MOLECULAR AND CELLULAR BIOLOGY

Expression of host defense peptides in the intestine of *Eimeria*-challenged chickens

S. Su,[*] D. M. Dwyer,[†] K. B. Miska,[‡] R. H. Fetterer,[§] M. C. Jenkins,[§] and E. A. Wong[*,1]

[*]*Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg 24061;* [†]*Department of Biological Sciences, Virginia Tech, Blacksburg 24061;* [‡]*Animal Biosciences and Biotechnology Laboratory, USDA/ARS, Beltsville MD 20705; and* [§]*Animal Parasitic Diseases Laboratory, USDA/ARS, Beltsville MD 20705*

ABSTRACT Avian coccidiosis is caused by the intracellular protozoan *Eimeria*, which produces intestinal lesions leading to weight gain depression. Current control methods include vaccination and anticoccidial drugs. An alternative approach involves modulating the immune system. The objective of this study was to profile the expression of host defense peptides such as avian beta-defensins (AvBDs) and liver expressed antimicrobial peptide 2 (LEAP2), which are part of the innate immune system. The mRNA expression of AvBD family members 1, 6, 8, 10, 11, 12, and 13 and LEAP2 was examined in chickens challenged with either *E. acervulina*, *E. maxima*, or *E. tenella*. The duodenum, jejunum, ileum, and ceca were collected 7 d post challenge. In study 1, *E. acervulina* challenge resulted in down-regulation of AvBD1, AvBD6, AvBD10, AvBD11, AvBD12, and AvBD13 in the duodenum. *E. maxima* challenge caused down-regulation of AvBD6, AvBD10, and AvBD11 in the duodenum, down-regulation of AvBD10 in the jejunum, but up-regulation of AvBD8 and AvBD13 in the ceca. *E. tenella* challenge showed no change in AvBD expression in any tissue. In study 2, which involved challenge with only *E. maxima*, there was down-regulation of AvBD1 in the ileum, AvBD11 in the jejunum and ileum, and LEAP2 in all 3 segments of the small intestine. The expression of LEAP2 was further examined by in situ hybridization in the jejunum of chickens from study 2. LEAP2 mRNA was expressed similarly in the enterocytes lining the villi, but not in the crypts of control and *Eimeria* challenged chickens. The lengths of the villi in the *Eimeria* challenged chickens were less than those in the control chickens, which may in part account for the observed down-regulation of LEAP2 mRNA quantified by PCR. Overall, the AvBD response to *Eimeria* challenge was not consistent; whereas LEAP2 was consistently down-regulated, which suggests that LEAP2 plays an important role in modulating an *Eimeria* infection.

Key words: *Eimeria*, host defense peptides, avian beta-defensins, LEAP2, in situ hybridization

2017 Poultry Science 96:2421–2427
http://dx.doi.org/10.3382/ps/pew468

INTRODUCTION

Coccidiosis is a common disease of poultry caused by the intestinal protozoan *Eimeria* (Conway and McKenzie, 2007). Infected birds usually show reduced feed efficiency and weight gain depression due to lesions in the intestinal mucosa (Kipper et al., 2013). Damage to the intestinal barrier also increases the mortality of the birds. *Eimeria* infection is responsible for losses of $3 billion annually in the poultry industry (Dalloul et al., 2007). The 3 species of *Eimeria* that mainly impact the poultry industry in the United States are *E. acervulina*, *E. maxima*, and *E. tenella*. These *Eimeria* species cause tissue-specific lesions, e.g., *E. acervulina* mainly affects the duodenum, *E. maxima* the jejunum, and *E. tenella* the ceca (Lillehoj and Trout, 1996).

Infection with *Eimeria* promotes both antibody and cell-mediated immune responses (reviewed in Chapman, 2014). Although antibodies can be abundantly produced locally, they cannot access and act on these intracellular pathogens (Lillehoj et al., 2004). Therefore, antibody-mediated responses play a minor role in protective immunity against coccidiosis. Cell-mediated immune responses are the major host immune response during *Eimeria* infection, and they also can provide protection against reinfection (Lillehoj and Trout, 1996; Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004). T- lymphocytes, natural killer cells, and macrophages are involved in the avian cellular immune response to *Eimeria* infection (Lillehoj and Trout, 1996; Dalloul et al., 2007). Host defense peptides not only show direct antimicrobial activity by disrupting membrane integrity but also enhance mucosal barrier

Published by Oxford University Press on behalf of Poultry Science Association 2017. This work is written by (a) US Government employee(s) and is in the public domain in the US.

Received April 22, 2016.
Accepted November 15, 2016.

[1]Corresponding author: ewong@vt.edu

function by inducing expression of mucins and tight junction proteins (Robinson et al., 2015).

