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ORIGINAL ARTICLE

# Differential gene expression of antimicrobial peptides β defensins in the gastrointestinal tract of *Salmonella* serovar Pullorum infected broiler chickens

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**Abstract** *Salmonella enterica* serovar Pullorum causes substantial mortality in chicks as well as results in persistent infection and vertical transmission in layer birds. An effective innate immune response in the early stages of infection could reduce bacterial colonization and mortality in chicks and persistency of infection in later stages. β Defensins (AvBDs) are now considered as one of the key components of innate immunity in avian species. In the present study, we quantified the mRNA expression levels of *AvBD*s (*1–14*) by real-time PCR in the gastrointestinal (GI) tissues (duodenum, jejunum, ileum and caecum) of 3-day-old broiler chicks after 24 h of oral infection with *Salmonella* Pullorum. Quantitative real-time PCR analysis revealed significant ($P<0.05$) upregulation of *AvBD3, 4, 5, 6* and *12* and a significant ($P<0.05$) down regulation in the expressions of *AvBD10, 11, 13* and *14* in one or few GI tissues, while no significant changes were observed for *AvBD1, 2, 7, 8* and *9* gene expressions in any of the GI tissues investigated upon infection with *S.* Pullorum. Most substantial change in gene expression was found for *AvBD5*, being significantly ($P<0.01$) upregulated in most of the GI tissues investigated. The differential expression levels of β defensins shed light on tailored innate immune response induced by *S.* Pullorum during the early stages of infection in chicks.

**Keywords** Chicken · β defensins · *Salmonella* · Innate immunity · Gene expression

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## Introduction

*Salmonella* Pullorum, a fowl-specific pathogen causes systemic salmonellosis resulting in substantial mortality in chicks (Shivaprasad 2000; Wigley et al. 2001). Persistence of infection or carrier-status and subsequent vertical transmission through eggs are the major concerns with *S.* Pullorum infections of birds more than a week old (Chappell et al. 2009). Growing concerns over the use of antibiotics demands the use of novel strategies to encounter infections. Thus, knowledge on innate immune mechanisms operating during bacterial infection will help in modulating them in favour of controlling initial bacterial colonization and later vertical transmission of infection.

Avian β defensins (AvBDs) are now considered as one of the key components of innate immunity in avian species. Defensins act as a first line of defense against invading pathogens and execute the anti-microbial activity by non-oxidative mechanisms (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 2003). A total of 14 β-defensin genes (*AvBD1-14*) have been identified in the chicken (Lynn et al. 2004, 2007; Xiao et al. 2004). Innate immune gene expressions have been studied for *Salmonella enteritidis* (SE) and *Salmonella typhimurium* infections in chicken (MacKinnon et al. 2009; Shaughnessy et al. 2009). Unlike infections with SE or *Salmonella* Typhimurium, *S.* Pullorum does not induce an inflammatory response in chicken intestine (Henderson et al. 1999; Kaiser et al. 2000) and other studies also suggested that the pattern of immune response induced by *S.* Pullorum invasion might be distinct (Chappell et al. 2009). Avian defensins have been linked to host resistance to Salmonella intestinal carriage (Sadeyen et al. 2004, 2006). Given the importance of β

defensins in Salmonella infection in the chicken and dearth of information on β defensins expression during *S*. Pullorum infection, the aim of this study was to examine the expression levels of all β defensins in the chicken (*Gallus domesticus*) gastro-intestinal (GI) tissues and to investigate whether these genes are differentially expressed in vivo during early stages of infection with *S*. Pullorum.

## Materials and methods

### Experimental birds

All the experiments were performed with prior approval of Institute Animal Ethics and Monitoring Committee. Day-old-commercial PB-1 (Punjab broiler-1) chicks were obtained from Project Directorate on Poultry, Hyderabad, India and were reared in experimental facility of Microbiolgy division, College of Veterinary Science, Hyderabad. Birds were given ad libitum access to water and feed. Chicks were confirmed for free status of Salmonella by culturing faecal samples in buffered peptone water (BPW) for overnight and spreading on brilliant green agar containing 100 μg/ml nalidixic acid.

