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The effect of intramuscular administration of colistin on the development and course of experimentally induced oedema disease in weaned piglets

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Abstract

Shiga-toxigenic E. coli (STEC) strains that produce Shiga toxin Stx2e cause oedema disease in weaned piglets. The purpose of the present study was to investigate the impact of Stx2e released in mesenteric lymph nodes on disease pathogenesis. Colistin and ampicillin were intramuscularly administered to piglets of the experimental group simultaneously challenged with STEC strain, type O139:F18ab, Stx2e+. Piglets of the control group were challenged with STEC only. The strain was naturally resistant to ampicillin and susceptible to colistin. After the challenge, colonisation of the intestines was observed in both antibiotic-treated piglets and control piglets without antibiotic treatment. Histochemistry and scanning electron microscopy revealed sporadic colonisation of the small intestine in the piglets. STEC was detected in the mesenteric lymph nodes of untreated piglets. The clinical manifestations of oedema disease were observed in both groups. In the antibiotic-treated group (11 piglets),
oedema disease developed in 10 piglets, 8 of which died or were euthanized ante finem. In the untreated group (11 piglets), oedema disease developed in 5 piglets, 4 of which died or were euthanized ante finem. We therefore propose that the STEC lysed by colistin suddenly released the toxin from bacterial cells immediately after their passage through the intestinal wall. That could explain a more severe course of oedema disease in the treated piglets. Even though high amounts of STEC were present in the lymph nodes of untreated piglets, the toxin was not released abruptly because the bacterial cells were not damaged.

Keywords: *Escherichia coli*, ED, STEC, colonising factors

**Introduction**

Oedema disease of swine is a toxaemia caused by shiga-toxigenic *E. coli* (STEC) colonising the small intestine and producing exotoxin. The causative agent of oedema disease is verotoxin Stx2e. Stx2e in pigs induces typical lesions, including fibrinoid necrosis of small arteries, brain haemorrhages and, at higher doses, surface necroses in the colonic epithelium as well as damage to kidney tissue (Gannon et al., 1989). Oedema in various parts of the body can be found on autopsy.

*E. coli* strains causing oedema disease colonise the intestines of piglets (Bertschinger and Gyles, 1994), and in some strains of STEC fimbrial antigen F18ab has been demonstrated (Rippinger et al., 1995; Wittig et al., 1995). Wittig et al. (1995) proposed that strains associated with oedema disease have the colonisation factor F18ab and adhere to the intestinal mucous membrane. STEC strains without known colonisation factors in piglets suffering from oedema disease have also been detected in other countries (Wittig et al., 1995; Osek, 1999; Osek, 2000; Alexa et al., 2002; Frydendahl, 2002; Cheng et al., 2006).
Timoney (1957) suggested that only a small proportion of the toxin present in intestinal lumen reaches the vascular system. The relatively long delay between colonisation of the intestine and the appearance of clinical symptoms of the disease is viewed as indicative of a slow passage of the toxin through the intestinal wall (Smith and Halls, 1968). Other than this, little is known about the absorption of functionally active Stx from the intestine (Gyles, 1994).

STEC is frequently isolated in pure culture from swollen and oedematous lymph nodes but not from other tissues or the blood of piglets suffering from oedema disease (Salajka and Salajkova, 1987). We expected that STEC colonising the lymph nodes of piglets can produce Shiga toxin there that is subsequently transported into the organs. We performed experiments with the aim to prevent STEC multiplication and Stx2e production in the lymph nodes of piglets without disturbing STEC colonisation of the intestines. For this purpose, we used parenteral administration of colistin, which does not pass through the intestinal wall. We supposed that its administration would not allow the toxin production by STEC that colonise the lymph nodes and that the course of oedema disease in the treated piglets would be milder than in untreated piglets. In contrast to our assumption, the antibiotic-treated piglets exhibited more severe signs of oedema disease.

Material and methods

E. coli strain

E. coli strain 11313 (O139:F18, stx2e+) isolated from a weaned piglet that had died of oedema disease was used for piglet infection. The strain was naturally susceptible to colistin and resistant to ampicillin. The culture was grown in culture medium containing 12.5 g of
acid casein hydrolysate, 12.5 g of enzymatic casein hydrolysate (Imuna, Sarisske Michalany, Slovak Republic) and 0.5 g of yeast extract (Oxoid) per 1 litre. The culture was incubated at 37°C with shaking for 16 h. A part of the culture for individual oral administration was pelleted by centrifugation and mixed into a neutral sterile puree (wheat flour and semolina). The paste contained $2 \times 10^{10}$ CFU/ml.

