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C-TERMINAL CLAW STABILISATION IN CORVUS AVIAN BETA DEFENSIN 7 AND ITS MUTATION STUDIES

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ABSTRACT: Antimicrobial peptides are considered as promising and potential next-generation antibiotics on account of their broad spectrum of antimicrobial activity and unique mode of microbial killing. For therapeutic application, it is essential to consider the hemolytic or cytotoxic property of AMPs along with antimicrobial function. In the current study, an avian beta-defensin 7 (AvBD7) gene was identified from *Corvus splendens*. The predicted amino acid sequence showed the presence of β defensin core motif and three disulphide bridges. We analysed the interaction of wild and mutant analogues of mature *Corvus* AvBD7 dimer with neutral 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) that mimics mammalian membranes using molecular dynamics simulation studies. Hydrophobic residues at the N-terminus of each monomer were found to provide stability in the dimeric form. In both monomer and dimer, Asp3 and Asp4 interact with Tyr40 and Ser41 thereby helping in maintaining a claw shape at the C-terminus. During simulation, we found that the C-terminus claw region of wild dimer remained intact than its mutant analogs, and also wild AvBD7 exhibited a greater propensity to bind POPC membrane than its mutant analogs. Based on the membrane-peptide distance and number of hydrogen bonds formed between peptide and membrane, we conclude that the alanine mutant might be least hemolytic as it showed reduced propensity to bind to POPC membrane.

INTRODUCTION: The alarming and rapid evolution of multidrug-resistant strains necessitates the development of novel therapeutic candidates possessing a reduced tendency to induce microbial resistance and least hemolytic and cytotoxic propensity. Antimicrobial peptides (AMPs) have been acknowledged as a promising target for prospective anti-infective agents due to their wide range of antimicrobial activity, low toxicity, and reduced susceptibility to gain drug resistance and multiple modes of killing [1-4].

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They are, in general small cationic peptides (<100 amino acid residues) that possess a significant portion of hydrophobic amino acid residues (≥30% or more) and the capability to fold into amphipathic conformations on interaction with membrane [5-8]. A major group of AMP found among vertebrates is defensin. They are characterized by the existence of a triple-stranded anti-parallel *β*-sheet structure with a less-conserved N-terminal helix of varying stability and three intramolecular disulfide bridges formed by 6-cysteine residues.

Out of the three different defensin subfamilies (α, β, and θ) that exist in vertebrates, only β-defensins have been reported in birds and are termed as avian beta-defensins (AvBDs). These classes of peptides are capable of killing bacteria, enveloped viruses, fungi, and eukaryotic parasites [9-12].

Though, the precise antimicrobial mechanisms of AMPs remain unclear, the cytoplasmic membrane is generally suggested as the primary target of antimicrobial peptides, and the accumulation of AMP on microbial membrane augment membrane permeability resulting in the release of cytoplasmic substances and cell death [13, 14]. It has been reported that features such as cationicity and hydrophobicity, and the potential for oligomerization play elementary roles in the microbicidal activity of these peptides by membrane disruption. Direct microbial killing is initiated by the electrostatic interactions between the peptides and the microbial membrane, and the hydrophobic residues of these peptides are responsible for the membrane permeabilization.

Although hydrophobicity and cationicity of the peptides are essential for their activity, beyond a limit these parameters do not improve antimicrobial activity [13, 15-22]. Highly hydrophobic peptides target neutrally charged mammalian cell membrane leading to a reduction in the selectivity of target [18, 23-26]. One of the major limitations in developing AMP as a therapeutic is peptide-mediated hemolysis or cytotoxicity against host cells [27].

Earlier studies have revealed that beta-defensins perform their activity in their most stable dimer form. Suresh and Verma recommended that in human β-defensin 2 (HBD-2), claw-shaped and positively charged tail region is important to hold on to the bacterial membrane for the initial interaction [28]. MD simulation studies using *Anas platyrhynchos* avian beta-defensin 2 (Apl_AvBD2), wild type and their more cationic *in-silico* mutants (replacing hydrophobic residues by cationic) revealed that C-terminal 'claw' which is significant for antimicrobial activity remained intact in the wild type throughout the simulation, but the shape of the claw showed alteration in all the mutants during simulation [15].