### Experimental infection with *S*. Pullorum and sample collection

*Salmonella enterica* serovar Pullorum, virulent field isolate, was obtained from the National Salmonella center, Indian Veterinary Research Institute, India. Bacteria were grown overnight in Luria-Bertani (LB) broth at 37°C in an orbital shaking incubator at 150 rpm. The bacteria were washed three times by pelleting at 10,000 × g for 10 min and resuspended in PBS. The concentration of suspension was determined by following standard plate count technique by plating serial dilutions of bacterial suspension and adjusted to $10^8$ CFU/ml. Chickens were randomly divided into two groups of 12 each at 3 days of age. One group was orally inoculated with 0.5 ml of the bacterial suspension containing $10^8$ CFU/ml and the control group was given 0.5 ml of PBS. A total of four chickens from each group were randomly chosen and were euthanized 24 h post-challenge. The entire GI tract was removed aseptically and a 50 mg piece of each tissue was removed from the middle of the duodenum (D), jejunum (J), ileum (I) and caecum (C). The tissues were washed in PBS and placed in a microcentrifuge tube containing 1 ml TRIzol and immediately processed. In addition, swabs from caecum of both groups were taken and cultured in BPW overnight and streaked on brilliant green agar plates containing 100 μg/ml nalidixic acid (37°C for 18–24 h) to confirm that control birds were uninfected and the challenged chicks were infected.

### RNA extraction and Reverse transcription (RT)-PCR

Total RNA was extracted from each tissue sample (50 mg) 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°C for 10 min with 5 U of RNase free DNase, following this step DNase was inactivated by incubation at 65°C for 10 min. Subsequently, first strand cDNA was synthesized from 1 μg of total RNA using oligo (dT) primer and MuMLV reverse transcriptase (MBI Fermentas, Canada) in a 20 μl reaction mixture following the recommendations of manufacturer.

### Quantitative analysis of AvBDs mRNA by real-time PCR

Relative quantification of chicken *AvBD*s (*1–14*) in GI tissues was done by real-time PCR using the Mx3000P QPCR system (Stratagene, USA) and primer pairs specific for the amplification of AvBD genes, as previously described (Ebers et al. 2009). The amplification was performed in a total volume of 25 μl, containing 1X QuantiTect SYBR Green PCR master mix (SYBR Green I dye, ROX passive reference dye, HotStartTaq DNA polymerase and dNTPs with dUTPs in optimized buffer, Qiagen GmBH, Germany), 10 pmol of each primer and 0.5 μl of cDNA template. All primer sequences are presented in Table 1. Thermal profile consisted of an initial denaturation at 94°C for 10 min,

**Table 1** Fold-changes in *AvBD* mRNA expression in gastrointestinal tissues of 3-day-old broiler chickens infected with *Salmonella nteric* serovar Pullorum. Data are expressed as $2^{-\Delta\Delta Ct}$. Positive values indicate infected chicken have an increase in respective AvBD gene expression compared to uninfected control in respective tissue. *Significantly different at $P<0.05$; ** Significantly different at $P<0.01$. Statistical analysis was performed on individual 40-ΔCt values. D: duodenum, J: jejunum, I: ileum, C: caecum

| Gene | D | J | I | C |
|---|---|---|---|---|
| *AvBD1* | 0.53 | 0.87 | 0.69 | 0.54 |
| *AvBD2* | 0.47 | 0.33 | −1.37 | 1.87 |
| *AvBD3* | 1.9 | 1.39 | −1.73* | 4.33** |
| *AvBD4* | 5.50* | −0.01 | −1.10 | −0.39 |
| *AvBD5* | 3.10* | 3.19** | 3.38* | −0.09 |
| *AvBD6* | 0.87* | 1.70 | −1.53** | −0.18 |
| *AvBD7* | 0.99 | −0.05 | −0.63 | 0.41 |
| *AvBD8* | 0.17 | −0.12 | −0.31 | 0.77 |
| *AvBD9* | 0.72 | 0.30 | −1.2 | −0.49 |
| *AvBD10* | −0.52 | 0.73 | 1.48 | −2.04* |
| *AvBD11* | −1.72** | 0.01 | −3.93** | −0.70 |
| *AvBD12* | 4.76* | 2.91** | 0.53 | −3.58 |
| *AvBD13* | 0.46 | 1.45 | −1.95* | 0.54 |
| *AvBD14* | 0.62 | 0.74 | −2.46* | −2.09* |

followed by 40 cycles of denaturation at 94°C for 30 s; annealing at 55–60°C for 30s and extension at 72°C for 30s. Dissociation analysis of amplification products was performed at the end of each PCR to confirm the specificity of amplicon. In each PCR reaction no template control was included to check contamination of master mix. Non-reverse transcribed RNA (10 ng) of each sample was used instead of cDNA to check contamination of samples with genomic DNA, failure of amplification confirms the purity of sample. To assess the efficiency of primers, standard curves for each primer pair were generated using serially diluted transcribed RNA sample. PCR efficiency was calculated from the slope of standard curves. 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 (MacKinnon et al. 2009). The comparative Ct method was used to express the fold-changes in gene expression and calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

