THE INFLUENCE OF NODULAR WORMS AND CARBOHYDRATES ON MORPHOLOGY AND PROLIFERATION OF EPITHELIAL CELLS IN THE LARGE INTESTINE OF PIGS

Saulius Petkevičius¹, Knud Erik Bach Knudsen²
¹Department of Infectious Diseases, Lithuanian Veterinary Academy, Tūžės 18, LT-47181 Kaunas, Lithuania, E-mail: saulius.petkevicius@lva.lt
²Department of Animal Health, Welfare and Nutrition, Danish Institute of Agricultural Sciences, Research Centre Foulum, DK-8830 Tjele, Denmark

Abstract. The influence of nodular worms Oesophagostomum dentatum infection and dietary carbohydrates on the morphology characteristics and epithelial cell proliferation in the large intestine of pigs was investigated. Thirty-two worm free weaners were randomly divided into four groups (A-D), of eight animals in each. Pigs in groups A (Control) and B (Infected) were fed Diet 1, and pigs in groups C (Control) and D (Infected) were fed Diet 2. The two diets were formulated: Diet 1 contained barley flour, oat husk meal plus soya bean meal (55% : 21% : 24%) and Diet 2 – barley flour, inulin and sugar beet fibre (SBF) (80.1% : 7% : 12.9%) plus soya bean meal (3:1). The two infected pig groups (B and D) were infected with 6000 infective larvae of O. dentatum and all 32 pigs were slaughtered 12 weeks p. i. The combination of O. dentatum infection and highly fermentable dietary carbohydrates affected the mucosal architecture, the epithelial cell proliferation and mucin secretion of the large intestine. These dietary carbohydrates in control pigs significantly influenced the tissue weight of caecum and colon. Infection with O. dentatum had significant effect on the gut wall architecture, because the changes in the affected gut sections corresponded directly to the number of worms present.

Keywords: Oesophagostomum dentatum, morphological and epithelial cell characteristics, dietary carbohydrates, pigs.

MAZGELINIŲ NEMATODŲ IR ANGLIAVANDENIŲ ĮTAKA KIAULIŲ STORŲ ŽARNŲ MORFOLOGIJAI IR EPITELIO LĄSTELIŲ PROLIFERACIJAI


Nustatyta, kad O. dentatum invazija ir lėngvai virškinami angliavandenai turėjo statistiškai reikšmingą įtakos kiaulų storų žarnų gleivinės morfologijai, mūčinių struktūrai ir epithelio ląstelių proliferacijai. Be to, veikiant tirpiams angliavandeniais labai nei netirpiams, padidėjo kiaulių aklosios ir gaubiančiosias žarnų masė bei raumeninis sluoksnis (p<0,01). Nustatyta, kad O. dentatum turėjo ženklų įtaką storų žarnų morfologijai, nes didžiausiai pakitiangi su kontrolinėmis kiaulėmis (p<0,05) buvo mazgelinių nematodų dislokacijos vietose, t. y. aklosioje žarnoje ir proksimalinėje gaubiančiosias žarnos dalinyje.

Raktazodžiai: Oesophagostomum dentatum, storų žarnų morfologija ir epithelio proliferacija, angliavandenai, kiaulės.

Introduction. It was shown, that diets with an increased content of fermentable carbohydrates have shown negative influence and a high content of digestion-resistant fibres provided favourable conditions for the establishment and persistence of O. dentatum in pigs (Petkevičius et al., 1995; 1997; 1999; 2000; 2001; 2003). However, the mechanisms behind the interactions between highly fermentable carbohydrates in the gastrointestinal content, the mucosa and the gastrointestinal parasites are not clear. Studies have shown that dietary constituents and microflora can influence the mucosa, alter the crypt-villus architecture and modify the mucin composition of the intestinal tract (Sharma et al., 1995; Sharma & Schumacher, 1995).