The current prevention and treatment of coccidiosis is through vaccination, anticoccidial drugs, prebiotics, probiotics, and natural compounds (Quiroz-Castaneda and Dantan-Gonzalez, 2015). Live, wild-type coccidial vaccines can induce a strong immune response in chickens, which results in reduced feed efficiency and body weight gain. One alternative is to use precocious strains, which are defined as completing the lifecycle from sporozoite to oocyst 30 h faster than parent strains. Precocious strains show attenuated virulence but can still induce immunity. One drawback to the use of precocious strains is the additional labor and time required for numerous rounds of selection through naïve birds (Sharman et al., 2010; Chapman, 2014; Fetterer et al., 2014). There are 2 major types of anticoccidial drugs: coccidiostats and coccidiocidals. Coccidiostats act by inhibiting the development of *Eimeria*, while coccidiocidals destroy the structural integrity of *Eimeria*. Although these are effective, the development of drug resistance and consumer concerns about chemical residues in poultry products have caused a decrease in the use of anticoccidial drugs (Quiroz-Castaneda and Dantan-Gonzalez, 2015). The use of natural alternatives such as fats, antioxidants, essential oils, herbal extracts, prebiotics, and probiotics have shown some promise in ameliorating the negative effects of coccidiosis (Bozkurt et al., 2014; Quiroz-Castaneda and Dantan-Gonzalez, 2015). The cost of production, however, of these natural alternatives is currently high, but the cost can be offset by increased performance and use of a more environmental and consumer friendly product.

An alternative approach to disease control involves enhancement of the immune system. Innate immunity is the first line of host defense against infections in vertebrate animals (Dziarski, 2013). Components of the innate immune system include: immune cells, host defense peptides (**HDP**), enzymes, and pro-inflammatory factors (Dziarski, 2013). Host defense peptides have antimicrobial and immunomodulatory properties and show broad-spectrum activity against a range of bacteria, fungi, and enveloped viruses (Robinson et al., 2015). Because HDP attach to and insert into membranes of microbes, there is a low risk of triggering resistance (Brogden, 2005; Melo et al., 2009). Thus HDP are attractive candidates for use in antimicrobial therapies.

In avians, 14 avian beta-defensins (**AvBD**), 4 cathelicidins (**CATH**), and liver expressed antimicrobial peptide-2 (**LEAP2**) have been identified as HDP (Cuperus et al., 2013; Zhang and Sunkara, 2014). In vitro studies showed some of these HDP have a direct negative effect on bacteria such as *Campylobacter* and *Salmonella* (Milona et al., 2007; Townes et al., 2009; van Dijk et al., 2012). Hong et al. (2012) examined the expression of AvBD in the jejunum of Cobb and Ross broilers following an *E. maxima* plus *Clostridium perfringens* challenge to induce necrotic enteritis. In uninfected chickens, abundance of AvBD8, AvBD10, and AvBD13 mRNA was high; AvBD1, AvBD6, AvBD9, AvBD11, and AvBD12 mRNA was moderate; and AvBD3 and AvBD4 mRNA was very low. Infected Cobb and Ross chickens showed different patterns of up-regulation and down-regulation of AvBD. The expression of AvBD during an *Eimeria* challenge has not been reported. Thus the objective of the current study was to compare the expression profiles of AvBD in the duodenum, jejunum, ileum, and ceca of chickens following challenge with *E. acervulina*, *E. maxima*, and *E. tenella*.

MATERIALS AND METHODS

Birds and Eimeria challenge

Study 1 Chickens used in this study were Ross Heritage broiler males (Longenecker's Hatchery, Elizabethtown, PA). *Eimeria* are all USDA strains: *E. acervulina* (USDA #12 isolate), *E. maxima* (USDA APU1 isolate), and *E. tenella* (Wampler isolate). Oocysts were maintained and isolated as previously described (Fetterer and Barfield, 2003). Broiler chickens were housed at the USDA-ARS facility (Beltsville, MD) from hatch and maintained coccidia-free in suspended wire cages. Chickens were given water and a standard starter-type corn-soybean meal, ad libitum. At 21 d of age, chicks were inoculated with *Eimeria* via gavage. The tissue samples were the same as those from the study reported by Su et al. (2015). In the first experiment, chickens were inoculated with *E. acervulina* (200,000 oocysts/chicken, n = 6) or *E. maxima* (10,000 oocysts/chicken, n = 6). Control 1 (n = 6) received no *Eimeria* oocysts. In the second experiment, chickens were inoculated with *E. tenella* (150,000 oocysts/chicken, n = 6). Control 2 (n = 6) received no *Eimeria* oocysts. Body weights of chickens were obtained on d of challenge (d 21) and 7 d post challenge.

Study 2 In a separate study, Ross Heritage broiler males (Longenecker's Hatchery, Elizabethtown, PA) were challenged with only *E. maxima*. Housing and challenge were as described for study 1. At 21 d of age, chicks (n = 12) were inoculated via gavage with a lower dose of *E. maxima* (USDA APU1 isolate, 1,000 oocysts/chicken). Control (n = 12) received no *Eimeria*. Body weights of chickens were obtained on d of challenge (d 21) and 7 d post challenge. Six control and 6 challenged chickens were randomly chosen for gene expression analysis.

