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Assessment of Toll-like Receptors in the Ileum of Weanling Pigs - Responses to Feed Antibiotic Chlortetracycline and Gnotobiotic Conditions

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Abstract

It has been suggested that changes in diet at weaning in pigs induce intestinal inflammation which may be mediated through toll-like receptors (TLRs). We hypothesized that the use of antibiotics as growth promoters and subsequent changes in intestinal microbiota may mediate changes in the expression of TLRs in the intestine. Thus, this study was performed to assess the changes in intestinal TLRs in weanling pigs in response to the use of chlortetracycline as a growth promotant, and under gnotobiotic conditions. Eighteen cesarean-derived half-sib piglets were divided into three groups; antibiotic-fed, control (normal-fed) and gnotobiotic groups.TLR-2, -4, -5 and -9 gene expression and abundance of TLR-2 and -9 proteins in the ileum of pigs was assessed at 5 wk of age. No significant differences ($p \geq 0.5$) in TLR-2, -4, -5 and -9 transcript levels and an abundance of TLR-2 and -9 proteins among three groups of pigs were observed.

Keywords: Toll-like receptors; Pigs; Ileum; Chlortetracycline; Growth promotant; Gnotobiotic

Introduction

The use of antibiotics in the diets of food animals began after the observation that sub-therapeutic levels of antibiotics supplements can improve feed conversion and growth rates. Although many studies have been conducted on how antibiotics improve growth rate, the exact mechanisms involved in growth promotion are still controversial. Some suggested mechanisms of action of feed antibiotics as growth promotant include suppression of subclinical infections, decrease in growth-depressing bacterial metabolites, consumption of nutrients by commensals and improvement in nutrient uptake [1,2]. It has been hypothesized that antibiotics, when used as growth promoters at weaning in piglets, cause a reduction in microbial load and make the intestinal microbial community more homogenous but no scientific studies have been reported so far to prove this presumption. The basis of this hypothesis is that the reduction in microbial load possibly helps in suppressing inflammation in the host intestinal tract and the surplus energy could be diverted for an increased growth rate [1,2]. However, in contrast to this hypothesis, a recent study elegantly shows that use of in-feed antibiotics in pigs shifts the bacterial phylotypes between antibiotic-fed and normal-fed pigs with a significant increase in *Proteobacteria* in antibiotic-fed pigs but it does not significantly decrease the bacterial diversity in antibiotic-fed pigs [3]. The routine use of antibiotics as feed additives may potentially diminish the therapeutic benefits of antibiotics for animal and human therapy. It is generally accepted that the low doses of antibiotics used in animal feeds select for resistant commensals in the intestine, which can transfer drug-resistant genes to human pathogens, and is thus a matter of great public health concern [3-6]. As a result, the European Union (EU) has enacted legislation since 1999 to restrict or ban the use of most feed antibiotics [7].

Intestinal epithelial cells (IECs) in both animals and humans are continually exposed to high numbers and diverse types of bacteria and have a front-line role in monitoring luminal microorganisms, and in responding to microbes and their ligands [8-10]. Toll-like receptors (TLRs) are pattern-recognition receptors and play a crucial role in the host defensive activity against microbial infections [11-13]. TLRs are expressed on different cell types in the body, but most highly expressed on cells involved in innate immunity such as macrophages, dendritic cells and epithelial cells where they participate in antibacterial, antiviral and antifungal host defenses [14-16]. TLRs specifically recognize microbial products/ligands. TLR2 recognizes lipotechoic acid (LTA) [17,18] whereas TLR4 mediates lipopolysacchride (LPS) stimulation leading to specific intracellular signal transduction pathways [19]. Mammalian TLR5 recognizes flagellin, a principal component of bacterial flagella [20,21]. TLR9 had been identified as the receptor for unmethylated CpG bacterial DNA [22].

The IECs constitutively express several functional TLRs, which appear to be key regulators of the luminal immune response system [16,23]. Inflammatory cytokines may up-regulate the expression of TLRs. Studies have shown that Th1 cytokine IFN-γ promotes TLR4/MD-2 signalling [24,25], whereas Th2 cytokines downregulate the TLR expression in the IECs [26]. Thus, the delicate balance between Th1 and Th2 cytokines mediated through TLRs may be responsible for the controlled state of inflammation on the host intestinal surfaces. Studies have associated intestinal microbiota in up-regulating the mucosal Th1

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cytokine profile by enhanced intestinal TLR expression, as in Crohn's disease [23,27,28]. The recognition of commensal bacteria by TLRs assists in intestinal epithelial homeostasis and protection from gut injury [29-31]. A recent study indicates that mice genetically-deficient in TLR5 have the features of pathological metabolic syndrome because of significant changes in the composition of the gut microflora [32]. These observations indicate that TLRs play a significant role in mediating and maintaining innate immune responses and inflammation at intestinal surfaces [32-35].

