Clinical efficacy of trimethoprim/sulfadiazine and procaine penicillin G in a *Streptococcus equi* subsp. *zooepidemicus* infection model in ponies

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Tissue chambers, implanted subcutaneously on both sides of the neck in eight ponies, were inoculated with *Streptococcus equi* subsp. *zooepidemicus* in order to compare the clinical efficacy of trimethoprim/sulfadiazine (TMP/SDZ) and penicillin G treatment in a purulent infection. The TMP/SDZ treatment consisted of one intravenous (i.v.) injection of 5 mg/kg TMP and 25 mg/kg SDZ and the same dose of TMP/SDZ per os (p.o.), both given 20 h after inoculation. The oral dose was then repeated every 12 h for 21 days. The penicillin treatment consisted of one i.v. injection of 20 000 IU/kg sodium penicillin G and intramuscular (i.m.) injection of 20 000 IU/kg procaine penicillin G, both given 20 h after infection. The i.m. dose was then repeated every 24 h for 21 days. Eight ponies, each with two tissue chambers, were used in a cross over design; in the first experiment the left tissue chamber (TC) was infected and in the second experiment the right. TMP/SDZ treatment resulted in a limited reduction of viable bacteria in the TC but did not eliminate the infection, resulting in abscessation in 10–42 days in all eight ponies. However, penicillin treatment eliminated the streptococci in seven of eight ponies, and only one pony suffered abscessation on day 10. This constitutes a significantly better efficacy of the penicillin treatment in this model. The most probable cause of the failure of TMP/SDZ to eliminate the streptococci is inhibition of the action of TMP/SDZ in the purulent TCF. Therefore, TMP/SDZ should not be used to treat purulent infections in secluded sites in horses.

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INTRODUCTION

Trimethoprim/sulfonamide combinations (TMPS) are often used for antibacterial treatment in horses because of their broad spectrum of activity and the convenience of oral administration (van Duijkeren *et al.*, 1994). Many bacteria are susceptible to TMPS *in vitro* and this includes *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*), a major pathogen in horses (Ensink *et al.*, 1993). The mechanism of action of sulfonamides is inhibited by an excess of *p*-aminobenzoic acid (PABA), such as found in tissue exudates (Prescott & Baggot, 1993). Thymidine is known to diminish the susceptibility of bacteria to trimethoprim (TMP) *in vitro* (Prescott & Baggot, 1993), and Greko *et al.* have shown that thymidine may also play a role in antagonizing the effect of TMPS *in vivo* (Greko *et al.*, 2002).

To determine whether TMPS is effective against *S. zooepidemicus in vivo* would require a study in patients. In horses this poses a problem, because it is rare to have a sufficient number of naturally infected animals available for these studies. Therefore, most studies are performed with standardized experimental infections. For this purpose the tissue chamber (TC) infection model has proved very useful (Beadle *et al.*, 1989; Ensink *et al.*, 1996a).

Before using TMPS in the TC infection model the concentrations of TMP and sulfadiazine (SDZ) that can be reached in tissue chambers with the proposed dose were evaluated. For this purpose a pharmacokinetic study was performed in healthy ponies that had tissue chambers implanted. The results of this study showed that this dose of 5 mg/kg TMP and 25 mg/kg SDZ, administered orally, produces concentrations in tissue chamber fluid (TCF) above the minimum inhibitory concentration (*MIC*) of *S. zooepidemicus* for the duration of the dosing interval (van Duijkeren *et al.*, 2002). Penicillins are often used to treat horses suffering from streptococcal infections. Therefore, intramuscular (i.m.) administration of procaine penicillin G was chosen as the control treatment in the infection model. Based on earlier

experiments, this treatment was expected to be effective against *S. zooepidemicus* in this infection model (Ensink *et al.*, 1996a,b).

The objective of our study was to compare the clinical efficacy of TMP/SDZ and procaine penicillin G in a *S. zooepidemicus* infection model in ponies.