The structure-activity relationship study of antimicrobial peptides helps us to design and develop many new analogs with improved activity, higher stability, and less toxicity to eukaryotic cells. Optimizing the natural AMP by insertion, deletion, and substitution of amino acid residues is one of the major approaches for peptide designing. Though the interactions of AMP with microbial membrane are of great concern, it is required to comprehend the effect of AMP on mammalian membranes as the AMP induced hemolysis of host cells restricts the use of these classes of defense peptides as potential therapeutics.

Production of antimicrobial peptides and proteins is one of the major non-oxidative antimicrobial mechanisms in birds as their heterophils lack superoxide ions and myeloperoxidase [29, 30]. Avian scavengers offer a significant ecosystem service role, including carcass removal, without them getting affected. Hence these birds may be considered as an important source of antimicrobial peptides. Till now, the whole genomic information on defensin clusters has only been reported in very few avian species [31, 32].

In the current study, we identified and sequenced the AvBD7 gene of *Corvus splendens* and also attempted to understand the structure-activity relationship of the corresponding peptide. In order to recognize the role of hydrophobicity in mediating the interaction of the peptide with a mammalian membrane, we compared the interaction of wild and two *in-silico* mutants of AvBD7 dimer (W39F and W39A) with model membrane using Molecular Dynamic simulation analysis.

MATERIALS AND METHODS:

Ethics Statement: All experimental animal procedures were approved by the Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Pookode, Kerala, India (Approval no. IAEC/COVAS/PKD/17/2019 dated 02.08.19).

Collection of Samples: Tissues/organs from live *Corvus splendens* were collected for this study. Birds were euthanized by intraperitoneal pentobarbitone sodium administration. The tissues were immediately dissected and placed in RNA later and stored at -20 °C or -80 °C until use.

RNA Extraction, Reverse Transcriptase Polymerase Chain Reaction Amplification, and Sequencing: Approximately 50-100 mg of tissue was homogenized in TRIzol reagent, and total cellular RNA was isolated using TRIzol (Invitrogen) method. The quality and concentration of RNA were assessed using a nanodrop

spectrophotometer and were separated on 2% agarose gel. Reverse transcription of the extracted RNA was performed in a 20µl reaction using SuperScript™ III First-Strand System for RT-PCR from Invitrogen. The synthesized first-strand cDNA was amplified by polymerase chain reaction using Taq Polymerase with Corvus specific AvBD7 primer. The PCR amplification was performed in an Eppendorf Master cycler in 25 ul volume. PCR products generated were then run on a 1% agarose-TAE gel. The amplified product was purified using Wizard® SV PCR Clean-Up system Kit, and the purified samples were sequenced. The sequences obtained were aligned using SEQUENCHER 4.1.2 (Gene Codes Inc.) The sequence was analyzed for its identity using NCBI-BLAST and submitted to GenBank.

Computational Analysis: Newly identified avian beta-defensin 7 (AvBD7) gene of *Corvus splendens* was compared with other organisms using Blast at NCBI (www.ncbi.nlm.nih.gov/genome). Since the antimicrobial activity lies in the peptide, we translate the nucleotide sequence to its corresponding peptide, and further analysis was made on the peptide. The experimentally obtained nucleotide sequence of the gene was translated using the translate tool of ExPASy [33]. The predicted sequence was compared with other protein sequences in the UniProt database to find out homologous sequence using Blastp at NCBI and was aligned with other organisms using ClustalW [34] using MEGA7 [35]. The signal peptide sequence of the identified AvBD7 sequence was predicted using SignalP 5.0 Server [36,] and disulfide connectivity among six cysteine residues was determined by DISULFIND server [37].

Molecular Dynamics: Homology modeling of wild type and *in-silico* mutated AvBD7 peptides were predicted with NMR structure of Chicken AvBD7 as a template using Phyre2 [38]. The best probable template PDB ID, c5lcsA showed 66% similarity with the query sequence, and the modeling pipeline of Phyre2 returned a structure with 98% modeling confidence. High modeling confidence suggests that the model is likely to adopt the predicted 3D structure.

The dimeric state of the modeled peptide AvBD7 (wild type) and its mutant W39F and W39A were generated using ZDOCK server [39]. Predicted 3D structures of AvBD7 monomer and dimers were visualized and analyzed using discovery studio visualizer Software (Accelrys Software Inc., 2007 Accelrys Discovery Studio Visualiser v 2.5.5. Accelrys Software Inc., San Diego). Hydrophobic Trp residue in the claw region of wild type protein was mutated to other hydrophobic residues such as Phenylalanine and Alanine, as expected there wasn't much change in the predicted conformation of the claw regions located at the C-terminal end.