All studies were carried out under protocols approved by the Beltsville Research Center Animal Care and Use Committee and conducted at the Animal Parasitic Disease Laboratory (USDA Agricultural Research Service, Beltsville, MD). Chickens were euthanized by cervical dislocation and intestinal segments were collected on d 28 (controls and 7 d post challenge). For both studies, each of the animals showed symptoms of *Eimeria* infection including intestinal lesions, loosening of the feces, and petechiae. Lesions were noted but not scored. Weight gain depression was used as an

Table 1. Forward and reverse primers for quantitative PCR.

Gene	Forward primer	Reverse primer
AvBD1	GAGTGGCTTCTGTGCATTTCTG	TTGAGCATTTCCCACTGATGAG
AvBD6	GCCCTACTTTTCCAGCCCTATT	GGCCCAGGAATGCAGACA
AvBD8	ATGCGCGTACCTAACAACGA	TGCCCAAAGGCTCTGGTATG
AvBD10	CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT
AvBD11	GGTACTGCATCCGTTCCAAAG	GCATGTTCCAAATGCAGCAA
AvBD12	TGTAACCACGACAGGGGATTG	GGGAGTTGGTGACAGAGGTTT
AvBD13	CAGCTGTGCAGGAACAACCA	CAGCTCTCCATGTGGAAGCA
LEAP2	CTCAGCCAGGTGTACTGTGCTT	CGTCATCCGCTTCAGTCTCA
β-actin	GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT

objective measure to assess the extent of *Eimeria* infection. Duodenum, jejunum, ileum, and ceca were collected for study 1 and only duodenum, jejunum, and ileum were collected for study 2. The contents of the intestine were removed and the tissue segments were immediately stored individually in RNAlater (Invitrogen, Grand Island, NY).

RNA Extraction and Quantitative Real-time PCR

The tissue samples were removed from RNAlater (Thermo Fisher Scientific, Pittsburgh, PA) and minced. For all samples, a 20 to 30 mg sample of tissue was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) and total RNA was extracted following the manufacturer's instructions of Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). RNA quantity and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and RNA quality was assessed by agarose-formaldehyde gel electrophoresis. The cDNA was synthesized from total RNA (500 ng) using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) and then diluted 1:30 for real-time PCR analysis. The 7 AvBD (AvBD1, AvBD6, AvBD8, AvBD10, AvBD11, AvBD12, and AvBD13) that showed the greatest expression in the intestine (Hong et al., 2012) were analyzed by quantitative real-time PCR. β-actin was chosen as the reference gene, because the Ct values for β-actin were the same for control and infected samples. In study 1, expression of 7 AvBD was examined, while for study 2, expression of 7 AvBD and LEAP2 was examined. The forward and reverse primers for the 7 AvBD, LEAP2, and β-actin are shown in Table 1. qPCR was performed on an Applied Biosystems 7500 system with Fast SYBR green (Thermo Fisher Scientific, Waltham, MA) using the following conditions for all genes: 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 seconds. Samples were run in duplicate and relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), as described in Su et al. (2015). The mean ΔCt of the control samples was used to calculate the $\Delta\Delta$Ct value, which was performed separately for each intestinal segment. *Eimeria* treatment and each gene are a group.

In situ Hybridization and Morphology Analysis

In situ hybridization (**ISH**) was performed using the RNAscope method (Advanced Cell Diagnostics, ACD, Newark, CA) as described by Wang et al. (2012). Three intestinal samples from both control and *E. maxima* challenged jejunal tissue were fixed in phosphate buffered 4% paraformaldehyde and embedded in paraffin. Sections (5 to 6 μm) were cut with a microtome and the expression of LEAP2 mRNA was assayed by ISH using a set of custom synthesized probes for chicken LEAP2 (ACD). The tissue sections were processed according to the manufacturer's directions using the HybEZ oven and the RNAscope 2.5 HD detection kit-Brown (ACD). Following RNA scope processing, the slides were stained with a 50% Gill #2 hematoxylin solution (Sigma Aldrich, St Louis, MO), rinsed in water, and then placed in 0.02% ammonia water. After air drying, a drop of Clear Mount solution (American Master Tech Scientific, Inc, Lodi, CA) was added and a coverslip was placed on top. Images were captured at various magnifications with a Nikon Eclipse 80i microscope and DS-Ri1 digital camera. Villus length was measured from the top of the intestinal crypt to the tip of the villus for intact villi ($n > 30$) from different samples using Infinity Analyze imaging software (Lumenera Co., Ottawa, Ontario).

