The antimicrobial potentialities of (Nk-lysin peptides of chicken, bovine, and human) against bacteria and *rotavirus*

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Abstract

For the first time, this study was carried out to investigate and evaluate the relative antibacterial activity of three different Nk-lysin peptides from human, chicken, and bovine activity compared to Gram-negative and Gram-positive bacteria as well as antiviral activity against *rotavirus* (strain SA-11) and finally mechanisms of action optionality. This report is the first of its kind that investigates the increased antimicrobial ability of (Nk-lysin + AgNPs) and (Nk-lysin + human IL-2) combinations against *S. typhi* activity by carrying out direct comparison under similar experimental settings. Our results showed that gram-negative and gram-positive microorganisms, including *Streptococcus pyogenes*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Shigella sonnei*, *Klebsiella pneumoniae* and *Salmonella typhimurium*, are susceptible to NK-lysin treatment. It was shown in our findings that there was equal potentiality in mixture (Nk-lysin + AgNPs) and (Nk-lysin + human IL-2) for preventing the growth of *S. typhi*, however, when added together, there was minor increase in the level of action. In our study, the TOHO-1 gene was absent in treated bacteria. Following treatment with Nk-lysin peptides, the beta-lactamases genes (CTX-M-1, M-8, and M-9) were not found in any bacterial strains. The examination did not find any of the plasmid mediated quinolone resistance genes in the bacterial strains as a response to NK-lysin treatment. Nonetheless, no study has been carried out in the past that characterized the antiviral activity of bovine, human and chicken Nk-lysin peptides. Hence, this is the foremost study on the enhanced antimicrobial activity of human, bovine and chicken Nk-lysin peptides against *Rotavirus* (strain SA-11). The findings of the study demonstrated that the powerful antiviral activities were exhibited by Nk-lysin peptides against *Rotavirus* (strain SA-11). Based on the comparison between these peptides, it can be concluded that there is an evident potent antiviral activity of bovine Nk-lysin against *Rotavirus* (strain SA-11) as it restrains infection by up to 90%. However, growth was restricted by 80% by chicken Nk-lysin and by 50% by the human peptide.

Introduction

The antimicrobial peptides (AMPs) are immune response molecules have been found in robust organisms and they are playing a fundamental role in first line of defense against invading pathogens to inhibit their fitness properties, moreover, they are also bearing immune-modulatory functions (Sugiarto and Yu, 2004). These The NK-lysin and granulysin are a cationic peptide with antibacterial activity that was firstly identified from pig gastrointestinal tract (Wang et al., 2006; Andersson et al., 1995), also they secreted from the granules of natural killer and cytotoxic T cells.

This protein is belonged to saposin-like protein family and referred to a granulysin in human. It possesses a strong positive charge contained conserved cysteines residues which responsible for disulfide bonds formation inside the amino acids chain, therefore, these structural characteristics lead to increase its antimicrobial potentialities (Gansert et al., 2006; Jacobs et al., 2006; Liepinsh et al., 1997; Lee et al., 2012; Munford et al., 1995; Stenger et al., 1998). A critical part is played by the number of non-polar amino acids at the side chain of Nk-lysin peptides in moving through the bacterial membrane, as it was indicated in the earlier study that these peptides consisted of 27-51% of non-polar amino acids within all animal species (Mahmoud and Yacoub, 2020).

It was noted by Sant et al. (2018) that peptide binding and disruption of cell membranes is facilitated by the existence of non-polar amino acids like Ile and Leu. It was reported earlier that Nk-lysin peptides are part of the saposin domain with minor changes in domain length. The domain was folded in 4-5 helical bundles consistent with the species, having three disulfide bonds between six cysteine residues that made the peptide conformational structure stable.

The topic of antibiotic resistance is continuously studied and published each year with new data that can change the way we think about this issue. The topics that have been studied most often in this field include β -lactam resistance and specifically β -lactamases, the enzymes that are capable of hydrolysing β -lactam antibiotics (Canton et al., 2012). Since the 1980s, the number of β -lactamases has increased significantly; however, alternative ways were taken into consideration by healthcare and pharmaceutical Industry to improve and invent new routes to combat and prevent multi-drug resistance pathogens with many interests by using healthy and non-toxic agents. Consequently, using silver nanoparticles taken up a substantial concern to prevent infection with highly significant bactericidal approach against Gram-Positive and Gram-negative bacteria (Dizaj et al., 2014; Bahadar et al., 2016; Bankier et al., 2019). Recently, combined silver nanoparticles with antimicrobial peptides or/and

antibiotic may lead to eliminate bacterial resistance and improve antimicrobial molecules and even new line of antibiotics significance (Allahverdiyev et al., 2011; Ghasemi and Jalal, 2016).

Several molecules like chemokines are exhibited antibacterial activities, in addition to their immune-regulatory properties (Cole et al., 2001; Yang et al., 2003). The binding affinity between these positive charged chemokines and negatively charged cell membrane bilayer phospholipids is the possible mode of action, thus causing membrane disruption (Zasloff, 2002, Yang et al., 2003; Mahmoud and Yacoub, 2020). An important role is performed by interleukin-2 in managing the immune system and it is also used as a medication to treat different oncological diseases (Gill et al., 2016; Mizui et al., 2016). It was demonstrated recently that this cytokine showed bacteriolytic activity (Levashov et al., 2012; Sedov et al., 2012; Levashov et al., 2015, 2016). It is not yet clear how this bacteriolytic activity is physiologically significant for this vital cytokine. A combination of interleukin and lysozyme also shows highest activity in the micro-molar range of antibiotics (Levashov et al., 2017).

It was found in various studies that there are antiviral effects of innate immune response molecules on enveloped and non-enveloped viruses, and it is assumed that membrane disruption is a mode of action induced against viral infection (Daher et al., 1986, Park et al., 2018).

Though the studies on Nk-lysin peptides have been carried out in terms of their antibacterial activity. Nonetheless, no study has been carried out in the past that characterized the antiviral activity of bovine, human and chicken Nk-lysin peptides. So, this study was designed to determine the antibacterial and antiviral activity of Nk-lysin peptides from chicken, bovine and human and their mode of action. In addition to examine the synergetic effect when combined with silver nanoparticles or human interleukin 2.

Material And Methods

The experiments were carried out in multiples of three and were repeated at three different times. Negative controls in all experiments for determining the impact of Nk-lysin peptides were bacterial cells cultivated in their absence.

Peptides

The synthesis of mature peptides of bovine, human and chicken Nk-lysin peptides was carried out by (GenScript USA, Inc.). HPLC was used to purify the three peptides up to 95%, and it was shown in mass spectrometry analysis that the peptides had a mass less than 1 Dalton of the theoretical value, as shown in Table 1. Sigma Aldrich (St Louis, MO, USA) provided the recombinant Human interleukin-2 and the Kanamycin. The antibiotics were dissolved in sterile water or 0.9% (w/v) NaCl and kept at -20°C to create a stock solution. This solution was used within 2 weeks of its creation. The concentrations determined show the number of antibiotics that are active in micrograms per unit volume (µg/ml).

Bacterial Cell Preparation

The following two gram-positive bacterial strains are utilized: *Streptococcus pyogenes* (ATCC 19615) and *Streptococcus mutans* (obtained from patient at King Abdul-Aziz hospital). The following five species of gram-negative strains are used: *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella oxytoca* (ATCC 49131), *Shigella sonnei* (ATCC 25931) and *Salmonella typhimurium* (ATCC 14028). Tryptic Soy Broth was used to grow all bacterial species overnight, after which the cells were washed using phosphate buffer saline (PBS, NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.4 g/L, KH₂PO₄ 0.24g/L), and then were diluted within the same buffer that consisted of 1/1000 TSB to OD_{600 nm} 0.08–0.1 (1×10⁸) colony forming units (CFU)/ml determined after performing retrospective plate counts on TS agar. Majority of the experiments employed this inoculum preparation, apart from when minimum inhibitory and bactericidal concentrations were being examined, which employed cells in Muller Hinton broth.

