ORIGINAL ARTICLE

Production of Novel Antimicrobial Peptide Human Beta-defensin 9 (hBD9) Using the Pet Sumo Expression System in *Escherichia coli* and Characterisation of Its Antimicrobial Activity

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ABSTRACT

Introduction: Defensins are small cationic polypeptides found in various organisms. They exhibit microbicidal activities against bacteria, fungi, mycobacteria, yeasts, and enveloped viruses. Defensins are divided into three groups known as α-, β-, θ-defensins, but only the first two have been isolated in humans. The human β-defensin-9 (hBD9) is a relatively new defensin isolated from a pool of human cells with unknown antimicrobial activities. **Methods:** In this study, *hBD9* gene was cloned into a pSUMO vector and co-expressed with a small ubiquitin-related modifier (SUMO) protein using an *Escherichia coli* expression system. To characterise its antimicrobial property, the soluble fraction of 6xHis-SUMO-hBD9 fusion protein was purified by nickel-charged iminodiacetic acid immobilized metal affinity chromatography (IMAC) followed by SUMO protease cleavage. hBD9 was recovered from the eluent of the second round of Ni-IDA IMAC. In this study, the antimicrobial activity of purified hBD9 against two common eye pathogenic bacteria, namely *Staphylococcus aureus* and *Pseudomonas aeruginosa* was determined. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the protein were also determined. **Results:** The result showed that hBD9 exhibited antimicrobial activity against *S. aureus* but not *P. aeruginosa*. All the hBD9 antimicrobial properties against the common bacteria were verified by conventional antimicrobial assays. **Conclusion:** The findings of this study further enhanced the understanding of hBD9 and its antimicrobial properties that provide essential information towards the discovery and development of a new antimicrobial peptide.

Keywords: Antimicrobial peptides (AMPs), Human beta defensin-9, Minimal bactericidal concentration, SUMO expression system

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INTRODUCTION

Antimicrobial peptides (AMPs) have attracted special interest of scientists in recent years due to their effectiveness in combating pathogens. A variety of AMPs have been isolated and purified from various organisms including humans (1). Defensin is a family of potent antibiotics that produced by neutrophils and macrophages. These molecules play essential roles in the host defence mechanism (2). The protective mechanism of these molecules includes binding to lipid bilayer membranes of pathogenic bacteria, fungi and viruses and subsequently leads to cell lysis and damage to the invading organisms (3, 4). However, the antimicrobial mechanism of defensins has not been fully clarified.

Human defensins are grouped under a family of cationic AMPs with sizes around 5 kDa and is characterised by a conserved six disulphide-linked cysteine motif (5). Based on the position of these disulphide bonds, defensins are divided into α-, β-, and θ-defensins (2). The first well characterised human defensin were β-defensins such as the human neutrophil proteins 1-3 extracted from neutrophil granules, which can be released upon phagocytosis of invading bacterium (6). A second class of human defensins known as β-defensins were later characterised from inducible tracheal AMPs from epithelial cells (7) and also cationic peptides isolated from bovine neutrophils (8). The first human β-defensin

(hBD1) was isolated from blood filtrates and urine, which has been found primarily expressed in kidneys and female urogenital tracts (9). Subsequently, human β-defensin 2 (hBD2) were isolated from the skin of patients with psoriasis and it was found to be expressed by various epithelial cells including the trachea, skin and lung tissues (10). hBD2 has been reported could actively kill Gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa*, and the yeast *Candida albicans*, but not Gram-positive bacteria such as *Staphylococcus aureus* (10). The human β-defensin 3 (hBD3) was discovered based on the similarity search using a genomic-based approach (9). *In vitro* antibacterial assay showed that hBD3 is active against clinical isolates such as *Staphylococcus aureus, Enterococcus faecium, Pseudomonas aeruginosa, Stenotrophomonas maltophilia*, and *Acinetobacter baumannii* (11). Other human β-defensins such as hBD4-8 were also discovered but they are not well characterised.

The human β-defensin 9 (hBD9) is a relatively new β-defensin with unknown characteristics. It was extracted as β-defensin AMP *DEFB109* from the ocular surface and its expression was characterised in the presence of ocular surface infection and/or inflammation (12). At the protein level, hBD9 has been localised to the surface of human cornea epithelium (12). Being a member of the human β-defensin family, it is also expected to have antimicrobial property. To the best of our knowledge, the antimicrobial activity of hBD9 has not been reported. Therefore, the aim of this study was to define the antimicrobial character of hBD9 for the first time. The antimicrobial details of hBD9 provide essential information for the development of anovel antimicrobial compound against pathogenic microorganisms such as Methicillin resistant *Staphylococcus aureus* (MRSA) that frequently infects lungs, eyes and joints (13).