Studies on intestinal mucosa are of special interest because changes in dietary composition may modify chemical composition of excreted materials by intestinal epithelium (Vahouny et al., 1985). Furthermore, studies on the influence of gastrointestinal parasites on the morphology of intestine are still scarce. Very little is known about factors that control nodular worms behaviour/survival in the gastrointestinal tract of the host, particularly in the nodule-free part of the large intestinal mucosa (Jensen & Christensen, 1997). However, it is clear that O. dentatum larvae must traverse the mucus layer in order to approach and penetrate the intestinal wall, and any diet induced alteration of the epithelial wall could potentially affect parasite establishment.
The aims of the present study were to investigate how infection with *O. dentatum* and intestinal luminal factors induced by diets with contrasting digestibility modify the morphological characteristics and epithelial cell proliferation in the large intestine of pigs. In our trial the two experimental diets were based on a standard barley flour formulation supplemented with a protein mixture and oat husk meal. The diets were then varied, with some pigs receiving this base diet plus high fermentable carbohydrates such as inulin and SBF. Because of the difference in site of carbohydrate degradation, we expected that the effect of inulin and SBF on mucosa would primarily be observed in the caecum and proximal colon and the effect of highly fermentable carbohydrates at the more distal parts of the large intestine.

**Materials and methods.** Thirty-two pigs Landrace/Yorkshire crosses were used at the experiment. Prior to infection, faecal examinations of all pigs were performed and helminth eggs were not found. At 13 weeks of age pigs were divided by stratified random sampling into 4 equal groups, 8 animals in each, according to bodyweight and sex as follows: 4 littermates were divided among of the groups A, B, C and D. Pigs were kept on a slat floor without bedding. The pigs had free access to water via drinking nipples. Pigs in groups A (control) and B (infected) were given Diet 1, and pigs in groups C (control) and D (infected) were given Diet 2. The two diets were formulated: Diet 1 contained barley flour, oat husk meal plus soya bean meal (55% : 21% : 24%) and Diet 2 - barley flour, inulin and SBF (80.1% : 7% : 12.9%) plus soya bean meal (3:1). After 3 weeks of adaptation to the diets the pigs in groups B and D were infected with 6,000 infective L	extsubscript{3} larvae of *O. dentatum*. All pigs were slaughtered at 12 weeks p. i.

At the end of the experiment the pigs were slaughtered. The samples were taken from the gut content and gut wall at various sites of the intestine. Oesophagostomum dentatum was collected from digesta and washings of the caecum and colon by modified agar-gel technique (Slotved et al. 1996). Gut tissue samples for microscopic measurements were taken from the caecum and the proximal (20%), the middle (50%) and the distal (80%) colon of the total colon.

Tissue samples for microscopy were taken from different sites of the large intestine immediately after slaughter. The intestinal tissue was cleaned of contents and subsequently weighed. The samples for microscopy were taken from the caecum, and the proximal, middle and distal colon as described by Brunsgaard (1997). The samples were taken in duplicate and immediately transferred to 10% neutral buffered formaldehyde. After 4h in the 10% neutral formaldehyde, the tissue samples were carefully cleaned of remaining digesta using deionized water and afterwards transferred to a fresh solution of 10% neutral formaldehyde. After a total of 24 h in the 10% neutral formaldehyde, these samples were dehydrated and infiltrated with paraffin wax. During the embedding, the tissue samples were oriented to obtain sections perpendicular to the mucosal surface. The slides were prepared and processed for carbohydrate histochemistry using dyes as described by Brunsgaard (1998). The dye methods were either the Periodic Acid-Shiff (PAS) reaction or Alcian Blue (AB) reaction at either pH 2.5 (AB 2.5) or pH 1.0 (AB 1.0) according to routine methods (Kiernan, 1990). Carbohydrate histochemistry on the PAS and AB stained samples was evaluated as previously described by Brunsgaard (1997; 1998). From each slide fifteen well-oriented crypts were selected and the area of mucin granules with a clear positive reaction for either neutral mucins, acidic mucins and sulfomucins was determined for each crypt using a computer-integrated microscope and an image analysis system with a video monitor (Quantimet 500 MC, Leica, Cambridge, UK). The slides for the volume, the height, the density of the crypts, the thickness of the muscularis externa were processed using the image analysis system as described by Brunsgaard (1998).

The samples in Clarke’s fixative were stained with Feulgen reaction, and the crypts were displayed by microdissection under a stereomicroscope as described by Goodlad (1994). The number of native mitosis in 20 crypts was then counted using a 40× objective on the light microscope.

