. Our analysis revealed that the β-defensin-9 was expressed in various organ and tissues of local chicken. The antimicrobial activity of synthetic chicken β-defensin-9 was studied against a wide set of infectious pathogens, including bacterial and fungal species. This peptide exhibited high antimicrobial activities against a broad spectrum of Gram-positive and Gram-negative bacteria representing concern to the public health. Interestingly, besides its broad spectrum of antibacterial activities, synthetic AvBD-9 has shown profound fungicidal potential against *C. albicans*, *A. flavus*, and *A. niger*. The analysis

revealed that the synthetic AvBD-9 was very efficacious anti-fungal agents causing more than 98% reduction in the fungi survival as compared to the control. The peptide was resistant to salt concentrations up to 50 mM NaCl, but lost its antimicrobial potential in a milieu containing higher salt concentrations (100 mM and 150 mM NaCl). This peptide displayed low haemolytic effects. Overall, our analyses showed that the synthetic avian β-defensin-9 has a potent antimicrobial activity against a broad spectrum of pathogens. Therefore, such peptides should be analyzed in more detail for their full potentials of the disease treatment in both humans and animals. It is likely that these bioactive peptides represent a possible alternative to antibiotics and can serve as natural antimicrobial agents. The hope also is that due to their naturally occurrence and natural properties, these peptides would not provoke bacterial resistance.

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Table 1. Primers and peptide sequences of chicken β-defensin-9 gene

mRNA	Forward Primer	Reverse Primer
AvBD-9-RT-PCR	5'- atgAGGAATCCTTTTCTTCCTTGTTGC -3'	5'- TTAGGAGCTAGGTGCCCATTTGCAGC -3'
AvBD-9-RT-PCR	5'- GCTTACAGCCAAGGAGATGCT-3'	5'-GGAGCTAGGTGCCCATTTGCA -3'
GAPDH ((Achanta et al. 2012))	5'- GCACGCCATCACTATCTTCC-3'	5'- CATCCACCGTCTTCTGTGTG-3'
sAvBD-9	MRILFFLVAVLFFLFQAAPAYSQEDADTLACRQSHGSCSFVACRAPSVDIGTCRGGKLKCCKW APSS	

RT-PCR: real time PCR; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; sAvBD-9: synthetic chicken β-defensin-9.

Table.2. The antimicrobial activities of synthetic chicken β-defensin-9- derived peptide

Organisms	**sAvBD-9**		**Tetracycline**	
Bacteria	MIC (µg/ml)	MBC or MFC (µg/ml)	MBC or MFC (µg/ml)	MIC (µg/ml)
S. bovis ATCC (49147)	6.25 ± 6.9	12.5± 1.7	25±0.0	12.5± 3.46
S. epidermidis ATCC (12228)	6.25 ± 3.0	12.5± 1.7	12.5±0.0	12.5± 1.73
S. aureus MRSA ATCC (43330)	6.25 ± 3.0	6.25 ±1.7	25± 1.73	25±0.0
E. faecalis ATCC (29212)	6.25 ± 1.53	6.25 ± 0.0	25±0.0	6.25±0.0
M. luteus ATCC (49732)	6.25 ± 1.15	12.5± 1.73	12.5±0.0	6.25±0.0
E. coli ATCC (25922)	6.25 ± 1.73	12.5± 1.73	12.5± 1.73	6.25±0.0
P. aeruginosa ATCC (27853)	6.25 ±6.93	12.5± 1.73	12.5± 1.73	12.5± 1.73
S. typhimurium ATCC (14028)	6.25 ± 3.0	12.5± 0.0	25±0.0	12.5±0.0
K. pneumonia ATCC (700603)	6.25 ± 3.46	6.25 ±0.0	25±0.0	6.25±0.0
Sh. sonnei ATCC (25931)	3.2 ± 1.73	6.25 ± 1.7	12.5±1.0	6.25±0.0
Fungi species			**Ketoconazole**	
C. albicans ATCC (10231)	6.25 ± 1.7	6.25 ± 1.73	10±0.0	10±0.0
A. flavus	3.2 ± 0.0	3.2 ± 1.73	10±0.0	10±0.0
A. niger	3.2 ± 0.58	6.25 ± 0.58	10±0.0	>10±0.0

sAvBD-9: synthetic chicken β-defensin-9; MIC: Minimal inhibition concentration; MBC: Minimal bacterial concentration; MFC: Minimal Fungal concentration.

