Efficacy of intramammary treatment with procaine penicillin G vs. procaine penicillin G plus neomycin in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria – a double blind field study

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The efficacy of intramammary treatments containing procaine penicillin G alone (treatment A) or a combination of procaine penicillin G and neomycin (treatment B) was compared in treating clinical bovine mastitis caused by gram-positive bacteria susceptible *in vitro* to penicillin G. Both treatments were supplemented with a single intramuscular injection of procaine penicillin G on the first day of treatment. The study was carried out using a double blind design on commercial dairy farms in Southern Finland. A total of 56 quarters were treated with treatment A and 61 with treatment B. The cure rates for both treatments were equal, which suggests that the use of the penicillin G–aminoglycoside combination does not increase the efficacy of the treatment over that achieved by using penicillin G alone in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria.

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INTRODUCTION

Aminoglycosides have been used since 1960 in the treatment of mastitis, mostly combined with penicillin G or other β-lactam antimicrobials (Whittem & Hanlon, 1997a). Theoretical grounds for the use of these combinations have been their wide spectrum and expected synergistic action against certain gram-positive bacteria. Synergies gained by using the combination have been well documented in the treatment of enterococcal infections in humans but not in other infections (Eliopoulos & Moellering, 1996). In principle, in order to obtain synergistic action the target bacteria should be susceptible *in vitro* to aminoglycosides (Eliopoulos & Moellering, 1996; Prescott, 2000a). The superiority of the combination over the use of a β-lactam alone in the treatment of mastitis has never been proved in clinical trials (Whittem & Hanlon, 1997a). In the US, combination preparations of this type were withdrawn from the market many years ago, due to the US Food and Drug Administration's strict position on this issue (Anonymous, 1993). In the European Union and elsewhere, intramammary treatments containing a fixed combination of β-lactam and aminoglycoside antibiotics are widely used (Whittem & Hanlon, 1997a).

Aminoglycosides are known to produce long-lasting residues in tissues, particularly in the kidneys of treated animals, which makes the use of these substances problematic in the treatment of food animals (Nouws & Ziv, 1978; Erskine *et al.*, 1992; Whittem & Hanlon, 1997b). Unnecessary use of antibiotic combinations may increase selection pressure for antibiotic resistance (Whittem & Hanlon, 1997b; Østerås *et al.*, 1999). If no clinical benefits are obtained from the use of a fixed combination of aminoglycoside and β-lactam antibiotics in treating mastitis, the aminoglycoside components could be removed from the preparations. This would diminish the risk of drug residues in milk and tissues, and reduce unnecessary use of wide-spectrum preparations in routine treatment of clinical mastitis.

The aim of this field study was to compare the efficacy of intramammary treatments with penicillin G alone with that of combination treatments with penicillin G and neomycin in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria.

MATERIALS AND METHODS

The study was carried out on commercial dairy farms in the practice areas of the Ambulatory Clinic of the Faculty of Veterinary Medicine and four municipal veterinarians in Southern

Finland during the years 1999–2000. Farmers contacted the veterinarians of the Ambulatory Clinic and the municipal veterinarians participating in the trial when a case of mastitis was detected in the herd. The attending veterinarian examined the cow clinically and estimated milk somatic cell count (SCC) using the California Mastitis Test (CMT). The history of the cow (identity, age, stage of lactation, etc.) was recorded. Local and systemic clinical signs, including rectal temperature and milk appearance, were observed and recorded on a form. Using the notes on the forms the signs were scored from 1 to 3, where 1 = clots and flakes seen in the milk but no other signs, 2 = body temperature 39.0–40.5 °C and/or slight anorexia/depression, swelling and/or tenderness in the affected quarter and moderate changes in milk appearance, and 3 = body temperature >40.5 °C and/or severe anorexia and depression and/or recumbent, severe swelling, firmness and soreness in the quarter and severe changes in the milk appearance. For statistical analyses, scores 2 and 3 were grouped together: 1 = mild signs, and 2 and 3 = moderate/severe signs. Before treatment, the veterinarian took an aseptic milk sample from the affected quarter(s) for bacteriological examination and *N*-acetyl-β-D-glucosaminidase (NAGase) activity determination. Cows with concomitant systemic disease, teat lesions or chronic mastitis, i.e. mastitis, which had persisted over a dry period, or had been treated at least two times during same lactation or had caused elevated SCC for a long period, i.e. months, were excluded from the trial.