MATERIALS AND METHODS

Ponies

Eight Shetland ponies (six stallions and two geldings) were used. The ponies were 2–13 years old and weighed 125–164 kg. Two tissue chambers (TCs), one on each side of the neck, just rostral to the scapula, had been implanted subcutaneously (Beadle *et al.*, 1989) in each pony. Eight weeks after implantation of the tissue chambers the ponies were used in the pharmacokinetic study on the distribution of TMPS into TCs (van Duijkeren *et al.*, 2002). Seven weeks later the left TC was used for the first infection, and the second infection, in the right TC, followed seven weeks after the first. The ponies were housed in individual box stalls with free access to water. They were fed 400 g of concentrates and a maintenance ration of grass silage twice daily.

Inoculation

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) originally isolated from a clinical case of endometritis was used as the inoculum. This organism was identified using the API 20 Strep system (BioMerieux sa, Marcy l' Etoile, France). The *MIC*s for this strain were 1 μg/mL of TMP alone, 16 μg/mL of SDZ alone, and 0.06/1.2 μg/mL when TMP/SDZ were used in combination. The *MIC* of penicillin G was ≤ 0.015 μg/mL (Ensink *et al.*, 1993). The organism had been stored at −70 °C in brain heart infusion (BHI) (Oxoid, Basingstoke, UK) and glycerine (1:1). To prepare the inoculum the organism was thawed and cultured in BHI at 37 °C in two stages for a total of 24 h. The overnight culture was centrifuged, the bacterial pellet was washed twice with 0.9% saline, and the organism was resuspended in phosphate-buffered saline (PBS, pH 7.2). A dilution in PBS was made to provide a concentration of approximately 1×10^6 cfu/mL. Two milliliters of this preparation were inoculated into each TC. The actual number of cfu inoculated per TC, confirmed by counting cfu after serial dilutions, was 1.1×10^8 in experiment 1, when all ponies had the left TC infected, and 5.4×10^7 in experiment 2, when all ponies had the right TC infected.

Medication

Eight ponies, each with two tissue chambers, were used in a cross over design, so that there were eight ponies for each treatment.

The TMP/SDZ treatment consisted of one intravenous (i.v.) injection of 5 mg/kg TMP and 25 mg/kg SDZ (Diatrim; Eurovet, Bladel, The Netherlands) and the same dose of TMP/SDZ p.o. (Sultrisan Orale Pasta, Anisane Pet Health Products, Raamsdonksveer, The Netherlands) both given 20 h after inoculation. The oral dose was then repeated every 12 h for 21 days. The ponies ate their concentrates immediately before oral administration and their grass silage was supplied immediately afterwards.

The penicillin treatment consisted of one i.v. injection of 20 000 IU/kg sodium penicillin G (Benzylpenicilline-Na; Eurovet) and i.m. injection of 20 000 IU/kg procaine penicillin G (Depocilline 300 000 IU/mL; Mycofarm, de Bilt, The Netherlands) both given 20 h after infection. The i.m. dose was then repeated every 24 h for 21 days. The daily dose (9–11 mL, depending on the body weight) was administered at a single injection site. Six different injection sites were used (the left and right pectoralis descendens muscle, the left and right semitendinosus muscle and the left and right biceps femoris muscle) so that the interval between subsequent injections in the same muscle was 6 days.

Evaluation

Clinical evaluation of the animals included taking the rectal temperature twice a day, checking for swelling around the TC, and making a note of the behaviour and appetite. TCF was sampled on days 0, 2, 8, 15, 21, and 29 by aspiration of 2 mL of TCF with a 5 mL syringe and a 18G needle. The samples were divided into aliquots for bacteriology and cell counting.

Aspirates of 0.5 mL TCF were taken for colony counts. The TCF was immediately added to 4.5 mL BHI and held at 4 °C for a maximum of 4 h. Colony counts were determined by a plate count method using 10-fold dilutions in 0.9% saline of the TCF in BHI. The detection limit of this plate count was 1×10^2 cfu/mL of TCF. For the purpose of calculation, samples with a count of $<1 \times 10^2$ cfu/mL were arbitrarily set at 10^0 cfu/mL. Furthermore, samples of 1 mL of TCF were taken from each TC on two occasions to check for contaminants. When organisms were cultured from TCF their identity was confirmed as *S. zooepidemicus* by checking for *β*-haemolysis, confirming the Lancefield group as group C with the Streptex system (Murex Diagnostics Ltd, Dartford, UK) and determining fermentation of lactose, sorbitol and salicin but not trehalose.