Small ubiquitin-related modifier (SUMO) is a ubiquitin-related protein that fuses covalently to other proteins (14). In the protein expression system, the protein of interest is fused to the N-terminal region of SUMO, which also functionally to protect the fusion protein with its chaperoning properties (15). The SUMO fusion system has been used to facilitate protein expression and purification in many instances including hBD4 (16) and CM4 (17) antimicrobial peptides, and reported to be highly successful (18). The presence of SUMO fusion tag in recombinant proteins has been reported to enhance protein solubility and the final yield (19, 20). Most importantly, SUMO protease digestion produces non-erroneous protein cleavage that releases the target protein with greater fidelity and efficiency. The SUMO protease is also more resilient for pH and urea, as well as more economical compared to enterokinase, factor Xa and thrombin (21). At the later stage, cleaved SUMO fusion tag can be easily removed by passing through the proteolytic reaction a second time on an IMAC column. Unbound collected flow through contains the protein of interest.

Omar (2016) reported that hBD9 peptide expression using a conventional pET fusion system produced hBD9 peptide with limited protein solubility and additional unwanted amino acids associated with the fusion of 6xHis-tag. Removal of the 6xHis-tag by enterokinase is non-specific and inefficient, causing increased in protein loss and incurred higher protein production cost. Additionally, protein stability and recovery are greatly reduced by narrow pH and urea tolerance range. Furthermore, isolation of the target protein following enterokinase cleavage required laborious purification steps. In this study, we expressed hBD9 using the SUMO-mediated protein expression system. The hBD9 was genetically fused at the C-terminus of 6xHis-taggged SUMO and expressed as a full-length recombinant fusion protein, 6xHis-SUMO-hBD9. The free hBD9 was liberated following enzymatic cleavage by SUMO protease. We also evaluated the bactericidal activity of hBD9 using two common eye pathogens.

MATERIALS AND METHODS

Bacterial strains, vectors and enzymes

Escherichia coli Mach1 (Invitrogen, Massachusetts, USA) was used for cloning and amplification of plasmid. *E. coli* strain BL21(DE3) (Invitrogen, Massachusetts, USA) was used as the host for protein expression and the pET SUMO vector (Invitrogen, Massachusetts, USA) was used as a system to produce the full-length 6xHis-tagged recombinant protein.

Preparation of recombinant plasmid

The DNA plasmid containing the gene encoding for full-length hBD9 (65 amino acid residues) was derived from previous work (22). The gene was amplified by PCR using the DNA plasmid as a template. Briefly, each PCR reaction was prepared by mixing the DNA plasmid (1 µL) with Dream Taq DNA polymerase (0.6 U, Thermo Scientific, Waltham, MA, USA). A pair of primers (forward primer: 5' GTCGCGGATCCAATGGGTTTGGGTCCT 3', reverse primer: 5'GTGGTGCTCGAGTTTCAAGTTAGG 3') were mixed with 25 µL of PCR reaction (10X Dream Taq buffer, 1 mM dNTP mix). The reaction was heated at 95°C (10 minutes) followed by 37 cycles of 95°C (1 minute), 58°C (1 minute), and 72°C (30 seconds). A final extension was performed at 72°C for 10 minutes. The PCR products were separated by 1.5% (*w/v*) agarose gel electrophoresis and ligated into pET SUMO vector according to the manufacturer's protocol (MCLAB, San Francisco, USA). The ligation mixture (5 µL) was introduced into *E. coli* Mach1 competent cells using the conventional heat-shock method. The plasmid transformed *E. coli* cells were grown at 37°C for 1 hour, shaking at 200 rpm, before spreading on LB agar plates supplemented with 50 µg/mL kanamycin. The LB agar

plates were incubated overnight at 37°C.

Colony selection and plasmid DNA isolation

Single colonies were randomly chosen and cultured overnight at 37°C in LB broth supplemented with kanamycin. Bacterial cells were pelleted by centrifugation at 8000 x g for 3 minutes and resuspended in DNA lysis buffer. The DNA plasmid was extracted using Qiagen Miniprep DNA extraction kit (Qiagen, Hilden, Germany). The DNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Scientific, Massachusetts, USA). The nucleotide sequence of the insert was confirmed by nucleotide sequencing.

