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

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3

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11

12

13   **Abstract**

14

15   Shiga-toxigenic *E. coli* (STEC) strains that produce Shiga toxin Stx2e cause oedema disease

16   in weaned piglets. The purpose of the present study was to investigate the impact of Stx2e

17   released in mesenteric lymph nodes on disease pathogenesis. Colistin and ampicillin were

18   intramuscularly administered to piglets of the experimental group simultaneously challenged

19   with STEC strain, type O139:F18ab, Stx2e+. Piglets of the control group were challenged

20   with STEC only. The strain was naturally resistant to ampicillin and susceptible to colistin.

21   After the challenge, colonisation of the intestines was observed in both antibiotic-treated

22   piglets and control piglets without antibiotic treatment. Histochemistry and scanning electron

23   microscopy revealed sporadic colonisation of the small intestine in the piglets. STEC was

24   detected in the mesenteric lymph nodes of untreated piglets. The clinical manifestations of

25   oedema disease were observed in both groups. In the antibiotic-treated group (11 piglets),

1

1 oedema disease developed in 10 piglets, 8 of which died or were euthanized *ante finem*. In the

2 untreated group (11 piglets), oedema disease developed in 5 piglets, 4 of which died or were

3 euthanized *ante finem*. We therefore propose that the STEC lysed by colistin suddenly

4 released the toxin from bacterial cells immediately after their passage through the intestinal

5 wall. That could explain a more severe course of oedema disease in the treated piglets. Even

6 though high amounts of STEC were present in the lymph nodes of untreated piglets, the toxin

7 was not released abruptly because the bacterial cells were not damaged.

8

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

10

11 **Introduction**

12 Oedema disease of swine is a toxaemia caused by shiga-toxigenic *E. coli* (STEC) colonising

13 the small intestine and producing exotoxin. The causative agent of oedema disease is

14 verotoxin Stx2e. Stx2e in pigs induces typical lesions, including fibrinoid necrosis of small

15 arteries, brain haemorrhages and, at higher doses, surface necroses in the colonic epithelium

16 as well as damage to kidney tissue (Gannon et al., 1989). Oedema in various parts of the

17 body can be found on autopsy.

18

19 *E. coli* strains causing oedema disease colonise the intestines of piglets (Bertschinger and

20 Gyles, 1994), and in some strains of STEC fimbrial antigen F18ab has been demonstrated

21 (Rippinger et al., 1995; Wittig et al., 1995). Wittig et al. (1995) proposed that strains

22 associated with oedema disease have the colonisation factor F18ab and adhere to the intestinal

23 mucous membrane. STEC strains without known colonisation factors in piglets suffering from

24 oedema disease have also been detected in other countries (Wittig et al., 1995; Osek, 1999;

25 Osek, 2000; Alexa et al., 2002; Frydendahl, 2002; Cheng et al., 2006).

2

1

2 Timoney (1957) suggested that only a small proportion of the toxin present in intestinal lumen

3 reaches the vascular system. The relatively long delay between colonisation of the intestine

4 and the appearance of clinical symptoms of the disease is viewed as indicative of a slow

5 passage of the toxin through the intestinal wall (Smith and Halls, 1968). Other than this, little

6 is known about the absorption of functionally active Stx from the intestine (Gyles, 1994).

7

8 STEC is frequently isolated in pure culture from swollen and oedematous lymph nodes but

9 not from other tissues or the blood of piglets suffering from oedema disease (Salajka and

10 Salajkova, 1987). We expected that STEC colonising the lymph nodes of piglets can produce

11 Shiga toxin there that is subsequently transported into the organs. We performed experiments

12 with the aim to prevent STEC multiplication and Stx2e production in the lymph nodes of

13 piglets without disturbing STEC colonisation of the intestines. For this purpose, we used

14 parenteral administration of colistin, which does not pass through the intestinal wall. We

15 supposed that its administration would not allow the toxin production by STEC that colonise

16 the lymph nodes and that the course of oedema disease in the treated piglets would be milder

17 than in untreated piglets. In contrast to our assumption, the antibiotic-treated piglets exhibited

18 more severe signs of oedema disease.