There are reports of a transient increase in mRNA coding for inflammatory cytokines in the intestine of weaned piglets [36,37], indicating that the weaning may be responsible for inducing low degree of inflammation in the intestine often mediated through changes in the TLR expression and signaling. Therefore, it is possible that weaning may be associated with changes in TLR expression in the intestinal tract. It is hypothesized that there may be changes in the expression of various TLRs and their signaling pathways in the pig intestinal wall due to the functional changes in intestinal microbiota induced by feeding antibiotics as growth promotants or because of absence of microbiota in germ-free animals. Furthermore, changes in TLR expression may affect the control of subclinical intestinal infections and inflammation, and thereby may influence animal performance. Several studies had been conducted for detecting TLRs on the intestinal tissues and cells of pigs using real-time RT-PCR (qRT-PCR) and/or immunohistochemical techniques [38-42]. A few recent studies [43-46] assessed the effects of fructo-oligosaccharides, commensals and germ-free conditions on the intestinal immune response genes including TLRs in pigs. However, to our knowledge no study has been conducted to investigate the changes in TLRs expression in weanling pigs in response to the feed antibiotic chlortetracycline, or under gnotobiotic conditions. This study aims to investigate the expression levels of toll-like receptors (TLR-2, -4, -5 and -9) on the ileal segments of weanling piglets in response to the feed antibiotic (chlortetracycline) and under gnotobiotic conditions using real time RT-PCR and immunohistochemical techniques.

Materials and Methods

Animals and treatments

All animal experiments and protocols used in this study were approved by the South Dakota State University (SDSU) Institutional Animal Care and Use Committee (IACUC). Three, line-13 healthy sows (Landrace X Yorkshire; AusGene International Inc., Grindley, IL), that tested negative for porcine respiratory and reproductive syndrome virus and rotavirus, were selected from the University Swine Unit at SDSU and inseminated with the semen of a single SP-1 (Duroc x Yorkshire) boar [47,48]. Half sib piglets from these sows were obtained 2 to 3 days before the expected parturition date by cesarean section. In total, 18 piglets, 6 from each of the 3 sows, were used for this study, and 2 pigs originating from each sow were assigned at random to gnotobiotic rearing or to nursing by either of 2 foster mothers. Six pigs (2 from each cesarean section-derived litter) were fostered onto each of 2 sows induced to farrow in synchrony with cesarean section delivery of the test animals, and their naturally born piglets were removed. The foster mothers were kept in separate isolation rooms, thus allowing the colonization of intestines of the pigs with the normal intestinal microbiota of the foster mother. Gnotobiotic pigs were raised in the Department of Veterinary and Biomedical Sciences gnotobiotic pig facility, which has been used successfully for various earlier studies [49,50]. The gnotobiotic piglets were supplied with commercial sterile milk replacer (Esbilac, PetAg Inc., Hempshire, IL) which was prorated based on animal age and expected energy requirements for 21 days. The compositions of the Esbilac milk replacer and animal feed used in this study have been described earlier [47].

Fostered 12 piglets nursed on two sows (6 piglets/each) were weaned after 21 days and regrouped as control (antibiotic-free) and antibiotic-fed groups (n=6). The fostered control (n=6) and antibiotic-fed (n=6) groups were formed by including 3 piglets from each foster sow. Fostering on nurse sows and redivision at weaning were done to nullify the effect of swine genetics and foster mother's microflora on the intestinal microflora composition of the experimental piglets. Gnotobiotic group pigs were removed from milk replacer after 21 days. All three groups of piglets were fed the same weanling diet as described earlier [47] ad libitum for 2 week, except for the addition of chlortetracycline at 50 ppm in the feed of the antibiotic-fed group, after which the piglets were euthanized at 5 week of age. It is difficult to maintain piglets in gnotobiotic units beyond 5 week of age because of their large size and body growth, thus the study was concluded at 5 week. Fecal samples from the gnotobiotic pigs were submitted to the SDSU Veterinary and Biomedical Sciences Diagnostic Laboratory for testing for aerobic and anaerobic microbes at 2 and 5 wk of age. No aerobic or anaerobic bacteria were detected by direct culture method, except for *Clostridium perfringens* in the samples collected at 5 weeks of age, and hence the term 'gnotobiotic pig' instead of 'germ-free pig' was used in this study.

Sample collection

One-third of the animals from each of the three groups were sacrificed on consecutive days for technical convenience. Piglets from each group were euthanized using a standard protocol approved by IACUC. After euthanasia, the abdomen was immediately opened, the mesentery was removed and the ileum bearing continuous Peyer's patch was located. A number of small caudal ileal segments were obtained from each piglet; rinsed in PBS (pH 7.3) and immediately snap frozen in liquid nitrogen and stored at -80°C. For immunohistochemistry, caudal segments of the ileum, 5 to 10 cm long, were obtained from each piglet and opened longitudinally. The digesta was removed by gentle washing in PBS (pH 7.3), and the tissue sample was immediately stapled at both ends onto a thick, polyethylene transparency sheet to preserve tissue morphology. All samples were immediately immersed in 10% buffered formalin for 48 h, followed by storage in 70% ethanol. Tissues were embedded in paraffin, and sections 5 µm in thickness were cut and laid on lysine-coated microscope slides (Fisher Scientific, Chicago, IL).