Statistical Analysis

PCR data were analyzed by ANOVA using JMP® Statistical Discovery Software from SAS (SAS Institute, Cary, NC). For study 1, control 1 and *E. acervulina*- and *E. maxima*-challenged chickens were analyzed separately from control 2 and *E. tenella*-challenged chickens. For gene expression of each *Eimeria* challenge, the model included the main effects of treatment, sorted by genes. Significance level was set at $P < 0.05$ when compared with the control. Villus length was analyzed by *t*-test.

RESULTS

Eimeria challenge caused the expected weight gain depression from d of challenge (d 21) to d of sample collection (d 28). In study 1, *E. acervulina*, *E. maxima*,

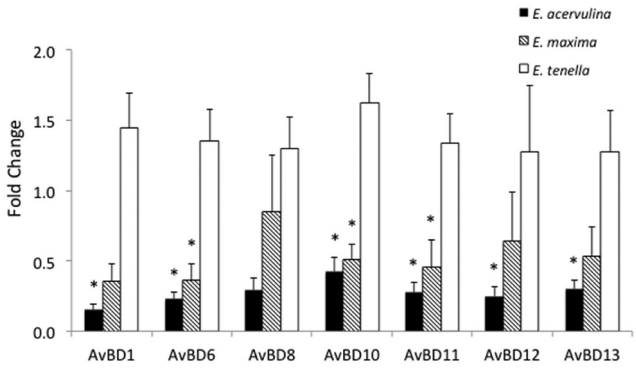

Figure 1. Expression of avian beta-defensins (AvBD) in the duodenum of *E. acervulina*, *E. maxima*, and *E. tenella*-challenged broilers (study 1). Doses were 200,000, 10,000, and 150,000 oocysts/chicken for *E. acervulina*, *E. maxima*, and *E. tenella*, respectively. Controls (non-challenged) are equal to a fold change of one for each AvBD. *indicates statistical significance from control at $P < 0.05$.

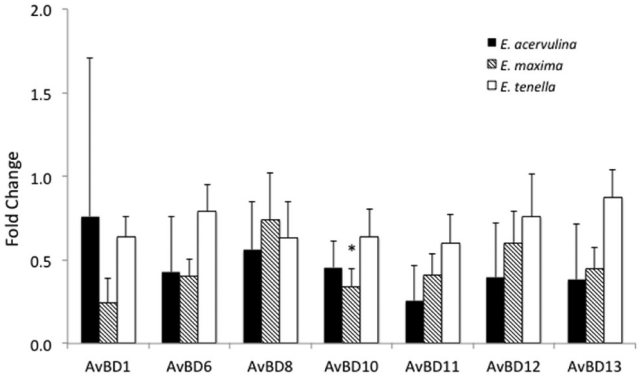

Figure 2. Expression of avian beta-defensins (AvBD) in the jejunum of *E. acervulina*, *E. maxima*, and *E. tenella* challenged broilers (study 1). Doses were 200,000, 10,000, and 150,000 oocysts/chicken for *E. acervulina*, *E. maxima*, and *E. tenella*, respectively. Controls (non-challenged) are equal to a fold change of one for each AvBD. *indicates statistical significance from control at $P < 0.05$.

and *E. tenella* challenge resulted in a 42, 30, and 24% weight gain depression (Su et al., 2015). In study 2, the control chickens (n = 6) weighed on average 817±24 g at d 21 (challenge d) and 1,311±47 g at d 28 (sampling d), for an average weight gain of 494±28 g/chicken. In contrast, the *E. maxima* challenged chickens (n = 6) weighed on average 725±58 g at d 21 and 1,144±79 g at d 28 for an average weight gain of 418±24 g, which equaled a 15% weight gain depression ($P < 0.05$).

In study 1, the expression of 7 AvBD was profiled in the small intestine and ceca of broilers challenged with *E. acervulina*, *E. maxima*, or *E. tenella.* In the duodenum (Figure 1), *E. acervulina* challenge resulted in the down-regulation of AvBD1, AvBD6, AvBD10, AvBD11, AvBD12, and AvBD13 to 15 to 42% of control. *E. maxima* challenge caused down-regulation of AvBD6, AvBD10, and AvBD11 to 37 to 51% of control, while *E. tenella* challenge had no effect on expression of any AvBD. In the jejunum (Figure 2), *E. maxima* caused down-regulation of AvBD10 to 34% of control. Both *E. acervulina* and *E. tenella* showed no changes in expression of AvBDs. In the ileum (Figure 3), none