All experiments were performed at least 3-times in doublets.

Figure legends

Figure 1. CLASTALW 2.0.12 multiple sequence alignment based on β-defensin-9 of local chicken (KR136304) and other avian species [(*Anas platyrhynchos* (mallard Duck, EF431957), *Coturnix coturnix* (quail, GQ985499), *Anser cygnoides* (goose, HQ909023)].

Figure 2. Phylogenetic relationship among β-desensin-9 of Saudi chicken, other related avian species and some of mammalian β-defensin-9. The phylogeny was inferred by using the Maximum likelihood method based on the Tamura-Nei model.

Figure 3. Amino acids sequence alignment of β-defensin-9 of Saudi chicken with related avian species. The signal and mature peptides of β-defensin-9 are underlined and the six conserved cysteines residues (C) are underlined in bold. The differences among avian species are highlighted in yellow.

Figure 4. Evaluating intrinsic disorder propensity of different defensins using the pre-residue disorder predictor PONDR® VSL2 (A) and a binary disorder predictor CH-plot (B).

Figure 5. The expression patterns of β-defenisn-9 in local chicken in different tissues. The expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase GAPDH as a reference gene. All assays were performed in three independent experiments and each point is the mean ± SE. The baseline is being 1 or -1, since the range is defined to be between +X and +1 for the up-regulated genes, -1 and -X for the down-regulated genes.

Figure 6. The kinetic inactivation of synthetic chicken β-defensin-9 peptide (sAvBD) against MRSA, *E. coli* and *C. albicans* species. All assays were performed in three independent experiments and each point is the mean ± SE ($P < 0.001$).

Figure 7. Effects of salinity on the antibacterial activity of synthetic chicken β-defensin-9-derived peptide (sAvBD9) against *E.coli*. All assays were performed in three independent experiments and each point is the mean ± SE ($P < 0.001$).

Figure 8. Heamolytic activities of synthetic chicken β-defensin-9-derived peptide (sAvBD-9) using the freshly isolated chicken erythrocytes. All assays were performed in three independent experiments and each point is the mean ± SE.

Figure 9. TEM micrographs of synthetic avian β-defensin-9 that exploit its size as marked by arrows.

Figure 10. TEM micrographs of bacteria treated with synthetic avian β-defensin-9 a) *S. aureus MRSA ATCC (43330)* as a control, bar 1 µm; b) *S. aureus MRSA* treated with 2.5×MIC, bar 0.2µm; c) *S. aureus MRSA* treated with 5×MIC, bar 0.2µm. Here, the arrows point to the damage of the bacterial cell membrane that promotes the cell lysis and leakage of the cytoplasmic continents.

Figure 11. TEM micrographs of bacteria treated with synthetic avian β-defensin-9 a) *S. sonnei ATCC (25931)* as a control, bar 0.2µm; b) *S. sonnei* treated with 2.5×MIC, bar 0.5µm; c) *S. sonnei* treated with 5×MIC, bar 0.5µm. Here, the arrows point the damage of the bacterial cell membrane that promotes the cell lysis and leakage of the cytoplasmic continents.