Antimicrobial Activity Assays

Minimum Inhibitory and Bactericidal Concentrations

In accordance with the Clinical Laboratory and Standard Institute (CLSI) broth micro-dilution technique as presented by Wiegand and Hancock (2008), the smallest inhibitory and highest bactericidal concentrations of Nk-lysin of bovine, chicken and human peptides were obtained for tested species. Here, Muller Hinton Broth comprising of 0.01% v/v acetic acid (Sigma Aldrich, St Louis, MO, USA) and 0.2% w/v bovine serum albumin (Sigma Aldrich; MHB) were used. Aerobic incubation of bacterial cells diluted to 0.5×10^8 CFU ml⁻¹ in MHB was carried out in microplate using equivalent volume (50 µl) of varied concentrations of Nk-lysin peptides of bovine, human and chicken, which were in the range of 0 to 150 µg/ml. After this, the plate was incubated at a temperature of 37°C. For each well, the optic densities were noted at 0 minutes and then at 1, 2 and 3 hours and overnight. MIC₅₀ values were used to denote the smallest peptide concentrates that decreased the growth of tested microorganisms to 50%.

Colony Count Assays

The testing of antimicrobial functions of the Nk-lysin of bovine, human and chicken peptides was carried out against *S. typhi* (ATCC 14028). The bacteria were kept in Tryptic Soy Broth at a temperature of 37°C and were grown to the mid logarithmic stage prior to their testing. To determine the activity of Nk-lysin peptides, colony count assays were carried out. That is, bacteria were pelleted and then re-suspended in a 10 mM sodium phosphate buffer at a pH of 7.0 and after being diluted to (0.D600 (0.08) bacterial culture) within MHB. 50 µl of Nk-lysin of chicken, human, and bovine peptides were combined with the same volume of bacterial culture, after which they were incubated at room temperature for 3 hours. The cultures (1 µl) were then diluted by 1000 times and spread over Muller-Hinton (MH) agar plates. They were then counted after 24 hours at room temperature to determine the bacteria that survived.

Growth Kinetic Activity

In accordance with the Clinical Laboratory and Standard Institute (CLSI) broth micro-dilution technique presented by Wiegand and Hancock (2008), the kinetic killing abilities of various Nk-lysin of bovine, human and chicken peptides were gauged. The Muller Hinton Broth was utilized which comprised of 0.01% v/v acetic acid (Sigma Aldrich, St Louis, MO, USA) and 0.2% w/v bovine serum albumin (Sigma Aldrich; MHB). Aerobic incubation of bacterial cells that had been diluted to 0.5×10^8 CFU ml⁻¹ in MHB was carried out in microplate with the same volume (50 µl) of 2 times MIC concentrations of Nk-lysin peptides of chicken, human and bovine. The plate was then incubated at room temperature. For each well, the optic densities were noted at 0 minutes and then at 1, 2, 3 and 4 hours. A distinct triplicate was used to repeat the kinetic potential of Nk-lysin peptides thrice.

Synthesis and Characterization of Silver Nanoparticles

The chemicals and reagents used will all be of analytical grade. An aqueous solution of sodium borohydride was included in an aqueous solution of silver nitrate (0.05M). A precipitate was created from the drop-wise addition while stirring constantly at room temperature. Once the sodium borohydride was fully inserted, the mixture was continuously stirred for another 10 minutes. The aqueous component will be sampled to supervise the reduction of Ag⁺ ions by sodium borohydride within the solution ([Diantoro et al., 2014](#)).

TEM Examination of Silver Nanoparticles

High resolution-transmission electron microscopy (HR-TEM; JEM-2100F, Jeol, Tokyo, Japan) instrument was used to differentiate the AgNPs which operated at a high voltage of 200 KV (Ramkumar et al., 2017). A sonicator (Branson 1510) was used to dissolve silver nano powders in 90% ethyl alcohol solution. After this, the suspended Ag NPs were placed over carbon coated copper grid and high resolution-transmission electron microscopy was used to assess the particle size as well as the surface morphology.

Combination effect of Nk-lysin Peptide and Silver Nanoparticles

The standard micro-dilution technique was used to distinctly identify MICs of Nk-lysin peptide and AgNPs. This method was also used to identify the synergistic impact of the Nk-lysin mixed with AgNPs. Dilution of the antibiotics used was carried out in geometric progression, which were mixed with AgNPs at silver concentrations using the MICs of AgNPs against the bacterial

strain being examined. The silver concentrations utilized were in the range of 0.6 to 5 mg/L, consistent with the bacterial strain employed. The bacterial suspension was fixed at O.D600 (0.08) into MHB, after which they were aerobically incubated in microplate using equal volumes (50 µl) of 1xMIC concentrations of Nk-lysin peptide. The plate was then incubated at room temperature and optic densities were obtained at 0 minutes, 1, 2 and 3 hours for every well.

Combination effect of Nk-lysin Peptide and Human IL-2

The standard micro-dilution technique was used to independently measure the least inhibitory concentration of Nk-lysin peptide and Human IL-2. This method was also used to measure the synergistic impact of the Nk-lysin peptide mixed with IL-2. The 1xMIC of Nk-lysin was mixed with IL-2 (2 ng/ml) against the bacterial strain being tested. Aerobic incubation of the bacterial suspension altered to O.D600 (0.08) into MHB was carried out in microplate with the same volume (50 µl) of 1xMIC concentrations of Nk-lysin peptide. The plate was then incubated at room temperature and the optic densities were noted at 0 minutes and then after 1, 2, 3, 4 and 24 hours for every well.

Mode of Action of NK-lysin Peptides

Leakage of Intracellular Contents

The protocol of (Carson et al., 2002; Yasser et al., 2019) was used with some changes to perform the assay for determining the loss of DNA/RNA. In short, 50 µl of bacteria was combined with the same volume of NK-lysin of bovine, human and chicken peptides at their 2x MIC and then incubated at room temperature. Sample collection was carried out at distinct time intervals (15, 30 and 60 minutes), which were diluted (1:10) and then filtered across 0.22µm pores (Merck, Tullagreen, Ireland). The measurement of the O.D 260 nm of the filtrates was carried out over Novus NanoDrop plate (Greiner Bio-one GmbH, Frickenhausen, Germany). The results were presented as a ratio to the original OD260 nm.

16S rRNA Amplification

Two universal primer sets (341F and 534 R (adjusted by Kornegay et al., 1993)) were used to magnify 500 bp of 16s rRNA gene of *S. typhi*. The forward primer had the following sequence: 5-CCT ACG GGA GGC AGC AG-3, whereas the reverse primer had the following: 5-ATT ACC GCG GCT GCT GGC-3. PCR amplification reactions were carried out in 50 ml of overall volume that comprised of 50 ng of template DNA, 10 pmol of every primer, 0.25 U of Taq DNA polymerase, 10 mM of Tris- HCl (pH.9.0), 250 mM of dNTPs mix, 30 mM of KCl, 1.5 mM of MgCl₂ and sterile nuclease free water to obtain the ultimate volume of 50 ml. The following cycling conditions were used to carry out the PCR reaction: pre-denaturing at 94⁰C for 5 minutes, denaturing at 94⁰C for 30 seconds, annealing at 60⁰C for 30 seconds and extension at 70⁰C for 1:30 minutes for 35 cycles and finally, an extension at 70⁰C for 10 minutes. An analysis of the amplified fragments was carried out in 1.5% agarose stained with ethidium bromide. In addition, a 100 bp DNA ladder was added in agarose gel electrophoresis so as to examine the size of amplificon product. Gel documentation system (a Ultra-Violet Products Ltd. (UVP, LLC Upland, CA)) was used to obtain the images, and the software available with the gel documentation system was used to determine the size of the amplicon.