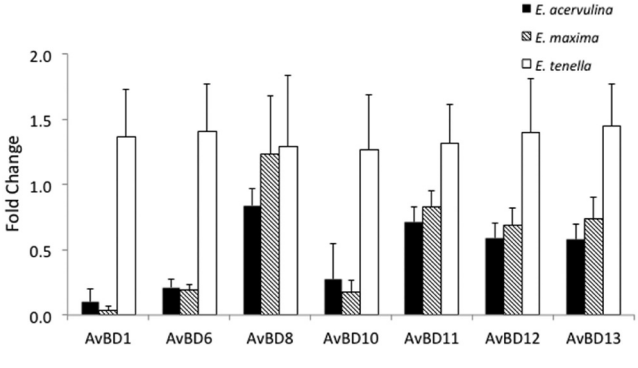

Figure 3. Expression of avian beta-defensins (AvBD) in the ileum of *E. acervulina*, *E. maxima*, and *E. tenella* challenged broilers (study 1). Doses were 200,000, 10,000, and 150,000 oocysts/chicken for *E. acervulina*, *E. maxima*, and *E. tenella*, respectively. Controls (non-challenged) are equal to a fold change of one for each AvBD.

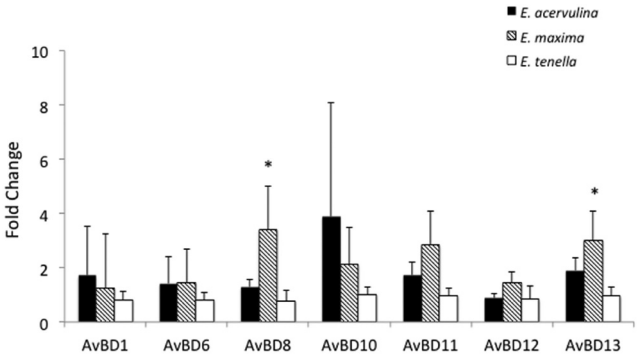

Figure 4. Expression of avian beta-defensins (AvBD) in the ceca of *E. acervulina*, *E. maxima*, and *E. tenella* challenged broilers (study 1). Doses were 200,000, 10,000, and 150,000 oocysts/chicken for *E. acervulina*, *E. maxima*, and *E. tenella*, respectively. Controls (non-challenged) are equal to a fold change of one for each AvBD. *indicates statistical significance from control at $P < 0.05$.

of the 3 *Eimeria* species caused changes in AvBD expression. In the ceca (Figure 4), *E. maxima* challenge resulted in an increase of 242 and 201% above control for AvBD8 and AvBD13, respectively. There were no changes in gene expression for *E. acervulina* or *E. tenella* challenge in the ceca.

In study 2, the expression of 7 AvBD plus LEAP2 was examined in the duodenum, jejunum, and ileum of *E. maxima* challenged chickens (Figure 5). In the duodenum, there was no change in the expression of the AvBD. In the jejunum, there was down-regulation of AvBD11 to 40% of control; and in the ileum there was down-regulation of AvBD1 and AvBD11 to 69 and 64% of control, respectively. LEAP2 was down-regulated to 46, 48, and 45% of control in the duodenum, jejunum, and ileum, respectively.

Expression of LEAP2 mRNA was evaluated by ISH in the jejunum of control and *E. maxima* challenged chickens from study 2 (Figure 6). LEAP2 mRNA (brown staining) was detected in the epithelial cells that line the villi, but not in the crypts of the jejunum of both the control and *E. maxima* challenged chickens. At the tips of the villi in the control tissues there was little to no staining for LEAP2 mRNA in the epithelial cells,

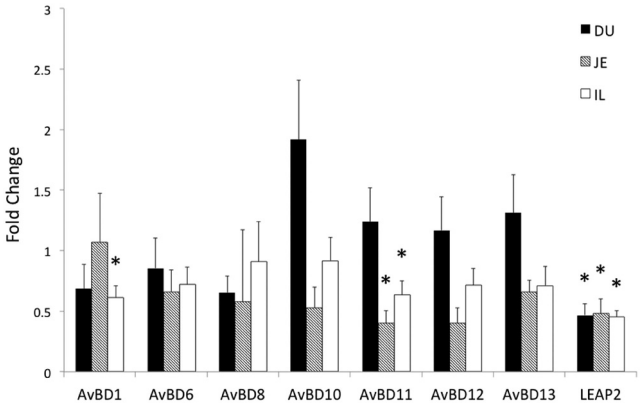

Figure 5. Expression of avian beta defensins and LEAP2 in the duodenum (DU), jejunum (JE), and ileum (IL) of *E. maxima* (1,000 oocysts/chicken) challenged broilers (study 2). Controls (non-challenged) are equal to a fold change of one for each AvBD. *indicates statistical significance from control at $P < 0.05$.

indicating that these cells were no longer expressing LEAP2 mRNA. The lengths of the villi also were examined in the histological sections. The villus length in the control chickens (1,210 ± 94 μm) was greater ($P < 0.001$) than that in the *E. maxima* challenged chickens (788 ± 141 μm).

