Expression of Avian β-Defensins in the chicken (Gallus domesticus) reproductive tract

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ABSTRACT

Avian β-defensins (AvβD) are antimicrobial peptides that play significant roles in the innate immune system in chickens. These genes clustered on chromosome 3 of the chicken genome encode a group of cationic antimicrobial peptides characteristic of β-defensins. Although many studies have reported the expression of AvβD in various chicken organs and the interactions between Salmonella and cells of the chicken gastrointestinal tract little is known about the function of these genes in the chicken reproductive tract. Therefore the aim of this study was to identify the types of avian β-defensins genes expressed in the reproductive organs of male and female chicken. Total RNA was extracted from ovaries, oviduct, testis and epididymis from one year old male and female chickens. The expression of AvβD in these reproductive organs was examined by reverse transcription PCR analysis.

Keywords: avian β-defensins, chicken, reproductive tract

INTRODUCTION

Recent studies have reported that the family of antimicrobial peptides defensins plays a significant role in the chicken innate immune system, providing the first line of defence against potential pathogens (Sugiarto and Yu, 2004; Higgs et al., 2005). Defensins constitute a large family of small cationic antimicrobial peptides, characterized by the presence of a conserved cysteine-rich defensin motif. These peptides are capable of killing a broad spectrum of pathogens, including bacteria, fungi, and certain enveloped viruses (Lehrer and Ganz, 2002; Thomma et al., 2002; Ganz, 2003) and thus play a critical role in defence and disease resistance by protecting the hosts against infections. In the chicken genome only the family of β-defensin exists, where it is known as

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gallinacins or avian β-defensins (Zhao et al., 2001). Avian β-defensins attack a wide range of microorganisms including Gram-positive and Gram negative bacteria, fungi, and yeast (Evans et al., 1995; Harmon, 1998; Sugiarto and Yu, 2004). A total of 13 avian β-defensin genes (AvβD -1 to -13) have been identified through in silico studies (Lynn et al., 2004; Xiao et al., 2004).

While there are many reports on the expression of avian β-defensin genes in various chicken organs and the interactions between Salmonella and the chicken gastrointestinal tract (Methner et al., 2004; Ledeboer et al., 2006) their expression in the chicken reproductive organs has not been studied extensively.

The aim of this study was therefore to examine the expression pattern of AvβD in the chicken (Gallus domesticus) reproductive tract in vivo and specifically in the chicken ovary, oviduct, testis and epididymis.

**MATERIAL AND METHODS**

**Livestock**

The poultry used in this study were supplied by a commercial supplier. The birds were kept in individual cages under a light regimen of 14 h light : 10 h dark. Feed and water were given ad libitum. One year old birds (Gold Line) were sacrificed by cervical dislocation. Chicken organs including total ovary, oviduct, testis and epididymis were removed from birds, snap frozen in liquid nitrogen and stored at -80°C until analysed.

**Salmonella test**

Experimental birds were tested for Salmonella species using Salmonella/Shigella (SS) agar (Sigma). Each bird was tested for Salmonella by plating faecal swabs on SS selective agar plates. Briefly 63gr of SS Agar were Suspend in 1000 ml of distilled water. The solution was boiled with frequent agitation to dissolve the medium completely and was mixed and poured into sterile petri plates. When cooled, different dilutions of faeces, resuspended in 1x PBS (Ambion), were poured into the plates. Using this medium growth of the Salmonella species is uninhibited and appears as a colorless colony with a black center.