19

20 **Material and methods**

21

22 *E. coli* strain

23 *E. coli* strain 11313 (O139:F18, stx2e+) isolated from a weaned piglet that had died of

24 oedema disease was used for piglet infection. The strain was naturally susceptible to colistin

25 and resistant to ampicillin. The culture was grown in culture medium containing 12.5 g of

3

1   acid casein hydrolysate, 12.5 g of enzymatic casein hydrolysate (Imuna, Sarisske Michalany,

2   Slovak Republic) and 0.5 g of yeast extract (Oxoid) per 1 litre. The culture was incubated at

3   37°C with shaking for 16 h. A part of the culture for individual oral administration was

4   pelleted by centrifugation and mixed into a neutral sterile puree (wheat flour and semolina).

5   The paste contained 2×10^{10} CFU/ml.

6

7   Animals

8   Animal handling complied with the legal directives of the Czech Republic and with the

9   Institution's policy. Piglets from a conventional herd (22 animals in 2 experiments), weaned

10 at 28 days of age were used in the experiments. The piglets were fed a diet composed of

11 wheat, barley, soybean meal and fish meal (19.2% N substances, 3.5% fat, 3.7% fibre). The

12 groups of piglets were kept in pens separated by metal bars. Experimental group piglets were

13 administered Ampisur (Ceva Sante Animale) (colistin and ampicillin) in the recommended

14 dose of 25,000 IU/kg of live body weight. Control group piglets were not treated.

15

16 Detection of piglets' susceptibility to intestinal colonisation with *E. coli* having colonisation

17 factors F18

18 To distinguish between piglets resistant and susceptible to intestinal colonisation by *E. coli*

19 with F18 adhesins, PCR was used for detecting the point mutation (G→A) of the *FUT1* gene

20 (fucosyltransferase gene) in the nucleotide sequence at position 307 (Meijerink et al., 2000).

21 For this purpose, ear skin samples were collected from each piglet. DNA was isolated from

22 25 mg of each sample of piglet auricle using the DNA Tissue Kit (Qiagen). The quality of the

23 isolated DNA was tested by agarose gel electrophoresis and the modified allele-specific PCR

24 method of Lee et al. (2002) was used. The forward primer to both alleles was **D(f)** 5'-GCC-

25 GCC-ACC-TCT-GTC-TGA-CCT-TC-3' , and 2 reverse primers were **R(r)** 5' CGG-CCG

4

1   TTG-AGC TGC-G**C** 3' for resistant pigs and **S(r)** 5' CGG-CCG TTG-AGC TGC-G**T**-3' for

2   susceptible pigs. The PCR was performed in 20 μl volume using the PCR Master Mix Kit

3   (Qiagen) according to the manufacturer's instructions. Ten pmol of each primer and 100 ng of

4   DNA were added to each PCR reaction. One hundred ng of the examined DNA was added to

5   the PCR. The PCR procedure included initial denaturation at 94°C/2 min and 38 cycles with

6   incubations at 95°C/15 s, 65°C/20 s and 72°C/40 s. After the final extension (5 min at 72°C),

7   the samples were cooled to 4°C and analysed by electrophoresis on 1.5% agarose gel.

8

9   Challenge of piglets

10   Both the groups (treated and untreated) were challenged with STEC strain 11313. All piglets

11   were orally infected with an STEC culture in a dose of 2×10^{11} CFU on the day after

12   weaning. The diet for piglets was supplemented with STEC culture of the same dose on each

13   of the following 3 days. The clinical health status of the piglets was regularly monitored for 3

14   weeks after infection. Rectal swabs were collected every day for bacteriological examination.

15   Animals that died or were euthanized *ante finem* were examined by autopsy; their intestines,

16   mesenteric lymph-nodes, livers, spleens and kidneys were examined by culture. Samples of

17   posterior jejunum and ileum were collected from the euthanized piglets for histology and

18   scanning electron microscopy.