Animals

Animal handling complied with the legal directives of the Czech Republic and with the Institution’s policy. Piglets from a conventional herd (22 animals in 2 experiments), weaned at 28 days of age were used in the experiments. The piglets were fed a diet composed of wheat, barley, soybean meal and fish meal (19.2% N substances, 3.5% fat, 3.7% fibre). The groups of piglets were kept in pens separated by metal bars. Experimental group piglets were administered Ampisur (Ceva Sante Animale) (colistin and ampicillin) in the recommended dose of 25,000 IU/kg of live body weight. Control group piglets were not treated.

Detection of piglets’ susceptibility to intestinal colonisation with \textit{E. coli} having colonisation factors F18

To distinguish between piglets resistant and susceptible to intestinal colonisation by \textit{E. coli} with F18 adhesins, PCR was used for detecting the point mutation (G→A) of the \textit{FUT1} gene (fucosyltransferase gene) in the nucleotide sequence at position 307 (Meijerink et al., 2000). For this purpose, ear skin samples were collected from each piglet. DNA was isolated from 25 mg of each sample of piglet auricle using the DNA Tissue Kit (Qiagen). The quality of the isolated DNA was tested by agarose gel electrophoresis and the modified allele-specific PCR method of Lee et al. (2002) was used. The forward primer to both alleles was \textit{D(f)} 5'-GCC-GCC-ACC-TCT-GTC-TGA-CCT-TC-3', and 2 reverse primers were \textit{R(r)} 5' CGG-CGG-
TTG-AGC TGC-GC 3' for resistant pigs and S(r) 5' CGG-CCG TTG-AGC TGC-GT-3' for susceptible pigs. The PCR was performed in 20 µl volume using the PCR Master Mix Kit (Qiagen) according to the manufacturer’s instructions. Ten pmol of each primer and 100 ng of DNA were added to each PCR reaction. One hundred ng of the examined DNA was added to the PCR. The PCR procedure included initial denaturation at 94°C/2 min and 38 cycles with incubations at 95°C/15 s, 65°C/20 s and 72°C/40 s. After the final extension (5 min at 72°C), the samples were cooled to 4°C and analysed by electrophoresis on 1.5% agarose gel.

Challenge of piglets

Both the groups (treated and untreated) were challenged with STEC strain 11313. All piglets were orally infected with an STEC culture in a dose of 2 x 10^{11} CFU on the day after weaning. The diet for piglets was supplemented with STEC culture of the same dose on each of the following 3 days. The clinical health status of the piglets was regularly monitored for 3 weeks after infection. Rectal swabs were collected every day for bacteriological examination. Animals that died or were euthanized ante finem were examined by autopsy; their intestines, mesenteric lymph-nodes, livers, spleens and kidneys were examined by culture. Samples of posterior jejunum and ileum were collected from the euthanized piglets for histology and scanning electron microscopy.

Microbiological examination

Rectal swabs from piglets or contents of jejunum and colon were diluted in PBS (pH 7.4) and spread on blood agar containing 5% lamb blood and on MacConkey agar and cultured in an incubator at 37 °C. After superficial burning and cutting, liver, spleen, kidney and mesenteric lymph nodes obtained from the euthanized piglets were smeared onto 5% lamb blood and on MacConkey agar. Ten randomly selected isolated colonies of *E. coli* from each swab were
examined and the percentages of haemolytic colonies were assessed. Haemolytic colonies were inoculated onto a nutrient broth (Imuna Šarišské Michalany, Slovakia). After incubation at 37°C for 16 h, intravital staining of cultures was performed by adding TTC (triphenyl-tetrazolium-chloride) for 1 h and heating to 100°C, and then these were examined by agglutination with antisera to O139 (Salajka et al., 1992). The percentage of the strain administered and present in faeces was calculated according to the numbers of haemolytic O139 type colonies in diluted culture on blood agar. Presence of the stx2e gene encoding the Shiga toxin was confirmed by PCR (Alexa et al., 2000).

Histological and immunohistochemical examinations
Immediately after slaughter, samples of intestines were collected and fixed in 10% neutral buffered formalin for 24 h. Paraffin sections 6 µm wide were mounted on silanised slides, deparaffinised and rehydrated. For conventional histopathological examinations, the sections were stained with haematoxylin-eosin.

