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Antimicrobial activity of recombinant *E. coli* expressed chicken AvBD-2 and its mRNA expression in Indian native Aseel and Kadaknath chicken breeds

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ABSTRACT

Avian β defensins (AvBD) are antimicrobial peptides that play a crucial role in the innate immune response in chickens. In the present study, chicken AvBD2 gene was cloned, expressed in *E. coli* system and the *in vitro* antimicrobial activity of recombinant peptide was evaluated. The entire mature peptide region of chicken AvBD2 region was amplified and cloned in pUC29 cloning vector. Further, the coding region was sub cloned in pET-28A expression vector. After transformation in *E. coli* cells, the peptide synthesis was induced and recombinant protein (7.7 kDa) was purified by using Ni-NTA affinity column. The recombinant chicken AvBD2 showed antibacterial activity against *S.* Pullorum. The minimum bactericidal concentration (MBC) of recombinant chicken AvBD2 evaluated by micro-broth dilution assay was 35 µg/ ml. We also quantified the expression of AvBD2 transcript expression in day-old spleen tissue of Indian native chicken breeds (Aseel and Kadaknath) and White Leghorn. Measurable AvBD2 mRNA expression was found in the spleen of all three breeds. However, no significant difference was found in AvBD2 gene expression between native chickens and White Leghorn.

Key words: Antimicrobials, AvBD, Chicken, Gene expression.

INTRODUCTION

Avian β defensins (AvBDs) are now recognized as an important effector component of innate immune system in avian species. Since the avian heterophils lack oxidative defense mechanisms involving superoxide ions and myeloperoxidase, anti-microbial activity executed via non-oxidative mechanisms by defensins play a pivotal role in defense against invading pathogens (Harmon, 1998; Sahl *et al.,* 2005). Some defensins are also chemoattractant for monocytes, lymphocytes and dendritic cells, thus acts as a link between innate and adaptive immune responses (Yang *et al.,* 1999; Ganz *et al.,* 2003). A total of 14 β defensin genes (*AvBD1-14*) have been identified in the chicken (Zhoa *et al.,* 2001; Lynn *et al.,* 2004, 2007; Xiao *et al.,* 2004). Antimicrobial activity of recombinant chicken, duck AvBD2 and quail AvBD10 have been documented earlier (Lynn *et al.,* 2004, 2007; Xiao *et al.,* 2004; Cao *et al.,* 2012; Ma *et al.,* 2009, 2012). However, there is paucity of information on chicken AvBD2. In the present study, we evaluated *in vitro* antimicrobial activity of recombinant *E. coli* expressed chicken AvBD2.

Expression profile of *AvBD* genes in various chicken organs have been documented (Van Dijk *et al.,* 2008). Moreover, AvBDs play a significant role in host resistance to *Salmonella* colonization (Sadeyen *et al.,* 2006). Indigenous chicken breeds are considered to be more disease resistant than their commercial counterparts and are genetically distinct (Rout *et al.,* 1992; Wimmers *et al.,* 2000). Further they are demonstrated to be more immune-competent, supported with higher complement activity, higher serum lysozyme level and better antibody response (Kundu *et al.,* 1999; Haunshi and Sharma, 2002; Baelmans *et al.,* 2005). As chicken strains show differences in susceptibility to a number of diseases, use of these genetic stocks in modern breeding program for improving disease resistance could be focused (Zekarias *et al.,* 2002). Hence, in the present study, we also quantified the *AvBD2* gene expression in the spleen of day-old Indian native chicks and White Leghorn chicks by real time PCR.

MATERIALS AND METHODS

Cloning, expression and purification of recombinant chicken AvBD2: The entire mature peptide region (40 amino acids) coded by exon 3 (119bp) of *AvBD2* gene was amplified from genomic DNA of White Leghorn bird by standard procedure using gene specific primers designed to include *Nde* I/ *Xho* I restriction sites in forward and reverse primers respectively (Table 1). The PCR product was purified and was cloned in pUC29 cloning vector after *Nde* I/ *Xho* I enzyme digestion following standard procedure. Positive clones after transformation in DH5á cells were selected by colony PCR. The presence of insert was checked by digestion of positive plasmids with *EcoR*I and *Hind* III restriction enzymes (sites present on the vector backbone outside the insert location)

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Table1: Primer sequences used to amplify chicken mature peptide region of *AvBD2* gene.