Furthermore, *S. zooepidemicus* recovered from tissue chambers were tested to determine the *MIC* of the bacteria for TMP, SDZ and penicillin G in the Etest (AB Biodisk, Solna, Sweden). For this test the organism was cultured in BHI, the overnight culture was diluted 1 in 1000, and 0.1 mL of this inoculum was applied to IsoSensiTest agar plates (Oxoid; diameter 14 cm) with 5% blood of sheep. Then the Etest strips were applied to the plate and the plates were incubated for 24 h at 37 °C. The resulting inhibition zones were read according to the manufacturer's instructions.

Total WBC counts in TCF were performed using a Coulter counter (type Industrial D, Coulter Counter, Luton, UK).

Treatment of abscessation

When antibiotic treatment in the TC model is not successful, abscessation of the TC occurs: the infection breaks through the

skin at the site of the scar from the implantation. In these cases the TC was removed in the standing animal using sedation with detomidine 10 μg/kg, (Domosedan; Orion Pharma Corporation, Espoo, Finland) and nalbufine 0.1 mg/kg (Nubain; Schering-Plough, Amstelveen, The Netherlands) and local analgesia (lidocaine HCl 2%, 20 mL; Eurovet). If no abscessation occurred during the experiment, ponies were monitored for at least another 14 weeks.

Data analysis

Differences in the efficacy of treatment, measured by the period from inoculation to abscessation, were subjected to survival analysis using the Kaplan–Meier test (SPSS computer-program; SPSS, Inc., Chicago, IL, USA). P values <0.05 were considered significant.

RESULTS

Infection of the TC resulted in fever (rectal temperature above 38.5 °C) and a painful swelling around the TC that was first seen between 12 and 18 h after inoculation. All ponies were reluctant to move the neck and some showed a swinging limb lameness of the forelimb. The ponies were slightly depressed, but they continued to eat their rations. These signs resolved within a week and no difference between the treatment groups became apparent in this period. No difference in reaction to the infection was seen between the first and second infection (both clinically and in the laboratory evaluations), so all data were pooled to be shown as a group of eight ponies on TMP/SDZ treatment and a group of eight ponies on penicillin G treatment.

Abscessation of the TC occurred in all the eight ponies on TMP/SDZ treatment, and one of the eight ponies on penicillin treatment. In these cases the TC was removed as described in Materials and methods. All abscesses healed quickly and uneventfully. The seven ponies that still had a TC at the end of the experiments were monitored for at least another 14 weeks. In this period no signs of infection were encountered. Table 1 shows the number of ponies that suffered abscessation in each treatment group, and the number of days post-infection that the abscess broke through the skin.

Penicillin G treatment caused a marked decrease of viable bacteria in the TC, while in the TMP/SDZ-treated group the number of cfu decreased only slightly and then rose again (Fig. 1). In ponies treated with penicillin, four ponies yielded TCF with no viable bacteria on day 8 and seven did so on day 15.

Table 1. Clinical efficacy of antibiotic treatments measured by prevention of abscessation

Treatment	*n*	No. of ponies showing abscessation	Day of abscessation
TMP/SDZ	8	8*	10–42
Penicillin	8	1*	10

*Difference between the groups were significant ($P < 0.001$).

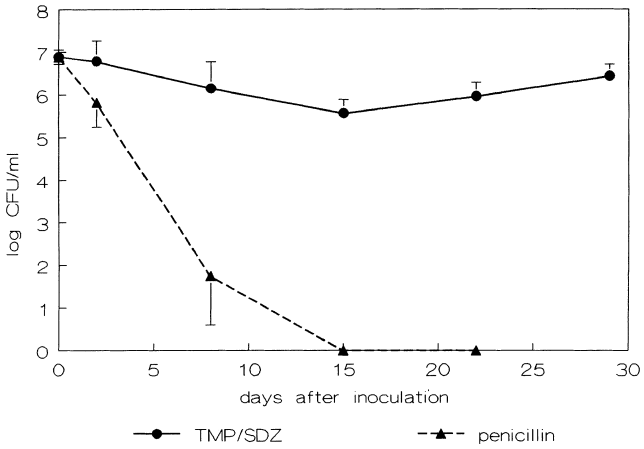

Fig. 1. Bacterial count in TCF in log cfu/mL (mean ± SD). TMP/SDZ (—•—) and penicillin (- - - - ▲ - - - -).