Bacterial Lytic Potential

Carson et al. (2002) was the one to first obtain the assay with certain modifications. Screening of the bacterial lytic potential of the Nk-lysin of human and bovine peptides was carried out using two distinct bacterial inoculums (O.D600 (0.5) and O.D600 (1.0)). In the bacterial culture, the Nk-lysin peptides (1xMIC) were added in the same volume. The microplate was subsequently incubated at room temperature. BioTek Instruments, Inc. reader was used to measure the micro-plates at varied time intervals (Yaser et al., 2019). The findings were presented as a ratio of O.D600 nm at every time interval in comparison to the OD600nm at 0 minutes (as a percentage).

Bacterial Membrane Damage with Fluorescent Microscopy

The *S. typhi* membrane damage was determined by incubating 6.5×10^6 CFU of *S. typhi* with 2x MIC of NK-lysin of bovine, chicken and human peptides in 10 mM phosphate buffer (pH 7.0), correspondingly at room temperature for one hour.

Fluorescent microscopy (Nikon, Carl Zeiss, Oberkochen, Germany) was used to observe the membranes once they were stained with the DAPI and PI for 1 hour, after which 10 µl of stained solution was added to the glass slide in dark area. This was then covered with over slip to observe the damaged *S. typhi* membranes after synthetic Nk-lysin peptides were used to treat them.

Detection of Beta-Lactamase Enzymes after Nk-lysin Peptides Challenge

Bacterial DNA Extraction

The following two gram-positive bacterial strains were used: *Streptococcus pyogenes* (ATCC 19615) and *Streptococcus mutans* (obtained from patient at King Abdul-Aziz hospital). The following five species of gram-negative strains are used: *Klebsiella oxytoca* (ATCC 49131), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 9027), *Shigella sonnei* (ATCC 25931) and *Salmonella typhimurium* (ATCC 14028). Luria Bertani (LB) agar was used to grow the bacterial strains at room temperature overnight. Inoculation of a single colony was performed to 5ml of LB broth, which was grown in a shaking incubator at room temperature for 16 to 18 hours. After this, genomic DNA was obtained with the help of QIAGEN genomic DNA extraction kit (QIAGEN, USA) in accordance with the recommendations of the manufacturer.

Detection and Characterization of Beta-Lactamase Enzymes

In order to amplify the target genes, PCR was performed to detect and characterize the beta-lactamase (bla) along with certain oligonucleotide primers as shown in Table 2. The PCR conditions achieved include an initial denaturation at 94°C for 5 min followed by 35 cycles; each for 30s at 94°C, specific annealing temperature for 30s as depicted in Table 2, and extension for 30s at 72°C followed by final extension for 10 min at 72°C. Once ethidium bromide staining was achieved, the PCR amplicons were inserted in 1.5% gel electrophoresis and then visualized using gel documentation system (Egual et al., 2017).

Characterization of Quinolone Resistance Genes

The genomic DNA was examined for presence of plasmid mediated quinolone resistance genes and quinolone resistance determining region (QRDR) using primer sets as shown in Table 2. The PCR conditions achieved include an initial denaturation at 94°C for 5 min followed by 35 cycles; each for 30s at 94°C, specific annealing temperature for 30s as depicted in Table 2, and extension for 30s at 72°C followed by final extension for 10 min at 72°C. Once ethidium bromide staining was achieved, the PCR amplicons were inserted in 1.5% gel electrophoresis and then visualized using gel documentation system (Egual et al., 2017).

Antiviral Activity of Nk-lysin Peptides

Cytotoxicity Experiments

The Nk-lysin of chicken, bovine and human peptides is examined in this study. They are prepared at 80 µg/ml concentration using sterile deionized H₂O. It was done according to Simoes et al. (1999). Briefly, bi-fold serial dilutions were carried out for 100 µL of the dissolved peptides and MA-104 cell monolayers were used to inoculate 100 µL of each dilution. An inverted light microscope was utilized to examine cell morphology of the cells daily for 6 days in order to evaluate cell toxicity of the tested peptide.

Rotavirus and Cell Culture Preparation

Rotavirus (strain SA-11) was activated by 10 µg/mL trypsin before propagated on MA-104 cells derived from kidney cells of African rhesus monkey. Cells were cultivated in tissue culture flasks encompassing less than 5% CO₂ placed at 37°C in Dulbecco's Minimal Essential Medium (DMEM; Sigma-Aldrich Co., St.Louis, MO) along with additional 1% antibiotic antimycotic solution and 10% heat inactivated fetal bovine serum (A5955, Sigma-Aldrich Co., St. Louis, MO). Centrifugation of rotavirus was performed at 300 ×g for 5 mins in order to remove cell debris and make them pure. The supernatant was utilized as a stock suspension after filtration through 0.2 µm membrane. The stock virus contained 10⁶–10⁷ TCID₅₀ per mL and were kept at -80°C for future use.

Antiviral Efficacy Experiment

In order to activate infectivity, the rotavirus was placed in trypsin solution of concentration 10 µg/mL at 37°C for 30 min. Hundred µl of the Nk-lysin peptides was mixed with 100 µl of activated rotavirus sa-11 (1×10^6 TCID₅₀/ml). The mixture was first incubated for at 37°C for only 1 hour. After that, the cells were placed into 96-well tissue culture plates (Nuncclon, Roskilde, Denmark) and then incubated until density of approximately 5.0×10^4 cells per well was achieved. After that, the cells were infected with 100 µl of 10-fold dilutions of treated (virus+tested peptide) and untreated rotavirus sa-11 followed by an incubation period encompassing 5% CO₂ atmosphere for 5 days at 37°C. The Cytopathic effect (CPE) was observed after microscopic monitoring of the cells. The viral concentration was represented as the infectious dose per millilitre of tissue culture (TCID₅₀/mL), equal to 50 percent of wells showing CPE at a specific dilution of the sample. The tissue culture infectious does that affected TCID₅₀ were determined using titration results obtained by the Reed–Muench method (Payment and Trudel 1993). Briefly, serial 10-fold dilutions of the virus sample were examined in 96-well tissue culture plates (Nuncclon, Roskilde, Denmark) comprising monolayers of MA-104 cells incubated with 5% CO₂ at 37°C. Eight wells were used to make sure sufficient assay precision is achieved and each of them was inoculated with 100 µl dilution. The viral cytopathic effects (CPE) of the cells were observed daily for 5 days. For the assessment of the TCID₅₀/mL, those highest dilutions were used in which 50% or higher wells were found positive.

Statistical analysis

The experiments were carried out in multiples of three and were repeated at three different times, and the data are presented as the means with standard error. A two-tailed test was performed on the data using Microsoft Excel 2020.

Results

Minimum Inhibitory and Bactericidal Concentrations

The aim of the study was to investigate and evaluate the relative antibacterial activity of three different Nk-lysin peptides from human, chicken, and bovine activity compared to Gram-negative and Gram-positive bacteria. The Figure 1 illustrates that the three tested Nk-lysin peptides showed strong antibacterial activity against *S. typhi* at 15 µg/ml at first three hours. Still the three peptides killed more than 95 % of bacterial cells evaluated at test conditions at the first hour of exposure.