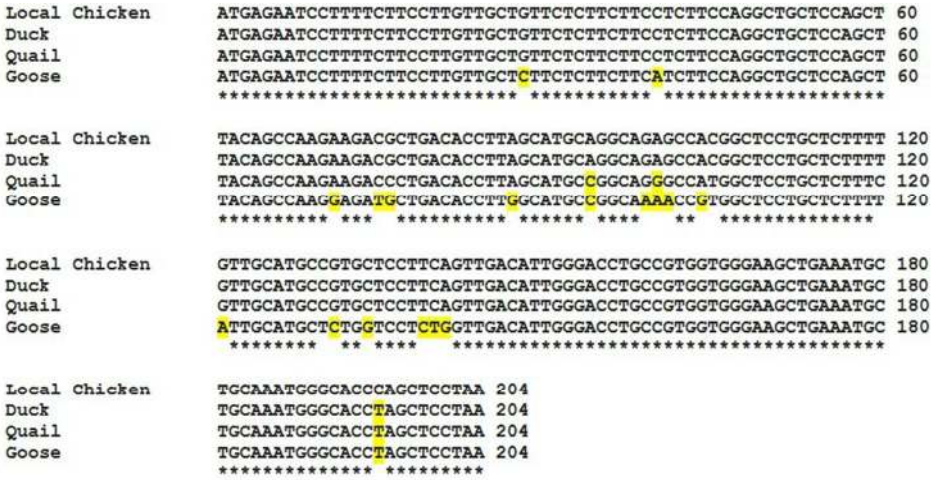

Figure 1
152x73mm (150 x 150 DPI)

Figure 2
82x78mm (300 x 300 DPI)

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Local Chicken   MRILFFLVAVLFFLFQAAPAYSQEDADTLACRQSHGSCSFVACRAPSVDIGTCRGGKLKC 60
Duck            MRILFFLVAVLFFLFQAAPAYSQEDADTLACRQSHGSCSFVACRAPSVDIGTCRGGKLKC 60
Quail           MRILFFLVAVLFFLFQAAPAYSQEDPDTLACRQGHGSCSFVACRAPSVDIGTCRGGKLKC 60
Goose           MRILFFLVALLFFIFQAAPAYSQGDADTLACRQNRGSCSFIACSGPLVDIGTCRGGKLKC 60
                ********* *** ********* * *******  ***** **  * **************

Local Chicken   CKWAPSS 67
Duck            CKWAPSS 67
Quail           CKWAPSS 67
Goose           CKWAPSS 67
                *******
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Figure 3
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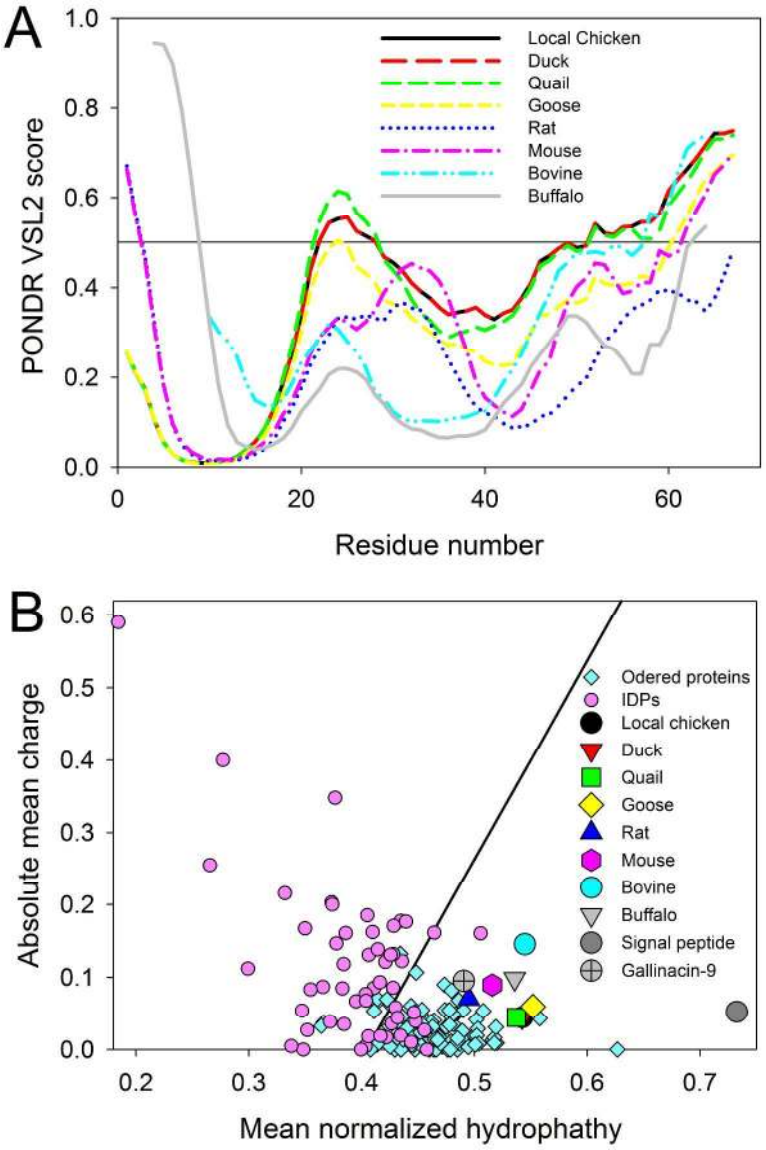

