Effect of deoxynivalenol on the levels of toll-like receptors 2 and 9 and their mRNA expression in enterocytes in the porcine large intestine: a preliminary study

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Abstract

Deoxynivalenol (DON), one of the most prevalent mycotoxins in the world, and is capable of inducing immune disorders in humans and animals. The aim of this study was to determine the effect of feed contaminated with DON on the number of TLR2- and TLR9-positive cells and their mRNA expression in the porcine large intestine. The experiment was conducted on two equal groups of pigs (n=4). The experimental group (E) was administered feed contaminated with DON (1008 μg/kg of feed) for 6 weeks, and the control group (C) was administered non-contaminated feed over the same period of time. A decrease in the expression of TLR2 mRNA was noted in the cecum. The percentage of TLR9-positive enterocytes increased in the ascending colon and decreased in the cecum. The results of this study indicate that DON can modify the local immune response by changing the expression of TLRs.

Key words: deoxynivalenol, toll-like receptors 2 and 9, large intestine, porcine

Introduction

Mycotoxins are secondary fungal metabolites that contaminate cereals and cereal products (Abysique et al. 2015). Deoxynivalenol is the most common group B trichothecene in cereals such as wheat, maize, barley, oats, rye, and it is less frequently isolated from rice and bananas (Binder 2007). Rodrigues and Naehrer (2012) analyzed 7049 cereal and feed samples in 2009-2011 and identified DON in 59% of the samples. The global prevalence of mycotoxins poses a significant risk to human and animal health (Fink-Gremmels 1999).

In humans and animals, high doses of DON provoke nausea, vomiting, sleepiness, diarrhea, leukocytosis, gastrointestinal bleeding and, in extreme cases, death (Bryden 2007). Exposure to moderate concentrations of this mycotoxin leads to anorexia, lower weight gains and lower feed efficiency (Pestka and Smolinski 2005). Similarly to other trichothecenes, DON exhibits tropism for rapidly proliferating and differentiating cells and tissues, in par-
ticular in the intestines and the immune system (Feinberg and McLaughlin 1989). Contaminated food or feed exposes the intestinal epithelium to high concentrations of potentially harmful mycotoxins (Bouhet and Oswald 2005). In the gastrointestinal tract, DON provokes shortening of intestinal villi, lysis and swelling of enterocytes (Kolf-Clauw et al. 2009). Caco-2 cells chronically exposed to DON induce the phosphorylation of mitogen-activated protein kinases (MAPKs) and lower transepithelial electrical resistance (TEER) (Sergent et al. 2006).

Trichothecenes target ribosomes at the molecular level. Those mycotoxins bind to the eukaryotic 60S ribosomal subunit (Middlebrook and Leatherman 1989). The above initiates a process known as the ribotoxic stress response which inhibits protein synthesis and activates MAPKs (Iordanov et al. 1997). After binding with ribosomes, DON inhibits ribosomal peptidyl transferase, which prevents the initiation and elongation of the translation process (Shifrin and Anderson 1999). The inhibition of protein synthesis induced by DON can also result from the degradation of 28S rRNA (Li and Pestka 2008). Zhou et al. (2003) demonstrated that DON can activate double-stranded RNA-associated protein kinase (PKR) which is responsible for the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) and inhibition of translation. He and Pestka (2010) reported that DON can bind with mRNA to activate microRNA molecules responsible for selective gene silencing and inhibiting protein synthesis.

Deoxynivalenol influences the transcription of genes encoding cytokines and chemokines by inducing and activating transcription factors such as NF-κB, CREB and AP-1 (Pestka 2010). It also stabilizes mRNA molecules and increases their concentrations inside cells (Chung et al. 2003).

Toll-like receptors act as pathogen recognition receptors (PRRs) in mammals where they are responsible for the recognition of pathogen-associated molecular patterns (PAMPs) (Akira and Takeda 2004). In pigs, TLRs are the first line of defense against pathogens, and their ligands are used as vaccine adjuvants (Uenishi and Shinkai 2009). The current state of knowledge indicates that TLRs and their signaling molecules play a key role in regulating gut inflammatory processes caused by invasive pathogens (Burkey et al. 2009). Toll-like receptor 2 plays a role in the recognition of bacterial cell wall components: peptidoglycan, lipoteichoic acid, lipoproteins, lipoarabinomannans, mycobacteria and zymosan (Wetzler 2003). By binding with ligands, TLR2 induces the secretion of TNF-α, IL-2, IL6 and IL-12 (Thoma-Uszynski et al. 2000). Toll-like receptor 9 is involved in the recognition of unmethylated CpG-dense sequences which are commonly found in the DNA of viruses, bacteria and their synthetic homologs (oligodeoxynucleotides). It is also present inside cells in endosomal compartments (Latz et al. 2004).