The human Nk-lysin peptide exhibited stronger antibacterial potential than peptides belonging to chicken, bovine after second and third hours of exposure and produced more than 60% and 50% reduction of bacterial survival at time intervals. Nk-lysin peptides of bovine and chicken exhibited strong antimicrobial activity (15 µg/ml) with less than 5 % survival rate overnight, while human peptide displayed (82% killing) overnight.

As shown in Table 3, all Nk-lysin peptides analysed in this current study possessed or had a wide range of against both Gram-positive and Gram-negative pathogens though to a certain degree. The MIC and MBC values of chicken Nk-lysin peptide ranged from 15 to 30 µg/ml depicted maximal activity against all tested bacteria. However, the same results were recorded for bovine and human Nk-lysin peptides, showing maximal activity (MIC 15 µg/ml), (MBC 15-70 µg/ml), respectively. However, these Nk-lysin peptides were found to be more efficient regarding bacterial inactivation as compared to the reference antibiotic but in case of *Sh. sonnei*, the reference antibiotic was more potent.

The Nk-lysin peptides followed a dose-dependent pattern to kill bacteria. Less than 50% of bacterial survival was produced by these three peptides at low concentration (0.350 µg/ml). Nk-lysin peptides of chicken and bovine introduced the strongest antibacterial effect than human analogue under test conditions. According to the results achieved, all the functional peptides encompass efficient antibacterial activates under a wide variety of peptide concentrations.

Colony Count Assays

The colony forming unit of *S. typhi* was determined using three of Nk-lysin peptides. The data for the activity of all three Nk-lysin peptides is shown in Figure 2. At 30 µg/ml concentration of chicken Nk-lysin an approximately 10-fold decrease in bacterial

counts is observed. However, at 25 µg/ml concentration around 8.6-fold decline in bacterial survival. A higher concentration of human Nk-lysin peptide (70 µg/ml) led to decrease bacteria growth by 4.6-fold.

Growth Kinetic Activity

To observe the time dependent changes in the bacterial growth, the bacterial concentration and growth inhibition rates were evaluated by measuring the OD at 600 nm at different time points as shown in Figure 3. The untreated bacterial growth curve reached the exponential phase rapidly and then followed a regular pattern comprising of a period of lag, an exponential phase followed by a stabilization phase.

In respect with Nk-lysin peptides of chicken and bovine the growth of *S. typhi* were decreased in lag phase at the first one hour, then enter exponential phase at the second hour with less than 20% survival rate for chicken and bovine peptides. The same scenario was detected at the third hour for both peptides. Later, complete inhibition was achieved after 4 hours of incubation at 2x MIC of chicken and bovine Nk-lysin.

For Human Nk-lysin peptide at first hour the growth of *S. typhi* was decline to 30% at lag phase, then was less than 40% at second and third three hours. At concentration of 2x MIC the bacterial growth was less than 25% after incubation for four hours. According to the data achieved, the bacterial growth inhibition not only depends on the peptide type but on peptide concentration as well.

In the case of *K. oxytoca*, the complete inhibition of growth was achieved after 4 h of incubation with the three Nk-lysin peptides and the same trend was observed concerning *St. mutans* that enter the dead phase dramatically after exposure of Nk-lysin peptides. However, the chicken Nk-lysin peptide illustrated more potent activity than other counterparts. After 3 h of incubation with Nk-lysin peptides, the growth of *Sh. sonnei*, *St. pyogenes*, *K. pneumoniae*, *K. aeruginosa* and *E. coli* were completely dead respectively.

Combination of Nk-lysin Peptide and Ag-NPs

The high magnification image of Ag-NPs achieved by using high resolution transmission electron microscope showed that the silver nanoparticles is spherical in shape with obvious size of 18.5 nm as shown in Figure 4. Whereas crystallographic planes of silver were apparent in the TEM electron micrograph of high resolution. These results were in agreement with (Anwar et al. 2015) who observed that the Ag NPs lattice space is around 0.24nm.

The antibacterial activity against *S. typhi* using the spherical silver nanoparticles with 18.5 nm in size was depicted in Figure 5. The figure emphasized that a potent antibacterial activity after the first time of exposure with AgNPs and continued had the same trend at overnight incubation. In our study the combination potential between AgNPs and Nk-lysin peptide exhibited strong effect against *S. typhi* at time interval as indicated in Figure 6. The results exhibited that Nk-lysin peptide and Nano-Ags possessed antibacterial effects and additive activities. Moreover, the results suggested that silver nanoparticles can serve as a combination therapeutic agent in order to treat infectious diseases caused by bacteria.

Combination of Nk-lysin Peptide and Human IL-2

For the determination of the antibacterial activity of the human interleukin-2 against *S. typhi* an inhibition assay was conducted. The case for interleukin-2 is depicted in (figure 7) which shoes that the antibacterial action of interleukin-2 increases with time and the maximum potential for antibacterial activity rose after 180 mins of incubating with *S. typhi* when compared to untreated bacterial culture against the one treated with IL-2, Determination of the combined activity of NK-lysin peptide and IL-2 was done against *S. typhi*. Figure 8 shows the antibacterial potential and the inhibition profile for IL-2 and NK-lysin peptide. The results showed both the peptides being almost equipotent in their antibacterial activity when tested alone, but the combined effect was additive with low increase in effectiveness

Mode of Action of NK-lysin Peptides

Leakage of Intracellular Contents

Nanodrop was used to quantify the release of nucleic acids from *S. typhi* after exposing the cells to NK lysin peptide which resulted in an O. D of 260nm and is shown in Figure 9. There were three different time manners used for recording the release of nucleic acids from *S. typhi* cells after membrane damage due to exposure to NK lysin peptide. After an incubation period of 15mins the NK lysin peptide from chickens showed higher activity compared to other similar peptides, but the activity of the NK lysin peptides had no significant difference at 30 min and 90 min, respectively. When NK lysin peptide isolated from humans were used the action of the peptide against *S. typhi* showed increase action after 15 min compared to increase in action of chicken NK lysin at 30 and 90 min of exposure. For the confirmation of such results gel electrophoresis quantification was used and subsequently 16s rRNA amplification was done to amplify at 500 bp, the visualization of these fragments was done using ethidium bromide stains on the specific DNA ladder.

Bacterial Lytic Potential

For the determination of the bacteriolytic potential of human and bovine peptides two different inoculums of bacteria which were the O.D600 (0.5) and O.D600 (1.0) were used for screening as seen in figures 10 and 11. The bacterial inoculum of *S. typhi* with concentration O.D600 (0.5) after being incubated for 1 hour shows the effect of human Nk-lysin peptide causing the survival rate of the cell to fall by 78%, similarly after 2 hours of incubation the probability for cell survival was reduced to less than 30%, this trend continued to be exhibited even after 3hrs with onset of minor modification in growth of cell for bovine Nk lysin peptide.

The bacterial survivability at O.D600 (1.0) was 27%, 34% and 35% after 1, 2 and 3 hours respectively passed. A similar trend was seen as in the case of bovine NK-lysin peptide, which is shown in figure 7, there is very little deviation. By incubating with 1xMIC of peptide the lysis of cell was 79%, 72% and 68% shown after 1, 2 and 3 hours have passed on the concentration of O.D600 (0.5). Similar to human NK lysin peptide at O.D 600 (1.0) there was significant decrease in growth by 74, 76 and 64% after 1, 2 and 3 hours passed respectively. The lysis activity of both bovine and human NK lysin peptides at their respective MICs was quite similar to each other as shown by both treatments as noted in figures 10 and 11.