Milk samples were cultured for bacteriological diagnosis in the laboratory of the Ambulatory Clinic by routine methods (Honkanen-Buzalski & Seuna, 1995). Production of β-lactamase by staphylococcal isolates was tested using a nitrocephin test (Myllys, 1995). Because of the study design and the requirement to conduct the study on commercial dairy farms, mastitis caused by penicillin-resistant organisms (penicillin-resistant staphylococci and coliform bacteria) were excluded, although they might have been susceptible to neomycin. NAGase activity of the milk samples taken at the day of diagnosis and at the follow-up visit 3–4 weeks post-treatment were determined as described earlier (Pyörälä & Pyörälä, 1997).

The study was carried out using a double blind design. Intramammary treatments, either with penicillin G (treatment A: Carepen®, procaine penicillin G 600 000 IU; Vetcare Oy, Salo, Finland) alone or with a combination of penicillin G and neomycin (treatment B: Neomast®, procaine penicillin G 500 000 IU and 300 mg neomycin; Pfizer GmbH, Freiburg, Germany), were administered to each diseased quarter once daily for four consecutive days. Both treatments were supplemented once by the attending veterinarian with procaine penicillin G (Penovet®, procaine penicillin G 300 mg/mL; Boehringer Ingelheim, Copenhagen, Denmark) 20 mg/kg body weight intramuscularly at the beginning of the treatment. Treatment was randomized according to cow identity numbers; cows with even numbers were given treatment A and those with odd numbers treatment B.

The outcome of the treatment was assessed 3–4 weeks (average 26 days) after the beginning of the treatment. On the follow-up visit, the cow was examined clinically and with CMT, and aseptic milk samples were taken from the affected quarter(s). Criteria to assess cure from mastitis were used as described by The European Agency for the Evaluation of Medicinal Products (EMEA, 2000). Bacteriological cure was assessed based on the results of the post-treatment milk samples: a quarter was classified as bacteriologically cured if no bacterial growth was found in the post-treatment milk sample. Quarters with growth of another bacteria than the original one (three cases in group A and eight in group B) were also classified as bacteriologically cured. Cure from inflammation was based on NAGase activity <40 U in the post-treatment milk sample (Pyörälä & Pyörälä, 1997). Clinical cure was assessed by clinical examination and a quarter was classified as clinically cured, if no systemic or local signs were detected and milk appearance was normal. In 11 cases growth of another bacteria interfered with clinical cure and these cases were classified as clinically cured, because the clinical signs in these cases most probably were caused by a new infection. Cure rates were calculated in which different criteria of cure were combined. The combinations, like clinical and bacteriological cure, were classified as cured, if both of the combined cure rates were classified as cured. Cure based on meeting all criteria (clinical and bacteriological cure and NAGase <40 U) was defined as complete cure.

The original material consisted of 167 quarter cases of clinical mastitis. The 50 excluded quarter cases were as follows: 19 quarters with mixed growth or no growth, six quarters with growth of coliform bacteria, one with *Enterococcus* sp., 19 with β-lactamase positive staphylococci and five quarters lacking information on follow-up visits and milk samples. The distribution of the mastitis-causing bacteria in the remaining 117 quarters in 96 cows from 68 farms was as follows: *Staphylococcus aureus* 19, coagulase-negative staphylococci (CNS) 28, *Streptococcus dysgalactiae* 24, and *Str. uberis* 46. All the isolated gram-positive bacteria included in the study were susceptible *in vitro* to penicillin G. Fifty-six quarters were treated with treatment A and 61 with treatment B. The mean or median values of age and stage of lactation of the cows and severity of mastitis determined with clinical criteria are shown in Table 1. Because of large amount of missing data, the results of CMT-testing were not used as criteria for severity of inflammation or cure from inflammation.