The objective of this study was to determine the influence of feed contaminated with DON at levels similar to the reference doses recommended by the European Commission (EC 2006) on the expression of TLR2 and TLR9 mRNA and the percentage of TLR2- and TLR9-positive enterocytes in the porcine cecum, ascending and descending colon. The human digestive system and the porcine gastrointestinal tract are characterized by significant anatomical and immunological similarities; therefore, the results of an experiment performed on a porcine model can be extrapolated to humans (Patterson et al. 2008, Meurens et al. 2012).

Materials and Methods

All procedures relating to animal handling and collection of samples for analysis were carried out with the consent of the Local Ethics Committee in Olsztyn, decision No. 45/2013 of 19 September 2013.

Animals

The experiment was performed on 8 crossbred (Polish Large White x Polish Landrace) weaners with an estimated initial body weight of 23 kg. The animals were randomly divided into two equal groups: the experimental group (E) (n=4) administered contaminated feed and the control group (C) (n=4) whose feed was not contaminated with mycotoxins. The groups were kept in separate pens with free access to water. Feeding times were restricted to 8 a.m. and 5 p.m. The experiment was conducted for 6 weeks, after which the animals were euthanized by intravenous administration of pentobarbital sodium (Fatro, Ozzano Emilia BO, Italy) and bleeding. Sections of the cecum, ascending and descending colon were collected immediately after cardiac arrest and were prepared for analyses.

Immunohistochemical evaluation of TLR2 and TLR9 expression

Tissue sections were fixed in 4% paraformaldehyde solution in phosphate buffer and embedded in paraffin. Two specimens for every analyzed segment of the large intestine were prepared and stained to
detect the presence of TLR2 and TLR9, respectively. Serial microtome sections with a thickness of 5 μm were placed on glass slides (Menzel-Glaser, Braunschweig, Germany) and deparaffinized in xylene (Polskie Odczynniki Chemiczne SA, Gliwice, Poland) and a series of graded ethanol solutions. Tissue sections were placed in citrate buffer (Sigma-Aldrich, Saint Louis, MO, USA) and microwaved for 20 minutes at 800 W to unmask antigens. The sections were coated with DAKO REAL™ Peroxidase Blocking Solution (DAKO, Glostrup, Denmark) and incubated for 15 minutes to block endogenous peroxidase activity. Non-specific antibody binding sites were blocked with 2.5% normal horse serum. The prepared sections were incubated overnight at a temperature of 4°C with primary antibodies: anti-porcine TLR9 (THU-A-TLR9 Cosmo Bio, Tokyo, Japan) and anti-porcine TLR2 (THU-A-TLR2 Cosmo Bio, Tokyo, Japan) diluted 1:200 and 1:300, respectively. After incubation, the specimens were rinsed three times in PBS (Sigma-Aldrich, Saint Louis, MO, USA) at 5 minute intervals. They were coated with secondary antibodies conjugated with horseradish peroxidase-labeled micro polymer (ImmPRESS™ HRP Universal Antibody, Vector Laboratories, Burlingame, CA, USA) and stained by incubation with DAB Universal Antibody, Vector Laboratories, Burlingame, CA, USA) for 3 minutes (DAKO, Glostrup, Denmark). The resulting specimens were rinsed in water and counterstained with Mayer’s hematoxylin (Sigma-Aldrich, Saint Louis, MO, USA). They were dehydrated in ethanol and xylene and mounted in DPX (Sigma-Aldrich, Saint Louis, MO, USA).

The preparations were scanned in a Panoramic MIDI digital slide scanner (3DHISTECH Ltd., Budapest, Hungary). The expression of TLR2 and TLR9 was evaluated in a Panoramic Viewer (3DHISTECH Ltd., Budapest, Hungary), a dedicated application for virtual microscopy. The percentage of enterocytes positive for TLR2 and TLR9 was determined in 8 randomly selected fields of view at 40x magnification. The results were expressed by the mean percentage of cells positive for TLR2 and TLR9.