Membrane Damage with Nk-lysin Peptides

To evaluate whether the NK lysin peptides alter morphology and survivability of *S. typhi*, a DAPI strain which specifically stains dead cells and is impermeable to live cells was used and the damage done to *S. typhi* cell membranes after being treated with NK lysin peptides was checked by using fluorescent microscopy technique. The results showed that when the peptide was not applied to the *S. typhi* cells their cell membranes remained intact for the majority, but as soon as treatment with 2xMIC of peptide was done most of the *S. typhi* cells stained brighter and were more permeable to the stain which indicates damage to the cell membrane (Figure 12). The damage done by chicken, bovine and human NK lysin peptide showed results consistent with the data obtained depending upon dose applied.

Detection of resistance to Beta Lactam and Quinolone genes

The treatment with NK lysin peptide determined the presence/absence of plasma mediated quinolone resistance and Beta-lactam genes for both gram negative and positive bacteria. The results shown in our findings were that the dominant TEM-1 gene was present in treated and untreated cells whereas the TOHO-1 gene was found in some bacteria but was absent in others table 4.

An interesting finding was that the presence of Beta-lactamase genes (CTX-M-1, M-8, and M-9) was not seen in any of the bacterial stains after being treated with NK-lysin peptide, in contrast their presence was found in all untreated cells (control) as seen in (figure 13 and 14). The presence chromosomal gene (Gyrase A) associated with topoisomerase 2 was indicated in treated and untreated bacteria both, whereas the presence of the tested genes associated with plasmid mediated quinolone resistance genes was detected in all cells irrespective of the fact whether they were treated or not. Our results showed that the beta-lactamase gene of (TEM) was observed in all cells irrespective of the fact whether they were treated or not. Whereas (Toho) gene was not explored in control cells or after exposure of Nk-lysin peptides.

Cytotoxicity and Antiviral Activity of Nk-lysin Peptides

To determine the cytotoxicity of all the NK lysin peptides (concentration 80microgram/ml), MA-104 cells are used which showed that all the peptides are not toxic and are safe. *Rotavirus* (strain SA-11) was used to evaluate the antiviral action of the three different peptides in vitro. The three peptides from chicken bovine and humans were combined in equal volumes with *rotavirus* and incubated for 1hour at 37 °C, no potential antiviral activity against any strain of rotavirus has been reported.

In the next process, we used MA-104 cells which were mixed with and without (mixture of virus and peptides) and were left to be incubated for 5 days at 37 °C and 5% CO₂. Table 3 shows that the NK-lysin peptides showed various antiviral activity against Rotavirus (strain SA-11). The bovine NK-lysin peptide showed potent antiviral activity by inhibition of infection by 90%, chicken NK-lysin peptide showed similar results with 80% inhibition whereas the inhibition by human NK-lysin peptide was the lowest with 50% inhibition as noticed in table 5.

Discussion

For the first time, this paper discussed the antimicrobial activity of Nk lysin peptide obtained from 3 different species namely chicken, bovine, and human. The antimicrobial activity and mechanism of action of these cationic peptides was also studied. The results showed high inhibition of bacterial activity after exposure to the NK lysin peptide especially at the first hour where it showed 95% inhibition rate, this held true especially for *S. typhi*. The results we obtained show strong activity of NK lysin peptide in other bacteria which were tested.

Synthetic NK lysin peptides (which corresponded with functional region helices 2 and 3) showed similar hydrophobicity (40-43%), net positive charge of (5.0-7.9) and basic residues of (20-30%) which might lead to high effectiveness against *S. aureus* and *E. coli* as reported by (Cheng et al., 2015). The high alpha helicity of the bovine peptide did not have much effect on *M. bovis* and *M. haemolytic* isolates (Cheng et al.,2016), but in contrast bovine NK lysin peptide was highly effective towards *H. Somni* isolates (Dassanayake et al.,2017,2018). Studies which were done on chicken NK lysin peptide show their antimicrobial activity is similar to other NK lysin peptides (Harwig, 1999; lehrar and Ganz, 2002; Goitsuka, 2007; Lee et al., 2012, 2014)

A research by (Jacobs et al., 2003) shows that the mammalian Nk-lysin peptide rapidly permeabilized the plasma membrane of the protozoan parasite *Trypanosoma cruzi* resulting in the release of cytosolic enzymes within minutes after exposure. They also found that the NK-lysin and NK-2 were even found to kill trypanosomes residing inside the human glioblastoma cell line 86HG39, but only NK-2 left the host cells apparently unharmed whereas Nk lysin was not safe.

Our results were demonstrated that using the Nk-lysin peptides have more antibacterial efficacy than the kanamycin (reference antibiotic). However, all MIC values of Nk-lysin peptides were recorded 15µg/ml for all tested bacteria. The MIC values of kanamycin was ranged from 7.8-62.5µg/ml. The Kanamycin was effective at concentration of 62.5 µg/ml for *S. typhi*, *St. pyogenes*, *St. mutans*, and *P. aeruginosa*, while it was significant in the case of *K. pneumonia* and *k. oxytoca* at concentration of 31.25 µg/ml. Whilst *Sh. Sonnei* was recorded 7.8 µg/ml as MIC of kanamycin.

A kinetic analysis of the three NK lysin peptides discussed in this study showed that chicken and bovine NK lysin peptides show similar activity against *S. typhi* with high lethality at first hour after treatment, whereas the human counterpart was not as effective and showed reducing the bacterial growth up to 75% after 4 hours when exposed to the same bacteria. The Nk-lysin of chicken peptides was remarkably more efficient compared to other bacteria being tested that did not survive for even 3 hours following exposure to the peptide, hence depicting its antibacterial potency. The changes in the kinetics that explain the antibacterial function of the peptide may be identified from its hydrophilic regions and from the positively charged amino acids that are mainly present on the surface area of these peptides and this may regulate the mode of action of majority of the AMPs.

The preceding findings were in agreement with the previous studies that had found intra-molecular structures of Nk-lysin to be connected through six cysteine residues by means of creating bridge to link units of (helical 1/helical 4/ helical 5) and (helical 2/helical 3). A helical peptide subsequently includes a Trp residue that provides greater affinity and more extensive insertion inside bacterial membranes (Torcato et al., 2013). Pheasant cathelicidin-1 (Pc-CATH1) showed similar data, where the growth of *E. coli* was restricted after 1 hour of exposure, and even after 6 hours of being exposed, the bacteria did not start growing (Wang et al., 2011).

Melimine peptide and its derivative were found by Yasir et al. (2019) to exhibit a powerful antimicrobial activity against *P. aeruginosa* because it is smaller in size (17-29 aa), which is possibly why it becomes capable of covering the cytoplasmic membrane of *P. aeruginosa*. It is indicated by the smaller length of the Mel4 peptide that it takes more time to move across the outer membrane or to interact with the inner membrane of *P. aeruginosa* to destroy the bacteria, or that it needs to align itself inside the membrane more effectively so as to start its activity.

A critical part is played by the number of non-polar amino acids at the side chain of Nk-lysin peptides in moving through the bacterial membrane, as it was indicated in the earlier study that these peptides consisted of 27-51% of non-polar amino acids within all animal species (Mahmoud and Yacoub, 2020). It was noted by Sant et al. (2018) that peptide binding and disruption of cell membranes is facilitated by the existence of non-polar amino acids like Ile and Leu. It was reported earlier that Nk-lysin peptides are part of the saposin domain with minor changes in domain length. The domain was folded in 4-5 helical bundles consistent with the species, having three disulphide bonds between six cysteine residues that made the peptide conformational structure stable. These findings were coherent with other studies which showed that there were five helical folded structures in a single globular chain in our peptide and stable disulphide bridges between six cys residues with respect to pig peptide, while human Nk-lysin domain was made up of four cysteine residues to create two disulphide bonds (Liepinsh et al., 1997; Zhai and Saier, 2000; Olmeda et al., 2013, Tammarozzi and Giuliatti, 2018).