Large scale expression of the fusion protein

Overnight culture (10 mL) of *E. coli* carrying the recombinant plasmid containing the coding sequence for 6xHis-SUMO-hBD9 was transferred into 1 L LB broth containing 50 µg/mL kanamycin. The culture was incubated for 3 hours until the OD_{600} reached 0.6 - 0.8 at 37°C. Protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 4 hours at 30°C, 200 rpm. The bacterial cells were harvested by centrifugation at 8000 x g for 20 minutes at 4°C. The pellet was transferred into a 50 mL falcon tube before resuspended in lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% (*v/v*) glycerol, 0.5% (*v/v*) Triton X-100, 10 mM imidazole, pH 7.8; 10 mL). Lysozyme (100 mg/mL stock solution; 20 µL) and DNase1 (10 mg/mL stock solution; 15 µL) were then added to the cell suspension and the mixture was gently agitated for 2 hours at 25 °C. The mixture was then sonicated on ice at 30 MHz (10 seconds for 10 cycles, with 20 seconds interval). The sonicated sample was centrifuged at 12,000 x g for 20 minutes at 4°C. The crude lysate containing the soluble protein was filtered with 0.45 µm syringe filter (Sartorius AG, Guttingen, Germany) before loading onto a HisTrap HP 1 mL immobilised metal affinity column (GE Healthcare, Uppsala, Sweden) that was mounted to an Akta Purifier (GE Healthcare, Uppsala, Sweden). After extensive washing with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4), the 6xhis-SUMO-hBD9 fusion protein was eluted by elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) using a constant flow rate of 1 mL/minute. Positive fractions containing the 6xhis-SUMO-hBD9 as analysed by SDS-PAGE were pooled and concentrated to ~10 mL using a Vivaspin 6 centrifugal protein concentrator with molecular weight cut-off of 10 kDa (Sartorius AG, Guttingen, Germany).

Enzymatic cleavage of 6xHis-SUMO-hBD9 and purification of hBD9

The SUMO fusion protein (200 µg) was digested with SUMO protease (1U) (MCLAB, San Francisco, USA) at 30°C for 2 hours to cleave the 6xHis-SUMO from hBD9. Afterwards, the SUMO protease digested fusion proteins were subjected to IMAC purification for a second round. The 6xHis-SUMO free hBD9 peptides were collected from the flow through (unbound fractions). The purified hBD9 was analysed using SDS-PAGE and the protein samples were stored at -20°C.

Antimicrobial assay of recombinant HBD9

In this study, antimicrobial assays were carried out to determine the antimicrobial activities of hBD9 against two common pathogens that cause eye infections. The micro dilution broth method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the hBD9 peptide. In this study, the former was determined as the minimum concentration of hBD9 that inhibited the growth of *Staphylococcus aureus* or *Pseudomonas aeruginosa* to a visible level while the latter is the minimum concentration of hBD9 that killed 99.9% of the same pathogens (23). In this study, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were grown overnight in LB broth. Different concentrations of hBD9 peptide (0, 9.4, 18.8, 37.5, 75.0, 150.0 µg/mL) were added to the overnight cultures at the exponential phase growth of bacteria at OD_{600} of 0.01 (~1 x 10^6 CFU/mL) in buffer and growth media. Determination of bacterial growth was done using qualitative observation. The mixtures were incubated at 37°C for 24 hours at 200 rpm in an orbital shaker. The MIC was determined by examining the tubes for visible growth (cloudiness). The cultures with and without visible growth were recorded as positive (+) and negative (-), respectively. The MBC was determined by plating the culture broth from the tubes with invisible growth of bacteria onto LB agar plates and incubated at 37°C for 18 hours. The minimum concentration of hBD9 which inhibited bacterial colony formation was expressed as MBC.

Kinetics of bactericidal efficacy

The MBC concentration of hBD9 was incubated with the overnight culture at exponential phase at OD_{600} of 0.01 (~1 x 10^6 CFU/mL) in buffer and growth media. The mixtures were incubated at 37°C for 8 hours at 200 rpm in an orbital shaker. After 0, 2, 4, 6 and 8 hours of incubations, an aliquot of each sample (100 µL) was 10-fold serially diluted with LB broth until 10^{-6} dilution and diluted samples (50 µL each) were plated on LB agar. The numbers of colony forming unit (CFU) were measured following overnight incubation at 37°C. In the negative control, the bacterial suspension (~1 x 10^6 CFU/mL) was incubated with the growth media in the absence of hBD9 protein. The curve that representing the bacteria survival at all time points was plotted to determine the best time point for kinetic killing.