Before immunohistochemical staining, heat induced antigen retrieval was performed in a microwave at high power (750 W) for 15 min (3x5 min) in citrate buffer, pH 6.0. The activity of endogenous peroxidase was blocked by 3% H₂O₂ for 15 min. Rabbit anti-O139 serum was used for detecting E. coli, with incubation at room temperature for 60 min. For visualisation of immunoreaction complexes, peroxidase conjugated affinity purified anti-rabbit IgG (code 611-903-002; Rockland, USA) was used according to the manufacturer’s operating protocols. Negative controls were obtained by replacement of primary antibody with a negative control serum. As a chromogen, 0.03% DAB (3,3'diaminobenzidine, Fluka Chemie AG, Buchs,
Switzerland) was used for 5 min at room temperature. The sections were counterstained with haematoxylin.

Jejunum samples were examined by scanning electron microscopy after platinum palladium metallisation using a Tesla BS300 scanning electron microscope.

Statistical analysis
The mean values and standard deviations were calculated for shedding of the challenge strain. Morbidity and mortality due to oedema disease were evaluated by Fisher’s test.

Results
By genotype PCR analysis, all piglets were found to be sensitive to the colonisation of intestines with F18 positive *E. coli* (data not shown). After STEC infection, intensive shedding of the challenge strain through faeces was noted in most piglets from both antibiotic-treated and untreated groups. The percentages of the challenge *E. coli* strain shed through faeces from antibiotic-treated piglets are presented in Fig. 1A. In the first experiment, intensive shedding of the challenge strain in most of the antibiotic-treated piglets was recorded on day 2 of infection, except for one piglet, in which the challenge strain prevailed on day 4 of infection. In the second experiment, the challenge strain predominated in all the antibiotic-treated piglets on day 1 of infection. In piglets that survived, shedding persisted up to day 9 after the start of infection. In the untreated piglets (Fig. 1B) intensive shedding of the challenge strain was observed in 3 and 4 piglets on days 1 and 2 of infection. With the exception of one piglet, the challenge strain outnumbered the other *E. coli* types on day 4 of
infection. Shedding of the challenge strain by the survived piglets was below the detection limit from day 10 after the start of infection.

In all euthanized (n=4) piglets from the antibiotic untreated group, STEC O139:F18ab, Stx2e+ was detected by culture in mesenteric lymph nodes. Only one antibiotic-treated animal was positive for STEC in mesenteric lymph nodes while the remaining (n=7) were negative.

The onset of clinical symptoms of oedema disease after infection and their duration in both the experiments are summarised in Fig. 2. In the antibiotic-treated groups, clinical symptoms of oedema disease were manifested in 10 piglets. Two of them had only moderate symptoms of the disease and these spontaneously disappeared. In the remaining 8 piglets, a rapid course of the disease was observed and the animals were euthanized ante finem within two days after the onset of clinical symptoms. The early symptoms of the disease, especially characterised by staggering, appeared on the third day after infection. Neural disorders rapidly impaired piglets affected by oedema disease and a loss of coordination and weakness developed. Oedema of the eyelids appeared in one piglet. Haemorrhagic diarrhoea manifested in one piglet on day 3 after infection. The health status of 2 piglets from the antibiotic-treated group remained good throughout the entire experiment. In the untreated groups, 5 piglets were affected by oedema disease. Four of them were euthanized ante finem. Moderate symptoms appeared in one piglet, which recovered 7 days later. In the antibiotic untreated groups, the health status of 6 piglets remained good over the entire experimental period. The differences in piglet mortality between groups were defined as P=0.099 and were non-significant; significant differences were found in morbidity between antibiotic-treated and untreated piglets (P<0.05).
Gross examination of euthanized piglets from both treated and untreated groups revealed oedema of the subcutis, particularly of the eyelids and mesocolon. Fibrin fibres were found on the peritoneum in some piglets. Symptoms of haemorrhagic enteritis and colitis were observed in one piglet with haemorrhagic diarrhoea from the antibiotic-treated group. Inflammatory infiltrate of the intestinal wall, local desquamation of epithelium and apical destruction of villi were found in the same piglet by histology. In the other piglets, histological examination revealed oedema and presence of increased amounts of infiltrate of the intestinal wall (lymphoid cells and polymorphonuclears), which reached the surface epithelium without alteration. Inflammatory lesions were not seen. No bacterial layers were detected by immunohistochemistry on small intestine mucosa, with the exception of a single piglet from the antibiotic-treated group affected by hemorrhagic enteritis, in which numerous *E. coli* O139 bacteria were present as well as destroyed mucosal tissue. Regarding the character of intestinal damage, it was not possible to unambiguously specify whether bacteria were attached to mucosa, or were present in mucus and detritus. The scanning electron microscopy examination of mucosa from euthanized piglets showed results similar to those of the immunohistochemical examination. Mucosa of jejunum and ileum was free from *E. coli* plaques in the majority of piglets. If bacteria were present on mucosa, they were rare. Areas of adhered bacteria were found in the caudal jejunum of one piglet only.