Total RNA and RNA amplification

Caudal ileal tissue samples stored at -80°C were minced using a scalpel and then ground to fine consistency in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 30 mg of sample using a Qiagen-RNeasy Mini Kit (Cat # 74104, Qiagen, Inc., Valencia, CA) as per the manufacturer's instructions. Each sample was treated with amplification-grade DNase I (Cat # 79254, Qiagen, Inc.,CA) to remove all genomic DNA contamination. The purified total RNA was then quantified using a Nano-drop ND-1000 spectrophotometer (Nanodrop, Wilmington, Delaware). RNA samples with ratios of approximately 1.8 (260/280) and above 2.0 (260/230) were chosen for analysis. Total RNA (1 µg) was reverse transcribed to cDNA and *in vitro* transcribed to amplified RNA (aRNA) using RNA ampULSE: Amplification Kit (Cat # KRE4057, Open Biosystems, Huntsville, AL) as per the manufacturer's protocol.

Real-time RT-PCR

cDNA was prepared from 1 µg of aRNA using Taqman reverse transcription reagents (Part # N808-0234, Applied Biosystems, Foster City, CA) in a 50 µl reaction volume. An equivalent volume of cDNA solution (5 µl) from each sample was used for quantitative real-time RT-PCR (qRT-PCR) with SYBR Green PCR Master Mix (Part # 4309155, Applied Biosystems, Foster City, CA) in a 25 µl volume. The PCR primers were designed using Beacon designer 3 (Premier Biosoft International, Palo Alto, CA) for porcine TLR4 (AY289532): forward 5'-ATCGCTGCTAACATCATC-3' and reverse 5'-GACTTCTCCAACTTCTGC-3' and for porcine TLR5 (AB208697): forward 5'-ACTCTCCCAAGCCCTAAG-3' and reverse 5'-GCAGCCACGATAGATAGC-3'. The PCR primers for porcine TLR2 (AB072190) were: forward 5' ACATGAAGATGATGTGGGCC-3' and reverse 5'-TAGGAGTCCTGCTCACTGTA-3' and for porcine TLR9 (AB071394) were: forward 5'-GTGGAACTGTTTTGGCATC-3' and reverse 5'-CACAGCACTCTGAGCTTTGT-3' [51]. The PCR primers for porcine β-actin (AY550069) were: forward 5'-GCCTCGCTGTCCACCTTCC-3' and reverse 5-TGCTGTCACCTTCACCGTTCC-3'. The PCR primer concentrations were optimized with 2 µl (10 µM working stock) for both forward and reverse primers for TLR 4 and 5 and with 1 µl (10 µM working stock) for swine β-actin, TLR 2 and 9. The qRT-PCR was performed on Stratagene Mx3000P (Stratagene, Cedar Creek, TX) real-time PCR machine. The thermal conditions were 10 minutes at 95°C, followed by 45 cycles of 15s at 95°C, 10s at 60°C and 5s at 72°C [52]. The primer efficiencies were determined by plotting the standard curves on serial dilutions of cDNA using same concentrations of primers. The sizes of PCR products were determined by running amplified samples on 1% agarose gel.

TLR immunohistochemistry (IHC)

Tissue sections (5 µm) were obtained from paraffin blocks prepared with formalin-fixed tissues. Tissue sections were incubated at 55°C for 30 minutes to improve adherence to slides and then de-paraffinized using xylene. For immunohistochemistry the sections were hydrated using descending grades of alcohol (100%, 95%, and 70%). Each treatment was carried out for 2 minutes and all washes were done in PBS for 5 minutes. Endogenous peroxidase was quenched by incubating the slides with 1ml of stock H_2O_2 (30%) and 1 ml of stock sodium azide (10%) to the 100 ml of PBS for 10 minutes. For unmasking the formalin fixed antigens, tissue sections were subjected to treatment with target retrieval solution (S3308, DakoCytomation, Carpinteria, CA) as per the manufacturer's protocol. Non-specific protein binding was blocked by treating the sections with 1% BSA in PBS for 30 minutes. Sections were washed in PBS three times and were incubated with corresponding goat anti-human TLR 2,4,5, and 9 antibodies (anti-TLR2: sc-8689; anti-TLR4: sc-12511; anti-TLR5: sc-8696; and anti-TLR9: sc-13215, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a concentration of 5 µg/ml in PBS with 1% BSA (100 µl/section), for 60 minutes [42]. The sections were then washed twice in PBS. Sections not treated with TLR antibody served as the control for primary antibody. Staining with normal goat immunoglobulin (goat IgG; cat # sc-2028, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), at 5 µg/ml in PBS with 1% BSA, also served as an isotype antibody negative control. Then sections were incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 5 µg/ml in PBS in 1% BSA (100 µl/section) for 30 minutes. After washing three times in PBS, the sections were treated with 3,3'-diaminobenzidine (DAB) (Cat # SK-4100, Vector laboratories, Inc. Burlingame, CA, USA) for seven minutes and counter-stained with Mayer's hematoxylin. The sections were dehydrated through ascending grades of alcohol (95%, 100%, and 100%), xylene and mounted permanently using Cytoseal 60 (Cat # 8310-4, Richard-Allan Scientific, MI). The specificity of each TLR antibody binding to the tissues was tested by pre-incubating TLR antibodies with the corresponding peptides (20 µg; sc-8689P and sc-13215P; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 500 µl of PBS for 2 hours, and then mixing with an equal volume of PBS containing 2% BSA to attain a final concentration of 5 µg/ml of each TLR in PBS with 1% BSA prior to applying on tissue sections [42].