Molecular dynamic simulation studies of AvBD7 wild and mutant dimers with the mammalian membrane were performed in order to understand the difference in their interaction with the membrane. Modeled structures of wild and mutated AvBD7 dimers were loaded on to the VMD software, the system was embedded in a membrane environment containing POPC with membrane dimensions of 55, 56 Å. The peptide in each system was reoriented on the top of the lipid bilayer system with the claw region facing the head region of POPC at a distance of 12Å from C alpha atom of the terminal residue.

The system in the membrane environment was solvated in two steps, and the distance between the maximum and minimum Z-coordinates and the water box edges in the z-axis was set to 15 Å. To solvate the membrane system a total of 5767 water molecules TIP3 model were placed below the membrane plane with: a water box dimensions of 57.62, 57.00, 15.86 (x,y,z in Å), and a total of 5767 water molecules TIP3 model were placed above the plane with: a water box of the dimensions 57.62, 57.00, 33.17 (x,y,z in Å). The system was neutralized using 20 Chloride ions and 16 sodium ions, making up a salt concentration of 0.15 mol/L.

Molecular dynamics was performed on this system using NAMD molecular dynamics package [40,] and CHARMM36 force field [41] was used in all MD simulations. Protein structure factors for the ionized structures were prepared using VMD [42] and the plugin QwikMD [43]. Energy Minimization of the systems was done for 2000 steps. This and all further downstream process were performed under explicit solvent using the TIP3 water in the NpT ensemble, a distance cut-off of 12.0 Å was applied to short-range non-bonded interactions, 10.0 Å for

the smothering functions, and Long-range electrostatic interactions were treated using the particle-mesh Ewald (PME) [44].

Annealing was performed with backbone restrained, explicit solvent in the NpT ensemble, for 0.29 ns of simulation, a temperature ramp from 60 K to 300 K was performed for 0.24 ns. The following parameters were used for further analysis. The pressure at 1 atm was maintained using Nosé-Hoover Langevin piston [45, 46]. The equations of motion were integrated using the r-RESPA multiple time-step schemes [40] to update the short-range interactions every 1 step and long-range electrostatic interactions every 2 steps. The time step of integration was chosen to be 2 fs for all simulations.

The Equilibration steps were performed with backbone restrained under explicit solvent in the NpT ensemble, consisted of 1.00 ns of simulation. The temperature was maintained at 300 K using Langevin dynamics. The MD of the final systems were performed with a temperature maintained at 300 K using Langevin dynamics and consisted of 10.0 ns of simulation, no atoms were constrained.

RESULTS AND DISCUSSION:

Identification of AvBD7 of *Corvus splendens*: The entire nucleotide sequence of the *Corvus* AvBD7 cDNAs included open reading frames (ORFs) of 201bp and 66 amino acids. The results of the Blastp revealed that the sequence showed 100% sequence identity with AvBD7 of *Corvus brachyrhynchos* from residues 1 to 62. However, AvBD7 of *C. splendens* possessed additional 4 residues at the C-terminal in comparison to *C. brachyrhynchos*. Alignment of the *AvBD7* protein with other related proteins was done by ClustalW; the presence of six cysteine residues is highlighted in red columns in **Fig. 1**.

The alignment showed the presence of a crucial structural component; β-Defensin core motif consists of six cysteine residues and GXC motifs that are conserved in all β-defensins [20, 47-50]. This indicates that the newly identified protein belongs to the defensin group.