**Discussion**

It is generally assumed that STEC colonise the small intestine and produce Shiga toxin Stx2e, which is absorbed into the bloodstream and distributed to other tissues. However, both the intestinal colonisation by STEC and the toxin transport across the gut epithelium into the
bloodstream pose several issues. Concerning the gut colonisation, except for a single animal, we did not observe any epithelium colonisation using histology and scanning electron microscopy. No bacteria were found associated with the epithelium. Even in the only animal, in which we found an epithelium colonisation, this was considerably different from the way of colonisation typical e.g. for the enterotoxigenic *E. coli*. The colonisation was limited to isolated areas and bacteria were present mostly in mucus. No bacterial plaques were found on the surface of epithelial cells.

The exact mechanism of Stx2e absorption into the blood vessels is not well understood. Although the toxin was found to bind to the surface membranes of the microvilli of the jejunum, ileum and colon, and receptors for Stx2e were found in the intestine of the pig, their role in absorption of Stx2e is unclear because the intraintestinal inoculation of pigs with large quantities of Stx2e did not result in oedema disease’s development (Waddell et al., 1996). Pathogenesis of oedema disease in pigs is still rather confusing and unclear.

Our results offer an alternative explanation for the pathogenesis of oedema disease in pigs. Although we did not observe any significant adhesion to the epithelium, the STEC overgrew other *E. coli* in the intestinal content because it was detected in faecal samples of both groups. Due to the fact that the faecal shedding was essentially the same in both groups of animals, this also confirmed that the parenterally administered colistin did not pass through the intestinal barrier and therefore did not affect intraintestinal multiplication of the challenge strain. In all antibiotic untreated piglets, colonisation of the mesenteric lymph nodes by the challenge strain was observed. This may appear unexpected since STEC are non-invasive extracellular bacteria and it is rather uncertain how the non-invasive *E. coli* could reach the mesenteric lymph nodes. It remains unclear whether and how F18ab fimbriae may participate
in the passage of STEC through mucosa. Initially, we expected that the parenteral
administration of colistin might prevent multiplication of *E. coli* in the lymph nodes and
therefore also might prevent the clinical signs of the disease. However, the numbers of
diseased piglets in the antibiotic-treated group were higher, the course of the disease was
more severe and more animals died in comparison with the untreated group, despite the fact
that colistin was apparently efficient. A similar effect has been observed in human infections
causd by STEC O157:H7, where the course of the disease was more complicated under
antibiotic therapy (Carter et al., 1987; Ostroff et al., 1989). We therefore propose that the
STEC lysed by colistin suddenly released the toxin from bacterial cells immediately after
their passage through the intestinal wall, which would explain a more severe course of
oedema disease in the treated piglets. Even though high amounts of STEC were present in the
lymph nodes from untreated piglets, the toxin was not released as abruptly because the
bacterial cells were not damaged. Our results did not rule out the possibility that the Stx2e
toxin is transported through the intestinal wall directly from bacteria colonising the intestinal
tract. Nevertheless, we propose that there exists an additional route of Stx2e release to the
bloodstream from the bacteria transported through mucosa where they are subsequently lysed
either abruptly by the action of colistin or slowly by the activity of the host’s immune system.
This allows the toxin to be released to the bloodstream and cause the clinical signs of oedema
disease.

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Fig. 1: Percentage of STEC O139 in total amount of *E. coli* shedding through faeces from piglets after challenge. The piglets were challenged on day 0 until day 3 with STEC O139:F18ab, stx2e+ in a dose of $2 \times 10^{11}$ CFU/day. A – antibiotic-treated group, B – untreated group.

Fig. 2: Onset and duration of oedema disease in piglets after challenge. A – antibiotic-treated group, B – untreated group. Only one and six piglets remained healthy during the trial in the antibiotic-treated group and untreated group, respectively. Morbidity differences between groups were $P<0.05$, mortality differences $P>0.05$. 
Figure 1B

Days after challenge

Percent of STEC O139
Figure 2A

Days after challenge

- Healthy
- Edema disease
- Death