Microbial resistance can be effectively overcome through strategies that are created on the basis of synergistic combinations of antimicrobial agents and also through drug designs developed on the basis of nanotechnology and phytomedicine (Krychowiak et al., 2018). Various studies have been carried out in the past few years that show that AgNPs may increase the antibacterial activities of antibiotics against susceptible as well as resistant bacteria, either in an additive manner or synergistically (Panáček et al., 2016). There has been widespread use of silver nanoparticles (AgNPs), which are known to have antimicrobial properties, in the field of medicine.

This report is the first of its kind that investigates the increased antimicrobial ability of (Nk-lysin+AgNPs) and (Nk-lysin+human IL-2) combinations against *S. typhi* activity by carrying out direct comparison under similar experimental settings. According to the findings, AgNPs exhibited only antibacterial effects and additive activity when mixed with these peptides as well as human IL-2. Antimicrobial activity of AgNPs has a complicated mechanism and is dependent on nanoparticles as well as silver ions that their surface releases. In addition, it interacts with various cellular components (Dakal et al., 2016). It was shown in our findings that there was equal potentiality in this mixture for preventing the growth of *S. typhi*, however, when added together, there was minor increase in the level of action. Hence, it was deduced that these combined molecules had an additive effect.

An important role is performed by interleukin-2 in managing the immune system and it is also used as a medication to treat different oncological diseases (Gill et al., 2016; Mizui et al., 2016). It was demonstrated recently that this cytokine showed bacteriolytic activity (Levashov et al., 2012; Sedov et al., 2012; Levashov et al., 2015, 2016). It is not yet clear how this bacteriolytic activity is physiologically significant for this vital cytokine. A combination of interleukin and lysozyme also shows highest activity in the micro-molar range of antibiotics (Levashov et al., 2017). This is, however, only correct when the concentrations of antibiotics and AgNPs attain their own minimum inhibitory concentrations (MICs), which refers to the concentrations at which the tested antibiotics or AgNPs attain antimicrobial activity on their own (Panáček et al., 2016).

In this combination, the additive activity has brought about major improvements in bactericidal activity irrespective of antibiotic resistance, and also in the ultimate multi-target antimicrobial effect. The conclusion that was derived from this was that AgNPs are effective as a combination therapeutic agent for treating infectious diseases caused by bacteria. Antibiotics with distinct methods of action against different bacterial strains depicted this additive effect (Shahverdi et al., 2007; Birla et al., 2009; Fayaz et al., 2010; Ghosh et al., 2012; Sathiyarayanan et al., 2013; Naqvi et al., 2013; Muhsin and Hachim, 2014).

A concentration-dependent release of DNA/RNA (260nm absorbing material) was brought about by the three distinct Nk-lysin peptides within 15 minutes of incubation, which was according to the findings of Minahk et al. (2000) and Yasser et al. (2019). It was shown that following treatment with antimicrobial peptides of Enterocin CRL35, Melimine and its derivative Mel4, there was a concentration-dependent release of DNA/RNA from *Listeria monocytogenes* and *P. aeruginosa*. No significant variation was noted between Nk-lysin peptides for the release of DNA/RNA after being exposed for 90 minutes. It was shown in the similar

findings of Yasser et al. (2019) that the extent of DNA/RNA release brought about by Mel4 was not the same as the amount released after being exposed to Melimine, even when the incubation times were larger.

A major advantage is possibly offered by their conformational structure to the antimicrobial effects of Nk-lysin and granulysin peptides since they have a similar pathway through which bacteria is inactivated by AMPs, which is interrupted by the physical interactions between the positively charged peptides and negatively charged cell membrane bilayer phospholipids, thus causing membrane disruption (Zasloff, 2002, Mahmoud and yacoub, 2020). Through this mode of action, DNA/RNA is released because of the bursting and disintegration of nucleic acid, which may happen during bacterial apoptosis-like death that is identical to eukaryotic cells that brought about physiological and biothermal variations following exposure of peptides (Dwyer et al., 2012). This was similarly attained by NK-2 that disintegrated the parasitic membrane to release the cytosolic marker protein. This suggests that both peptides, mammalian NK-lysin and NK-2, target the plasma membrane of the parasite (Jacobs et al., 2003).

It was deduced from our findings that a bacterial lytic potential was used by these Nk-lysin peptides against high inoculum of *S. typhi*, where there was an 80% decline in bacterial growth following incubation of 1xMIC. According to these findings, even when there is high bacteria culture, a strong antibacterial effect is exhibited by Nk-lysin peptides, as depicted in Figure 7. It may be their small sizes (30 amino acids) and structure that gives rise to this potent activity; hence, this finding was coherent with other studies that the amino acid length should be around 15-20 residues for the peptides to cover bacterial cytoplasmic membranes (Popot and Engleman, 2000; Mackenzie, 2006; Beevers and Dixon, 2010).

The topic of antibiotic resistance is continuously studied and published each year with new data that can change the way we think about this issue. The topics that have been studied most often in this field include β -lactam resistance and specifically β -lactamases, the enzymes that are capable of hydrolysing β -lactam antibiotics (Canton et al., 2012). Since the 1980s, the number of β -lactamases has increased significantly; however, this increase is almost entirely because of class A and D β -lactamases (Bush and Jacoby, 2010). From the class A β -lactamases, extended-spectrum β -lactamases (ESBLs) that are able to hydrolyse expanded spectrum cephalosporins (for example cefotaxime, ceftriaxone, ceftazidime, or cefepime) and monobactams (aztreonam) are cause of a major public health concern (Coque et al., 2008; Pitout and Laupland, 2008).

Class A ESBLs essentially comprise of TEM, CTX-M, SHV, GES and VEB enzymes, out of which the greatest number of variants defined in the previous years are part of the CTX-M family (total of 123 variants till the year 2011) (Rice et al., 2008). Therefore, this study identified the existence or lack of beta-lactam and plasmid mediated quinolone resistance genes following treatment with Nk-lysin peptides. In our study, the presence of dominant TEM-1 gene was noted in all untreated and treated bacteria, while TOHO-1 gene was absent in all bacteria.

In addition, following treatment with Nk-lysin peptides, the beta-lactamases genes (CTX-M-1, M-8, and M-9) were not found in any bacterial strains; however, they were observed in all untreated bacteria. The examination did not find any of the tested plasmid mediated quinolone resistance genes in all of the bacterial strains that went through treatment or were untreated. The lack of plasmid mediated quinolone resistance genes and beta-lactam is due to plasmid curing phenomena and down-regulation of beta-lactamase gene expression enzymes (CTX-M1,M-8 and M-9) after being exposed to Nk-lysin peptides. This mode of action has never been noted for these peptides and for other innate immunity molecules.

A plasmid cured derivative is often sought with certain plasmid-containing bacteria so that a direct comparison can be performed between the two. There are certain plasmids that go through spontaneous segregation and deletion. But most of them are quite stable and need curing agents or other conditions (increased growth temperature, thymin starvation) to enhance the frequency of spontaneous segregation so that majority of the plasmids can enter the bacterial host chromosome. When this happens, the plasmid would no longer be present as a covalently closed circular (CCC) molecule (Carol et al., 1984; Trevors, 1986).