Statistical differences in the cure rates between the treatment groups were tested using logistic regression. Because of the small number of farms or cows, which appeared more than once in the material (17 of 68 farms had more than one cow in the material and 22 of 95 cows had more than one inflamed quarter), cows from the same farm, different quarters from the same cow and treatment by the same veterinarian were treated as if they were independent observations. The statistics were run with equal results also in a material, where only one cow per farm and one quarter per cow was included (Beaudeau *et al.*, 1996). The factors initially included in the model were the treatments (A or B), parity (first or subsequent), infecting organism (*S. aureus*, CNS, *Str. dysgalactiae*, or *Str. uberis*), and stage of lactation (1–60 days postpartum or >60 days postpartum).

Table 1. Descriptive statistics of the study cows and severity of mastitis determined with clinical criteria in treatment group A (intramammaries containing 600 000 IU procaine penicillin G) and B (intramammaries containing 500 000 IU procaine penicillin G and 300 mg neomycin). Both treatments were supplemented with procaine penicillin G 20 mg/kg body weight intramuscularly once on the first day of treatment

Variable	Treatment A	Treatment B
Number of quarters	56	61
Median age of the cow, years	3.0	4.0
Percentage in first lactation	25.5	18.6
Median days in milk	37.0	29.0
Percentage clinical signs, moderate or severe	71.4	63.4
Percentage with a raised body temperature	8.9	9.8
Mean clinical sign score at the day of diagnosis	1.5	1.5
Mean milk NAGase activity at the day of diagnosis	338 U	451 U
Min. and max. values of milk NAGase at the day of diagnosis	8–2310	23–2720

The effects of adding clinical signs (score mild or moderate/severe, presence of elevated body temperature or milk NAGase value) to the model were then assessed using a likelihood ratio test. Finally the model was further reduced with nonsignificant variables (stage of lactation). Parity, even nonsignificant, was left in the model, because cure rates in *S. aureus* mastitis differ between first and subsequent parities (Pyörälä *et al.*, 2000), and in the final model, treatment, parity and infecting organism were included. The model was tested with and without the interaction terms between treatment groups and infecting organisms. All the different cure rates were tested separately and in combination with each other (Table 2). Statistical differences between the treatment groups in the cure rates of mastitis caused by different bacteria were tested using Fisher's exact chi-square test. The similarity of the two treatment groups was tested using chi-square test. The groups were not statistically different.

RESULTS

The cure rates for the treatment group A and B are presented in Table 2. Cure rates are shown in Table 3 according to the causing organism. The treatment did not affect any of the cure rates (bacteriological, clinical or cure based on NAGase value) (Table 4). The log-likelihood ratio of the models with and without the interaction terms was not statistically significant ($P = 0.105$), which means that the effects of treatments were not statistically different within the groups of the infecting organisms. The bacteriological and clinical cure rates of CNS mastitis were significantly higher than those of *S. aureus* mastitis, and the cure rate based on NAGase value in *Str. dysgalactiae* mastitis was significantly higher compared with *S. aureus* mastitis (Table 4).

DISCUSSION

The cure rates in clinical mastitis caused by penicillin-susceptible gram-positive agents using penicillin G alone or combined with neomycin were equal, which implies that use of the combination treatment does not increase the efficacy of the treatment. Similar results were found in a recent study by Ødegaard and Sviland (2001), where intramammary therapies containing penicillin G alone or penicillin G and another aminoglycoside, dihydrostreptomycin, were compared. In that study, 218 quarters infected with bacteria susceptible to penicillin G *in vitro* were treated with intramammaries containing either 300 000 IU of procaine penicillin G and 250 mg dihydrostreptomycin, 300 000 IU of procaine penicillin G alone or 500 000 IU of procaine penicillin G alone. No statistical differences in the cure rates between the treatment groups were observed. Several field studies were conducted in which a penicillin treatment in clinical and subclinical mastitis was compared with a penicillin–aminoglycoside combination in an open study design or even in a nonrandomized set-up (Postle & Natzke, 1974; Pyörälä & Syväjärvi, 1987; Jarp *et al.*, 1989; McDougall, 1998). None of these studies showed any advantage in using the combination over using penicillin G alone.