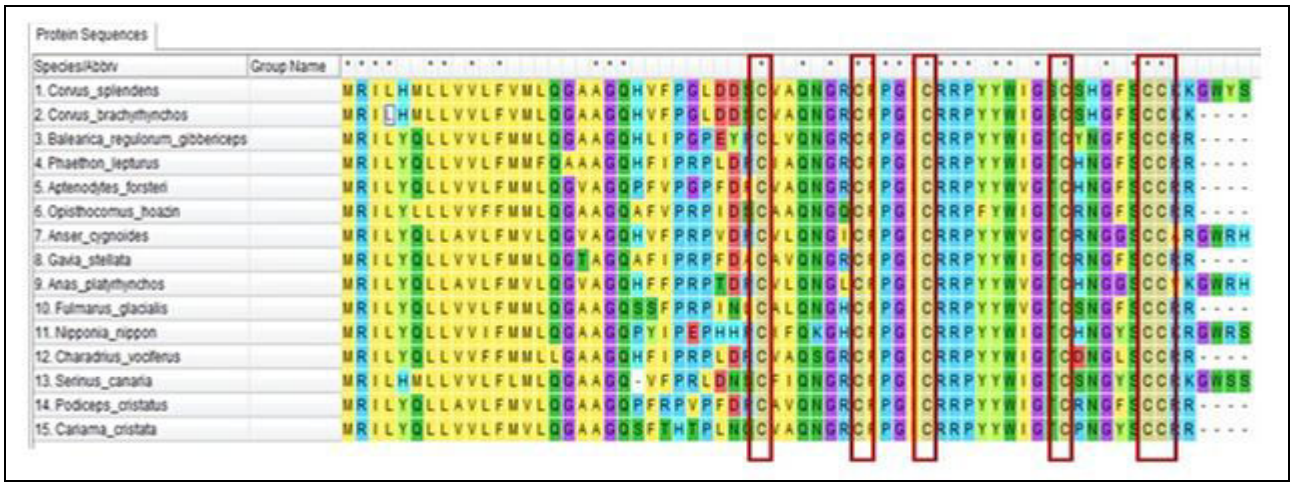

FIG. 1: ALIGNMENT RESULT OF *CORVUS* AVBD7

The signal peptide cleavage site of this gene was predicted between the positions 20 and 21 amino acid residues (between AAG-QH residues). Defensins are usually characterized by 3 disulphide bonds; disulphide bonding analysis of our peptide **Fig. 2** also showed the presence of three disulphide bridges [51-53].

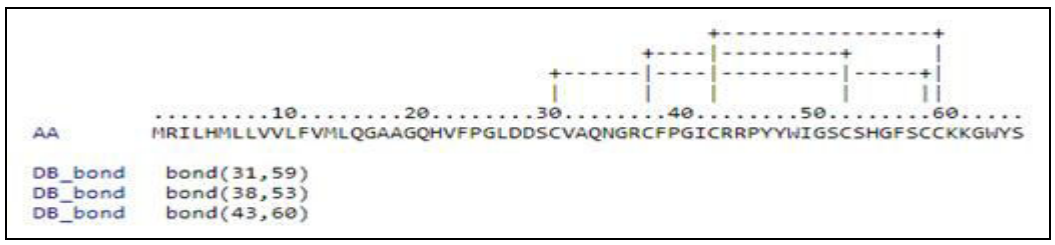

FIG. 2: DISULPHIDE CONNECTIVITY PREDICTION USING DISULPHIND

The AvBD7 of *C. splendens* identified in this study was found to possess 5 cationic residues (3Arg and 2 Lys) and 2 anionic residues (Asp). The overall surface charge of folded AvBD7 monomer was found to be +3.08, whereas folded dimer had an overall surface charge +5.9. Minimum cationicity required for AMPs to exhibit its activity has been reported as +2 [1, 54, 55]. As dimerization showed a significant increase in the cationicity, dimer-membrane interaction may be stronger than monomer-membrane interactions and probably more active than monomer. Oligomerization, which brings about an increase in cationicity may be carried out by augmentation in the local concentration of the peptides in response to several environmental factors [28, 56]. Oligomeric structure of HBD-2 possesses uniform positively charged outer surface which is capable of eliciting electrostatic charge based permeabilization of the membrane rather than pore formation mechanism [57]. It has been reported that human α-defensin hNP3 crystal structure revealed its dimeric state possessing 6 beta-strands [58].

Homology modeling of AvBD7 monomers (both wild and mutants) was carried out using Phyre2 software, and the dimeric forms were created using ZDOCK software. Preliminary analysis of the modeled monomeric structure revealed 4 distinct geometric faces **Fig. 3A,** out of which face 2 and 3 seems to have a role in membrane interaction and dimerization, respectively. Face1 and face2 harbour positively charged residues along with few hydrophobic residues, face1 is near to N-terminal region and contains positively charged Arg12 and a hydrophobic Phe14, the face2, which is adjacent to face1 encloses Arg19, Arg20, and Pro21 residue. Face3 in contrast, is distinct by the presence of several hydrophobic residues (Gly1, Leu2, Gly16, Ile17, Cys18, Trp24, Ile25, Gly31, Phe32, Cys34, and Trp39).