More concerns are in a position to search for reliable inhibitors for β -lactamase enzymes are given additional energy or force by the continued weakness of the antibiotic discovery pipeline for Gram-negative bacteria (Falagas et al., 2016;Theuretzbacher et al., 2018). In addition, the combinations of susceptible β -lactams with mechanism based β -lactamase inhibitors represent the major strategy for combatting β -lactamase-mediated resistance (Drawz and Bonomo, 2010).Moreover, using Nk-lysin treatment

may also lead to genetic re-organization of beta-lactamases enzymes during cell replication, which may cause a change in its location or integration of transposon elements. These enzymes were differentiated through β -lactamase genes (*bla*) location in plasmids or chromosomes. However, this genetic feature is not used anymore because it is possible to mobilize and integrate chromosomal *bla* genes into plasmids or transposons; however, a reverse case of initially described plasmid-mediated β -lactamases into the chromosome is also noted excessively (Toleman et al., 2006; Coelho et al., 2010). Furthermore, protein regulation features (constitutive or inducible expression) are also noted with respect to various β -lactamases groups (Livermore, 1995); however, this is dependent on the adjacent genes.

Bla genes may be absent in certain isolates with decreased susceptibility in *Salmonella* possibly because of weak sensitivity of phenotypic resistance detection approaches, lack of or down-regulation of the creation of outer membrane porins (Delcou, 2009), changes in the beta-lactam targets (PBPs) (Sun et al., 2014), varied *ampC* beta lactamases (Jacoby, 2009) and overexpression of efflux may cause the decrease in susceptibility. There are certain genetic elements and plasmids with *bla*CTX-M genes that also include other resistance genes, such as those encoding *AmpC* β -lactamases (plasmid *blaAmpC*) and carbapenemases, methylases affecting aminoglycosides or plasmid-mediated quinolone resistance (PMQR) genes (Egualle et al., 2017).

These genes may also benefit *bla*CTX-M for maintenance because of the co-selection procedures (Canton et al., 2012); therefore, it may cause few CTX-M genes to be lost in the process of cell division. IS located upstream, like *ISEcp1*, have demonstrated the experimental mobilization of *bla*CTX-Ms genes (Lartigue et al., 2006). There are various IS that have been recognized upstream of the *bla*CTX-Ms genes, such as *ISEcp1*, *IS10*, *ISCR1* and *IS26*. Though there are other IS elements that have also been identified upstream, they were because of ensuing integration activities. For example, *IS1* and *IS10*, and also *IS26* and *ISCR1*, have been found to disrupt *ISEcp1* (Eckert et al., 2006; Bae et al., 2008). It has been asserted in other studies that spacer sequences between *ISEcp1* and *bla*CTX-M genes are linked to cephalosporin MIC values (Ma et al., 2011) and may be an outcome of a single transposition incident (Canton et al., 2012).

Though the studies on Nk-lysin peptides have been carried out in terms of their antibacterial activity, the potential antiviral activity of other innate immunity molecules, apart from Nk-lysin, has been demonstrated in the latest studies. For example, antiviral activity of the human cathelicidin LL-37 was observed against, IAV, adenovirus, HIV and respiratory syncytial virus (Bergman et al., 2007; Barlow et al., 2011; Currie et al., 2016). Furthermore, it has also been determined that defensins, such as α - and β -defensins, have antiviral activity against IAV (Doss et al., 2009).

Extensive studies have been carried out on chicken cathelicidins, which have been found to exhibit various activities (Peng et al., 2020). In addition to broad-spectrum antibacterial activity, they also possessed immunomodulatory functions; hence, they may be able to activate phagocytosis, neutralize LPS-induced immune responses and improve DNA-induced TLR21 activation (Veldhuizen et al., 2013; Coorens et al., 2015; Coorens et al., 2017; Peng et al., 2020).

Nonetheless, no study has been carried out in the past that characterized the antiviral activity of bovine, human and chicken Nk-lysin peptides. Hence, this is the foremost study on the enhanced antimicrobial activity of human, bovine and chicken Nk-lysin peptides against *Rotavirus* (strain SA-11). The findings of the study demonstrated that the powerful antiviral activities were exhibited by Nk-lysin peptides against *Rotavirus* (strain SA-11). On the basis of the comparison between these peptides, it can be concluded that there is an evident potent antiviral activity of bovine Nk-lysin against *Rotavirus* (strain SA-11) as it restrains infection by up to 90%. However, growth was restricted by 80% by chicken Nk-lysin and by 50% by the human peptide.

It was found in various studies that there are antiviral effects of innate immune response molecules on enveloped and non-enveloped viruses, and it is assumed that membrane disruption is one of the antiviral methods exhibited by defensins against enveloped viruses, similar to the antibacterial mechanism, however, this is not exhibited directly (Daher et al., 1986; Park et al., 2018). A recent study by Falco et al. (2019) showed that powerful antiviral activity was shown by Nk-lysin short peptide of the turbot (*Scophthalmus maximus*) against spring viremia of carp virus (SVCV) by not just restricting viral particles from binding to host cells, but also preventing the integration of virus and cell membranes that needs a low pH.

It seems that the other antiviral mechanism of these peptides depends on their specific binding on particular viral proteins or the non-specific lectin-like binding to the envelope glycoproteins of viruses (Smith and Nemerow, 2008; Smith et al., 2010; Nguyen et

al., 2010; Gounder et al., 2012; Flatt et al., 2013; Tenge et al., 2014). Taking into account this mechanism, defensins may exhibit inhibitory effects by blocking the fundamental interaction between influenza glycoprotein hemagglutinin and cellular receptor sialic acid (Leikina et al., 2005, Park et al., 2018).

Conclusion

For the first time, this paper discussed the antimicrobial activity of Nk lysin peptide obtained from 3 different species namely chicken, bovine, and human as well as antiviral activity against *Rotavirus* (strain SA-11). The mechanisms of action of these peptides were also determined. Additionally, the antimicrobial activities of (Nk-lysin+AgNPs) and (Nk-lysin+human IL-2) combinations against *S. typhimurium* were tested. Our results showed that gram-negative and gram-positive microorganisms, including *Streptococcus pyogenes*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Shigella sonnei*, *Klebsiella pneumoniae* and *Salmonella typhimurium*, are susceptible to NK-lysin treatment. An interesting finding was down-regulation of beta-lactamase gene (CTX-M-1, M-8, and M-9) in response to treatment with NK-lysin peptide. None of plasmid mediated quinolone resistance genes were found in the bacterial strains as response to NK-lysin treatment. The findings of the study demonstrated that the powerful antiviral activities were exhibited by Nk-lysin peptides against *Rotavirus* (strain SA-11) for the first time and lead to restrains infection by up to 90%, 80% and 50% regarding Nk-lysin of chicken, bovine and human, respectively.

Declarations

Conflict of interest

All the authors declare no conflict of interest . All the authors declare that all data used in this study were filled in United states patent and trademark office under application number (17213483).