Killing percentage was calculated using formula below:

$$\frac{\text{Number of colonies (control cells - treated cells)}}{\text{Number of colonies from the control cells}} \times 100\%$$

RESULTS

Construction, expression and purification of 6x-His-SUMO-hBD9 and hBD9

The coding fragment of hBD9 was successfully ligated via a TA cloning approach into the Champion™ pET SUMO vector to create a recombinant plasmid, pET-SUMO-hBD9 (Fig. 1) that carries the coding sequence for 6xHis-SUMO-hBD9 and the orientation of the insert was confirmed by the nucleotide sequencing. Fig. 2 shows the nucleotide and amino acid sequences of the SUMO fusion protein. The SUMO modifier contains a polyhistidine-tag that was fused to its N-terminus.

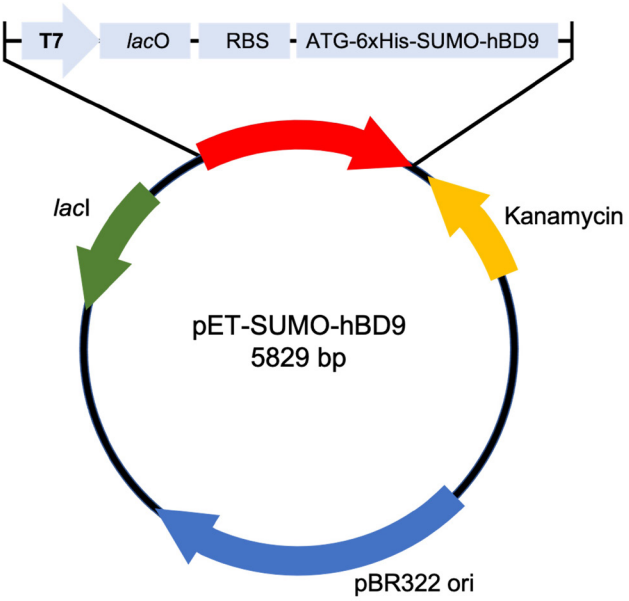

Fig. 1: A schematic diagram of the expression vector pET-SUMO-hBD9. Human β-defensin 9 was expressed as a SUMO fusion protein tagged with 6xHis at its N-terminus.

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atg ggc agc agc cat cat cat cat cat cac ggc agc ggc ctg gtg ccg cgc ggc agc gct
 M   G   S   S   H   H   H   H   H   H   G   S   G   L   V   P   R   G   S   A
agc atg tcg gac tca gaa gtc aat caa gaa gct aag cca gag gtc aag cca gaa gtc aag
 S   M   S   D   S   E   V   N   Q   E   A   K   P   E   V   K   P   E   V   K
cct gag act cac atc aat tta aag gtg tcc gat gga tct tca gag atc ttc ttc aag atc
 P   E   T   H   I   N   L   K   V   S   D   G   S   S   E   I   F   F   K   I
aaa aag acc act cct tta aga agg ctg atg gaa gcg ttc gct aaa aga cag ggt aag gaa
 K   K   T   T   P   L   R   R   L   M   E   A   F   A   K   R   Q   G   K   E
atg gac tcc tta aga ttc ttg tac gac ggt att aga att caa gct gat cag acc cct gaa
 M   D   S   L   R   F   L   Y   D   G   I   R   I   Q   A   D   Q   T   P   E
gat ttg gac atg gag gat aac gat att att gag gct cac aga gaa cag att ggt ggt ggt
 D   L   D   M   E   D   N   D   I   I   E   A   H   R   E   Q   I   G   G   G
ctg ggt ccg gcg gaa ggt cac tgc ctg aac ctg ttc ggt gtt tgc cgt acc gac gtt tgc
 L   G   P   A   E   G   H   C   L   N   L   F   G   V   C   R   T   D   V   C
aac atc gtt gaa gac cag atc ggt gcg tgc cgt cgt cgt atg aaa tgc tgc cgt gcg tgg
 N   I   V   E   D   Q   I   G   A   C   R   R   R   M   K   C   C   R   A   W
tgg att ctg atg ccg atc ccg acc ccg ctg atc atg agc gac tac cag gaa ccg ctg aaa
 W   I   L   M   P   I   P   T   P   L   I   M   S   D   Y   Q   E   P   L   K
ccg aac ctg aaa taa
 P   N   L   K   *
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Fig. 2: Nucleotide and amino acid sequences of the human β-defensin 9 SUMO fusion protein. Nucleotide sequence of the construct is displayed in small letters. The amino acid sequence of the construct is displayed in capital letters. The 6x His-tag (italic) and the preceding sequence are underlined. The SUMO modifier and hBD9 amino acid sequences are coloured in green and red, respectively. The stop codon is marked with an asterisk. The theoretical molecular weight for the construct is 20770.76 Da. The molecular weight for free hBD9 is 6920.23 Da.