Earlier studies reveal that C-terminal claw-like structure in both Human beta-defensin 2 and avian b-defensin Apl_AvBD2 are highly cationic and is important for initial binding to the anionic bacterial membrane [15, 28]. It is interesting to note that as in the case of Apl_AvBD2 both monomer and dimer of modeled AvBD7 of *Corvus splendens* also possessed the distinct C- terminal claw. In this peptide, claw region contained hydrophobic residues Tpr39, Tyr40, and Ser41, moreover, Tyr40 and Ser41 were found to be interacting with Asp3 and Asp4 on the N terminus of the same monomer **Fig. 3B**. This interaction seems to have a role in maintaining the shape of the hydrophobic claw on the C-terminus. This claw region is adjacent to face 2, the dimer was formed in such a way that the claw regions of both the monomer were positioned on one side of the dimer **Fig. 3C**. This claw region is reported to have a significant role in the initial interaction with bacterial membrane [15].

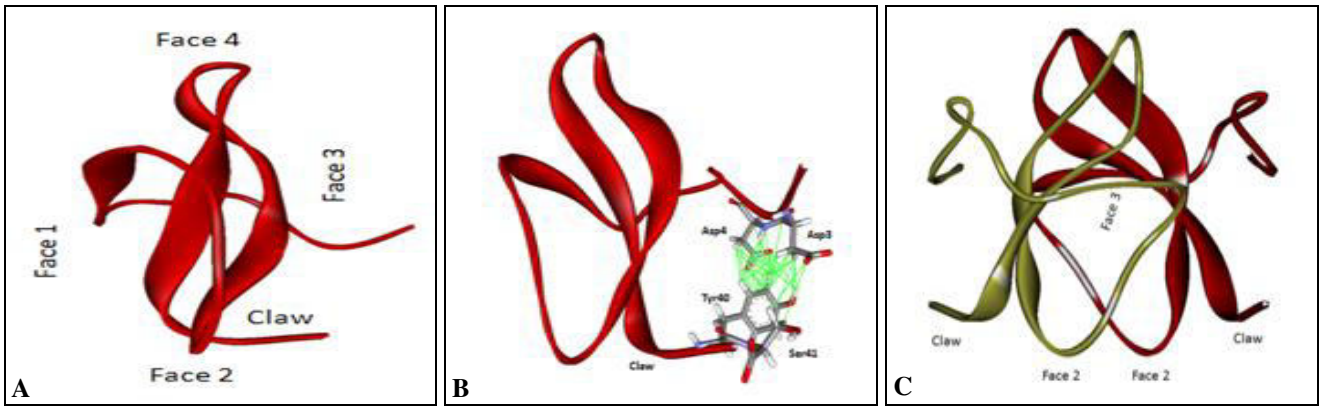

FIG. 3A: *CORVUS* AVBD7 SHOWING 4 DISTINCT FACES. B: MONOMER SHOWING INTERACTION BETWEEN ASP3 AND ASP4 IN THE N-TERMINUS WITH HYDROPHOBIC RESIDUES AT C-TERMINUS. C: AVBD7 DIMER

Monomeric human beta-defensin 2 (HBD2) exhibits amphiphilic nature having cationic and hydrophobic residues on either surface, and the hydrophobic residues are involved in dimerization [28]. Interaction of hydrophobic residues on face 3 with the bulk aqueous solvent may be the driving force to form dimers. In the dimeric form, Face3 of two monomers interacted with each other by 1455 Van der Waals contacts (4A cutoff). Apart from the hydrophobic residues on Face3, Arg19 and Arg20

on Face2 and Trp39 present on the claw region were also found to be involved in interfacial bonding through Van der Waals forces. The first two residues at the N-terminus (Gly1 and Leu2) of one monomer clinged the residues present in beta-sheets of other monomers, thus imparting stability to AvBD7s dimeric form **Fig. 4**. Within 5A cut-off Gly1 of one monomer was seen interacting with Pro15, Gly16, and Phe32 of the second monomer by Van der Waals force, whereas Leu2 interacted with more number of residues, namely Pro15, Gly16, Ile17, Gly31, Phe32 and Ser33 within the same cut-off. The dimer which is devoid of Gly1, Leu2 and Asp3 at the N terminus was found to be less stable in the aqueous phase (data not given). Overall, N-terminal resides seen to have a significant role in maintaining the stability of the dimeric form.