The main reason for the use of β-lactam–aminoglycoside combinations is its wide spectrum, where the aminoglycoside component is thought to be effective against coliform bacteria. The high spontaneous cure rate known in coliform mastitis

Table 2. The cure rates of clinical mastitis caused by gram-positive bacteria susceptible *in vitro* to penicillin G in treatment group A (intramammaries containing 600 000 IU procaine penicillin G) and B (intramammaries containing 500 000 IU procaine penicillin G and 300 mg neomycin). Both treatments were supplemented with procaine penicillin G 20 mg/kg body weight intramuscularly once on the first day of treatment

Cure rates	Treatment A		Treatment B		
	Quarters	Cured *n* (%)	Quarters	Cured *n* (%)	*P*-value
Clinical cure	56	42 (75.0)	61	45 (73.8)	0.708
Bacteriological cure	56	41 (73.2)	61	48 (78.7)	0.514
Post-treatment NAGase <40 U	52	26 (50.0)	56	26 (46.4)	0.457
Clinical + bacteriological cure	56	38 (67.9)	61	44 (72.1)	0.699
Clinical cure + NAGase <40 U	52	26 (50.0)	56	24 (42.9)	0.256
Bacteriological cure + NAGase <40 U	52	23 (44.2)	56	24 (42.9)	0.684
Complete cure	52	23 (44.2)	56	23 (41.1)	0.568

Table 3. Bacteriological cure rates of clinical mastitis caused by gram-positive bacteria *in vitro* susceptible to penicillin G in treatment group A (intramammaries containing 600 000 IU procaine penicillin G) and B (intramammaries containing 500 000 IU procaine penicillin G and 300 mg neomycin). Both treatments were supplemented with procaine penicillin G 20 mg/kg body weight intramuscularly once on the first day of treatment

	Bacteriological cure				
	Treatment A		Treatment B		
Micro organism	Treated quarters	Cured *n* (%)	Treated quarters	Cured *n* (%)	*P*-value
S. aureus	10	3 (30.0)	9	4 (44.4)	0.650
CNS	13	8 (61.5)	15	14 (93.3)	0.069
Str. dysgalactiae	9	8 (88.9)	15	12 (80.0)	1.000
Str. uberis	24	22 (91.7)	22	18 (81.8)	0.405
Total	56	41 (73.2)	61	48 (78.7)	0.514

Table 4. Variables affecting cure rates of clinical mastitis caused by gram-positive bacteria *in vitro* susceptible to penicillin G in treatment group A (intramammaries containing 600 000 IU procaine penicillin G) and B (intramammaries containing 500 000 IU procaine penicillin G and 300 mg neomycin). Both treatments were supplemented with procaine penicillin G 20 mg/kg body weight intramuscularly once on the first day of treatment

Cure rate	Variable	Odds ratio	95% CI	*P*-value
Bacteriological	Treatment group*	1.370	0.532–3.526	0.514
	CNS†	9.936	2.763–35.729	0.000
	Str. dysgalactiae†	2.008	0.560–7.194	0.284
	Str. uberis†	1.344	0.334–5.402	0.677
	Parity‡	2.313	0.580–9.225	0.235
Clinical	Treatment group*	0.842	0.343–2.069	0.708
	CNS†	4.693	1.463–15.061	0.009
	Str. dysgalactiae†	0.560	0.153–2.049	0.381
	Str. uberis†	0.924	0.272–3.136	0.898
	Parity‡	0.995	0.309–3.208	0.993
NAGase <40 U	Treatment group*	0.722	0.306–1.703	0.457
	CNS†	1.605	0.467–5.518	0.453
	Str. dysgalactiae†	0.142	0.043–0.470	0.001
	Str. uberis†	0.792	0.266–2.361	0.675
	Parity‡	1.211	0.397–3.698	0.737

*Treatment B compared with treatment A.
†Compared with *S. aureus*.
‡First parity compared with subsequent parities.

makes treatment with antimicrobials generally questionable (Pyörälä & Pyörälä, 1998; Wilson *et al.*, 1999). In severe coliform mastitis, systemic antimicrobial treatment may be beneficial to the outcome (Wenz *et al.*, 2001; Rantala *et al.*, 2002). The use of aminoglycosides in coliform mastitis lacks scientific support (Jones & Ward, 1990; Erskine *et al.*, 1992). The few cases of coliform mastitis, excluded from our study material, were all cured irrespective of the nature of the treatment.