**Statistical analysis**

The data on the influence of diet and *O. dentatum* on the body weight and the tissue weight of the large intestine were analyzed by a simple analysis of variance (ANOVA).

Nodular worm numbers, development stages and location were calculated according Petkevičius et al. (1995). All statistics on the large intestine morphology and epithelial cell proliferation were done using the sample means generated from the 15 (20 for mitotic counts) individual measurements. These data was analyzed by a multiple ANOVA. The effects of infection, diet and section were analyzed as fixed and the effect of the individual pig was analyzed as random. Comparison of the means of the main effects were performed using Fisher’s *t*-test with a significance level of 0.05. The test was performed on the least square means.

**Results.** There were no clinical signs of parasitism in any of the pigs during the experiment. The total mean live weight (standard deviation-SD) of the pigs at the start of the experiment was 55.5 kg (1.9) for groups A-D, respectively. There was a significant increase in the weight of pigs between groups A-D due to infection, diet or sex of the pigs (*P*<0.05). The total mean weight of the caecum and colon significantly increased in the control pigs fed Diet 2 compared with the group fed Diet 1 (*P*<0.05). It appeared, that in the control pigs fed by the Diet 2 tissue weight at the proximal and middle colon increased highly significantly (*P*<0.01) compared to the corresponding sections of the pigs fed by the Diet 1.

No worms were recovered from the non-infected pigs. The total caecal (Ce) worm burden was not significantly
affected by the diet. In the anterior and middle parts of the large intestine (Co1-3), pigs fed Diet 1 had statistically higher worm burdens compared to the pigs on Diet 2; the mean (SD) of nodular worm numbers recovered was 4969 (1247) for the pigs on Diet 1, compared with ones 536 (269) on Diet 2 \((P<0.001)\). The mean location of \textit{O. dentatum} in the sections of the large intestine was 2.6 (0.4) and 3.2 (0.5) \((P<0.05)\). All \textit{O. dentatum} worms recovered were adults and in all diet groups were an equal distribution of females and males.

The crypt volume of \textit{O. dentatum} infected pigs fed by the Diet 1 averaged 37808 \(\mu\text{m}^2\) which was significantly higher than crypt volume in the control pigs fed Diet 1 \((P<0.01)\). Crypt volume and height increased significantly from the caecum to the distal parts of the colon. At the different sections of the large intestine the highest average crypt volume 46,689 \(\mu\text{m}^2\) was found at the distal colon of the infected pigs fed by the Diet 1, which was extremely much higher \((P<0.001)\) compared with the other three groups.

At the infected pigs fed by the Diet 1 and Diet 2 crypt height had a tendency to increase compared with the non-infected pigs and the differences were statistically significant \((P<0.05)\). The average crypt height was 387 \(\mu\text{m}\) at the caecum of non-infected pigs fed Diet 1 which increased significantly at the distal colon \((P<0.05)\). In infected pigs fed Diet 1 crypt height averaged 441\(\mu\text{m}\) in the caecum and 635\(\mu\text{m}\) at the distal colon, which was much higher compared with the other groups \((P<0.01)\).

The crypt density significantly decreased in infected pigs compared with non-infected control. It was apparent, that infection with \textit{O. dentatum} decreased the mean crypt density by 20.3\% in pigs fed Diet 1 \((P<0.01)\) and by 15.7\% in pigs fed Diet 2 \((P<0.05)\) compared with the non-infected pigs. The regional differences in crypt density were 18-21\% lower at the proximal colon \((P<0.05)\) of infected pigs on both experimental diets and 26.5\% at the distal colon \((P<0.01)\) of infected pigs fed Diet 1 compared with non-infected pigs, respectively.

The mean thickness of muscularis externa significantly increased in infected pigs compared with non-infected control. The thickness of muscularis externa of non-infected pigs decreased 12.7-24.1\% from the caecum to the proximal colon and increased significantly in the distal parts of colon where in the Diet 1 group thickness averaged 637 \(\mu\text{m}\) \((P<0.01)\). The thickness of muscularis externa at the proximal and medial colon was significantly higher \((P<0.01)\) in infected pigs fed Diet 1 and significantly higher \((P<0.05)\) in infected pigs fed Diet 2 compared to the corresponding non-infected pigs. It appeared that significant increment of thickness of intestinal wall corresponded to the sections of the large intestine where highest worm burdens of \textit{O. dentatum} were found.