DISCUSSION

Eimeria is a protozoa that invades intestinal epithelial cells and induces an immune response. The antimicrobial peptide LEAP2 was down-regulated in the small intestine following challenge with *E. acervulina*, *E. maxima*, *E. tenella*, and *E. praecox* (Casterlow et al., 2011; Sumners et al., 2011; Paris and Wong, 2013; Su et al., 2014, 2015; Yin et al., 2015). Using a necrotic enteritis model induced by initial challenge with *E. maxima* followed by challenge with *Clostridium perfringens*, Hong et al. (2012) reported the expression of HDP in the jejunum of both Cobb and Ross broilers. In Cobb broilers showing necrotic enteritis, AvBD3, AvBD4, and AvBD12 were down-regulated, while AvBD8, AvBD11, and AvBD13 were up-regulated. Ross broilers showed a different pattern with only AvBD12 down-regulated and AvBD1, AvBD6, AvBD8, and AvBD10 up-regulated. Because expression of AvBD in birds infected with only *E. maxima* was not reported in Hong et al (2012), changes in gene expression cannot be definitively attributed to the effect of *Eimeria*, *Clostridium*, or both. Since we observed only a few changes in AvBD expression following *Eimeria* challenge, most of the changes in AvBD expression reported by Hong et al. (2012) during necrotic enteritis are likely due to the secondary *Clostridium* infection.

In our studies, we compared the expression of AvBD in chickens infected with 3 different *Eimeria* species. Our results in study 1 showed that AvBD were down-regulated in *E. acervulina* and *E. maxima* infected chickens in different intestinal segments, similar to the down-regulation reported for LEAP2 (Su et al., 2015). There was common down-regulation of LEAP2, AvBD6, AvBD10, and AvBD11 in the duodenum of *E. acervulina* and *E. maxima* infected chickens. *E. acervulina* also showed down-regulation of AvBD1, AvBD12, and AvBD13 in the duodenum and no effect in the jejunum, ileum, or ceca. This is consistent with *E. acervulina* causing lesions in the duodenum. *E. maxima* also caused down-regulation of AvBD10 and LEAP2 (Su et al., 2015) in the jejunum, which is the site for *E. maxima* lesions. Interestingly in the ceca of *E. maxima* challenged broilers, there was up-regulation of AvBD8 and AvBD13. *E. tenella* challenged broilers had no effect on AvBD expression in any intestinal segment and caused down-regulation of LEAP2 only in the ceca (Su et al., 2015).

In study 2, *E. maxima* challenge resulted in a different pattern of AvBD expression. There was no change in gene expression in the duodenum and only down-regulation of AvBD1 in the ileum and AvBD11 in the jejunum and ileum. In contrast, in study 1 AvBD1 showed no changes, and AvBD11 was down-regulated only in the duodenum. Consistent with previous

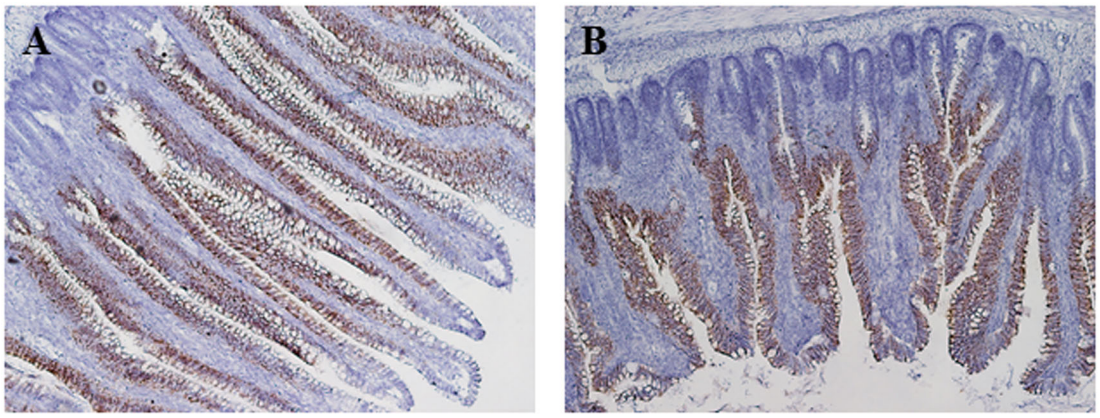

Figure 6. In situ hybridization analysis of LEAP2 mRNA. Formalin fixed paraffin embedded jejunal samples from control (A) and *E. maxima* challenged (B) chickens from study 2 were assayed by in situ hybridization using the RNA scope 2.5 HD kit (Brown). Brown staining revealed the presence of LEAP2 mRNA. The tissues were counterstained with 50% hematoxylin. Images were captured using 100x magnification.

studies, LEAP2 was down-regulated in all 3 segments of the small intestine (Su et al., 2014, 2015). In this study, ceca were not collected. The difference in AvBD results may be partly due to the challenge dose. In study 1, 10,000 *E. maxima* oocysts were gavaged, whereas in study 2, only 1,000 *E. maxima* oocysts were gavaged. The different *E. maxima* dosages were consistent with the weight gain depression observed. Study 1 resulted in 30% weight gain depression and study 2 resulted in 15% weight gain depression. These results show that the AvBD response to *Eimeria* challenge was not always consistent, in contrast to the consistent down-regulation of LEAP2.