Synergy is given as another ground for using β-lactam–aminoglycoside combinations. Among mastitis pathogens, this synergy has been shown *in vitro* for *S. aureus*, *Str. uberis* and *Str. dysgalactiae* (Rosselet *et al.*, 1977; Franklin *et al.*, 1984; Lohuis *et al.*, 1995a,b). In some experiments it has been suggested that the synergy is dependent on the concentrations of the two drugs and the bacterial strains, some of them being susceptible to the synergistic effect, while others are not (Franklin *et al.*, 1984). The mechanism of synergy is classically thought to be caused by the breakdown of bacterial cell wall by β-lactam, which enhances bacterial uptake of aminoglycoside and its access to the bacterial cytoplasm, where it binds to the 30S ribosome (Eliopoulos & Moellering, 1996). More recent studies, however, have shown that the primary mechanism of action of aminoglycosides may not be inhibition of protein synthesis by binding to the bacterial 30S subunit, but through competitive displacement of cell biofilm-associated Mg^{2+} and Ca^{2+} that links the polysaccharides of adjacent lipopolysaccharide molecules (Stratton, 1996). Low concentrations of β-lactam in combination with an aminoglycoside may stimulate increased production of biofilm and thus enhance the effect of the aminoglycoside by providing a better target, whereas higher concentrations, which disrupt the cell wall, lessen the biofilm target and thus effectiveness of the aminoglycoside (Stratton, 1996). The mechanisms of possible synergistic effects may be more complicated than previously thought, which may help in explaining why the assumed synergies in treatment of mastitis have not been supported by *in vivo* studies.

The pharmacological properties of aminoglycosides are very different from those of β-lactams. β-Lactams are time-dependent drugs (Livermore & Williams, 1996; Prescott, 2000b). In successful treatment with a β-lactam a constant concentration above minimum inhibitory concentration level is needed for a sufficient length of time, whereas aminoglycosides are concentration-dependent with a marked postantibiotic effect, and the higher the peak concentration, the more efficacious the treatment (Stratton, 1996; Prescott, 2000a). Extending the duration of the treatment does not increase the efficacy, but may enhance the nephrotoxic and ototoxic properties of aminoglycosides. Furthermore, aminoglycosides are known to bind to tissues and cause long-lasting residues in tissues, and consequently are not very suitable antibiotics for food animal use (Nouws & Ziv, 1978; Erskine *et al.*, 1992; Whittem & Hanlon, 1997b). In mastitis, drug absorption from the udder can be increased because of the breakdown in the integrity of the blood–milk barrier, and drug persistence in the body may be prolonged (Nouws & Ziv, 1978). In mastitic cows, aminoglycoside residues in the kidney have been found 60 h after intramammary treatment (Nouws & Ziv, 1978). Gentamicin has been found in urine 14 days after intramammary infusion (Erskine *et al.*, 1992).

It seems that aminoglycosides have been introduced into mastitis preparations in the absence of clinical evidence of enhanced efficacy, and based purely on theoretical assumptions and *in vitro* studies (Rosselet *et al.*, 1977; Franklin *et al.*, 1984). Most of the combination preparations for mastitis treatment were introduced into the market decades ago, with minimal requirements for efficacy data from the drug registration authorities. In some countries even new combination preparations have

been introduced more recently (Malinowski *et al.*, 1993). In the EU guideline for fixed antibiotic combinations (EMEA, 1994), the efficacy of a combination must be tested against that of one of its components before it can be authorized. Considering the risks of using aminoglycosides in the routine treatment of mastitis, the results of previously published studies and the results of this rather small study with a double-blind design, the traditional use of penicillin–aminoglycoside combinations in mastitis treatment should be discontinued.

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