The epithelial cell proliferation as determined as the average number of native mitoses, decreased significantly \((P=0.01)\) from the caecum (ranged 12.9-16.3 \(\text{N}/\text{crypt}\)) to the distal parts of colon (ranged 7.4-9.7 \(\text{N}/\text{crypt}\)). The mitotic count at the proximal and medial colon was significantly lower \((P<0.05)\) in infected pigs fed Diet 1 compared to the corresponding non-infected pigs.

There was significant influence of infection in the pigs fed Diet 1 compared to non-infected group on the total amount of neutral, acidic and sulfomucins. The mucin staining volume, however, for neutral, acidic and sulfomucins in the crypts of all pigs increased highly significantly from the caecum to the distal colon \((P<0.01)\). The mean staining volume of neutral, acidic and sulfomucins was lowest in the caecum for all three mucin types and increased significantly \((P<0.01)\) in infected pigs fed Diet 1 compared to the pigs fed Diet 1 in the non-infected group.

The total mucin staining volume of total crypt volume \((\%)\) in infected and non-infected pigs was significantly higher for neutral mucins compared to acidic and sulfomucins. When accounting for the regional difference in crypt size, the mucin staining volume of total crypt volume \((\%)\) of the three mucin types was lowest in caecum and increased towards to the distal colon \((P<0.01)\). The mucin staining volume of total crypt volume was significantly higher at the proximal colon in infected pigs fed Diet 1 \((P<0.05)\) compared to non-infected pigs on the same diet. In infected pigs fed Diet 1 and 2, the mucin staining volume of total crypt volume in the proximal colon was significantly larger than in the caecum for all three mucin types.

\textbf{Discussion.} The results of this experiment demonstrated the interaction between type of dietary carbohydrates and parasitic infection, affecting the morphological characteristics of the large intestine in growing pigs. The results of this investigation are in concert with our previous findings (Petkevičius et al., 1995; 1997; 1999; 2000; 2001; 2003). In addition, the present study indicates that the morphological characteristics and proliferation of the epithelial cells layer in the large intestine of pigs is significantly influenced by \textit{O. dentatum} infection, while the diet containing carbohydrates with contrasting properties had relatively lower impact. In the present study pigs fed the diet containing carbohydrates not digestible in the small intestine but fermentable in the large intestine (inulin and SBF) had significantly higher relative and absolute tissue weight of the caecum and colon compared to the pigs fed the diet composed of resistant to fermentation carbohydrates. This difference was highly significant in proximal colon, the main site for carbohydrates degradation. Moreover, there was a significant influence of the diet on establishment and persistence of \textit{O. dentatum} infection, when the pigs fed Diet 1 had significantly higher worm numbers compared with pigs fed Diet 2. Infection had a significant influence in the proximal and distal colon on crypt volume, height and density, and on muscularis externa at the proximal and middle colon. However, these parameters appeared unaffected by the diet. The infected pigs fed Diet 1 had a more profound influence on the gut wall architecture compared with the infected pigs fed Diet 2, and the
dietary difference in the proximal and middle colon reached 12-18%. The difference in the gut wall architecture corresponded to the worm number in the mentioned sections of the large intestine. Symons (1978) suggested that the intestinal mucosa tends to respond to various stimuli and Riecken (1988) pointed out that physiologically, there is a balance of cell proliferation and exfoliation which maintains normal intestinal mucosal structure. This balance, however, may be disturbed by the gastrointestinal parasites, leading to three principally different responses: mucosal atrophy; mucosal hypertrophy and hyperplasia; and mucosal transformation of the hyperregenerative type. In the small intestine a common effect of gastrointestinal helminths influence on mucosa is villous atrophy associated with crypt hyperplasia (Roy et al., 1996). Many factors affect the degree of villous atrophy and crypt hyperplasia: the species of parasite involved, the age of the host at the time of infection, the number of parasites present and the nutritional status of the host; the latter is one of the most important factors influencing the severity of a helminth infection (Martin, 1981). According Hoste et al. (1988; 1995) the structural and functional changes found in the distal part of intestine in gastrointestinal parasite infection include hypertrophy and hyperplasia of the crypts associated with eventual intestinal adaptation, with possible compensatory absorption. A direct or indirect action of worms on the production of epithelial cells was suggested by Hoste et al. (1988) and Hoste & Mallet (1990) in experimental rabbits infection with Trichostrongylus colubriformis, for Nematodirus spathiger infection (Hoste et al., 1993), and for sheep infection with Ostertagia circumcincta (Scott et al., 1998). In all these reports the pathophysiological effects were associated with histopathological changes in the intestine, when marked crypt hyperplasia and major reduction of enzyme activities was found.