Gene name	Primer sequence (5'-3')	Accession no.
Forward*	CAGTGACATATGTTGTCTTCGCCCCG	AF033336
Reverse**	TCACTGCTCGAGCCATTTGCAGCAGG	

Shaded sequence region include *Nde I and **Xho I restriction enzyme digestion site.

digestion. Two positive clones were sequenced in either direction using vector specific primers in automated gene sequencer (ABI prism, model 377, version 3.0). The nucleotide sequence was aligned with AF033336 to confirm the sequence identity.

The coding region (143 bp including restriction enzyme region sites) of chicken *AvBD2* mature peptide was sub-cloned into *Nde* I/ *Xho* I restriction sites in pET-28A expression vector (Novagen, USA) adopting standard cloning procedures. The recombinant plasmid (pET-28A-Gga_AvBD2) DNA was transformed into competent BL21 (DE3) (Promega, WI) *E. coli* cells and transformants were grown on Luria Bertani (LB) plates containing 50µg/ml Kanamycin. Single colony from the plate was inoculated into LB broth and incubated at 37^{0}C until the OD600 reached 0.5. For checking the production of recombinant chicken AvBD2 protein, log phase cultures were induced at 37^{0}C by adding IPTG (Promega, USA) to a final concentration of 1 mM. Aliquots collected at 0 to 5h post induction were analyzed on a 12% SDS-PAGE. Recombinant chicken AvBD2 was purified from 100ml of induced cultures under denaturing conditions by nickel-nitrilotriacetic (Ni-NTA) acid resin affinity chromatography by using Qiagen kit following manufacturer's instructions. The eluted recombinant protein was checked in SDS-PAGE and quantified by Bradford assay (Bradford, 1976).

Antimicrobial activity of recombinant chicken AvBD2: Minimum Bactricidal Concentration (MBC) of recombinant chicken AvBD2 was determined by microtitre broth dilution method. Virulent isolate of *Salmonella enterica* serovar Pullorum obtained from the National Salmonella Center, Indian Veterinary Research Institute, India was used. Mid-log-phase cultures of the test organisms were diluted in LB broth to reach a density of 10^6 CFU/ml. 10µl of this diluted culture was treated with 250µl recombinant chicken AvBD2 (5-50 µg/ml in PBS; with 136 mM NaCl and 2.68 mM KCl) in a 96-well micro titer plate for 3 h at 37°C. LB broth (100µl) was added after 3 h and plates were further incubated for 12 h. The bacterial growth was detected by measuring the absorbance at 570 nm. The growth inhibition was further confirmed by plating the contents of the wells, showing no visible growth of bacteria, onto LB agar plates and incubating at 37°C for 12-18 h. The MBC was calculated as the lowest concentration of the recombinant chicken AvBD2 that prevents any residual colony formation.

Experimental birds: The present experiment was performed with the approval of Institute Animal Ethics and Monitoring Committee. Day-old chicks of Indian native breeds (Aseel and Kadaknath) and White Leghorn were obtained from Project Directorate on Poultry, Hyderabad. A total of six chicks from each breed were randomly chosen and were euthanized. Spleen tissue from all chicks were aseptically collected and processed immediately.

RNA extraction and Reverse transcription (RT)-PCR: Total RNA was extracted from each tissue sample (50mg) using TRIzol reagent (Invitrogen), according to the manufacturer's instruction. To avoid the possible traces of genomic DNA, 5 µg of each RNA sample was incubated at 37^{0}C for 10 min with 5 U of RNase free DNase, following this step DNase was inactivated by incubation at 65^{0}C for 10 min. Subsequently, first strand cDNA was synthesized from 1ig of total RNA using oligo (dT) primer and MuMLV reverse transcriptase (MBI Fermentas, USA) in a 20 µl reaction mixture following the recommendations of manufacturer.

Quantitative analysis of *AvBD2* mRNA by real-time PCR: The constitutive expression of mRNA of *AvBD 2* was quantified by SYBR green method by using Mx-3000P spectrofluorometric thermocycler (Stratagene, USA) and primer pairs specific for the amplification of *AvBD* genes, as previously described (Kannaki *et al.,* 2012). The chicken β actin gene was used as an endogenous control. Primer amplification efficiencies were assessed for each gene from the standard curve generated by using serial tenfold dilution of transcribed RNA. Regression analysis of the Ct values of standard curve was done to calculate slope and amplification efficiency and found close to 100%. The resulting threshold cycle (Ct, a fractional PCR cycle number at which the change in reporter dye (DRn) passes the significant threshold) values were normalized to the endogenous control, β actin (ΔCt = Ct value of target gene-Ct value of β actin). To convey the inverse relationship between starting template concentration and Ct value, results were expressed and analyzed as 40-ΔCt values, interpreted as higher 40-ΔCt value implying greater gene expression. Analysis of 40-ΔCt mean values was carried out using one-way ANOVA with Tukey's post-hoc test using SPSS 10.0.1 to identify significant difference in gene expression among the chicken breeds (SPSS Inc, Chicago, IL). Values were considered significant at $P<0.05$. Fold change in *AvBD* gene expression in native chicken breeds were calculated by $2^{-\Delta\Delta}$ Ct method (Livak and Schmittgen, 2001) using White Leghorn as calibrator.