The pET-SUMO-hBD9 plasmid was introduced into *E. coli* strain BL21(DE3) by heat-shock at 42°C. The SUMO fusion protein 6xHis-SUMO-hBD9 was successfully expressed upon induction with 1 mM IPTG (Fig. 3). Following the first round of IMAC, SDS-PAGE analysis shows that the eluted fractions (Fig. 3a, lanes 9-13) contain a dominant protein band ~23 kDa, a size which is overall in good agreement with the calculated molecular weight of 6xHis-SUMO-hBD9 (~21 kDa). The positive fractions were combined, concentrated, and digested with SUMO protease. Fig. 3b shows that 1 hour of incubation with SUMO protease (100U/µL) at 30°C was sufficiently to digest 200 µg of SUMO fusion protein. The freed hBD9 was recovered with a second round of IMAC. As shown in Fig. 3c, the free hBD9 was collected from the flow-through (lanes 3-4). The SUMO modifier containing the polyhistidine-tag was eluted by elution buffer containing 500 mM imidazole (lane 10).

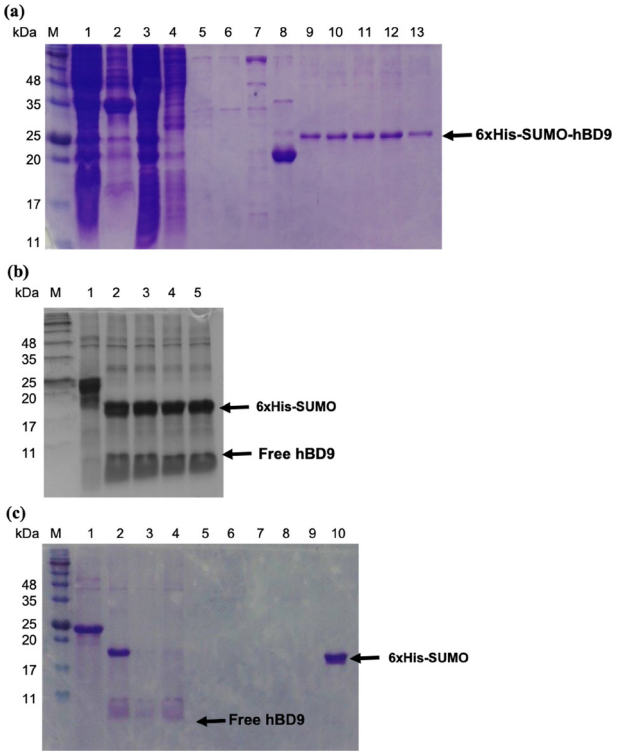

Fig. 3: SDS-Polyacrylamide gel electrophoresis analysis of *Escherichia coli* expressed and purified human β-defensin 9 (hBD9). (a) Purification of the SUMO fusion protein with immobilised metal affinity chromatography (IMAC). Lane M; protein ladders in kDa; lane 2; insoluble fraction, lane 3; crude lysate, lanes 4 – 6; unbound proteins, lanes 7-13; imidazole eluted fractions. (b) SUMO protease digestion with different periods of incubation. Lane M; protein markers in kDa, lane 1; uncleaved sample, lanes 2-4; incubation of 1, 2, 4 and 6 hours, respectively. (c) reverse purification of hBD9 with a second round of IMAC. Lane M; protein markers in kDa, lane 1; intact 6xHis-SUMO-hBD9, lane 2; SUMO protease cleaved products, lanes 3 – 6; unbound protein (free hBD9), lanes 7 – 10; imidazole eluted bound protein (6xHis-SUMO). The arrows indicate the location of purified proteins.

Determination of minimal inhibitory concentration and minimal bacterial concentration of human β-defensin 9

The antimicrobial activity of hBD9 was assessed by microbial inhibitory concentration (MIC) by incubating 1 x 10^6 CFU/mL of *S. aureus* and *P. aeruginosa* with a range of hBD9 concentrations. The bacterial cultures were incubated for 4, 8 and 24 hours at 37°C after addition of hBD9. After 4 hours of incubation, culture media of *S. aureus* with hBD9 concentrations lower than 150 µg/mL turned turbid, indicating continuous growth of bacteria (Fig. 4a and b). The culture media with hBD9 concentrations of 150 and 300 µg/mL remained clear up to 24 hours of incubation (Fig. 4c). The result indicates that the MIC of hBD9 to inhibit the growth of 1 x 10^6 CFU/mL of *S. aureus* was 150 µg/mL. With respect to *P. aeruginosa*, the final concentration of hBD9 was increased to 400 µg/mL following optimisations. Unfortunately, none of the hBD9 concentration used in this study was able to inhibit the growth of the *P. aeruginosa* at an initial CFU of 1 x 10^6/mL. As shown in Fig. 4d-f, all culture media turned cloudy after 4 hours of incubation even at the highest hBD9 concentration of 400 µg/mL. As a result, the MIC of hBD9 towards *P. aeruginosa* could not be determined in this study.