19

20   Microbiological examination

21   Rectal swabs from piglets or contents of jejunum and colon were diluted in PBS (pH 7.4) and

22   spread on blood agar containing 5% lamb blood and on MacConkey agar and cultured in an

23   incubator at 37 °C. After superficial burning and cutting, liver, spleen, kidney and mesenteric

24   lymph nodes obtained from the euthanized piglets were smeared onto 5% lamb blood and on

25   MacConkey agar. Ten randomly selected isolated colonies of *E. coli* from each swab were

5

1 examined and the percentages of haemolytic colonies were assessed. Haemolytic colonies

2 were inoculated onto a nutrient broth (Imuna Šarišské Michalany, Slovakia). After incubation

3 at 37°C for 16 h, intravital staining of cultures was performed by adding TTC (triphenyl-

4 tetrazolium-chloride) for 1 h and heating to 100°C, and then these were examined by

5 agglutination with antisera to O139 (Salajka et al., 1992). The percentage of the strain

6 administered and present in faeces was calculated according to the numbers of haemolytic

7 O139 type colonies in diluted culture on blood agar. Presence of the *stx2e* gene encoding the

8 Shiga toxin was confirmed by PCR (Alexa et al., 2000).

9

10

11

12 Histological and immunohistochemical examinations

13 Immediately after slaughter, samples of intestines were collected and fixed in 10% neutral

14 buffered formalin for 24 h. Paraffin sections 6 μm wide were mounted on silanised slides,

15 deparaffinised and rehydrated. For conventional histopathological examinations, the sections

16 were stained with haematoxylin-eosin.

17 Before immunohistochemical staining, heat induced antigen retrieval was performed in a

18 microwave at high power (750 W) for 15 min (3x5 min) in citrate buffer, pH 6.0. The activity

19 of endogenous peroxidase was blocked by 3% H_2O_2 for 15 min. Rabbit anti-O139 serum was

20 used for detecting *E. coli*, with incubation at room temperature for 60 min. For visualisation

21 of immunoreaction complexes, peroxidase conjugated affinity purified anti-rabbit IgG (code

22 611-903-002; Rockland, USA) was used according to the manufacturer's operating protocols.

23 Negative controls were obtained by replacement of primary antibody with a negative control

24 serum. As a chromogen, 0.03% DAB (3,3'diaminobenzidine, Fluka Chemie AG, Buchs,

6

1 Switzerland) was used for 5 min at room temperature. The sections were counterstained with

2 haematoxylin.

3 Jejunum samples were examined by scanning electron microscopy after platinum palladium

4 metallisation using a Tesla BS300 scanning electron microscope.

5

6 Statistical analysis

7 The mean values and standard deviations were calculated for shedding of the challenge strain.

8 Morbidity and mortality due to oedema disease were evaluated by Fisher's test.

9

10

11

12 **Results**

13

14 By genotype PCR analysis, all piglets were found to be sensitive to the colonisation of

15 intestines with F18 positive *E. coli* (data not shown). After STEC infection, intensive

16 shedding of the challenge strain through faeces was noted in most piglets from both

17 antibiotic-treated and untreated groups. The percentages of the challenge *E. coli* strain shed

18 through faeces from antibiotic-treated piglets are presented in Fig. 1A. In the first experiment,

19 intensive shedding of the challenge strain in most of the antibiotic-treated piglets was

20 recorded on day 2 of infection, except for one piglet, in which the challenge strain prevailed

21 on day 4 of infection. In the second experiment, the challenge strain predominated in all the

22 antibiotic-treated piglets on day 1 of infection. In piglets that survived, shedding persisted up

23 to day 9 after the start of infection. In the untreated piglets (Fig. 1B) intensive shedding of the

24 challenge strain was observed in 3 and 4 piglets on days 1 and 2 of infection. With the

25 exception of one piglet, the challenge strain outnumbered the other *E. coli* types on day 4 of

7

1   infection. Shedding of the challenge strain by the survived piglets was below the detection

2   limit from day 10 after the start of infection.

3

4   In all euthanized (n=4) piglets from the antibiotic untreated group, STEC O139:F18ab,

5   Stx2e+ was detected by culture in mesenteric lymph nodes. Only one antibiotic-treated animal

6   was positive for STEC in mesenteric lymph nodes while the remaining (n=7) were negative.