**Analysis of TLR2 and TLR9 mRNA expression**

Total RNA was isolated from mechanically homogenized tissues immediately after collection, with the use of the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer’s instructions. The quantity and quality of isolated RNA was checked spectrophotometrically (BioSpectrometer, Eppendorf, Hamburg, Germany). Isolated RNA (1 μg) was converted into cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA). The results of gene expression were normalized relative to the reference gene (β-actin). To compare the expression of the analyzed genes, the reaction was optimized to produce equivalent efficiencies. Comparable efficiency was validated by performing serial dilutions of the obtained cDNA and determining the threshold cycles (Ct) for every dilution. The slope of the obtained curve (representing cDNA concentration vs. Ct) was used to calculate reaction efficiency based on the formula $E = 10^{\frac{-1}{\text{curve slope}}}$.

Real-time PCR was carried out in a Rotor Gene 6000 system (Qiagen, Venlo, Netherlands) with a Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and specific primers: swine TLR2, TLR2-S: ACATGAAGATGATGTGGGCC (sense) and TLR2-A: TAGGAGTCCTGCTCACTGTA (antisense); swine TLR9, TLR9-S: GTGGAAC-TGTITTGGGATTC (sense) and TLR9-A: CACAG-CACTCTGAGCTTGT (antisense); swine β-actin (housekeeping gene), B-ACTIN-S: TGGCAT-TGTCACTGAGCTTGT (sense) and B-ACTIN-A: AGGGCGATGATCTTGATCT (antisense) (Tohno et al. 2005, Shimosato et al. 2005). The following protocol was used: incubation with uracil-DNA glycosylase (UDG) at 50C for 2 minutes and initial denaturation at 95C for 10 s. This was followed by 40 amplification cycles with the following sequence: denaturation at 95C for 15 s, annealing at 57C for 30 s, and extension at 72C for 30 s.

**Analysis of mycotoxin concentrations in feed**

Deoxynivalenol was isolated from feed with the use of immunoaffinity columns (DON-Test™ Don Testing System VICAM, Watertown, USA). All extraction procedures were performed in accordance with the recommendations of the column manufacturer. Chromatographic analyses were conducted with the use of the Agilent 1100 series HPLC system equipped with 4.6 x 100 mm (3.5 fm) Eclipse Plus C18 columns. The mobile phase was a water and acetonitrile mixture with a 90:10 solvent ratio. Flow rate was 0.6 ml/min. The mycotoxin was identified with the use of a diode detector at 220 nm wavelength.

Zearalenone was isolated from feed with the use of the Zearala-Test™ (VICAM, Watertown, USA). All extraction procedures were performed in accordance with the recommendations of the column manufacturer. Chromatographic analyses were conducted with the use of an Agilent 1100 series HPLC system in accordance with the procedure described by Zwierzchowski et al. (2004).
Table 1. Percentages of TLR2- and TLR9-positive cells, including mean values, standard deviation and P values, in the evaluated sections of the large intestine. * – significant at \( p \leq 0.05 \).

<table>
<thead>
<tr>
<th>Group</th>
<th>Ascending colon</th>
<th>Descending colon</th>
<th>Cecum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig No.</td>
<td>E</td>
<td>C</td>
<td>E</td>
</tr>
<tr>
<td>1</td>
<td>7.778</td>
<td>8.214</td>
<td>7.122</td>
</tr>
<tr>
<td>4</td>
<td>10.535</td>
<td>6.218</td>
<td>6.123</td>
</tr>
<tr>
<td>Mean</td>
<td>12.866</td>
<td>9.091</td>
<td>10.094</td>
</tr>
<tr>
<td>SD</td>
<td>8.408</td>
<td>2.453</td>
<td>8.283</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td></td>
<td>0.421</td>
</tr>
<tr>
<td>Group</td>
<td>Ascending colon</td>
<td>Descending colon</td>
<td>Cecum</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
<td>-----------------</td>
<td>-------</td>
</tr>
<tr>
<td>Pig No.</td>
<td>E</td>
<td>C</td>
<td>E</td>
</tr>
<tr>
<td>1</td>
<td>85.972</td>
<td>72.167</td>
<td>80.469</td>
</tr>
<tr>
<td>2</td>
<td>84.508</td>
<td>70.964</td>
<td>64.269</td>
</tr>
<tr>
<td>3</td>
<td>87.392</td>
<td>63.069</td>
<td>43.109</td>
</tr>
<tr>
<td>4</td>
<td>76.392</td>
<td>79.874</td>
<td>60.371</td>
</tr>
<tr>
<td>Mean</td>
<td>83.566*</td>
<td>71.516</td>
<td>62.052</td>
</tr>
<tr>
<td>SD</td>
<td>4.925</td>
<td>5.959</td>
<td>15.337</td>
</tr>
<tr>
<td>( p ) value</td>
<td></td>
<td></td>
<td>0.029</td>
</tr>
</tbody>
</table>