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Tables

Table.1. The amino acids sequence of the three Nk-lysin peptides

Peptide name	Amino Acid Sequence	M.W
Chicken-Nk-lysin	PDEDAINNALNKVCSTGRRQRSICKQLLKK	3399.92
Bovine- Nk-lysin	RPSKNVIIHVTSNVCSKMGLWSILCNQMMK	3419.16
Human-Nk-lysin	PTQRSVSNAATRVCRTGRSRWRDVCRNFMR	3568.07

Table. 2. The primers list for detection and characterization of beta-lactamases and quinolone resistance genes

Gene	Forward Primer (5' to 3')	Reverse primer (5' to 3')	amplicon size (bp)	References
BLATem	ATGAGTATTCAACATTTCCG	GACAGTTACCAATGCTTAATCA	869	Hendriksen et al., 2009
CTX-M1	GACGATGTCACTGGCTGAGC	AGCCGCCGACGCTAATACA	499	Hendriksen et al., 2009
TOHO1	GCGACCTGGTTAACTACAATCC	CGGTAGTATTGCCCTTAAGCC	351	Hendriksen et al., 2009
CTXM8	CGCTTT GCCATGTGCAGCACC	GCT CAGTACGATCGAGCC	307	Hendriksen et al., 2009
CTXM9	GCTGGAGAAAAGCAGCGGAG	GTAAGCTGACGCAACGTCTG	474	Hendriksen et al., 2009
Gyrase A	AAATCTGCCCGTGTCTGTTGGT	GCCATACCTACTGCGATACC	344	Harrois et al., 2014
QnrA	ATTTCTCACGCCAGGATTTG	GATCGGCCAAAGGTTAGGTCA	516	Piddock, 2006
QnrB	GATCGTGAAAGCCAGAAAGG	ACGATGCCTGGTAGTTGTCC	469	Piddock, 2006
QnrD	CGAGATCAATTTACGGGGAATA	AACAAGCTGAAGCGCCTG	565	Kruger et al, 2004
QnrS	ACGACATTCGTCAACTGCAA	TAAATTGGCACCCCTGTAGGC	417	Piddock, 2006

Table. 3. Antimicrobial activity of Nk-lysin peptides of chicken, bovine and human against Gram-positive and Gram-negative bacteria

Bacterial strain	Nk-lysin Chicken		Nk-lysin Bovine		Nk-lysin Human		Kanamycin	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
<i>S.typhimrium</i> <i>ATCC 14028</i>	15±0.098	30±0.10	15±0.10	25±0.12	15±0.090	70±0.092	62.5±0.00	62.5±0.00
<i>K.Pneumonia</i> <i>(isolate)</i>	15±0.030	25±0.010	15±0.03	25±0.010	15±0.040	15±0.040	31.25±0.00	31.25±0.00
<i>St. pyogenes</i> <i>ATCC 19615</i>	15±0.00	25±0.010	15±0.00	25±0.010	15±0.00	25±0.010	62.5±0.00	62.5±0.00
<i>St. mutans</i> <i>(isolate)</i>	15±0.00	15±0.00	15±0.0	15±0.0	15±0.010	25±0.012	62.5±0.023	62.5±0.23
<i>E. coli</i> <i>ATCC 11775</i>	15±0.012	25±0.010	15±0.012	25±0.010	15±0.014	25±0.012	15.62±0.011	15.62±0.00
<i>K. oxytoca</i> <i>ATCC 49131</i>	15±0.015	25±0.10	15±0.015	25±0.10	15±0.012	15±0.012	31.25±0.00	31.25±0.00
<i>P.aeruginosa</i> <i>ATCC 9027</i>	15±0.012	25±0.013	15±0.012	25±0.013	15±0.010	15±0.010	62.5±0.00	62.5±0.00
<i>Sh. sonnei</i> <i>ATCC 25931</i>	15±0.033	25±0.010	15±0.033	25±0.010	15±0.025	25±0.012	7.81±0.026	7.81±0.026

Table.4. Detection of beta-lactam and quinolone resistance genes after treatment with Nk-lysin peptides

Gene name	Untreated bacteria	treated bacteria
BLATem	(+) all	(+) all
TOHO1	(-) all	(-) all
CTX-M1	(+) all	(-) all
CTXM8	(+) all	(-) all
CTXM9	(+) all	(-) all
Gyrase A	(+) all	(+) all
QnrA	(-) all	(-) all
QnrB	(-) all	(-) all
QnrD	(-) all	(-) all
QnrS	(-) all	(-) all

(+) all, the gene is present in all bacteria

(-) all, the gene is absent in all bacteria

Table.5. The antiviral activity of Nk-lysin peptides of chicken, bovine and human at concentration (80µg/ml) against Rotavirus sa-11

Tested extract	TCID ₅₀ /ml	Log ₁₀ TCID ₅₀ /ml	Log reduction	% reduction
Initial titre	1.95×10 ⁶	6.29	N.A	N.A
cNk-lysin	4.11×10 ⁵	5.61	0.7	79%
bNk-lysin	1.95×10 ⁵	5.29	1.00	90%
hNk-lysin	1.00×10 ⁶	6.00	0.29	49%

Figures

Figure 1

Antimicrobial activity of Nk-lysin peptides of chicken, bovine and human against *S. typhi*. Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 2

Colony counting assay of Nk-lysin peptides of chicken, bovine, human against *S. typhi*. Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 3

Growth kinetic activities of 2xMIC of chicken, bovine and human Nk-lysin peptide against *Streptococcus pyogenes* (ATCC 19615) and *Streptococcus mutans* (isolate), *Escherichia coli* (ATCC 11775), *Klebsiella oxytoca* (ATCC 49131), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhimurium* (ATCC 14028), *Klebsiella Pneumonia* (isolate) and *Shigella sonnei* (ATCC 25931). Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 4

The TEM micrograph of the aggregation of suspended silver nanoparticles in water which spherical in shape and ranging from 18 ± 5 nm in diameter (bar 2 nm) which showed characterization of prepared silver nanoparticles.

Figure 5

Antimicrobial activity of nanosilver particles against *Salmonella typhimurium* (ATCC 14028). Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 6

The activity of human of Nk-lysin peptide and nanosilver particles (hNk-lysin+Nanosilver) against Salmonella typhimurium (ATCC 14028). Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 7

Antimicrobial activity of human IL-2 against *Salmonella typhimurium* (ATCC 14028) . Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 8

The activity of human of Nk-lysin peptide and Human IL-2 (+hNk-lysin+human IL-2) against *Salmonella typhimurium* (ATCC 14028). Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 9

DNA/RNA release of *Salmonella typhimurium* (ATCC 14028) bacterial cells after incubation with (Nk-lysin peptides of chicken, bovine, human) at 37°C. Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 10

The bacterial lytic effect of 1x MIC bovine Nk-lysin peptide against *Salmonella typhimurium* (ATCC 14028) at different inoculum concentrations. Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

Figure 11

The bacterial lytic effect of 1x MIC bovine Nk-lysin peptide against *Salmonella typhimurium* (ATCC 14028) at different inoculum concentrations. Data presented as means ($\pm$ SD) of three independent repeats in triplicate.

cNk-lysin

bNk-lysin

hNk-lysin

Bacterial control

Figure 12

The membrane damage *Salmonella typhimurium* (ATCC 14028) following treatment with Nk-lysin peptides of chicken, bovine and human. Green fluorescence indicates live bacteria.

Figure 13

The amplified fragment of CTX-M1 gene with 499 bp, where M (DNA ladder =100 bp), 1(*S. typhi* ATCC 14028 untreated and treated cells), 2 (*P. aeruginosa* ATCC 9027 untreated and treated cells), 3 (*Klebsiella oxytoca* ATCC 49131 untreated and treated cells), 4 (*St. pyrogens* ATCC 19615 untreated and treated cells) and arrows indicate gene in untreated bacteria whereas empty well indicates treated bacteria after challenge with Nk-lysin.

Figure 14

The amplified fragment of CTX-M8 gene with 307 bp, where M (DNA ladder =100 bp), 1(*S. typhi* ATCC 14028 untreated and treated cells), 2 (*P. aeruginosa* ATCC 9027 untreated and treated cells), 3 (*Klebsiella oxytoca* ATCC 49131 untreated and treated cells), 4 (*St. pyrogens* ATCC 19615 untreated and treated cells) and arrows indicate gene in untreated bacteria whereas empty well indicates treated bacteria after challenge with Nk-lysin.

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