**DNA isolation, PCR amplification, cloning and sequencing**

Genomic DNA was isolated from chicken liver using the NucleoSpin Tissue kit (Macherey Nagel, Germany) according to the manufacturer’s instructions. The integrity of the DNA samples was examined by electrophoresis through a 1,5% TBE agarose gel. For amplification of the 13 AvβD genes specific primers were designed based on the nucleotide sequences of the chicken avian β-defensins genes reported in the GenBank database. The sequences of these primers are presented in Table 1. PCR amplification was performed using 0.1μg genomic DNA as template, 200nM primers each, 1mM dNTPs and 1 unit of Taq DNA Polymerase Recombinant (Invitrogen) in 50μl
total volume reaction. PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 30sec, 55°C (for AvβD -2, -4, -5, -6, -12) or 57°C (for AvβD -1, -3, -9) or 60°C (for AvβD -7, -8, -10, -11) for 30sec, 72°C for 2 min and a final extension period at 72°C for 10 min. The PCR products were separated on a 1% TBE agarose gel, cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen) according to the manufacturer’s protocol and sequenced.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>cDNA (bp)</th>
<th>DNA (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvβD1</td>
<td>GAAACCATTGTCAGCCCTGT</td>
<td>TCAGCCCATATTCTTTTC</td>
<td>187</td>
<td>0.7</td>
</tr>
<tr>
<td>AvβD2</td>
<td>TTTCTCCAGGGTTTGCTTTCG</td>
<td>CTTCTTGCTGCTGAGGCTTT</td>
<td>231</td>
<td>1</td>
</tr>
<tr>
<td>AvβD3</td>
<td>TGTAATCCATCCTCCCCCTTC</td>
<td>GGGCAATACACCTCATATGC</td>
<td>177</td>
<td>2.5</td>
</tr>
<tr>
<td>AvβD4</td>
<td>CATCTCATGTGCTTCTTCGTCG</td>
<td>ACAATGGTGTTCCCCCAATCCAAC</td>
<td>321</td>
<td>0.9</td>
</tr>
<tr>
<td>AvβD5</td>
<td>CTGCCACGAAAGAAGAAAACTGG</td>
<td>TGAACGTGAAAGGGACATCAGAG</td>
<td>300</td>
<td>1.1</td>
</tr>
<tr>
<td>AvβD6</td>
<td>AGGATTTCACATCCCCGCTGTC</td>
<td>CAGGAGAAGCCAGTGATGCATC</td>
<td>249</td>
<td>1.2</td>
</tr>
<tr>
<td>AvβD7</td>
<td>CTGCCCTGCTCTCTCTTG</td>
<td>CATTGGTAGCTGCGAGGAGA</td>
<td>230</td>
<td>0.6</td>
</tr>
<tr>
<td>AvβD8</td>
<td>ACAGTGTGAGCAGGGAGGAGGGA</td>
<td>CTTCTTGCTGCTGCTTTGGTG</td>
<td>261</td>
<td>0.9</td>
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<tr>
<td>AvβD9</td>
<td>GCAAAGCTATTTCCACACGAG</td>
<td>AGCATTTCAGCTCTCCACCAC</td>
<td>211</td>
<td>1.8</td>
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<tr>
<td>AvβD10</td>
<td>GCTCAGCGACCCACTTTC</td>
<td>GTGCTGTCACAGGGCAAT</td>
<td>189</td>
<td>1.9</td>
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<tr>
<td>AvβD11</td>
<td>CAGAATTGCAGAAGGCCCA</td>
<td>TTCTACGTCGCTGCTG</td>
<td>240</td>
<td>1.2</td>
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<tr>
<td>AvβD12</td>
<td>CCCAGCAGGACCCAAAACTAG</td>
<td>GTGAAATCCACAGCCATAGAG</td>
<td>335</td>
<td>0.7</td>
</tr>
</tbody>
</table>

RNA Isolation and cDNA Synthesis

Total RNA was isolated from chicken ovaries, oviduct, testis and epididymis snap-frozen in liquid nitrogen. Initially, the tissues were ground to a fine powder, and the RNA was extracted using the TRI Reagent (Ambion) according to the manufacturer’s instructions. To reduce degradation, RNase inhibitor (Invitrogen) was added to each sample (1 U/µg of RNA) before storage at -80°C. All samples were pretreated, before reverse transcription (RT), with DNase (Fermentas) at a concentration of 1 U/µg of RNA. One microgram of total RNA was reverse transcribed to cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s instructions. 2 µl of the RT products were subjected to PCR amplification for each of the AvβD genes, using the primer pairs and the conditions detailed above. All primer pairs were designed to cross intron sequences to aid in distinguishing PCR products amplified from cDNA vs. genomic DNA (Table 1). Amplification of 18s ribosomal DNA was performed using the Classic 18S Internal Standard primer pair (Ambion) and an annealing temperature of 57°C. In control samples, reverse transcriptase was omitted to demonstrate that PCR amplification was not due to contamination with genomic DNA. RT-PCR products were resolved by electrophoresis using 1% TBE agarose gels, visualised with ethidium bromide and imaged under UV illumination. All cDNA products were identified by DNA sequencing or restriction analyses. All expression experiments were performed in triplicate.
RESULTS AND DISCUSSION

Recent studies have reported that AvβD -1 to -7 are predominantly expressed in bone marrow and respiratory tract, whereas AvβD -8 to -13 are restricted to the liver and urogenital tract (Xiao et al., 2004). Expression of AvβD -3 was observed in the ovary of immature chicken (Zhao et al., 2001) and more recently expression of AvβD -4, -7 and -9 were observed in the ovary of 38-day-old chicken (Milona et al., 2007). In addition, expression of AvβD -1, -2, and -3 was observed in the chicken vaginal mucosa (Yoshimura et al., 2006), while the expression of AvβD -1, -2, -7, -8, -10, and -12 in the theca layer and AvβD -1, -8, -10, and -12 in the granulosa layer was identified in white and yellow chicken follicles (Subedi et al., 2007).