Analysis

TLR-2,-4,-5 and -9 gene expression changes in all experimental groups were calculated using $2^{-\Delta\Delta Ct}$ method [53] and the data was analyzed using the Student's paired t-test. P-values < 0.05 were considered significant and results were expressed as mean ± standard error (S.E). The immunohistochemically stained cross sections of ileum were divided into eight different compartments, namely the dome epithelial surface, the villous epithelial surface, the villus goblet cells, the crypt epithelial cells, the dome, the corona, the follicle, and the inter-follicular area for the convenience of making comparisons [47,54,55], and each region of the tissue was evaluated independently for TLR protein abundance. Three fields (10X objective) were scanned from each tissue compartment to ensure that equal areas were included for regional comparisons of ileal tissues from different treatment groups. The compartments were scored based on the intensity of DAB coloration as visualized under the light microscope under 10X as described earlier [47]. The intensity of TLR staining was scored as (0) for no staining, (1) for weak, (2) for moderate and (3) for heavy staining for all TLRs. IHC scoring of TLR staining was subjective, but all the measurements were made by a single person blinded to treatments. Similar scoring methods have been used by others and us for tissue IHC staining comparison for TLRs [56] and lectin binding [47] respectively.

In this study, our major research question was to determine whether gnotobiotic and antibiotic-fed pigs significantly differed from the control animals in the expression and abundance of different TLRs in the ileum. As the ranked data obtained from staining intensities from all three groups was not normally distributed, data was analyzed using nonparametric ANOVA by the Wilcoxon/Kruskal-Wallis test as described in our earlier study [47] using JMP IN version 5.1 (The Statistical Discovery Software,Thompson/Brooks/Cole, SAS Institute inc., Cary, NC, USA). A p value of < 0.05 was considered significant and the results were expressed as mean ± standard error (SE).

Results

TLR Real-Time RT-PCR

All the animals from three groups showed the expression of TLR-2, -4, -5 and -9 in the ileum based on the real-time RT-PCR (qRT-PCR) results (Figure 1A). TLR-2, -4, -5 and -9 PCR products were of the expected sizes of 109, 179, 187, and 199 bps respectively, as confirmed by running PCR products on 1% agarose gel (Figure 1B). Furthermore, melting curve analysis for various TLRs and β-actin PCR products confirmed the specifity of qRT-PCR. We tested the primer efficiencies of TLR-2, -4, -5, -9 and β-actin primers using serial dilutions of cDNA. The PCR efficiency for TLR primers ranged from 90% to 107% indicating that the $2^{-\Delta\Delta Ct}$ method can be used for calculating fold changes in the gene expression among various groups. The β-actin was used as a house keeping gene for the normalization