### Statistical analysis

All expression data are relative mRNA levels presented as mean 40-ΔCt values±SEM. Student's *t*-test with two tailed analysis was performed to evaluate differences between the infected and uninfected groups. Values of ($P<0.05$) were considered statistically significant. For presentation purpose the fold-changes are calculated as $2^{-\Delta\Delta Ct}$. However, statistical analysis was not performed on the fold-changes but only performed on the individual 40-ΔCt values for each *AvBD* gene.

## Results

All experimental chicks before the challenge study were free from Salmonellae. In all the chicks challenged with *S.* Pullorum, the organism was detected in cloacal swabs, in contrast, it was not detected in samples from control birds. Efficiencies of primer pairs specific for *AvBD* genes, calculated from the slope of standard curves ranged from 92% to 100%. *AvBD* gene expression analyzed by real-time PCR in control and infected chicken GI tissues are presented in Fig. 1. The fold-changes in *AvBD* gene expressions in infected group as calculated by $2^{-\Delta\Delta Ct}$ method are presented in Table 1.

Expression analysis revealed that all the 14 *AvBD* genes were detectable in GI tissues in both uninfected and infected groups, while expressions of *AvBD2* and *7* were at very low level in the both groups. In the present study, quantitative real-time PCR analysis revealed significant upregulation of *AvBD3* in caecum (4.3 fold; $P=0.02$), *AvBD4* in duodenum (5.5 fold; $P=0.005$), *AvBD5* in duodenum (3.1 fold; $P=0.04$), jejunum (3.19 fold; $P=0.007$) and ileum (3.38 fold; $P=0.01$), *AvBD6* in duodenum (0.87 fold; $P=0.01$) and *AvBD12* in duodenum (4.76 fold; $P=0.01$) and jejunum (2.91 fold; $P=0.003$). Significant down regulation in the expressions of *AvBD10* in caecum (2.04 fold; $P=0.02$), *AvBD11* in duodenum (1.72 fold; $P=0.002$) and ileum (3.93 fold; $P=0.02$), *AvBD13* in ileum (1.95 fold; $P=0.02$) and *AvBD14* in ileum (2.46 fold; $P=0.014$) and caecum (2.09 fold; $P=0.03$) were observed. Whereas no significant changes were observed for *AvBD1, 2, 7, 8* and *9* gene expressions in any of the GI tissues investigated upon infection with *S.* Pullorum. Most substantial changes in gene expression were found for *AvBD5*, being significantly ($P<0.05$) upregulated in most of the GI tissues investigated.

## Discussion

Innate immune system is crucial in very young chickens as the acquired immune system fully develops only after the first week of age (Bar-Shira et al. 2003). Growing evidences have demonstrated the significant role of β defensins in neonatal chicken in the control of infection (Meade et al. 2009a). In the present study we investigated the mRNA expression levels of chicken β defensins in GI tissues upon *S. Pullorum infection after 24 h in 3-day-old-broiler chicken.*

Expression analysis revealed that out of 14 *AvBD* genes, all were detectable in GI tissues of both uninfected and infected groups, while the gene expressions of *AvBD2, 7* were at very low level in both the groups. Differential constitutive expression of *AvBD* genes in developing chicken embryonic tissues as well as neonatal tissues have been demonstrated earlier (Lynn et al. 2004; Bar-Shira and Friedman 2006; Ma et al. 2008; Meade et al. 2009a). Our finding of low expression of *AvBD2* in GI tissues is in consistence with earlier reports from chicken (Lynn et al. 2004) and duck (Soman et al. 2009) suggesting the lack of its role in primary defense in the digestive tract. Minor discrepancies were observed in constitutive expression pattern of β defensin genes between the present results and earlier studies. It can be speculated that these discrepancies between these studies reflect differences between the experimental conditions such as variation in breeds, age and techniques used (semi-quantitative RT-PCR vs real-time PCR).