Despite the decrease in cfu, one pony on penicillin treatment suffered abscessation during treatment. In this pony the course of the infection was unusual. The bacterial count declined as quickly as in the other penicillin-treated ponies, with the number of cfu per milliliter below the limit of detection on day 8, but on day 10 the infection broke through skin. As the fistula was small, and the discharge of TCF less than usual, the TC was left in place for the time being. The fistula healed and the TC yielded sterile TCF at days 15 and 22. Nevertheless on day 57 abscessation occurred and aspiration of TCF just before removal of the TC yielded high numbers of *S. zooepidemicus*. Day 10 was counted as the day of abscessation in this pony and the results from later TCF samples were not included in the tables. In the other seven ponies on penicillin treatment swelling and fever had subsided by the end of treatment. All eight ponies on TMP/SDZ showed abscessation between days 10 and 42. The difference in the number of ponies suffering abscessation between the TMP/SDZ-treated group and the penicillin-treated group was significant ($P < 0.001$).

Table 2 shows the WBC count in TCF. The WBC count rose quickly in both treatment groups to levels above 100×10^9 cells/l. These large numbers of white blood cells are characteristic of a purulent infection. There was a large variation between individual animals.

Table 2. White blood cell count in TCF (10^9 cells/L)

	TMP/SDZ treatment		Penicillin treatment	
Day	*n*	Mean ± SD	*n*	Mean ± SD
0	8	1.4 ± 1.3	8	2.1 ± 2.5
2	8	172.2 ± 73.8	8	162.3 ± 79.4
8	8	144.9 ± 85.5	8	77.5 ± 38.6
15	3	239.7 ± 116.2	7	121.9 ± 84.9
22	3	274.3 ± 76.3	7	142.2 ± 119.9
29	3	ND1	7	ND2
69	0	ND3	7	16.2 ± 26.5

ND1: sample taken for bacteriology only, no cell count performed.
ND2: tissue chamber present but no sample taken.
ND3: no tissue chamber present.

Table 3. Minimum inhibitory concentration (*MIC*) (mean ± SD) of bacteria recovered from tissue chambers in μg/mL

	TMP/SDZ treatment				Penicillin treatment			
Day	*n*	TMP	SDZ	Penicillin	*n*	TMP	SDZ	Penicillin
0	3*	0.75 ± 0.0	13.3 ± 2.3	0.012 ± 0.0	3*	0.75 ± 0.0	13.3 ± 2.3	0.012 ± 0.0
2	8	0.75 ± 0.0	11.5 ± 1.4	0.012 ± 0.0	8	0.75 ± 0.19	13.0 ± 3.5	0.012 ± 0.001
8	8	0.78 ± 0.09	12.0 ± 2.1	0.012 ± 0.0	8	0.72 ± 0.16	12.0 ± 3.0	0.012 ± 0.0
15	3	0.75 ± 0.0	9.3 ± 2.3	0.012 ± 0.0	7	Neg	Neg	Neg
22	3	0.67 ± 0.14	10.7 ± 4.6	0.012 ± 0.0	7	Neg	Neg	Neg
29	3	0.75 ± 0.0	10.7 ± 2.3	0.012 ± 0.0	7	ND	ND	ND

*The strain used for inoculation was tested in triplicate.
ND, no sample taken; Neg, no bacteria cultured.
Note: The *MIC*s given for TMP and SDZ are for the individual antimicrobial drugs, the *MIC*s of these drugs when tested in combination for this strain of *S. zooepidemicus* are much lower: 0.06 μg/mL for TMP and 1.2 μg/mL for SDZ (Ensink *et al.*, 1993).