FIG. 4: WILD DIMER SHOWING INTERACTION BETWEEN MONOMERS. RESIDUE GLY1 AND LEU2 (BLUE STICK) OF ONE MONOMER (RED TUBE) IS SEEN IN CLOSE CONTACT WITH SEVERAL RESIDUE (GREY STICKS) ON THE BETA SHEET OF THE SECOND MONOMER (YELLOW SOLID RIBBON)

TABLE 1: RESIDUES INVOLVED IN THE INTERFACIAL INTERACTIONS IN DIMERS

Initial frame (before simulation)					
Wild		**W39F**		**W39A**	
Monomer1	**Monomer2**	**Monomer1**	**Monomer2**	**Monomer1**	**Monomer2**
Gly1	Leu2	Gly1	Gly1	Gly16	Gly1
Leu2	Pro15	Gly16	Leu2	Ile17	Ser5
Pro15	Gly16	Ile17	Gly16	Trp24	Gly16
Gly16	Ile17	Cys18	Ile17	Ser27	Ile17
Ile17	Arg19	Arg19	Cys18	Hsd30	Trp24
Arg19	Trp24	Tyr22	Arg19	Gly31	Gly26
Arg20	Gly31	Trp24	Pro21	Ser33	Ser27
Trp24	Phe32	Gly26	Tyr23		Gly31
Gly26	Ser33	Ser27	Trp24		Phe32
Gly31	Trp39	Gly31	Gly31		Ser33
Phe32		Phe32	Phe32		
Ser33		Phe39	Ser33		
Trp39		Ser41	Gly38		
			Ser41		
Final frame (after simulation)					
Wild		**W39F**		**W39A**	
Monomer1	**Monomer2**	**Monomer1**	**Monomer1**	**Monomer2**	**Monomer1**
Ile17	Gly16	Asp4	Ile17	Gly16	Pro15
Arg19	Ile17	Ile17	Arg19	Ile17	Ile17
Tyr23	Arg19	Arg19	Arg20	Trp24	Trp24
Trp24	Tyr23	Trp24	Trp24	Ile25	Gly26
Tyr40	Trp24	Gly26	Ser27	Ser27	Ser27
Ser41	Phe32	Ser27	Gly31	Gly31	Hsd30
	Tyr40			Phe32	Gly31
	Ser41			Ser33	Phe32
				Tyr40	Ser33

After dimerization, face2, which carries more cationic residues compared to face1, was also seen to be exposed on the surface of the dimer. Positively charged residues and few hydrophobic residues are said to be the prerequisite for membrane interacting peptides [59-61]. In concordance to this, similar residues were found to be present on both face1 and face2 of our peptide.

It is interesting to observe that these faces remained exposed even after dimerization. It was found that the accessible surface area for monomer and dimer are 3024.6 A^2 and 4740.87 $A^{2,}$ respectively. Increased accessible surface area and masking of

hydrophobic residues indicate the dimer resulted in the information of more hydrogen bonds with water.

To gain insights into the role of hydrophobicity of AvBD7 in mammalian membrane interaction, Trp39 at the C-terminal claw region was mutated to highly hydrophobic Phe (W39F) and mid-range hydrophobic Ala(W39A). The residues involved in the interfacial interaction of wild and mutant dimers are mentioned in **Table 1**. Gly16, Ile17, and Trp24 were found in the interfacial region before simulation in all three forms. In W39F mutant, five hydrogen bonds (between Trp24-Cys18, Gly1-Pro21, Gly1-Gly38, Arg19-Gly1, and Tyr22-Gly1) were also identified apart from Van der Waals interaction. As W39F, W39A dimer also possessed 5 hydrogen bonds in the interfacial region, between Trp24-Gly1, Gly31-Trp24, Gly31-Ser33, Gly31-Gly31, and Hsd30-Gly16. W39A dimer was further stabilized by pi-stacking between the Trp39 of both monomers.

It has been reported that highly hydrophobic AMPs can easily interact with zwitterionic mammalian membrane and induce hemolysis[59, 62]. Even though hydrophobicity of AMP had the least role in the binding and permeabilization of Phosphatidyl-glycerol (PG) membrane, in zwitterionic membrane consisting of PC only, there was an approximately 300 fold difference in the permeabilization capacity between highest and least hydrophobic AMPs [54]. The majority of the AMPs are not recommended as the lead for therapeutic development as they induce hemolysis or exhibit toxicity against mammalian cells.