7

8   The onset of clinical symptoms of oedema disease after infection and their duration in both

9   the experiments are summarised in Fig. 2. In the antibiotic-treated groups, clinical symptoms

10   of oedema disease were manifested in 10 piglets. Two of them had only moderate symptoms

11   of the disease and these spontaneously disappeared. In the remaining 8 piglets, a rapid course

12   of the disease was observed and the animals were euthanized *ante finem* within two days after

13   the onset of clinical symptoms. The early symptoms of the disease, especially characterised

14   by staggering, appeared on the third day after infection. Neural disorders rapidly impaired

15   piglets affected by oedema disease and a loss of coordination and weakness developed.

16   Oedema of the eyelids appeared in one piglet. Haemorrhagic diarrhoea manifested in one

17   piglet on day 3 after infection. The health status of 2 piglets from the antibiotic-treated group

18   remained good throughout the entire experiment. In the untreated groups, 5 piglets were

19   affected by oedema disease. Four of them were euthanized *ante finem*. Moderate symptoms

20   appeared in one piglet, which recovered 7 days later. In the antibiotic untreated groups, the

21   health status of 6 piglets remained good over the entire experimental period. The differences

22   in piglet mortality between groups were defined as P=0.099 and were non-significant;

23   significant differences were found in morbidity between antibiotic-treated and untreated

24   piglets (P<0.05).

25

8

1 Gross examination of euthanized piglets from both treated and untreated groups revealed

2 oedema of the subcutis, particularly of the eyelids and mesocolon. Fibrin fibres were found on

3 the peritoneum in some piglets. Symptoms of haemorrhagic enteritis and colitis were

4 observed in one piglet with haemorrhagic diarrhoea from the antibiotic-treated group.

5 Inflammatory infiltrate of the intestinal wall, local desquamation of epithelium and apical

6 destruction of villi were found in the same piglet by histology. In the other piglets,

7 histological examination revealed oedema and presence of increased amounts of infiltrate of

8 the intestinal wall (lymphoid cells and polymorphonuclears), which reached the surface

9 epithelium without alteration. Inflammatory lesions were not seen. No bacterial layers were

10 detected by immunohistochemistry on small intestine mucosa, with the exception of a single

11 piglet from the antibiotic-treated group affected by hemorrhagic enteritis, in which numerous

12 *E. coli* O139 bacteria were present as well as destroyed mucosal tissue. Regarding the

13 character of intestinal damage, it was not possible to unambiguously specify whether bacteria

14 were attached to mucosa, or were present in mucus and detritus. The scanning electron

15 microscopy examination of mucosa from euthanized piglets showed results similar to those of

16 the immunohistochemical examination. Mucosa of jejunum and ileum was free from *E. coli*

17 plaques in the majority of piglets. If bacteria were present on mucosa, they were rare. Areas of

18 adhered bacteria were found in the caudal jejunum of one piglet only.

19

20

21 **Discussion**

22

23 It is generally assumed that STEC colonise the small intestine and produce Shiga toxin Stx2e,

24 which is absorbed into the bloodstream and distributed to other tissues. However, both the

25 intestinal colonisation by STEC and the toxin transport across the gut epithelium into the

9

1 bloodstream pose several issues. Concerning the gut colonisation, except for a single animal,

2 we did not observe any epithelium colonisation using histology and scanning electron

3 microscopy. No bacteria were found associated with the epithelium. Even in the only animal,

4 in which we found an epithelium colonisation, this was considerably different from the way

5 of colonisation typical e.g. for the enterotoxigenic *E. coli*. The colonisation was limited to

6 isolated areas and bacteria were present mostly in mucus. No bacterial plaques were found on

7 the surface of epithelial cells.

8

9 The exact mechanism of Stx2e absorption into the blood vessels is not well understood.

10 Although the toxin was found to bind to the surface membranes of the microvilli of the

11 jejunum, ileum and colon, and receptors for Stx2e were found in the intestine of the pig, their

12 role in absorption of Stx2e is unclear because the intraintestinal inoculation of pigs with large

13 quantities of Stx2e did not result in oedema disease's development (Waddell et al.*,* 1996).

14 Pathogenesis of oedema disease in pigs is still rather confusing and unclear.

15

16 Our results offer an alternative explanation for the pathogenesis of oedema disease in pigs.

17 Although we did not observe any significant adhesion to the epithelium, the STEC overgrew

18 other *E. coli* in the intestinal content because it was detected in faecal samples of both groups.