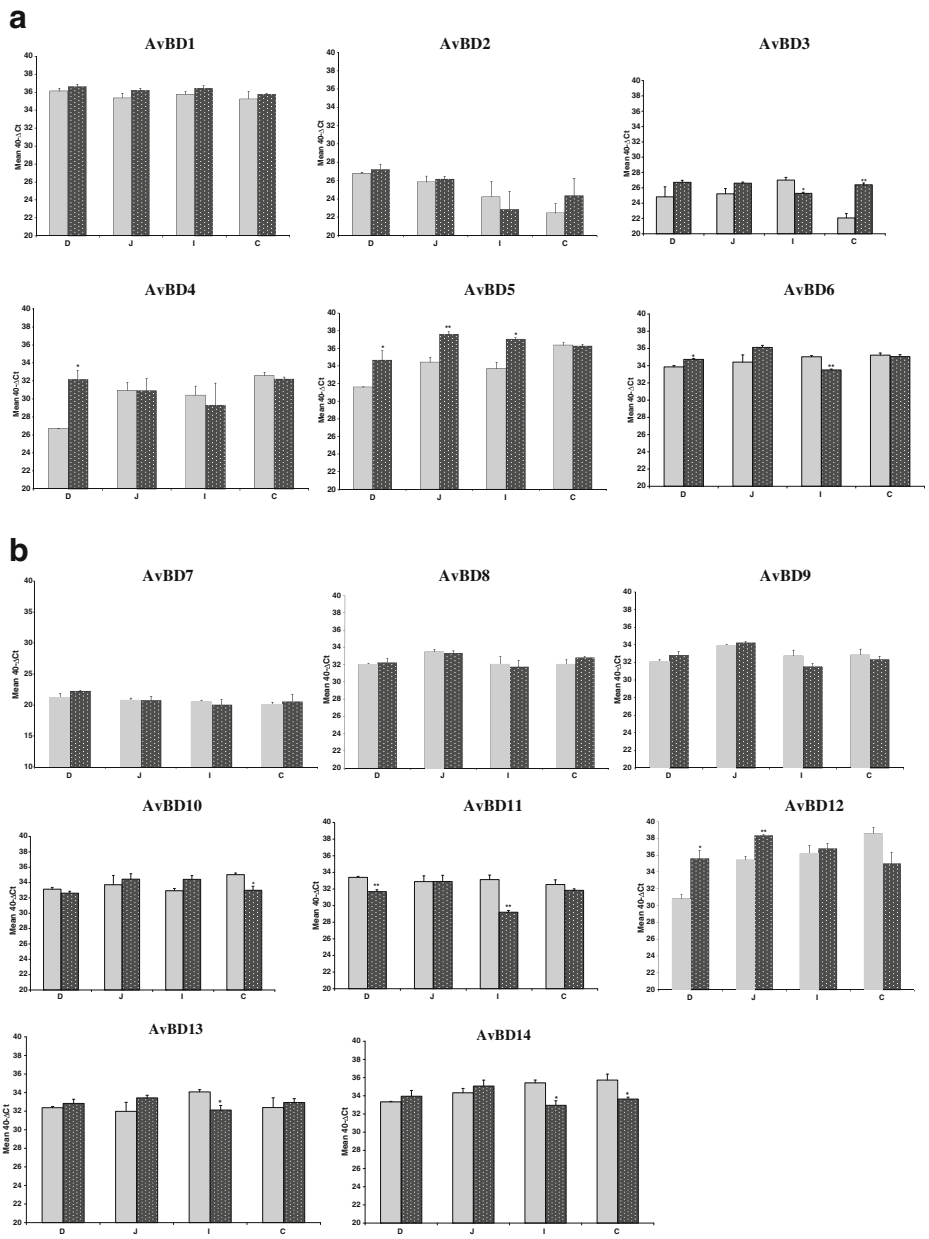


**Fig. 1** Differential expression of *AvBD (1–14)* mRNA in the duodenum (D), jejunum (J), ileum (I) and caecum (C) of uninfected chickens and *Salmonella enterica* serovar Pullorum infected broiler chickens after 24 h. β actin was used as normalizer gene for real-time qRT-PCR analysis. The data are mean 40-ΔCt±SEM values from four individual chickens. *Significantly different at $P<0.05$; ** Significant different at $P<0.01$. – uninfected, -infected with *S.* Pullorum