RESULTS AND DISCUSSION

Cloning, expression and purification of recombinant chicken AvBD2: A 143bp (119bp of target gene+primer sequence with RE sites) PCR product of chicken *AvBD2* was

amplified from chicken genomic DNA (Fig. 1). The PCR product was cloned into pUC29 cloning vector by restriction enzymes digestion and ligation. Positive clones checked by *EcoR*I and *Hind* III RE digestion released ~250bp insert from the 2.5Kb cloning vector (Fig. 2). Sequence analysis of cloned insert showed 100% similarity with published chicken *AvBD2* coding sequence (AF033336). The insert was sub cloned in pET-28A expression vector and positive clone was selected for induction of recombinant protein. Induction of the recombinant chicken AvBD2 did not affect the viability of *E. coli* cells transformed with pET-28A-Gga_AvBD2 constructs. SDS-PAGE analysis of the bacterial pellet obtained from cultures transformed with recombinant plasmid showed presence of a 7.7 kDa additional protein (Fig. 3). No toxic effect of the protein was observed in the un-induced and induced cultures during the 5 h growth period studied. The growth kinetics of the un-induced cultures were similar in both transformed and control cells. In induced cultures, the overall growth was low; however, the bacterial growth trends in the control and transformed cells were not significantly different.

Analysis of the supernatants and pellet fraction of bacterial cells after disruption by sonication indicated that all the recombinant chicken AvBD2 peptide was present in the pellet fraction in an insoluble form. So a purification scheme using denaturing conditions was employed and the protein was purified to more than 90% purity as evidenced by the coommasie staining of the SDS-PAGE gels after electrophoresis (Fig. 4). The yield of the purified protein was about 1-2 mg from a liter of induced *E. coli* culture.

Antimicrobial activity of recombinant chicken AvBD2: The recombinant chicken AvBD2 showed antibacterial activity against *S.* Pullorum. The minimum bactericidal concentration (MBC) of recombinant chicken AvBD2

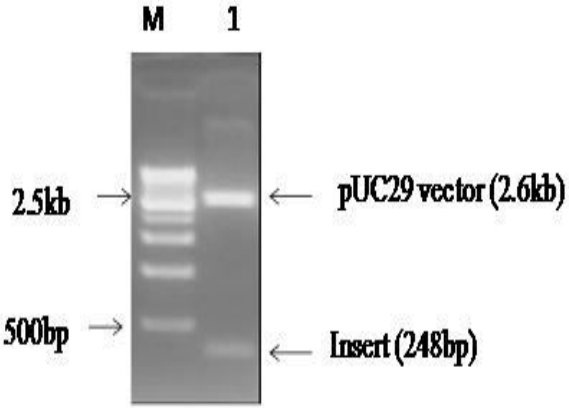

Fig. 2: Restriction enzyme analysis of recombinant pUC29 cloning vector.
Lane M: 100bp ladder; Lane 1: EcoRI/ Hind III digested recombinant pUC29 vector and insert (248bp)

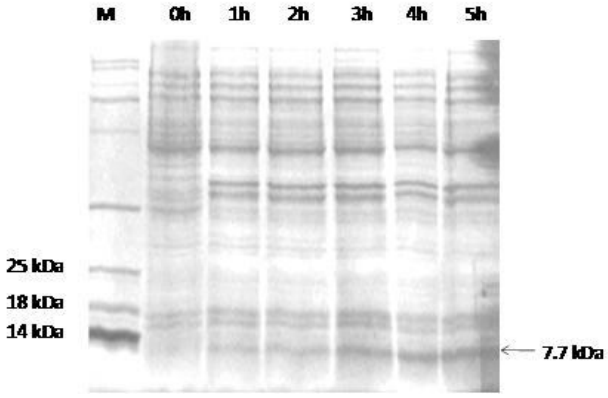

Fig. 3: SDS-PAGE analysis of induced recombinant pET28A expression vector.
Lane M: Protein marker (Chromous biotech