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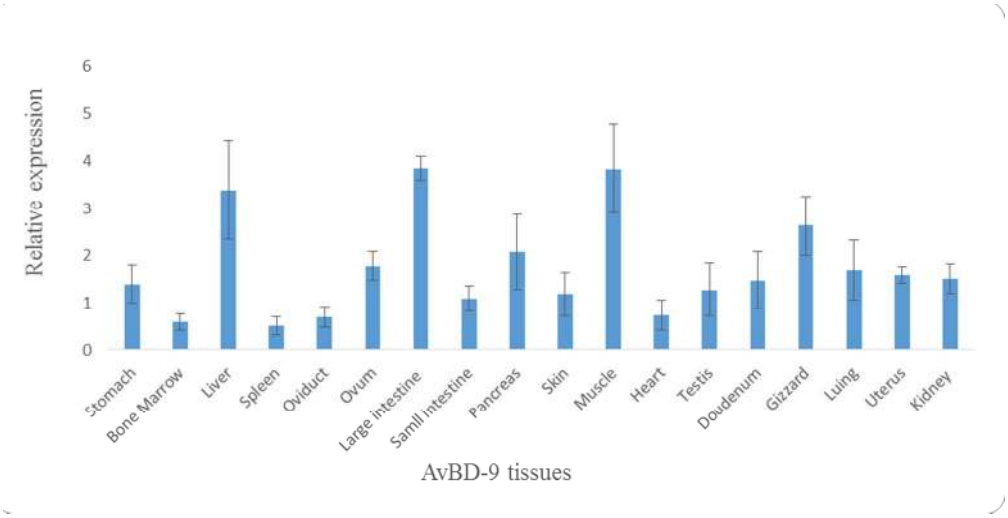

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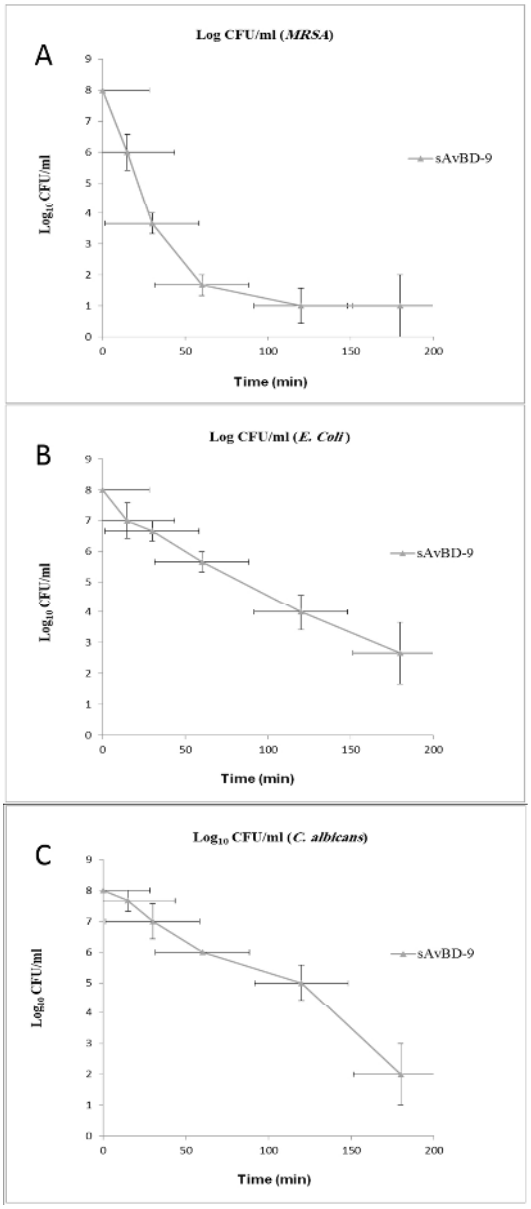