The *MIC* values of penicillin G, TMP and SDZ were estimated for *S. zooepidemicus* recovered from tissue chambers during the experiments, using an agar diffusion susceptibility test (E-test, AB Biodisk), and are shown in Table 3. The organism was recovered from all ponies in the TMP/SDZ group up to the day of abscessation and from all ponies in the penicillin group on day 2 and on day 8, although on day 8 the number was below 100 cfu/mL in four of the eight ponies. The *MIC*s of penicillin, TMP and SDZ of the organism recovered from the different treatment groups remained unchanged during the experiment and were in accordance with the results obtained with the same strain of *S. zooepidemicus* when tested in the agar dilution test (Ensink *et al.*, 1993).

DISCUSSION

The objective of this study was to compare the clinical efficacy of oral TMP/SDZ as compared with intramuscular procaine penicillin G, both administered for 3 weeks. TMP/SDZ resulted in an initial reduction of viable bacteria, whereas in untreated animals, only increasing numbers of bacteria can be expected (Ensink *et al.*, 1996a). However, from day 15 the number of bacteria rose again and TMP/SDZ treatment failed to eliminate the infection in all eight ponies. Penicillin treatment caused a larger reduction of viable bacteria in the TC and eliminated the infection in seven of the eight ponies. This constitutes a significantly better clinical efficacy of penicillin, when compared with TMP/SDZ. In earlier studies with the infection model, failure to eliminate the infection has been encountered only with treatment for 1 week or less (with cephapirin: Beadle *et al.*, 1989; or pivampicillin or ampicillin: Ensink *et al.*, 1996a).

The TMP/SDZ-treatment used in this study resulted in failure: streptococci survived despite treatment of the animals with an appropriate dose of antibacterial drugs to which the streptococci are susceptible. The dosing regimen used in this study should be considered adequate. As soon as symptoms of infection were apparent an i.v. injection of TMP/SDZ was given to reach high concentrations quickly, and they were maintained for 21 days by oral administration q12h. This dosage regimen should ensure concentrations above *MIC* in the TCF from 4 h after the initiation of treatment (van Duijkeren *et al.*, 2002). Failure to eliminate the pathogen despite initial improvement during antimicrobial treatment has also been encountered in other studies with infection models in horses using penicillin, TMP/SDZ and cephapirin (Varma *et al.*, 1982; Bertone *et al.*, 1987; Beadle *et al.*, 1989). There are a number of possible explanations for the survival of streptococci: lower concentrations of TMP and SDZ in infected than in uninfected tissue chambers, sequestration in inflammatory exudate, an increase in *MIC* during antibiotic treatment, or a higher *MIC* of streptococci in infected TCF than in the *in vitro* medium.

From the study on the distribution of TMP and SDZ into subcutaneous tissue chambers (van Duijkeren *et al.*, 2002) the concentrations in uninfected tissue chambers are known. As the *MIC* for the *S. zooepidemicus*-strain used in this study is as low as 0.06/1.2 μg/mL of TMP/SDZ, concentrations in uninfected TCs remain above 10 times *MIC* during treatment. Data of TMP and SDZ concentrations in infected TCs in horses are currently lacking but there are research data that allow a prediction of these concentrations. One factor that may influence concentrations in infected TCs is the fact that the pH in infected TCs is lower, decreasing to 6.9 on day 3, than in uninfected TCs where pH is 7.2 (Ensink *et al.*, 1996a). This low pH in infected TCF would lead to low concentrations of SDZ, which is a weak acid (with pKa 6.4), but higher concentrations of TMP, which is a weak base (with pKa 7.6). Clarke *et al.* (1989) compared TMP and SDZ concentrations in infected and uninfected tissue chambers in cattle, and found higher concentrations of both compounds in infected TCF. The possible explanations for the higher concentrations stated in this study were the higher total protein concentration in infected TCF and the decreased diffusional barrier in inflamed tissues. Also, in horses, Bertone *et al.* (1988) showed that TMP/SDZ, in the same oral dose as the dose used in our study, produced adequate concentrations in joints experimentally infected with *Staphylococcus aureus*. On the strength of these data we assume that concentrations in infected tissue chambers, too, were above *MIC* for the *S. zooepidemicus*.