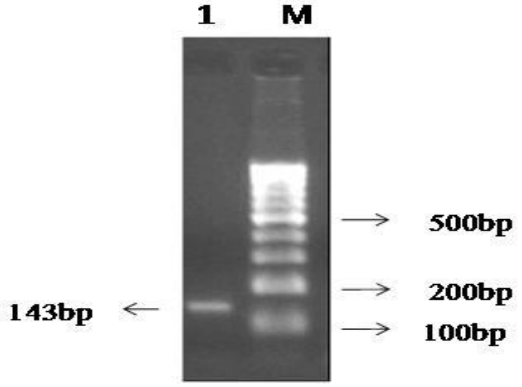

Fig. 1: PCR amplification of exon 3 of chicken AvBD2 mature peptide gene region.
Lane M: 100bp ladder; Lane 1: PCR product

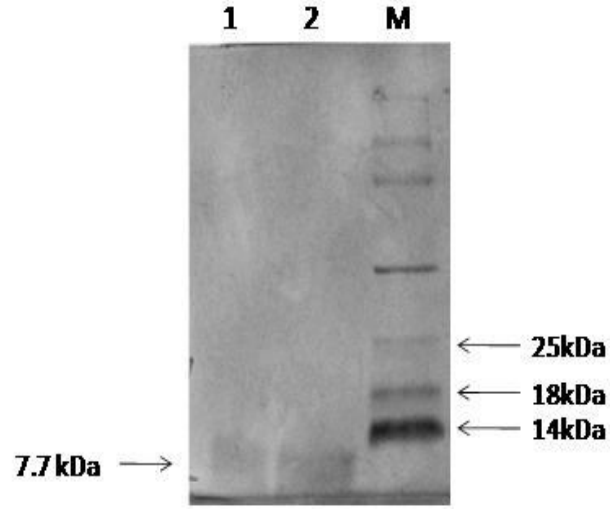

Fig. 4: SDS-PAGE analysis of purified recombinant chicken AvBD2 peptide

evaluated by micro-broth dilution assay was 35 µg/ ml (Fig. 5).

Quantitative analysis of *AvBD2* mRNA by real-time PCR: Measurable *AvBD2* mRNA expression was found in the spleen of all three breeds. However, no significant difference was found in AvBD2 gene expression between native chickens and White Leghorn (Table 2).

In the present study, chicken AvBD2 mature peptide encoding gene region was cloned, expressed in *E. coli* system and its antimicrobial activity was evaluated. Antimicrobial potency of recombinant protein has been shown previously not to be significantly altered by the presence of a His-tag (Yenugu *et al.,* 2003). However, expression of the anti-microbial proteins in recombinant form in prokaryotic system can be difficult due to their toxicity to the bacterial cells (Piers *et al.,* 1993). Toxic effects of chicken AvBD2 were not observed in this study. This may be because the protein formed insoluble inclusion bodies during recombinant expression. The level of expression of the mature protein, with an N-terminal His tag, was considerably low. Earlier study reported that addition of a part of signal peptide to duck AvBD2 mature peptide increased the level of expression of recombinant protein in *E. coli* system (Soman *et al.,* 2009). However, in the present study, that step was not attempted.

Earlier studies on antimicrobial activity of avianβ-defensins indicate that they are active against both Gram-positive and Gram negative bacteria (Evans *et al.,* 1995; van Dijk *et al.,* 2008). The Gga_AvBD2 is active against *E. coli* and exerts the antimicrobial effect at peptide concentrations

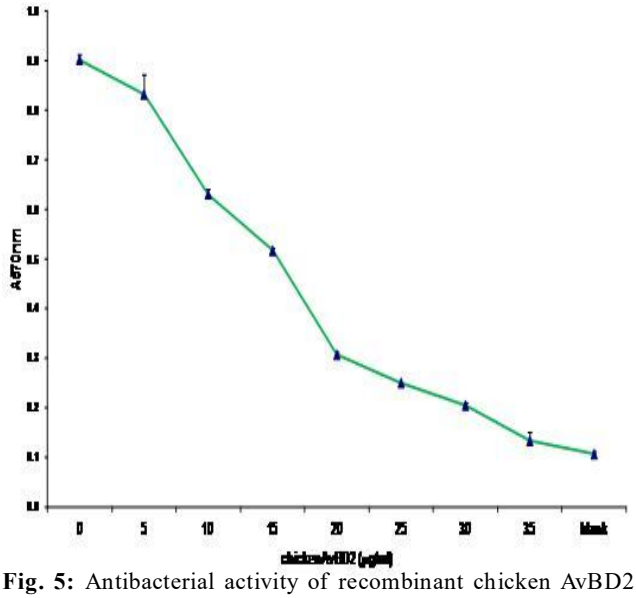

Fig. 5: Antibacterial activity of recombinant chicken AvBD2 evaluated by microbroth dilution assay.Data are mean absorbance (OD) at 570 nm ±SE.