In the present study, quantitative real-time PCR analysis revealed significant upregulation of *AvBD3, 4, 5, 6* and *12* and a significant down regulation in the expressions of *AvBD10, 11, 13* and *14* in one or few GI tissues, while no significant changes were observed for *AvBD1, 2, 7, 8* and *9* gene expressions in any of the GI tissues investigated upon infection with *S.* Pullorum. Single nucleotide polymorphisms in *AvBD3* and *AvBD7* in broilers were found to be associated with higher antibody levels of *S. enterica* serovar *Enteritidis* vaccination (Hasenstein and Lamont 2007). The present results are consistent with an earlier study that reported upregulation of *AvBD3* and *12* gene expressions upon *S.* Typhimurium infection in chicken (Meade et al. 2009b). Interestingly, in the present study *AvBD3* gene expression was significantly upregulated in caecum whereas downregulated in ileum possibly reflecting the site of colonization in caecum and the host's response to that. Upregulation of *AvBD4* and *AvBD6* in duodenum upon *S.* Pullorum in the present study may be important in encountering initial bacterial intestinal colonization. An earlier study demonstrated bactericidal activity of recombinant chicken *AvBD4, AvBD7* and *AvBD9* peptides against *Salmonella* spp. (Milona et al. 2007), however, they observed no changes in the gene expression of *AvBD7* and *9* in small intestine of chicken upon oral challenge with *Salmonella* spp in similarity to the present results. Hence, this can be speculated as immune evasion strategy employed by the pathogen. Akbari et al. (2008) also reported that *AvBD1* and *2* gene expressions were not changed in caecal tonsils upon infection

with *S.* Typhimurium. Recently Derache et al. (2009) also observed no change in *AvBD2* gene expression in intestinal epithelial cells upon Salmonella challenge in in vitro assay. However, in contrast to our results, Sadeyen et al. (2006) earlier reported increases in the expression levels of *AvBD1* and *AvBD2* in the cecal tonsils of chicken inbred lines with Salmonella resistant trait. The observed discrepancy may be a result of differences in bacterial strains (SE, versus S. Pullorum).

Higgs et al. (2005) showed the strong expression of *AvBD11* in small intestine of day-old broiler and demonstrated the antimicrobial activity of synthetic AvBD11 peptide against a range of pathogens including Salmonella and found to be most active against bacteria residing in the intestine. AvBD13 modulates the adaptive immune responses of chickens in vivo and in vitro (Yang et al. 2007). However, in the present study *AVBD11*, *13* and *14* gene expressions were significantly downregulated in GI tissues upon *S.* Pullorum infection. Similar down regulation of *AvBD3, 8, 13* and *14* gene expressions were found in *Campylobacter jejuni* infection model in chicken (Meade et al. 2009b). In mouse and rat models, studies have shown that defensins could be downregulated by *Salmonella* in the intestine (Rodenburg et al. 2007; Salzman et al. 2003). The present results together with earlier reports confounds earlier speculation of this downregulation as immune evasion mechanism employed by *S*almonella to avoid inflammatory reaction in initial bacterial colonization phase.

Recognition of pathogen-associated molecular pattern (PAMP) by toll-like receptors (TLR) activates nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), leading to the up-regulation of beta defensin-2 (Vora et al. 2004). Recently a study suggested that AvBD13 maybe an endogenous ligand for TLR4 and that AvBD13 enhances the proliferation of monocytes via the NF-$\kappa$B pathway (Yang et al. 2010). We observed significant upregulation of *TLR2, 4* and *21* gene expressions in GI tract tissues of 3-day-old broiler chicks infected with S. Pullorum (unpublished data). It is likely that LPS, flagellin, and/or secreted virulence factors of *Salmonella* function as PAMP to trigger the expression of TLRs and subsequent AvBDs by complex interlinked pathways. It appears that the β defensin genes play a role in the transition from an innate immune response to an adaptive response in the newly hatched birds apart from having direct anti-microbial activity (Ganz 2003; Bar-Shira and Friedman 2006).

In summary, this study provides some intriguing insights into the pattern of innate immune gene expressions in the GI tissues in response to *S.* Pullorum infection. Furthermore, it identifies some key β defensins that are differentially regulated during the early stages of bacterial invasion of GI tract. Future works can concentrate on further elucidation of immune pathways TLR signaling and TLR and AvBD interlinks during the course of systemic salmonellosis. Modulation of these β defensins during early stages to check the initial bacterial colonization will help to reduce mortality in chicks and carrier state in adult birds.

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