Figure 6
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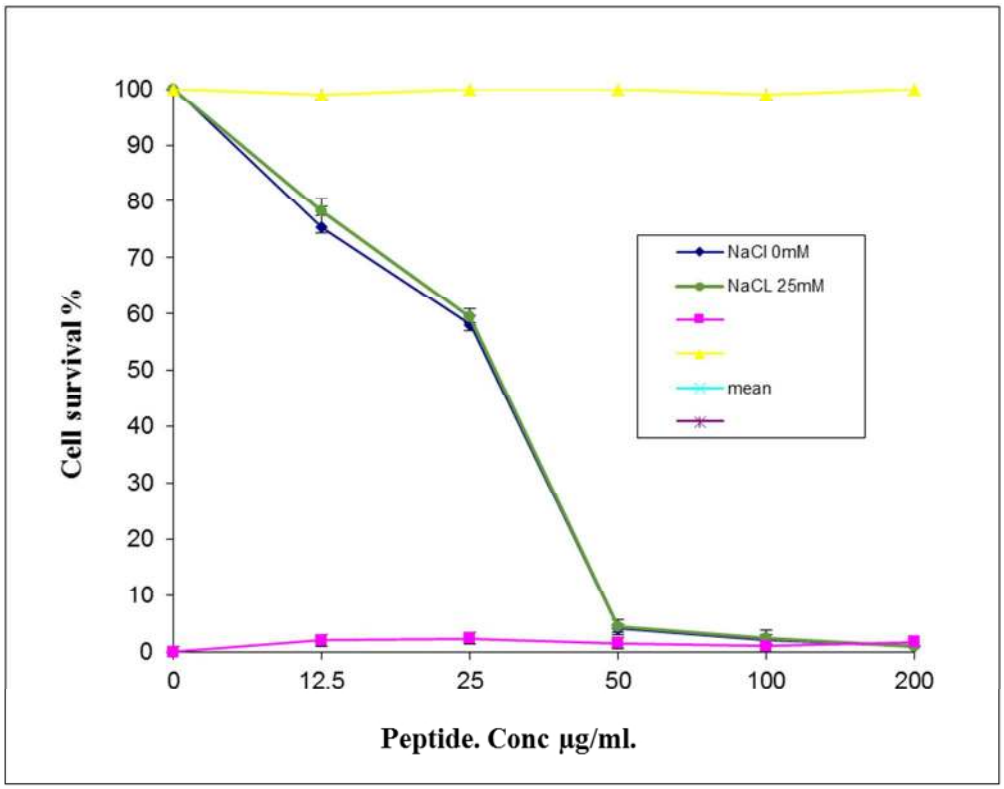