Based on the result obtained from MIC determination assay, the antimicrobial activity of hBD9 was further characterised by determination of minimum bactericidal concentration (MBC). As shown in Fig. 5, sub-culturing of these culture broth gave rise to bacterial colonies at all hBD9 concentrations except at 300 µg/mL (Fig. 5b and c). The result also implies that a minimum incubation period of 8 hours was required to kill all residual of *S. aureus*. The MBC of hBD9 was not determined for *P. aeruginosa* because MIC could not be determined for this pathogen.

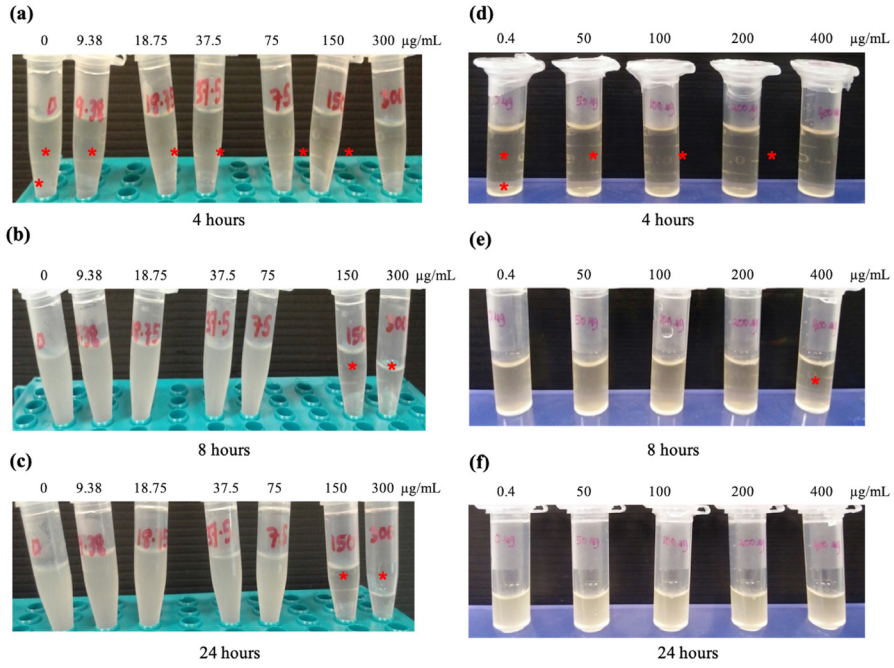

Fig. 4: Determination minimal inhibitory concentration (MIC) of human β-Defensin 9 (hBD9) against two common eye pathogens. Tubes (a) – (c) and (d) to (f) contains 1 x 10^6 CFU/mL of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. hBD9 peptide at various concentrations were transferred to these tubes, which were then incubated for 4, 8 and 24 hours at 37°C. The MIC was determined based on turbidity of the broth visually examined by naked eyes. Tubes marked with asterisks (*) indicate clear visibility.