Fumonisins B<sub>1</sub> and B<sub>2</sub> were isolated with the use of the FumoniTest™ WB (VICAM, Watertown, USA). All extraction procedures were performed in accordance with the recommendations of the column manufacturer. Chromatographic analyses were conducted with the use of the Agilent 1100 series HPLC system, and fumonisin derivatives were obtained from o-phthaldialdehyde in the presence of mercaptoethanol. Separation was performed on a reversed phase C<sub>18</sub> column. The mobile phase was a mixture of ethanol and 0.1 M sodium dihydrogen phosphate (80:20) with a pH of 3.5 (Wiśniewska-Dmytrow et al. 1999).

Ochratoxin A was isolated with the use of immunoaffinity columns (OchraTest™ WB Mycotoxin Testing System, VICAM, Watertown, USA) in accordance with the manufacturer’s guidelines. Chromatographic analyses were conducted with the use of the Agilent 1100 series HPLC system. The analyzed extract, in the amount of 100 μl, was applied to the Poroshell RP C<sub>18</sub> column. The elution mixture was composed of 0.25 N H<sub>3</sub>PO<sub>4</sub>, acetonitrile and 2-propanol (55:19:28), and the flow rate was set at 0.9 ml/min. Ochratoxin A was identified with a fluorometer at 330/460 nm wavelength (Czerwiecki et al. 2004).

Fig. 1. A – Percentage of TLR2-positive enterocytes in the analyzed sections of the large intestine. B – Percentage of TLR9-positive enterocytes in the analyzed sections of the large intestine. * – significant at \( p \leq 0.05 \).
**Statistical analysis**

The percentage of enterocytes positive for TLR2 and TLR9 was expressed by mean values (\(\bar{x}\)) with standard deviation (SD). Data were processed using the Statistica program (StatSoft Inc., USA). The assumption that dependent variables in groups E and C have normal distribution was verified using the Kolmogorov-Smirnov test. The equality of variances between groups was checked using Levene’s test. Differences between groups were determined using Student’s t-test. The results were significant at \(p \leq 0.05\) and highly significant at \(p \leq 0.01\).

The relative expression of TLR2 and TLR9 mRNA in the examined segments of the large intestine was represented by mean values (\(\bar{x}\)) with standard deviation (SD). Data were processed using the Statistica program (StatSoft Inc., USA). The assumption that the analyzed parameters have normal distribution in groups E and C was ruled out using the Kolmogorov-Smirnov test. Statistically significant differences were identified by the Wilcoxon rank-sum test. The results were significant at \(p \leq 0.05\) and highly significant at \(p \leq 0.01\).

**Results**

Feed naturally contaminated with mycotoxin was supplied by a commercial feed mill. The presence of DON, zearalenone (ZEN), fumonisins B₁ (FB₁) and B₂ (FB₂), and ochratoxin (OTA) in feed was determined by HPLC-FLD/UV. Feed was contaminated with 1008 µg/kg DON. The remaining mycotoxins were below the limit of detection (LOD). The feed administered to group C was supplied by the same feed manufacturer. It was analyzed for the presence of DON, ZEN, FB₁, FB₂ and OTA. The results were below LOD values.

A significant (\(p \leq 0.05\)) increase in the percentage of TLR9-positive enterocytes was observed in the ascending colon in group E relative to group C. A significant (\(p \leq 0.05\)) decrease in the percentage of TLR9-positive cells was noted in the cecum in group E. Significant differences were not observed in the percentage of TLR9-positive enterocytes in the descending colon or in the percentage of TLR9-positive enterocytes in all analyzed segments of the large intestine. The percentages of TLR2- and TLR9-positive cells, including mean values, SD values and \(p\) values in groups E and C in the investigated sections of the large intestine, are presented in Table 1 and Fig. 1.

A statistical analysis of the relative expression of TLR2 and TLR9 mRNA revealed a significant (\(p \leq 0.05\)) decrease in TLR2 levels in the cecum in group E. Significant differences in gene expression were not observed in the remaining segments of the large intestine. Mean values with standard deviation for TLR2 and TLR9 are presented in Fig. 2.

**Discussion**

The aim of this study was to determine the influence of feed contaminated with DON at levels similar to the reference doses recommended by the European Commission on the expression of TLR2 and TLR9 mRNA and the percentage of TLR2- and TLR9-positive enterocytes in the porcine large intestine.