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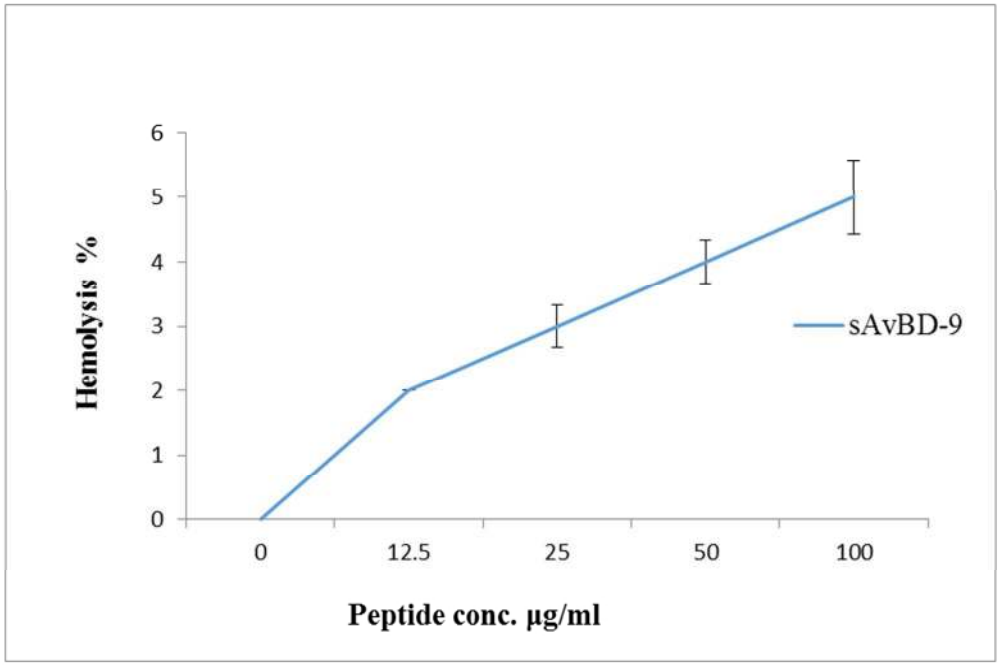

Figure 8
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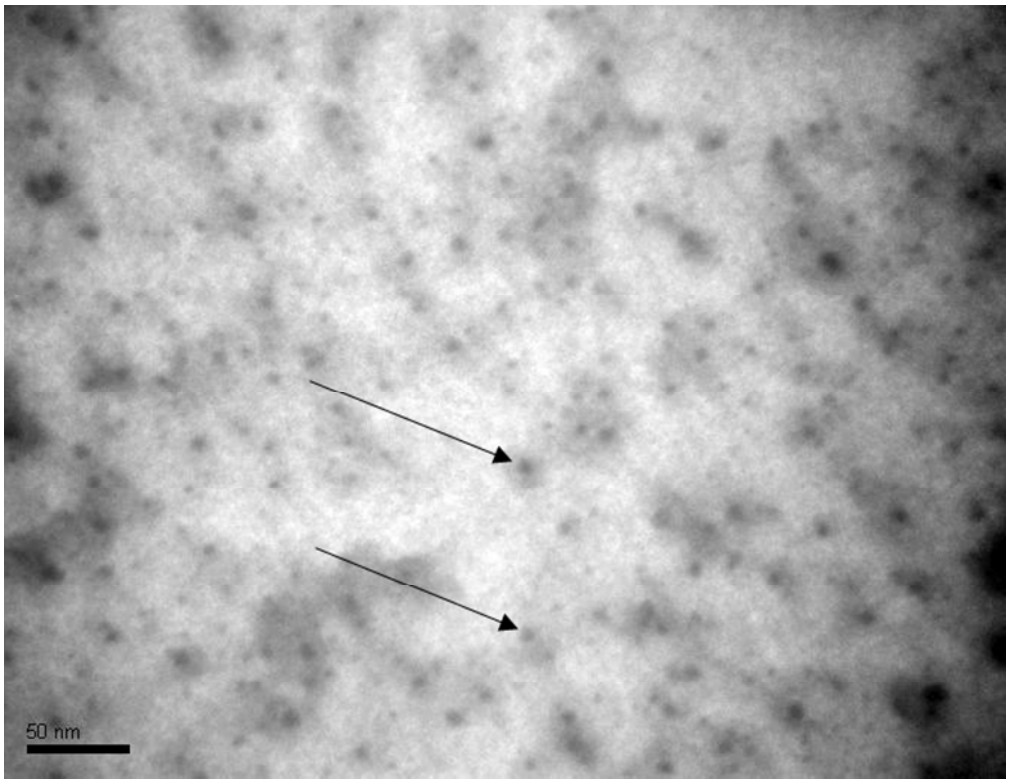