In the present experiment the proliferative activity was much lower in the distal part of the large intestine as compared to the proximal parts and in infected pigs fed Diet 1 shown significant influence on the epithelial cell proliferation. The decline in the proliferative activity along the large intestine is undoubtedly related to the process of microbial fermentation of carbohydrates and as consequence of increased short-chain fatty acids (SCFA) production (Brunsgaard, 1997). Several investigations have shown that SCFA products derived from microbial fermentation of carbohydrates – stimulate cellular proliferation in the epithelium of the hindgut (Sakata, 1987; Sakata, 1989; Frenkel et al., 1994). Furthermore, Huby et al. (1999) have pointed to excretory/secretory products of T. colubriformis as stimulators for the epithelial cell proliferation when the number of native mitosis in the intestinal crypt cells are increased. In addition, there are some reports showing that Nippostrongylus brasiliensis induce not only an increase in the number of cells undergoing mitosis in the crypts of the intestine of rodents (Symons, 1965; 1978), as for T. colubriformis in ruminants and rabbits (Barker, 1975; Hoste et al., 1988; Hoste, 1989), but also a hypertrophy of muscularis externa of the gut mucosa (Symons, 1962). Some studies on Trichuris suis infection in pigs have shown that the results of nematode infestation was elongation of large intestinal crypts, excessive mucus production and desquamation of epithelial cells (Beer & Lean, 1973; Hall et al., 1976).

The present results reveal that infection with O. dentatum significantly increased mucus volume at the proximal and the distal colon compare with non-infected pigs indicating a greater production and secretion of mucus in infected pigs compared with non-infected pigs. This difference may relate to the production and release of SCFA in the large intestine (Sakata & Setoyama, 1995; Hicks et al., 2000). However, the lower volume of mucin granules in the caecum and proximal colon may indicate a more rapid secretion of goblet cell mucins. Studies on rats suggest that a high concentration of SCFA in the caecum stimulate mucin release (Sakata & Setoyama, 1995). In infected pigs fed a diet containing non-fermentable carbohydrates a significant increase in acidic and sulfomucins volume occurred, whereas the volume of neutral mucins was highest at non-infected pigs. This is in agreement with findings of Miller & Nawa (1979), which showed that in response to N. brasiliensis infection the mucin quality changed from a neutral to acidic mucins by day 14 of infection. In addition, More et al. (1987) have pointed out that low dietary fibre content modifies the nature of the mucins secreted, when the content of neutral mucins was reduced significantly. The mucins contribute to lubrication of mucosal surfaces, and may serve as a mechanism of defence from parasitic infection and as a protection against undigested residues (Turck et al., 1993). The amount of mucin produced may be of importance as a protective layer between intestinal tissue and the luminal contents in preventing intestinal helminth infections. There are two groups of mucins: (1) secreted mucins, the main component of mucus gels, which are extremely large glycoproteins and donate the necessary viscoelastic properties to the protective barrier and (2) membrane-bound mucins, which are smaller than excreted mucins, highly glycosylated and do not form extracellular gels (Hicks et al., 2000). This biochemical specialization provides the flexibility that enables mucins gels to be exquisitely responsive for parasite infections. Increased turnover of intestinal mucin permitted establishment of gastrointestinal helminths (Miller, 1984) and lend further support to the concept that mucins serves an important function in protection against intestinal helminths by binding and entrapment (Miller, 1987). Some studies have been designed to analyse how helminth parasites may influence the content of mucins. Dobson (1967) have found that proliferation of the mucins cells in the gut and the diarrhoea is common features of sheep infected by the adult worms of Oesophagostomum columbianum. Following primary infection with Trichostrongylus colubriformis in quinea pigs pronounced goblet cell hyperplasia developed and the proportion of sulphomucin in these cells increased (Manjili et al., 1998). However, mucus and mucins could act not only as a mechanical barrier, but also as a matrix