Real-time PCR revealed a significant decrease in the relative expression of TLR2 in the cecum. Immunohistochemical evaluation of the number of TLR2- and TLR9-positive enterocytes demonstrated that the percentage of TLR9-positive cells increased significantly in the ascending colon and decreased in the cecum.
The results of this study indicate that DON can modify the local immune response by changing the expression of TLR2 and TLR9 in the large intestine.

There is growing evidence to indicate that porcine tissues harbor various TLRs whose expression is influenced by a host of biological and chemical factors. There is a general scarcity of published data relating to the effect of mycotoxins on the expression and reactivity of TLRs in vivo.

Uddin et al. (2013) observed that mRNA expression patterns in all members of the TLR family in the porcine gut increases with age because gut-associated lymphoid tissue (GALT) is continuously exposed to commensal and pathogenic microflora, which creates a balance between tolerance and immunity. These authors also noted that the levels of TLR2, TLR3 and TLR9 proteins in the porcine gut do not always correspond to mRNA expression levels, which is consistent with our findings. In contrast, Hausmann et al. (2002) reported that macrophages isolated from humans with inflamed intestinal mucosa were characterized by higher levels of TLR2 and TLR4 proteins and mRNA expression. A study of the IPEC-1 cell line exposed to ZEN demonstrated an increase in the mRNA expression of genes encoding TLR1-TLR10 and inflammatory cytokines, but only in the presence of the Escherichia coli K88 ligand (Taranu et al. 2015). In an in vitro study of the RAW 264.7 mouse macrophage cell line, Pestka and Zou (2006) observed that cell incubation with LPS, the TLR4 ligand, increased macrophage sensitivity to DON, which elevated the expression of IL-1β, IL-6 and TNFα mRNA in the evaluated cells. Somewhat different results were reported by Sugiyama et al. (2016) in an in vitro study of macrophages stimulated with LPS where DON decreased the expression of myeloid differentiation factor 88 (MyD88), the adaptor protein for most TLRs which induces nuclear factor κB (NF-κB), the protein responsible for macrophage activation and inflammatory states.

Numerous studies investigating TLR expression in the large intestine have been performed on patients diagnosed with inflammatory bowel disease (IBD). It is believed that IBD is caused by abnormal gut microflora, increased intestinal permeability and changes in TLR expression (Kassinen et al. 2007, Camilleri and Gorman 2007, Brint et al. 2011). Belmonte et al. (2012) demonstrated that IBD patients suffering from constipation alternating with diarrhea had higher TLR2 and TLR4 expression in colonic enterocytes. Mice with turned off TLR2 gene were more sensitive to colitis induced by dextran sulfate sodium (DSS) (Cario et al. 2010). Ligand-specific stimulation of TLR2 activates PKC-α/δ, which increases TEER and leads to the redistribution of ZO-1, the key tight junction (TJ) component (Cario 2010). Toll-like receptor 9 also plays a key role in IBD. Sánchez-Muñoz et al. (2011) reported higher expression of TLR9 mRNA in 51 patients diagnosed with ulcerative colitis relative to healthy subjects. A study of mice with induced colitis also revealed that the TLR9 ligand intensifies inflammatory states (Obermeier et al. 2002). In contrast, CpG oligonucleotides alleviated symptoms of colitis in mice with turned off IL-10 gene (Rachmilewitz et al 2002). Rose et al. (2012) also reported a higher risk of colitis and significantly slower intestinal healing in mice with turned off TLR9 gene.

The current state of knowledge does not explain which types of changes related to TLR expression in the intestines are implicated in IBD. It can be assumed that the entire spectrum of disorders associated with IBD is characterized by abnormal TLR expression. The described in vivo experiment was performed on a porcine model where enterocytes were continuously exposed to intestinal microflora and DON. The number of TLR9-positive cells increased in the ascending colon and decreased in the cecum. The expression of TLR2 mRNA decreased in the cecum. Our findings suggest that DON is able to modify the expression of TLR2 and TLR9 mRNA in vivo. The analyzed mycotoxin decomposes intestinal microflora (Piotrowska et al. 2014), decreases TEER and increases intestinal permeability (Pinton and Oswald 2014), which could indicate that it induces and/or exacerbates immune disorders characteristic of IBD. Our results are relevant for future research which should focus on mycotoxin influence on the family of TLR proteins and the profile of proinflammatory and anti-inflammatory cytokines in the gastrointestinal tract.

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