Because HDP are part of the innate immune system, they would be expected to serve as the first line of defense during a pathogen challenge. Sumners et al. (2011) examined the temporal changes in expression of LEAP 2 and cathelicidin 3 in the duodenum and jejunum of broilers challenged with 50,000 or 500,000 *E. praecox* oocysts at daily intervals from d 1 to d 7 post challenge. They found that the lower dose of *E. praecox* caused down-regulation of LEAP2 in the duodenum at d 3 and the duodenum and jejunum at d 4 post challenge. At the higher *Eimeria* dose, LEAP2 was down-regulated in the duodenum at d 3, d 4, and d 5. Cathelicidin 3 was down-regulated in the jejunum at only d 3 following challenge with the low dose of *Eimeria*. Thus the down-regulation of LEAP2 and cathelicidin 3 does not occur immediately after challenge, but requires 3 d post challenge to develop. In this study, expression of the AvBD was not examined. It would be interesting to analyze the expression of AvBD at different d after challenge.

In situ hybridization was used to examine LEAP2 mRNA expression because it has the advantage of being able to identify individual cells expressing LEAP2 mRNA, which cannot be revealed by qPCR. Our results show that LEAP2 mRNA was expressed in the epithelial cells lining the villi in both the control and *E. maxima* challenged chickens. In addition, LEAP2 mRNA was not localized to cells in the crypt, which is the site for intestinal stem cells in mammals (Carulli et al., 2014). This result is consistent with the results of Howard et al. (2010), who showed by immunohistochemical analysis that LEAP2 protein was localized to epithelial cells lining the colonic crypts and the proximal and distal tubules of the kidney.

Morphological analysis of the villi of control and *E. maxima* challenged chickens in study 2 revealed that although the jejunal villi in *E. maxima* challenged chickens were shorter than villi in control chickens, the epithelial cells of both villi were functional as shown by similar expression of LEAP2 mRNA by ISH in the epithelial cells. Furthermore, the samples analyzed for AvBD mRNA expression in study 1 were the same samples previously analyzed for expression of amino acid, peptide, and monosaccharide transporters (Su et al., 2015). This study showed that some transporters had unchanged expression following *Eimeria* challenge, while others showed increased or decreased expression. These results demonstrate that the intestinal epithelial cells in both of our studies were intact and functional following *Eimeria* challenge. Analysis of the villi from study 2 showed that the villi from the *E. maxima* challenged chickens were on average 65% of the length of control chickens. Because there appeared to be no qualitative difference in intensity of ISH staining for LEAP2 in the jejunum between control and *E. maxima* challenged chickens, the down-regulation of LEAP2 to 48% of control as quantified by qPCR may in part be attributable to the shortening of the villi.

In summary, at 7 d post challenge, LEAP2 was consistently down-regulated following *Eimeria* challenge, whereas the AvBD response was variable and may be dose dependent. LEAP2 mRNA was expressed similarly in the epithelial cells lining the villi of control and *E. maxima* challenged chickens.

ACKNOWLEDGMENTS

Funding for this work was provided in part by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture. SS was supported by a fellowship from the John Lee Pratt Animal Nutrition Program in the College of Agriculture and Life Sciences at Virginia Tech. We thank H. Zhang for assistance with RNAscope and villus measurements.

REFERENCES

Bozkurt, M., N. Aysul, K. Kucukyilmaz, S. Aypak, G. Ege, A. U. Catli, H. Aksit, F. Coven, K. Seyrek, and M. Cinar. 2014. Efficacy of in-feed preparations of an anticoccidial, multienzyme, prebiotic, probiotic, and herbal essential oil mixture in healthy and Eimeria spp.-infected broilers. Poult. Sci. 93: 389–399.

Brogden, K. A. 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3:238–250.

Carulli, A. J., L. C. Samuelson, and S. Schnell. 2014. Unraveling intestinal stem cell behavior with models of crypt dynamics. Integr. Biol. 6:243–257.

Casterlow, S., H. Li, E. R. Gilbert, R. A. Dalloul, A. P. McElroy, D. A. Emmerson, and E. A. Wong. 2011. An antimicrobial peptide is downregulated in the small intestine of Eimeria maxima-infected chickens. Poult. Sci. 90:1212–1219.

Chapman, H. D. 2014. Milestones in avian coccidiosis research: a review. Poult. Sci. 93:501–511.

Conway, D. P., and M. E. McKenzie. 2007. Poultry Coccidiosis: Diagnostic and Testing Procedure. 3rd ed. Blackwell Pub., Ames, Iowa.