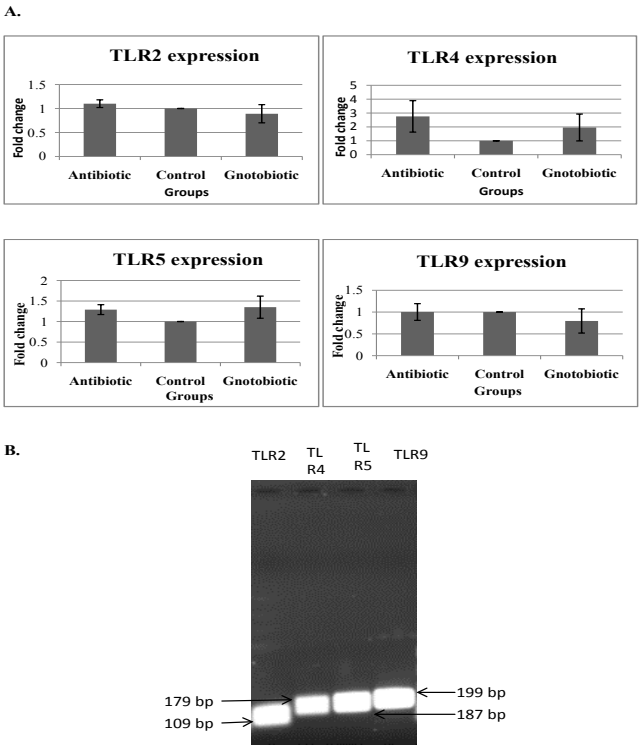

Figure 1: Real-time RT-PCR analysis for TLR-2, -4, -5 and -9 expression. Ileal tissue samples were collected from different groups of pigs at 5 weeks of age, snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted from frozen tissue samples and was used to prepare amplified RNA (aRNA). aRNA was reverse transcribed to cDNA and was used for quantitative real-time RT-PCR (qRT-PCR). The β-actin was used as a house keeping/ reference gene and its expression was used for normalization of TLRs. (A). The fold change is obtained by normalizing the target gene to the reference gene for both treated and control pigs using $2^{-\Delta\Delta Ct}$ method. Fold change = $2^{-(Ct \text{ value of target gene of treated animal - Ct value of reference gene of treated animal}) - (Ct \text{ value of target gene of control animal – Ct value of reference gene of control animal})}$. The data was analyzed using the Student's paired t-test and P-values < 0.05 were considered statistical significant. P values for TLR-2 expression diffferences between antibiotics (A) vs control (C), antibiotics (A) vs gnotobiotics (G) and control (C) vs gnotobiotics (G) groups were 0.30, 0.54 and 0.61 respectively. P values between A-C, A-G and C-G groups for TLR-4 expression differences were 0.19, 0.20 and 0.37; for TLR-5 expression were 0.09, 0.53 and 0.26, and for TLR-9 expression were 0.99, 0.05 and 0.50 respectively. (B). TLR-2, -4, -5 and -9 qRT-PCR products were run on 1% agarose gel. The PCR products of expected sizes of 109, 179, 187, and 199 bps were obtained.

of TLR gene expression. Relative fold TLR gene expression changes in antibiotic and gnotobiotic group pigs were compared to control group pigs. qRT-PCR analysis showed that the expression levels of TLR-2, -4, -5, and -9 did not differ significantly ($p>0.05$) between antibiotic vs control, gnotobiotic vs control and gnotobiotic versus antibiotic group pigs (Figure 1A).

TLR immunohistochemistry

As qRT-PCR for various TLRs was performed using the whole ileal tissues, it did not discriminate where and which cell types mainly express TLRs in the ileal tissues. Furthermore, this method does not provide information on the abundance of various TLR proteins in these tissues as amount of TLR transcripts may not fully correlate to TLR protein levels. Therefore, we sought to check the abundance of various TLR proteins in the ileal tissues by immunohistochemistry. As no porcine specific TLR antibodies were commercially available, we used goat anti-human TLR-2, -4, -5, and -9 polyclonal antibodies for the detection of porcine TLR proteins in the ileal tissues from all the three different groups of pigs. The background staining was minimal or absent in all the tissues from different animals and there was a distinct differential staining pattern for both the TLR-2 and TLR-9. TLR-2 and TLR-9 antibodies specifically stained ileal tissues in all three groups of animals (Figure 2), while TLR-4 and TLR-5 antibodies failed to stain any of the ileal tissues. Both TLR-2 and -9 antibodies stained the cells in villus and dome epithelium as well as in the follicular regions in all the animals from three groups. The negative controls without primary antibody (not shown) and with normal goat IgG alone did not show any tissue staining, indicating the specificity of both TLR-2 and TLR-9 staining (Figure 2). Further, the blocking of the TLR-2 and TLR-9 antibodies with their respective blocking peptides resulted in the absence of any staining on the ileal tissues indicating anti body specificity for TLRs only (Figure 2). In some animals, visual examination of ileal tissue sections stained with both TLR-2 and -9 antibodies (Figure 2) showed lesser staining in gnotobiotic pigs compared to antibiotic fed and control animals. However, statistical analysis of TLR-2 and -9 staining intensities of tissues from different groups did not reveal any significant differences between the control vs gnotobiotic and antibiotic vs gnotobiotic groups in various compartments of the ileum (Table 1a and 1b). These findings supported the data obtained using qRT-PCR method.

Discussion

This study was undertaken to investigate changes in transcript and protein levels of bacteria-specific TLRs in the ileum of weanling piglets fed on chlortetracycline as growth promotant and under gnotobiotic conditions. Piglets were delivered by cesarean surgery and were segregated into control, antibiotic-fed and gnotobiotic groups in such