19 Due to the fact that the faecal shedding was essentially the same in both groups of animals,

20 this also confirmed that the parenterally administered colistin did not pass through the

21 intestinal barrier and therefore did not affect intraintestinal multiplication of the challenge

22 strain. In all antibiotic untreated piglets, colonisation of the mesenteric lymph nodes by the

23 challenge strain was observed. This may appear unexpected since STEC are non-invasive

24 extracellular bacteria and it is rather uncertain how the non-invasive *E. coli* could reach the

25 mesenteric lymph nodes. It remains unclear whether and how F18ab fimbriae may participate

1 in the passage of STEC through mucosa. Initially, we expected that the parenteral

2 administration of colistin might prevent multiplication of *E. coli* in the lymph nodes and

3 therefore also might prevent the clinical signs of the disease. However, the numbers of

4 diseased piglets in the antibiotic-treated group were higher, the course of the disease was

5 more severe and more animals died in comparison with the untreated group, despite the fact

6 that colistin was apparently efficient. A similar effect has been observed in human infections

7 caused by STEC O157:H7, where the course of the disease was more complicated under

8 antibiotic therapy (Carter et al., 1987; Ostroff et al., 1989). We therefore propose that the

9 STEC lysed by colistin suddenly released the toxin from bacterial cells immediately after

10 their passage through the intestinal wall, which would explain a more severe course of

11 oedema disease in the treated piglets. Even though high amounts of STEC were present in the

12 lymph nodes from untreated piglets, the toxin was not released as abruptly because the

13 bacterial cells were not damaged. Our results did not rule out the possibility that the Stx2e

14 toxin is transported through the intestinal wall directly from bacteria colonising the intestinal

15 tract. Nevertheless, we propose that there exists an additional route of Stx2e release to the

16 bloodstream from the bacteria transported through mucosa where they are subsequently lysed

17 either abruptly by the action of colistin or slowly by the activity of the host's immune system.

18 This allows the toxin to be released to the bloodstream and cause the clinical signs of oedema

19 disease.