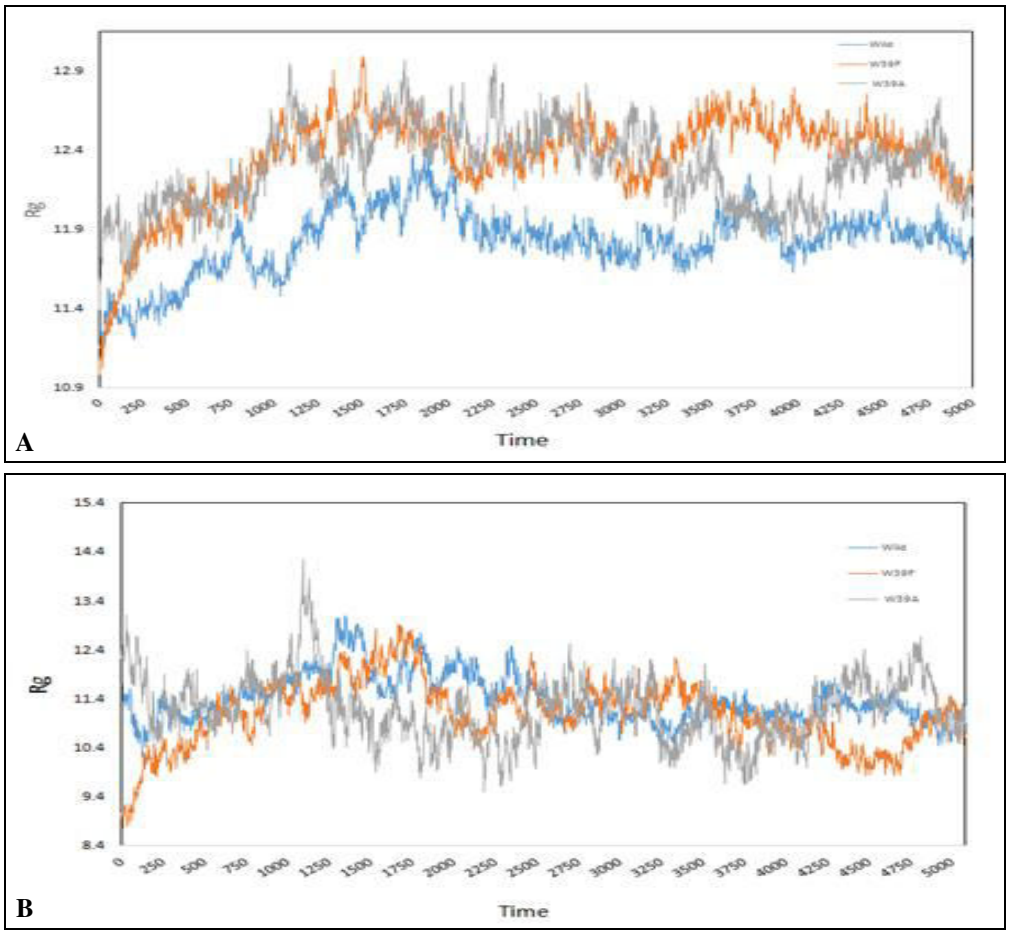

FIG. 5: RADIUS OF GYRATION (RG) OF CORVUS AVBD7 DIMERS. A) RG OF WILD AVBD7 DIMER AND ITS MUTANTS OVER 10NS OF MD SIMULATION. B) RG OF CLAW REGION (RESIDUE 36-41) OF WILD AND ITS MUTANTS

Dimeric forms (both wild and mutants) were only selected for MD analysis as beta-defensins are stable and efficient in this form. We performed a 10 ns MD simulation of both wild and mutant analogs of modeled AvBD7 in the presence of a POPC membrane. Several initial orientations of the dimer (both wild and mutants) with respect to membrane head regions were studied. Analysis of the MD results of wild and mutant dimers showed that the interfacial interactions between the dimers after the

MD run were seen reduced in **Table 1**. After MD, W39A was found to have more interacting residues in the interface that may impart more stability to alanine mutant than others. Different parameters, such as gyration of peptides (Rg), hydrogen bonding, and lipid peptide distance in both monomers and dimers, were considered during the analysis of the MD run.