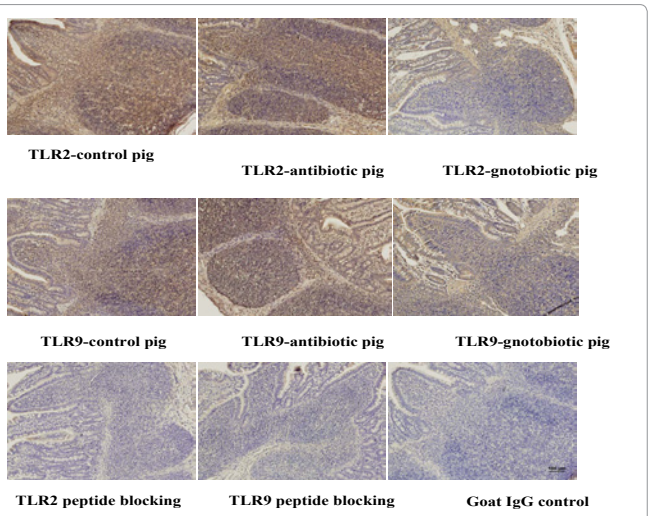

Figure 2: Detection of TLR-2 and TLR-9 proteins on porcine ileal tissues by immunohistochemical (IHC) staining. Ileal tissue sections were prepared and stained as described in the materials and methods. Briefly, tissue sections were treated with Dako target retrieval solution for unmasking the tissue antigens. After blocking, tissue sections were incubated with goat anti-human TLR-2, -4, -5 and -9 antibodies. Normal goat IgG treatment served as an isotype antibody negative control. Folllowing 3X washings, tissue sections were incubated with HRP-conjugated rabbit anti-goat IgG antibody, treated with DAB and counter-stained with Mayer's hematoxylin. The sections were dehydrated and mounted permanently. The specificity of each TLR antibody was tested by pre-incubating TLR antibodies with the corresponding peptides and this treatment completely abrogated the TLR staining. The tissue sections from various treatment groups showed TLR-2 and TLR-9 specific staining. No TLR-4 and -5 specific tissue staining was observed.

Tissue compartment	TLR2$		
	Control	Antibiotic	Gnotobiotic
Dome epithelial surface	1.75 ± 0.25	1.50 ± 0.29	0.75 ± 0.48*
Villous epithelial surface	1.75 ± 0.25	1.75 ± 0.25	1 ± 0.41*
Villous goblet cells	0.50 ± 0.50	0.33 ± 0.33	0.25 ± 0.25*
Crypt epithelial surface	0.75 ± 0.48	1 ± 0	0.25 ± 0.25
Dome	2.20 ± 0.37	2 ± 0.45	1.40 ± 0.24
Corona	1.80 ± 0.37	1.20 ± 0.49	1.20 ± 0.37
Follicle	1.83 ± 0.17	1.80 ± 0.37	1.50 ± 0.22
Inter follicular area	0.67 ± 0.21	0.80 ± 0.37	0.67 ± 0.21

*Data from 3 or 4 animals only as in some tissues epithelial layer was damaged.
$- Statistical differences between the control vs antibiotics, control vs gnotobiotic, and antibiotic vs gnotobiotic groups were calculated and P-value of ≤0.05 was considered significant. No significant differences in various compartments were obtained as P-values between different treatments were ≥0.05.

Table 1a: TLR2 immunohistochemistry. TLR2 immunohistochemistry data.

Tissue compartment	TLR9$		
	Control	Antibiotic	Gnotobiotic
Dome epithelial surface	1.75 ± 0.25	1.50 ± 0.29	0.50 ± 0.28*
Villous epithelial surface	1.75 ± 0.25	1.50 ± 0.29	0.75 ± 0.25*
Villous goblet cells	0.50 ± 0.50	0 ± 0	0.25 ± 0.25*
Crypt epithelial surface	0.50 ± 0.29	1 ± 0	0 ± 0
Dome	1.60 ± 0.24	1.20 ± 0.20	1 ± 0
Corona	1.20 ± 0.20	0.80 ± 0.37	0.80 ± 0.19
Follicle	1.33 ± 0.21	1.40 ± 0.24	1 ± 0
Inter follicular area	0.67 ± 0.21	0.60 ± 0.24	0.67 ± 0.21

*Data from 3 or 4 animals only as in some tissues epithelial layer was damaged.
$- Statistical differences between the control vs antibiotics, control vs gnotobiotic, and antibiotic vs gnotobiotic groups were calculated and P-value of ≤0.05 was considered significant. No significant differences in various compartments were obtained as P-values between different treatments were ≥0.05.

Table 1b: TLR9 immunohistochemistry. TLR9 immunohistochemistry data.

a manner that each group was allotted an equal number of half-sibs at random to minimize the genetic variation among the experimental subjects. Host genetics has been shown to play an important role on the intestinal microbiota composition [57,58]. Thus, to minimize the effect of differences in intestinal microbiota of the host, if any, on the intestinal microbiota composition of experimental animals, piglets kept on two foster mothers were segregated into control and antibiotic-fed groups in equal proportions.