References:

- Brunsgaard, 1997
- Sakata & Setoyama, 1995
- Miller & Nawa, 1979
- Turck et al., 1993
- Miller, 1987
- Dobson, 1967
- Manjili et al., 1998
in which other components such as Ig, complement, lysozyme or lactoperoxidase responding as the host-effector interact with the parasite. The exact mechanisms regulating mucus secretion are not established. Evidence has been presented that mucus production and release are stimulated locally by SCFA (Sakata & Setoyama, 1995), hormones (Finnie et al., 1996) and neurotransmitters (Phillips & Wilson, 1993). There are some findings that qualitative changes in the viscosity and composition of the mucous barrier could have a negative influence on parasite because the mucus is of nutritional value to the parasite, particularly to worms which ingest superficial mucus and this produce morphological damage to the worm gut cells (Ogilvie & Love, 1974; Miller & Huntley, 1982). Moreover, the mucus composition could be changed by the activity of glycoproteins from the parasitic gastrointestinal helminths (Smith et al., 1994) or by glycosyltransferases and glycosidases from the microflora of the host gut surface (Freeman et al., 1980). The diet and age of the host affect the composition of mucous glycoproteins from the pig colon and this changes in colonic mucus composition may affect a mammal’s ability to defend itself against intestinal infection (Turck et al., 1993; Brunsgaard, 1998).

The studies regarding the pathology of the O. dentatum infections have been focused primarily on description of the process of nodule formation around larval stages during migration through the mucosal layer, and the level of cellular infiltration (Stewart & Gasbarre, 1989). The mechanisms by which larvae of O. dentatum breach the mucosal barrier and the process of nodule formation around larval stages during migration through the intestinal wall of infected animals are only partially understood (Jensen & Christensen, 1997). Still very little is known about factors controlling nodular worms behaviour/survival in the gastrointestinal tract of the host, particularly in the nodule-free part of the large intestinal mucosa. However, it is clear that O. dentatum larvae must traverse the mucous layer in order to penetrate the intestinal wall. It was demonstrated that parasites secrete mucin-degrading enzymes, enabling the penetration of protective mucous gels that overlie the mucosal surfaces of their potential hosts (Hicks et al., 2000). In recent studies performed by Leonhard-Marek & Daugschies (1997) it was found that O. dentatum larvae produce acute inflammatory reaction during the penetration in and out the intestinal wall on days 2 and 14 p.i. During the histotrophic development of the parasite on day 7 p.i this reaction was significantly declined. Typical feature of the pathological process of O. dentatum infection is formation of subepithelial granuloma in the large intestine, where active part is played by the lipid mediators, delivered from the nodular worms (Daugschies & Rutkovski, 1998; Daugschies & Joachim, 2000). Such bioactive materials could have influence on various physiology functions of the hindgut - secretion, absorption and motility. Furthermore, it was pointed that nematodes incorporate fatty acids and lipids from their environment (Frayha & Smyth, 1983) and the source of fatty acids in intestinal content influences the relative fatty acid contents of the nodular parasite and may support establishment and development of different stages of Oesophagostomum in the host (Joachim et al., 2000). The products excreted from O. dentatum and the inflammation surrounding the larvae could have influence on the colonic epithelial proliferation and crypt elongation in sites distant from parasite induced nodules (Jensen & Christensen, 1997).

In conclusion, this study shows that the infection with O. dentatum and composition of the dietary carbohydrates affects the mucosal architecture, mucin secretion and the epithelial cell proliferation in the large intestine of growing pigs. The content of dietary carbohydrates significantly influenced the tissue weight of caecum and colon, and the establishment and persistence of O. dentatum infection. The difference in the gut wall architecture corresponded to the worm counts in the mentioned sections of the large intestine. Since dietary factors that either affect the production of mucin or enhance it’s degradation would make the intestinal mucosa susceptible to pathogenic parasites, it is possible that appropriate modifications of dietary carbohydrates may modulate persistence of O. dentatum and thus protect pigs against infection.

References