Wild type AvBD7 was found to have the least average Rg compared to its mutant analogs **Fig. 5A**. The decreased Rg value of wild indicates that it was more compact than its analogs. Analysis of Rg of the claw (36-41 residues) separately showed that W39A claw exhibited greater variation in its radius of gyration which may be due to its flexible claw, whereas Rg for wild showed the least variation **Fig. 5B**. Hence, we predict that the claw region of the wild dimer remains more intact during the simulation study compared to its mutant analogs.

Even though the mammalian membrane is neutral in charge, cationic AMPs can interact with the anionic PO4 head group of the lipid layer through hydrogen bond formed by basic amino acids [63, 64]. Wild type dimer formed a greater number of hydrogen bonds followed by W39F. From the graph, **Fig. 6** it is understood that wild type showed a greater tendency to bind to the mammalian membrane, thus exhibiting a greater propensity for mammalian membrane. Trp39 present in the wild type may be the contributing factor for its higher propensity towards the neutral membrane. By comparing peptide-membrane distance and number of hydrogen bonds, the alanine mutant showed the least tendency to bind to the POPC membrane.

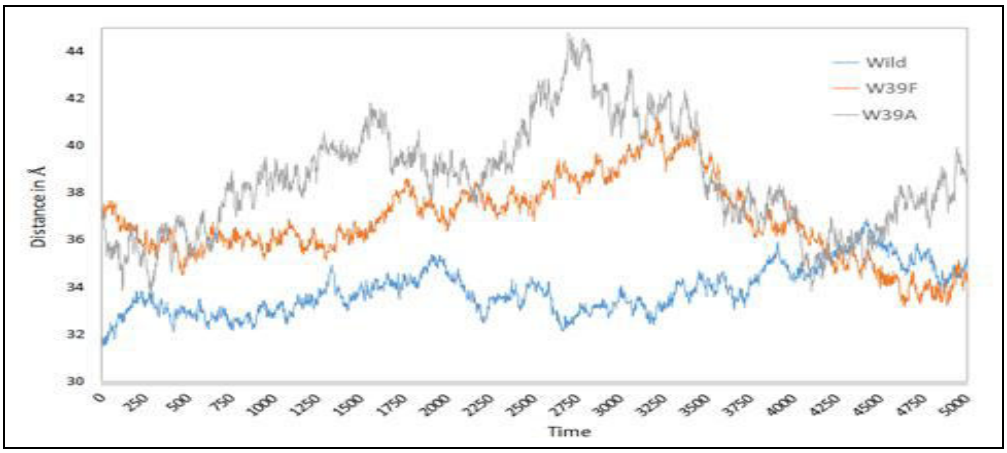

FIG. 6: PEPTIDE LIPID DISTANCE OF DIMERS OVER 10NS OF MD SIMULATION

Aliste *et al*., observed from their studies that trp residues in the pentapeptides interact with the choline groups of lipid bilayer through cation-π interaction [65]. The presence of two Trp at both claw region in the wild dimer might be the reason for its greater propensity to bind to the mammalian membrane.

It has been reported that hemolytic peptides interact strongly with the neutrally charged POPC membrane, while the non-hemolytic peptides exhibit the least interaction [62]. Chen *et al*., reported in his study using α helical antimicrobial peptide, $V13K_L$ that increased hydrophobicity induced by the replacement of alanine residues by leucine residues enhances the hemolytic activity of the peptide [66]. From our MD simulation study, we also found that alanine mutant showed the least tendency to bind with POPC membrane. Hence, conclude that W39A mutant due to its reduced hydrophobicity may be considered as least hemolytic among these.

CONCLUSION: Most of the AMPs are not recommended for therapeutic use as they induce hemolysis or exhibit toxicity against mammalian cells. In this study, we identified and sequenced the AvBD7 gene of *Corvus splendens* and attempted to develop the least hemolytic analog of this peptide using MD simulation. The translated sequence showed the presence of beta-defensin core motif and disulphide pairing, a characteristic of the beta-defensin family of peptides.

The results indicate that the residues at the N-terminus possess a significant role in determining the stability of the dimer and maintaining the C-terminal claw.

The C-terminal claw region maintained its shape in both wild AvBD7 and its mutants, but the claw region of W39A dimer remained more flexible when compared to others. Studies of *Corvus* AvBD7 with POPC membrane revealed that a single amino acid substitution in the crucial location of AMPs could alter its interaction with the mammalian membrane, thereby enhance or reduce the hemolytic activity. We found the alanine mutant as the least hemolytic.

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CONFLICTS OF INTEREST: The authors have no conflicts of interest.

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