20

21

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11

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3

4

5

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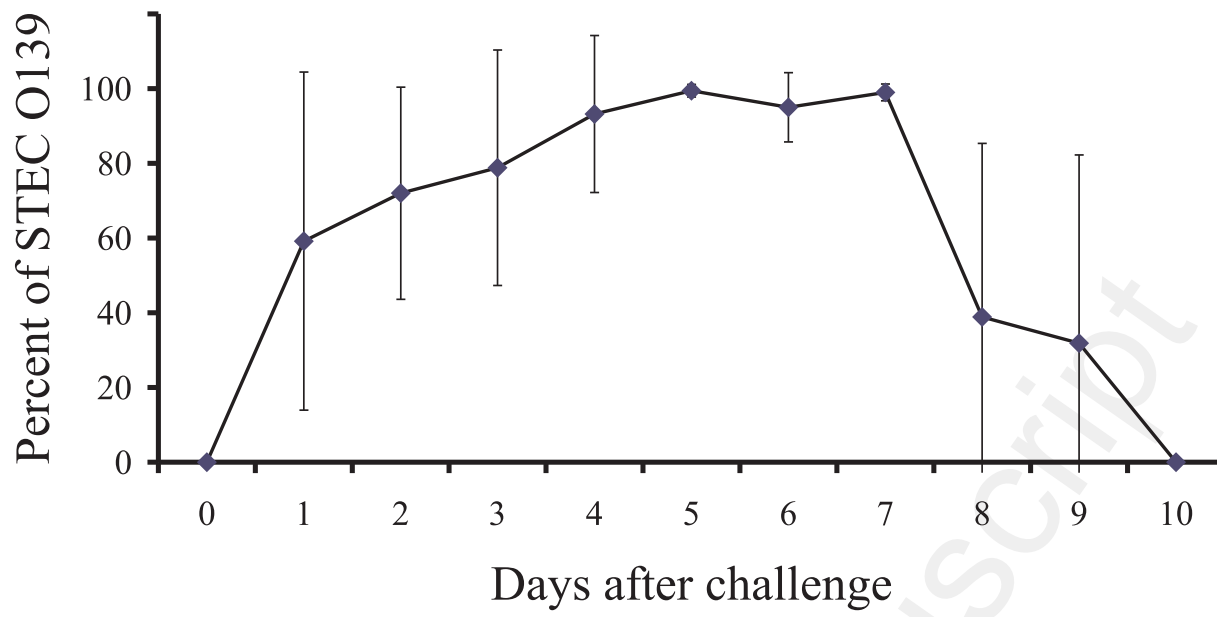
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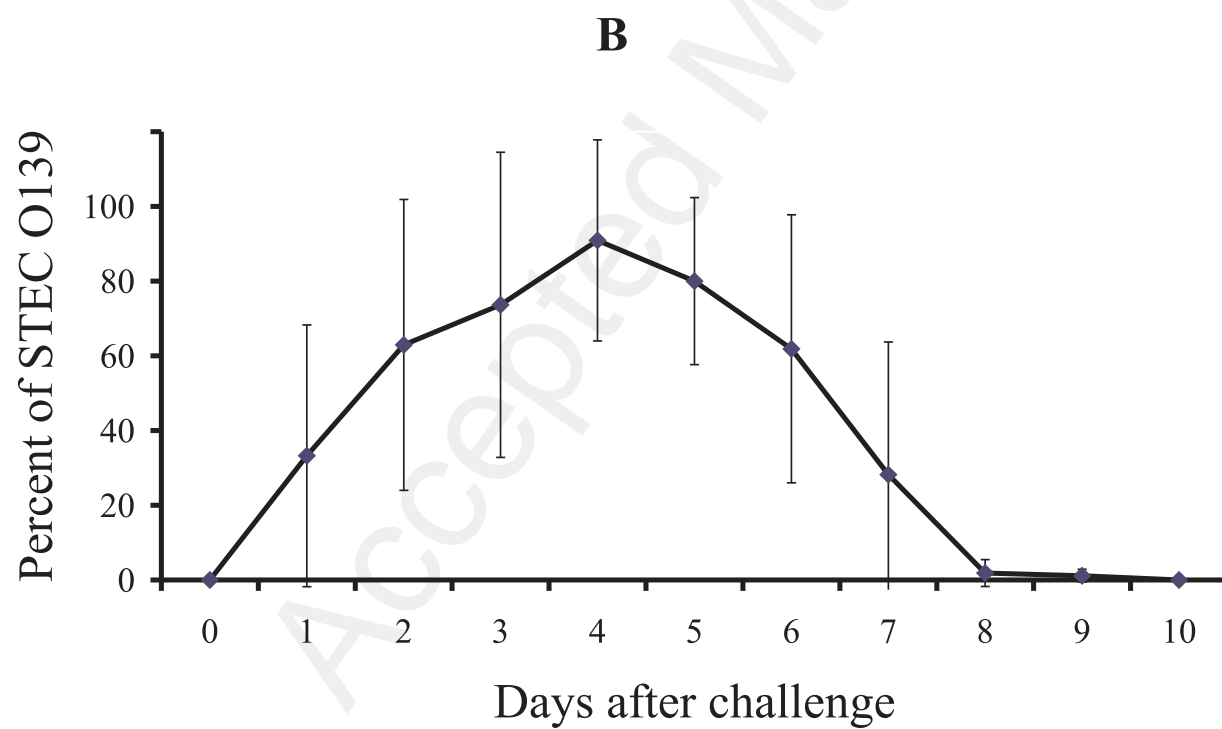
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14

1   Fig. 1: Percentage of STEC O139 in total amount of *E. coli* shedding through faeces from

2   piglets after challenge. The piglets were challenged on day 0 until day 3 with STEC

3   O139:F18ab, stx2e+ in a dose of 2 x 10^{11} CFU/day. A – antibiotic-treated group, B –

4   untreated group.

5

6   Fig. 2: Onset and duration of oedema disease in piglets after challenge. A – antibiotic-treated

7   group, B – untreated group. Only one and six piglets remained healthy during the trial in the

8   antibiotic-treated group and untreated group, respectively. Morbidity differences between

9   groups were P<0.05, mortality differences P>0.05.

15

A

B

A

B