In addition it has been reported that synthesis of AvβD antimicrobial peptides increases in response to challenge with Salmonella serovars (Yoshimura et al., 2006; Milona et al., 2007). Therefore one of the primary objectives of this study was to include in the expression experiments only Salmonella free birds. Using the SS selection method described in materials and methods, we isolated Salmonella free birds. These birds were further investigated for the expression of AvβD genes. Birds that were found to be infected by Salmonella species were excluded from the expression analysis experiments.

The aim of this study was to investigate the expression pattern of the 13 chicken AvβD genes reported to date in the avian reproductive tract. For this reason the initial experiments were focused on identifying the optimal conditions for the PCR amplification of these genes. As illustrated in Figure 1 this was successful for AvβD -1 to -12. However, we were unable to confirm AvβD -13 amplification in the chicken genome, despite using a number of different PCR primers, and genomic DNA extracted from different chicken tissues. In addition no amplification was successful either using cDNA as template. For this reason AvβD -13 was excluded from the expression analysis studies.

Figure 1. PCR amplification of avian β-defensins (AvBD) genes in chicken genomic DNA
Prior to expression analysis experiments, the quality of all the synthesized cDNA samples was investigated by amplification of the 18s ribosomal RNA. As illustrated in Figure 2, the cDNAs synthesized from ovary, oviduct, testis and epididymis RNA respectively, were of very good quality as was observed by amplification of a strong band of the expected size (489bp). This was confirmed by cloning and sequencing of the PCR product.

As illustrated in Figure 2, the expression analysis data revealed that out of the 12 $\gamma$-genes that were investigated by RT-PCR, 11 $\gamma$-genes were expressed in chicken ovary. $\gamma$-11 was the only one that was found not to be expressed in this tissue. Very low levels of expression were observed for $\gamma$-7 and very high levels of mRNA were observed for $\gamma$-9 and -10. The RT-PCR analysis data also showed that all the 12 $\gamma$-genes were expressed in the chicken oviduct. Very low levels of expression were observed for $\gamma$-7. In contrast, very high levels of expression were observed for $\gamma$-9, -10, -11, -12.

Expression analysis of $\gamma$-D in the male reproductive tract, as illustrated in Figure 2, revealed that in chicken testis all the $\gamma$-D were expressed. Very high levels of mRNA were observed for $\gamma$-D -7, -9, -10 and -11. Interestingly the RT-PCR analysis for $\gamma$-D -12 using the primer pair detailed in Table 1, revealed the presence of two major bands in chicken testis. This must be due to alternative splicing of the chicken $\gamma$-D -12 gene. Further analysis is underway to determine the role and function of the second $\gamma$-D -12 mRNA transcript.

RT-PCR analysis in the chicken epididymis revealed that out of the 12 $\gamma$-D
genes examined 11 genes were expressed and only AvβD -5 transcripts were not detected. Very low levels of mRNA were observed for AvβD -4, while very high levels of expression were observed for AvβD -9 and -11.

Collectively the RT-PCR data revealed that two genes, AvβD -9 and AvβD -10 were expressed in very high levels in the chicken reproductive tract, while AvβD -11 was also expressed in very high levels in the oviduct, testis and epididymis and interestingly it was not expressed in ovary. Very low levels of mRNA expression were observed in all reproductive organs for AvβD -4.

Our expression analysis data are in consistent with previous reports which indicated mRNA transcripts of AvβD -3, -4, -7 and -9 in the chicken ovary (Zhao et al., 2001; Milona et al., 2007) and expression of AvβD -1, -2 and -3 in the chicken oviduct (Yoshimura et al., 2006). However we extended the expression pattern of 12 avian β-defensins in the reproductive tract in both male and female birds. These novel findings provide evidence to suggest that the avian β-defensins host defence peptides play a significant role in the chicken reproductive tract and that they probably function *in vivo* to protect the female reproductive tract for successful follicular development, successful fertilization and also the newly forming egg from microbial infection. In addition the expression of AvβD in the male reproductive tract such as testis and especially the epididymis suggest that these peptides function to prevent male infection that may affect temporary or permanent fertility and have a significant role in avian sperm maturation and Capacitation.

**CONCLUSIONS**

To our knowledge, this is the first report to examine the mRNA expression patterns of the avian β-defensins genes in the reproductive tract of both male and female chicken. Noteworthy findings of the present study are:

(1) Out of the 12 AvβD examined, all the genes were expressed in the chicken oviduct and testis and 11 AvβD were expressed in the chicken ovary and epididymis.

(2) Very high levels of expression were observed for AvβD -9 and -10 in all reproductive organs examined.

(3) Very low levels of mRNA were observed for AvβD -4 in the chicken reproductive tract.

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