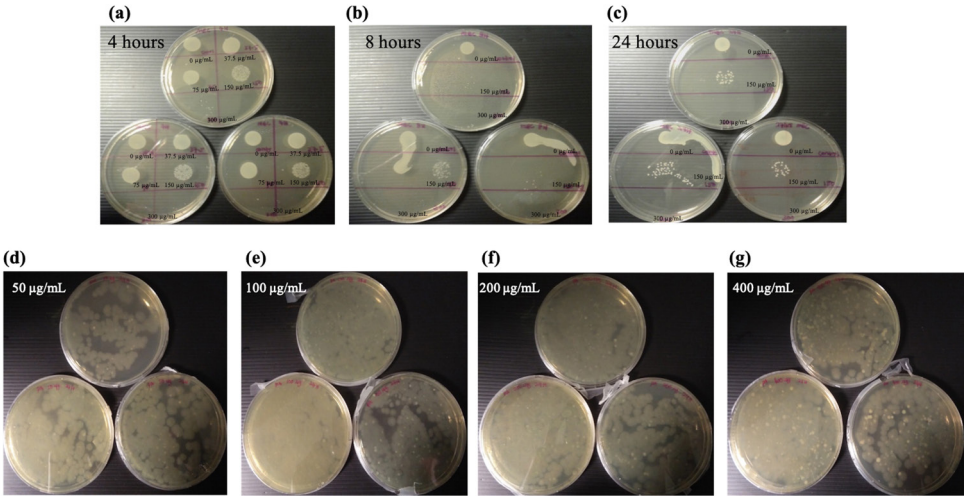

Fig. 5: Determination minimal bactericidal concentration (MBC) of human β-Defensin 9 (hBD9) against *S. aureus* and *P. aeruginosa*. (a) MBC determination for *S. aureus* and, (b) MBC determination of *P. aeruginosa*. Culture media were plated after (a) 4 hours, (b) 8 hours, and (c) 24 hours of incubation. Each time point of study was carried out in triplicate. Plating areas correspond to their respectively concentrations were labelled.

Kinetics of bactericidal assay

Following determination of MBC of hBD9 towards *S. aureus*, the kinetic of bactericidal of hBD9 at 300 µg/mL was determined after 0, 2, 4, 6 and 8 hours of incubation with 1 x 10^6 CFU/mL. The culture medium at each time point was ten-fold serially diluted to 1 x 10^{-6} and plated on agar plates (Fig. 6). The numbers of colony forming unit (CFU) were determined following overnight incubation at 37°C. Fig. 7 shows the curve of the percentage of bacterial survival against each time point to determine the incubation time required for kinetic killing of *S. aureus* and *P. aeruginosa* relative to negative controls. The results show that less than 5% of *S. aureus* survived after 4 hours of incubation with 300 µg/mL hBD9. Similarly, the kinetic killing assay was also carried out for *P. aeruginosa* using the highest concentration of 400 µg/mL hBD9 (Fig. 6). As expected, hBD9 did not show any antimicrobial effect against *P. aeruginosa* under our experimental condition. As a result, the kinetic killing of hBD9 towards *P. aeruginosa* could not be determined in this study.

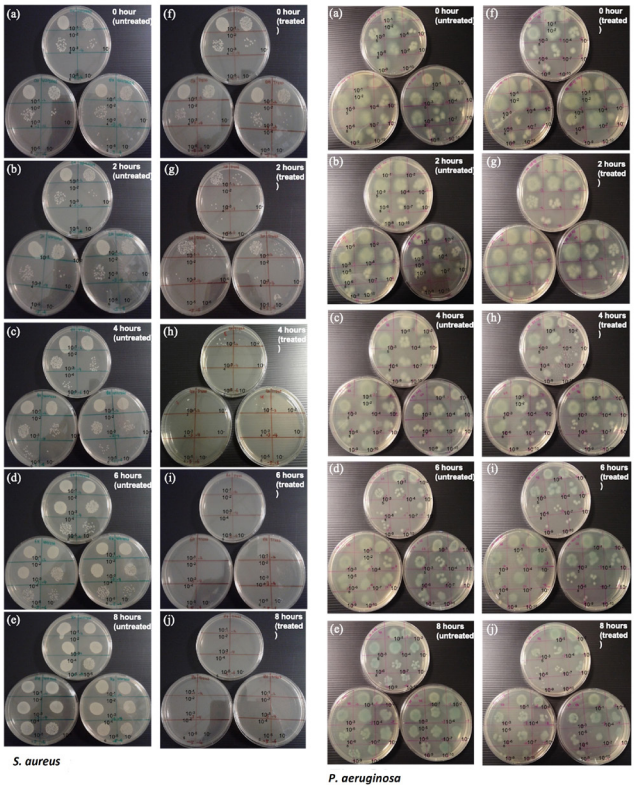

Fig. 6: Kinetic killing of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by human-defensin 9 (hBD9). Left panel: (a) to (e) are hBD9 untreated samples that were plated from culture broth containing initial 1 x 10^6 CFU/mL and incubated for (a) 0, (b) 2, (c) 4, (d) 6, and (e) 8 hours. (f) to (i) are hBD9 treated samples that were plated from culture broth containing initial 1 x 10^6 CFU/mL *S. aureus* and 300 g/mL hBD9 and incubated for (f) 0, (g) 2, (h) 4, (i) 6, and (j) 8 hours. Right panel: (a) to (e) are hBD9 untreated samples that were plated from culture broth containing initial 1 x 10^6 CFU/mL and incubated for (a) 0, (b) 2, (c) 4, (d) 6, and (e) 8 hours. (f) to (i) are hBD9 treated samples that were plated from culture broth containing initial 1 x 10^6 CFU/mL *Pseudomonas aeruginosa* and 400 g/mL hBD9 and incubated for (f) 0, (g) 2, (h) 4, (i) 6, and (j) 8 hours. The grids on each plate are labelled with respective 10-fold serial dilutions. Plating from each time point was carried out in triplicates.