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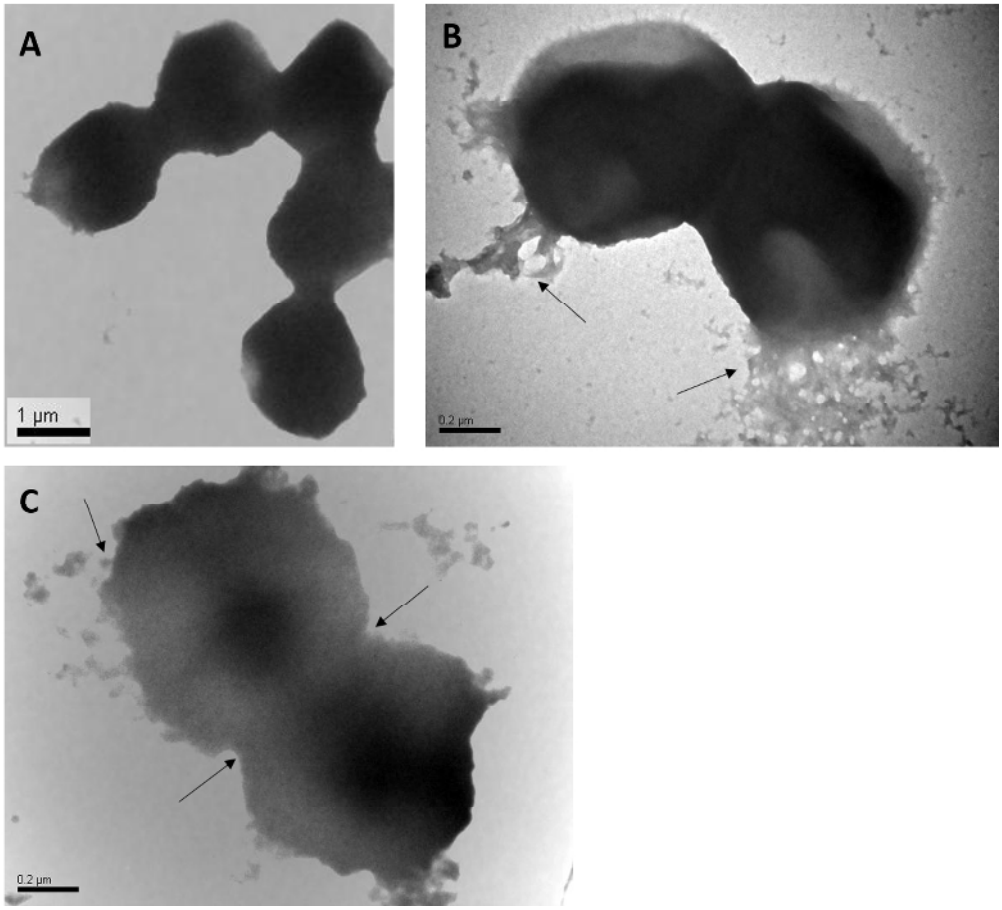

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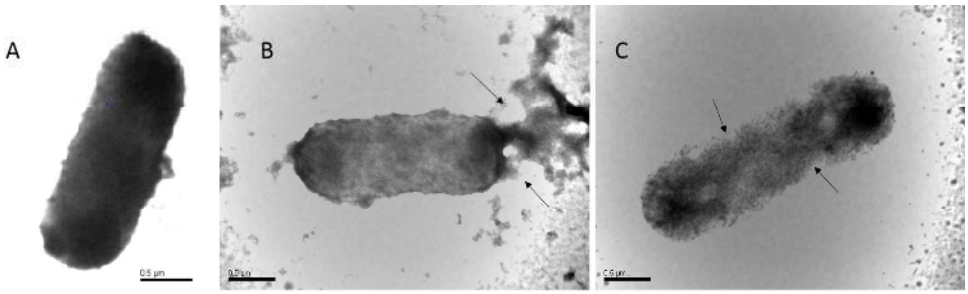

Figure 11
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