Feeding Esbilac (sterile milk replacer) to the gnotobiotic piglets was the best available alternative because it had been successfully used in other studies to maintain gnotobiotic pigs [49,50] and was found beneficial to weight gains in pigs [59]. Among the wide array of feed antibiotics, chlorteteracycline was chosen for this study primarily due to its relatively frequent usage for growth promotion in the pork industry [60-62]. Secondly, chlortetracycline was found to promote a greater growth rate in pigs compared with tylosin and carbadox in an experiment conducted at SDSU prior to this study (unpublished data), and therefore, the growth performance of pigs was not measured directly in this study. Tissue samples in this study were obtained from distal ileum since the microbial load and rate of nutrient absorption is higher in ileum compared to the proximal small intestine [2]. Tissue samples were obtained from distal ileum immediately upon sacrificing the animals to preserve tissue morphology and transcriptome integrity. Tissue sections were obtained from the same region of ileum for both real-time RT-PCR and immunohistochemistry to keep experimental variations to the minimum.

Among the thirteen different TLRs described in scientific literature [63], only four bacteria-specific TLRs (TLR-2, -4, -5 and -9) were selected for this study. The diverse intestinal microflora expresses different types of TLR ligands and regulates the development and expansion of lymphoid tissues and gut immunity including the expression of various TLRs [8,10,12,13]. Furthermore, previous studies have indicated that intestinal microbiota and bacterial TLR ligands may regulate the expression of various TLRs [8,10,12,30]. As the use of antibiotics in the feed affects the microbial diversity in the gut [2,48], it was hypothesized that the use of antibiotics as growth promotants in the weanling pigs or absence of intestinal microbiota/gnotobiotic conditions may affect the expression of bacteria-related TLRs *viz.* TLR2,-4,-5 and -9. In this study, our intention was to keep the gnotobiotic pigs as germ-free pigs during the whole course of the study. However, although these pigs remained germ-free up to 2 weeks of age, they were found to be contaminated with low levels of *C. perfringens* at 5-week of age as assessed by direct culture method. We could not determine whether these pigs got contaminated just before the termination of the experiment or at an earlier time point. Thus, it is important to interpret the data presented in this study keeping in mind that gnotobiotic pigs were contaminated with *C. perfringens* and were not germ-free.

Many earlier studies have confirmed that linearly amplified RNA (aRNA) generates gene expression profile comparable with unamplified total RNA in both qRT-PCR and microarray expression studies [64,65]. Thus, qRT-PCR method perfomed in this study using aRNA as template was appropriate and should not result in any bias in TLR expression in various ileal tissues. Further, the results obtained through qRT-PCR were validated by immunohistochemical staining for various TLRs. Analysis of qRT-PCR results of this study showed that all the four TLRs (-2, -4, -5 and -9) mRNAs were expressed in the pig ileum at a detectable level in all the three groups of pigs. These findings are consistent with previous studies [42,45,46] which showed the presence of TLR-2, TLR-4 and TLR-9 in the pig small intestine, but are in contrast with another study which indicated the absence of TLR4 message in the small intestine [66]. In the present study, based on the qRT-PCR data analysis, no significant changes in the expression of TLR-2, -4, -5 and -9 were observed among normal-fed, antibiotic-fed and gnotobiotic animal groups. In an earlier study [46], expression of TLR-2 was significantly increased in the germ-free pigs conventionalized with sow feces compared to germ-free animals; however, no significant differences in TLR-4 and TLR-9 were observed. Gnotobiotic group animals in the present study were contaminated with *C. perfringens* and this might have caused the up-regulation of TLRs in these pigs resulting in no significant differences in TLR expression and abundance between gnotobiotic and control group pigs.

To further confirm the qRT-PCR results, abundance of various TLR proteins was assessed by imunohistochemical (IHC) staining of ileal tissues. No porcine TLR-2, -4, -5 and -9 specific antibodies were commercially available; however, human TLR-2, -4, -5 and -9 show satisfactory homology to porcine TLRs both at DNA sequence and protein levels [39,66-68]. Therefore, we expected that polyclonal antibodies against human TLRs would recognize porcine TLRs in IHC staining. An earlier study supports this notion where human TLR4 specific polyclonal antibodies recognized porcine TLR4 in the intestine [42]. In this study, both human TLR-2 and -9 antibodies very effectively detected the presence of porcine TLR-2 and -9 in the ileal tissues from all the three groups of animals; however, we were unable to detect porcine TLR-4 and -5 with polyclonal human TLR-4 and -5 antibodies. One possible explanation might be a longer fixation of ileal tissues for

48 hours in formalin in the present study compared to 16 hours used in earlier studies [42,69]. This might have resulted in non-retrieval of TLR-4 and -5 antigens with the treatment procedure used in this study. It might also be possible that goat anti-human TLR-4 and -5 antibodies used in this study did not recognize or cross-react with porcine TLR-4 and -5 on the fixed tissues during immunohistochemical staining. Finally, there may be a possibility that TLR-4 and -5 transcripts are poorly translated in the intestinal tissues. Pre-incubation of TLR-2 and TLR-9 antibodies with their respective peptides completely abolished the staining for TLRs confirming the specificities of TLR-2 and -9 antibodies. Use of normal goat IgG at the same concentration as TLR antibodies (5 ug/ml) did not result in any staining on the ileal tissues further confirming the specificities of TLR-2 and -9 antibodies. These observations were consistent with earlier reports [38,40,52] which showed the presence of both TLR-2 and -9 in the ileum of pigs.