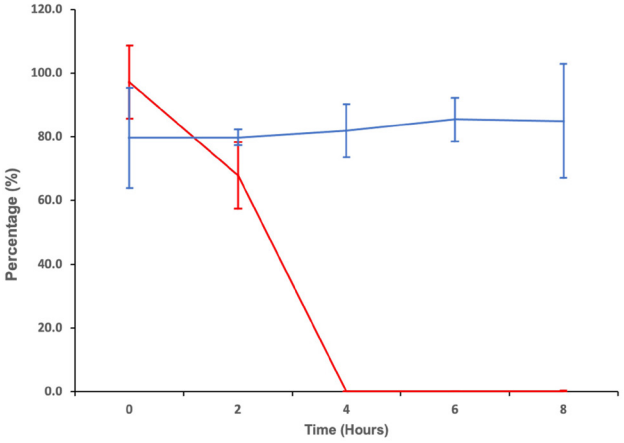

Fig. 7: Kinetics of bactericidal assay. Average percentages of *S. aureus* (red line) and *P. aeruginosa* (blue line) survival against hBD9 at different time points.

DISCUSSION

In a previous study reported by Omar (24), DEFB-109 (*hBD9* gene) was cloned into a conventional pET polyhistidine-tag fusion system for hBD9 expression. Removal of polyhistidine-tag was achieved by enterokinase (EK) which recognised a cleavage motif of (Asp) 4Lys (DDDDK) preceding hBD9 peptide with non-native amino acid residues as a linker in between the EK cleavage site and hBD9. Therefore, the purified hBD9 contained unwanted amino acid residues which may affect the yield, solubility, stability and the activities of hBD9. In this study, SUMO fusion tag fused at the N-terminus of hBD9 was cleaved off by SUMO protease. This enzyme recognises the 3D conformation of the SUMO modifier, and as such, does not cleave erroneously at other site within the recombinant protein (25). As reported in previous studies (26,27), the SUMO protease cleaves the C-termini of SUMO and also deconjugates it from the side-chain of lysine residues within modified proteins. Furthermore, SUMO fusion tag expression system was also reported to enhance the expression and solubility of recombinant target proteins comprise only its native amino acid residues (15, 28).

The MIC of *S. aureus* and *P. aeruginosa* was determined with a range of hBD9 concentrations. The MIC was determined based on the turbidity of the bacterial culture media as described earlier. After obtaining the MIC, MBC was determined. It was determined from broth dilution of MIC assays by sub-culturing the medium from the negative tubes (no visible growth) to agar plates without hBD9 and incubated overnight at 37°C. However, MIC and MBC of hBD9 for *P. aeruginosa* could not be determined in this study. Therefore, kinetic killing of hBD9 against *P. aeruginosa* was unable to be concluded. As a result, *P. aeruginosa* might resistant to hBD9.

Microorganisms that resistant to defensins have been reported. Gram-negative bacteria with reduced negative charges of the outer membrane, and limit the interaction of cationic defensins with their membrane targets (29). *P. aeruginosa* modify Lipid A by regulating PhoP-PhoQ and PmrA-PmrB regulatory systems. Alteration of Lipid A by increasing the number of Lipid A acryl tails is able to reduce the electrostatic interaction between cationic defensins to the outer membrane (30). However, not all the Gram-negative bacteria have the same antimicrobial-peptide-resistance mechanisms. In future study, more investigation is needed in order to explain the resistance of *P. aeruginosa* against hBD9.

CONCLUSION

In conclusion, the efficiency of hBD9 production has been improved by using the SUMO fusion protein expression system. The customised protein expression and purification protocol also shortened the time and lowered the cost of producing the hBD9 protein at a laboratory scale. From this work, hBD9 peptide showed antimicrobial activity towards *S. aureus* but not *P. aeruginosa*. Our finding in this research indicates that the effective dose of hBD9 to kill the bacteria was 300 µg/mL. This study shows that hBD9 is less effective in antimicrobial activity as compared to others hBDs because of higher concentrations and longer time are required to exhibits its antimicrobial activity. Postulate antimicrobial may not be its main effect.

ACKNOWLEDGEMENT

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