Cuperus, T., M. Coorens, A. van Dijk, and H. P. Haagsman. 2013. Avian host defense peptides. Dev. Comp. Immunol. 41:352–369.

Dalloul, R. A., T. W. Bliss, Y. H. Hong, I. Ben-Chouikha, D. W. Park, C. L. Keeler, and H. S. Lillehoj. 2007. Unique responses of the avian macrophage to different species of Eimeria. Mol. Immunol. 44:558–566.

Dziarski, R. 2013. Chapter 6: Innate Immunity. Pages xvii, 826 p. in Schaechter's Mechanisms of Microbial Disease. M Schaechter, N C Engleberg, V J DiRita, and T Dermody eds. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Fetterer, R. H., and R. C. Barfield. 2003. Characterization of a developmentally regulated oocyst protein from Eimeria tenella. J. Parasitol. 89:553–564.

Fetterer, R. H., M. C. Jenkins, K. B. Miska, and R. C. Barfield. 2014. Evaluation of an experimental irradiated oocyst vaccine

to protect broiler chicks against avian coccidiosis. Avian. Dis. 58:391–397.

Hong, Y. H., W. Song, S. H. Lee, and H. S. Lillehoj. 2012. Differential gene expression profiles of beta-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult. Sci. 91:1081–1088.

Howard, A., C. Townes, P. Milona, C. J. Nile, G. Michailidis, and J. Hall. 2010. Expression and functional analyses of liver expressed antimicrobial peptide-2 (LEAP-2) variant forms in human tissues. Cell. Immunol. 261:128–133.

Kipper, M., I. Andretta, C. R. Lehnen, P. A. Lovatto, and S. G. Monteiro. 2013. Meta-analysis of the performance variation in broilers experimentally challenged by Eimeria spp. Vet. Parasitol. 196:77–84.

Lillehoj, H. S., and E. P. Lillehoj. 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian. Dis. 44:408–425.

Lillehoj, H. S., W. Min, and R. A. Dalloul. 2004. Recent progress on the cytokine regulation of intestinal immune responses to Eimeria. Poult. Sci. 83:611–623.

Lillehoj, H. S., and J. M. Trout. 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin. Microbiol. Rev. 9:349–360.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25:402–408.

Melo, M. N., R. Ferre, and M. A. Castanho. 2009. Antimicrobial peptides: Linking partition, activity and high membrane-bound concentrations. Nat. Rev. Microbiol. 7:245–250.

Milona, P., C. L. Townes, R. M. Bevan, and J. Hall. 2007. The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against Salmonella serovars. Biochem. Biophys. Res. Commun. 356:169–174.

Paris, N. E., and E. A. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult. Sci. 92:1331–1335.

Quiroz-Castaneda, R. E., and E. Dantan-Gonzalez. 2015. Control of avian coccidiosis: Future and present natural alternatives. Biomed. Res. Int. 2015:430610.

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the intestinal barrier function by host defense peptides. Front. Vet. Sci. 2:57.

Sharman, P. A., N. C. Smith, M. G. Wallach, and M. Katrib. 2010. Chasing the golden egg: Vaccination against poultry coccidiosis. Parasite Immunol. 32:590–598.

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2014. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult. Sci. 93:1217–1226.

Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp. Parasitol. 150: 13–21.

Sumners, L. H., K. B. Miska, M. C. Jenkins, R. H. Fetterer, C. M. Cox, S. Kim, and R. A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during *Eimeria praecox* infection in chickens. Exp. Parasitol. 127:714–718.

Townes, C. L., G. Michailidis, and J. Hall. 2009. The interaction of the antimicrobial peptide cLEAP-2 and the bacterial membrane. Biochem. Biophys. Res. Commun. 387:500–503.

van Dijk, A., M. Herrebout, M. H. Tersteeg-Zijderveld, J. L. Tjeerdsma-van Bokhoven, N. Bleumink-Pluym, A. J. Jansman, E. J. Veldhuizen, and H. P. Haagsman. 2012. Campylobacter jejuni is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. Vet. Microbiol. 160:347–354.

Wang, F., J. Flanagan, N. Su, L. C. Wang, S. Bui, A. Nielson, X. Y. Wu, H. T. Vo, X. J. Ma, and Y. L. Luo. 2012. RNAscope a novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 14:22–29.

Yin, H., L. H. Sumners, R. A. Dalloul, K. B. Miska, R. H. Fetterer, M. C. Jenkins, Q. Zhu, and E. A. Wong. 2015. Changes in expression of an antimicrobial peptide, digestive enzymes, and nutrient transporters in the intestine of E. praecox-infected chickens. Poult. Sci. 94:1521–1526.

Zhang, G., and L. T. Sunkara. 2014. Avian antimicrobial host defense peptides: From biology to therapeutic applications. Pharmaceuticals. 7:220–247.