A second reason for treatment failure may be sequestration of streptococci in inflammatory exudate (fibrin clots), which protects them from the antibiotics. There are no data available to

quantify the influence that fibrin clots may have on the ability of the individual antimicrobial drugs to reach the bacteria, but in general this would have played a similar role in both treatment groups.

The third explanation for treatment failure could be an increase in *MIC* of the streptococci during treatment. Therefore, the susceptibility of the *S. zooepidemicus* to penicillin, TMP and SDZ was monitored throughout our experiment. *MIC*s of TMP and SDZ were not increased when determined at the end of the experiment. Also we did not find increased *MIC*s of penicillin in ponies treated with penicillin. This is in contrast with earlier experiments where the *MIC* of ampicillin did seem to increase during treatment (Ensink *et al.*, 1996a). In this study we found no indication that selection for less susceptible individuals in the inoculum does occur in animals treated with either TMP/SDZ or penicillin.

The most probable cause of treatment failure of TMPS is inhibition of the action of TMPS by PABA in the TCF. The *MIC* values of TMP/SDZ for the *S. zooepidemicus* used in this study have been determined *in vitro*, on IsoSensiTest agar with 5% horse blood (Ensink *et al.*, 1993). In *in vitro* susceptibility tests *MIC*s may vary with the medium used. Therefore, it is possible that the *MIC*s would be different if TCF were used as an *in vitro* medium (Woolcock & Mutimer, 1983). Determination of *MIC*s both on traditional culture media and in TCF are useful to provide a link between *in vitro* susceptibility of bacteria and their reaction to antimicrobial drugs in an infection model (Shojaee Aliabadi & Lees, 2002). This would be especially true for TMPS where high concentrations of PABA or thymidine will counteract the competitive inhibition of the biosynthesis of tetrahydrofolate coenzymes necessary for bacterial metabolism (Greko *et al.*, 2002).

In the penicillin-treated group, abscessation of the TC occurred in only one pony. In this pony the course of the infection was unusual: the bacterial count declined quickly, with the number of cfu per milliliter below the limit of detection on day 8, but on day 10 the infection broke through skin. As the fistula was small, and the discharge of TCF less than usual, the TC was left in place for the time being. The fistula healed and the TC yielded sterile TCF at days 15 and 22. Nevertheless on day 57 abscessation occurred and aspiration of TCF just before removal of the TC yielded high numbers of *S. zooepidemicus*. A possible explanation for the recurrence of the infection may be that the bacteria were eliminated from the lumen of the TC that is sampled, but they survived in a space that was initially closed off from the lumen of the TC and later spread to this lumen. The reason that the penicillin treatment was unable to eliminate the *S. zooepidemicus* in this space may be that the bacteria survived in necrotic tissue.

The dose of procaine penicillin G used in this study was the dose commonly used in our clinic, 20 000 IU/kg once a day. This is lower than the advised dose, which is 20 000 IU/kg twice a day (Sweeney & Boy, 1993). However, an earlier study showed that administration of procaine penicillin once daily produces stable steady state concentrations in TCF and that a dose as low as 12 000 IU/kg produces concentrations of 10 times *MIC* for the duration of the treatment (Ensink *et al.*, 1996b) and can be effective against *S. zooepidemicus* in the infection model (Ensink *et al.*, 1996a). Therefore, we did not administer the procaine penicillin twice a day so as not to increase the discomfort to the animals from i.m. injections. The results from this study show that, for very susceptible bacteria like *S. zooepidemicus*, administering procaine penicillin once daily is adequate.

Earlier research has shown that a treatment with pivampicillin p.o. is able to completely eliminate the streptococci from the TC, when given for 3 weeks (Ensink *et al.*, 1996a). In the present study, treatment with penicillin by i.m. injection for 3 weeks also proved successful in seven of the eight animals. TMP/SDZ, on the contrary, failed to eliminate the *S. zooepidemicus* from the purulent TCF, resulting in treatment failure in all the eight ponies. Therefore, TMP/SDZ should not be used to treat infections involving a bacterial pathogen in a lumen of purulent fluid, such as an abscess, arthritis or pleuritis.

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