It is important to note that in this study only distal ileum tissues were used for determining the TLR expression and no tissues from other intestinal locations were tested. It is certainly possible that the use of chlortetracycline as growth promotant or germ-free conditions may have different effects on the TLR expression in different intestinal locations as physiology and composition of microflora in different intestinal compartments vary significantly. Thus, the findings of the present study are only applicable to distal ileum and can not be generalized to the other intestinal locations. As we did not find any significant differences in TLR expression both at mRNA and protein levels in this study, one may argue that either a treatment group or subclinical chronic infection group which shows the upregulation of TLRs or inflammatory changes in the intestine could have been included as a biological positive control. However, as all the tissues from control group animals showed the expression of all the TLRs tested here, these tissues acted as technical positive control for the TLR expression in this study. As it is very difficult to maintain gnotobiotic pigs beyond 5-week period in gnotobiotic units, a sub-clinical chronic infection control group was also not included in this study. Another important criterion for the determination of the inflammatory changes in the animals is to measure the levels of certain proteins such as inflammatory cytokines or c-reactive proteins in the serum of experimental animals. Unfortunately, we did not collect blood from the experimental animals upon completion of this study so we were unable to deterimine possible inflammatory changes based on the serum analysis.

Some studies indicate that intestinal microbiota may play a role in activating and maintaining TLR expression and homeostasis in the intestine [8,12,30,70]. Our earlier studies and another recent study show that normal-fed control pigs contain a different intestinal bacterial phylotypes as compared to antibiotic-fed pigs and the use of antibiotics has been shown to change the nature of microbial community [2,3,48]. Previous studies have found that TLR-4 is upregulated in both Crohn's disease and ulcerative colitis, whereas, TLR-2 and TLR-5 remain unchanged [23,71]. These studies support the hypothesis that presumably there would be a lower expression of TLRs in the antibiotic and gnotobiotic pigs compared to the control pigs because of changes in microbial populations. However, the findings of the present study in contrast do not support this hypothesis as no significant differences in TLRs expression (TLR-2, -4, -5 and -9) were observed in antibiotic and control pigs compared to gnotobiotic animals. It was worth noticing that some of the ileal tissues obtained from gnotobiotic group pigs showed lower expression of TLR proteins upon immunohistochemical staining indicating that normal intestinal microbiota could play some role in regulating the TLR expression in these pigs. However, these findings were complicated because of contamination of pigs with *C. perfringens* based on the direct culture report [47]. It is well established that large number of bacteria in the gastro-intestinal tract can not be cultured and can only be detected by other indirect methods including16S rRNA based approach [48]. We did not attempt to detect the non-culturable bacteria in contaminated gnotobiotic pigs by indirect methods so the possibility that these pigs were also contaminated by some other bacterial species can not be ruled out. It might also be possible that *C. perfringens* infection alone or along with other unculturable bacteria could trigger significant upregulation of TLRs in these pigs and thus, no inference could be drawn here with regard to the role of intestinal microbes in regulating the expression of TLRs in the pig intestine. Further studies should be conducted using germ-free animals to address this issue.

As no significant changes in TLR expression both at transcript and protein levels were observed in the present study, one may argue that this may be due to unavailability of biologically-active antibiotic in the feed or due to absence of significant changes in the microbial population in the intestine. All the ileal tissues used in this study were obtained from the same previous study where we checked for the changes in the expression of carbohydrate moieties on the ileal surface and changes in the microbial community in response to chlortetracycline and gnotobiotic conditions [47,48]. In the first report [47], we showed that chlortetracycline as a growth promotant and gnotobiotic conditions induced biologically-relevant and significant changes in the lectin binding profile of the ileum. In the second report [48], we showed that chlortetracycline induced significant changes in the composition of the microbial population in the antibiotic-fed animals. These studies [47,48] confirm that no changes in the TLRs expression observed in the present study were not due to absence of biological activity of chlortetracycline in the animal feed or because of no changes in the intestinal microbial populations. We therefore conclude that chlortetracycline exerts its growth promoting effects by inducing salient changes in the microbial composition with no detectable levels of alterations in the expression of TLRs.

To our knowledge this is the first study undertaken to estimate the levels of TLR-2, -4,-5 and -9 mRNAs and protein expression (TLR-2 and -9) on the ileal compartment of the small intestine of pigs in response to use of feed antibiotics and gnotobiotic conditions. The study addresses the impact of continual use of antibiotics in livestock feeds from an immunological perspective. The results of this study emphasize that the use of antibiotics as growth promotant and intestinal microbiota overall do not play a major role in regulating TLR expression in the ileum of weanling pigs. Further studies are needed to elucidate the actual mechanisms of growth promotion and immune regulation in weanling pigs involving other TLRs.

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