Table2. Expression of *AvBD2* mRNA in spleen of Aseel, Kadaknath and White Leghorn quantified by real-time PCR

Gene	Aseel		Kadaknath	White Leghorn	
	Mean±SEM	FD	Mean±SEM	FD	Mean±SEM
AvBD2	29.54±3.21	-0.72	30.32±0.75	0.06	30.26±1.97

Values are expressed as mean 40-ÄCt±SEM (n=6 for each breed). FD: fold difference in gene expression in comparison to White Leghorn. Positive values indicate the higher gene expression and negative represents lower gene expression in native breeds in comparison to White Leghorn

of 16 µg/ml (Evans *et al.,* 1995). Further, it is bactericidal against G (+) pathogen *L. monocytogenes*, however it is not effective against *Candida albicans* (Evans *et al.,* 1994; Harwig *et al.,* 1994). The Apl_AvBD2 inhibits the growth of *E. coli* at a concentration of 25 µg/ml (3.7 µM). However, the Mga_AvBD2, which has a very high amino acid similarity with Apl_AvBD2 (83.3% of the mature peptide region), did not kill *E. coli* (Evans *et al.,* 1994). Predicted amino acid sequence analysis indicated that the major variations in the two proteins lie in the N-terminal region, which forms the ûrst β-sheet in the secondary structure prediction. The recombinant Apl_AvBD2 also demonstrated potent antibacterial activity against Gram-positive bacteria *M. luteus* at the same concentration as that was for *E. coli*. Avian β-defensin molecules are subjected to adaptive evolution in nature in synchrony with the host-speciûc pathogens (Maxwll *et al.,* 2003; Semple *et al.,* 2006; Higgs *et al.,* 2007; Peschel, 2002). In the present study, the recombinant Gga_AvBD2 was found effective against *S.* Pullorum at a concentration of 35 µg/ml. The higher bactericidal concentration observed in this study as against reported against *E. coli* may be due to the variation in bacterial strains or secondary structure of recombinant peptide. Antimicrobial peptides must first be attracted to bacterial surfaces and the obvious mechanism is through electrostatic bonding between the peptides and structures on the bacterial surface. *Salmonella* spp. is known to resist cationic antimicrobial peptide activity by reducing the negative charge of their outer membranes through modifications of the anionic membrane molecules including LPS, with positively charged substituent (Peschel, 2002). Earlier study showed that recombinant AvBD9 is potent against *Salmonella* sp. than AvBD4 and 7, and attributed this to reduced charge (+4) of AvBD9 (Milona *et al.,* 2007). This may be one of the reasons for higher concentration required against *S.* Pullorum as AvBD2 has significantly higher charge (+9.2). This higher cationic charge may favour killing other microbes. Further experiments are required to evaluate the antimicrobial activity against range of microbes.

Salt sensitivity is one of the key features of β-defensins, and many of these peptides are inactivated at physiological concentration of sodium chloride (~150 mM or 300 mOsm) (Tomita *et al.,* 2000). In this study, bacteria

were treated with the Gga_AvBD2 in PBS, which had a sodium chloride concentration of 136 mM, and the protein exhibited antibacterial activity against *S*. Pullorum. This indicates that the chicken AvBD2 is fairly salt resistant, though the resistance cannot be compared with that exhibited by the Apa_AvBD103b at 160 mM (348 mOsm) (Thouzeau *et al.,* 2007). Information on the salt sensitivity of Gga_AvBD2 and closely related Mga_AvBD2, and Apl_AvBD2 is currently not available. Among the chicken â-defensins previously studied, only AvBD9 has found to be relatively salt resistant at 150 mM concentration of sodium chloride (Van Dijk *et al.,* 2007).

In the present study we also quantified the constitutive expression of AvBD2 transcripts in spleen of Indian native chicken breeds (Aseel and Kadaknath) and White Leghorn. The results were in consistent to the earlier findings of expression of AvBD2 in chicken spleen (Zhoa *et al.,* 2001; Lynn *et al.,* 2004; van Dijk *et al.,* 2008), duck (Ma *et al.,* 2009; Soman *et al.,* 2009) and zebra finch spleen (Hellgren and ekblom, 2010). Expression of AvBD2 transcripts in day-old-chicks of all three breeds in the absence of infection suggests the innate preparedness of younger chicken to encounter pathogens immediately post-hatch. Further studies are needed to examine their expression in response to pathogen assault.

CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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