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Treatment of mild to moderate clinical bovine mastitis caused by gram-positive bacteria: A noninferiority randomized trial of local penicillin treatment alone or combined with systemic treatment

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ABSTRACT

Bovine mastitis is one of the most important diseases in modern dairy farming, as it leads to reduced welfare and milk production and increased need for antibiotic use. Clinical mastitis in Denmark is most often treated with a combination of local and systemic treatment with penicillin. The objective of this randomized clinical trial was to assess whether worse results could be expected with local intramammary treatment with penicillin compared with a combination of local and systemic treatment with penicillin in terms of the bacteriological cure of mild and moderate clinical mastitis cases caused by gram-positive bacteria. We carried out a noninferiority trial with a noninferiority margin set to a relative reduction in bacteriological cure of 15% between these 2 treatment groups to assess the effect of reducing the total antibiotic use by a factor of 16 for each treated case. Clinical mastitis cases from 12 Danish dairy farms were considered for enrollment. On-farm selection of gram-positive cases was carried out by the farm personnel within the first 24 h after a clinical mastitis case was detected. A single farm used bacterial culture results from the on-farm veterinarian, whereas the other 11 farms were provided with an on-farm test to distinguish gram-positive bacteria from gram-negative or samples without bacterial growth. Cases with suspected gram-positive bacteria were allocated to a treatment group: either local or combination. Bacteriological cure was assessed based on the bacterial species identified in the milk sample from the clinical mastitis case and 2 follow-up samples collected approximately 2 and 3 wk after ended treatment. Identification of bacteria was carried out using MALDI-TOF on bacterial culture growth. Noninferiority was assessed using unadjusted cure rates and adjusted cure rates from a multivariable mixed logistic regression model. Of the 1,972 clinical mastitis cases registered, 345 (18%) met all criteria for inclusion (full data). The data set was further reduced to 265 cases for the multivariable analysis to include only complete registrations. *Streptococcus uberis* was the most commonly isolated pathogen. Noninferiority was demonstrated for both unadjusted and adjusted cure rates. The unadjusted cure rates were 76.8% and 83.1% for the local and combined treatments, respectively (full data). The pathogen and somatic cell count before the clinical case had an effect on the efficacy of treatment; thus efficient treatment protocols should be herd- and case-specific. The effect of pathogen and somatic cell count on treatment efficacy was similar irrespective of the treatment protocol. We conclude that bacteriological cure of local penicillin treatment for mild and moderate clinical mastitis cases was noninferior to the combination of local and systemic treatment using a 15% noninferiority margin. This suggests that a potential 16-fold reduction in antimicrobial use per mastitis treatment can be achieved with no adverse effect on cure rate.

Key words: intramammary treatment, intramuscular treatment, on-farm test, bacteriological cure

INTRODUCTION

Mastitis is one of the most important diseases in dairy cattle worldwide, and it is often treated with antibiotics. Due to a general focus on antimicrobial resistance as well as an industry goal to reduce antibiotic consumption in dairy cattle, treatment strategies should be optimized at herd level to ensure prudent antibiotic use. In addition to prevention of mastitis, a more prudent use of antibiotics could be achieved through evidence-based mastitis treatment (Vries et al., 2016; Ruegg, 2018).

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In Denmark, mastitis is the major cause of antibiotic use in adult dairy cattle (DANMAP, 2018), and it is mainly treated with penicillin (procaine benzylpenicillin or penthamate hydroiodide), as other antibiotics may only be used under special circumstances due to certain regulations (Wilm et al., 2021). Even though local (intramammary, **IMM**) administration of antibiotics for mastitis is the most common treatment worldwide (Pyörälä, 2009), the penicillin products used in Danish dairy herds are primarily prescribed for systemic (intramuscular, i.m.) administration (DANMAP, 2020). Systemic mastitis treatment is common practice in Scandinavian countries, and in Denmark it is primarily used in combination with local administration (Rajala-Schultz et al., 2021; Wilm et al., 2021). The effects of systemic or combined local and systemic administration of antibiotics are well documented, especially when it comes to mastitis pathogens that can be intracellular, such as *Staphylococcus aureus* (Taponen et al., 2003; Pyörälä, 2009). However, systemic treatments contain larger amounts of active compound compared with local treatments (Hillerton and Kliem, 2002; Pyörälä, 2009). For example, based on information from the summaries of product characteristics (European Medicines Agency; https://medicines.health.europa.eu/veterinary/en), we estimated that a traditional Danish combination treatment with IMM procaine benzylpenicillin and i.m. penethamate hydroiodide contains around 16 times the amount of antibiotic compound compared with local treatment alone. This was calculated as follows. Assuming that a Holstein dairy cow has a weight of 600 kg, the i.m. treatment comprises 9 g of penethamate hydroiodide per day (15 mg/kg × 600 kg). The IMM treatment comprises 600 mg (0.6 g) of procaine benzylpenicillin per day. According to the summary of product characteristics, the recommended treatment duration is 3 to 5 d for both local and systemic treatments; thus the treatment length was considered the same for both treatments. The daily amount of antibiotic active compound is thus 0.6 g for the local treatment and 9.6 g for the combination treatment, corresponding to a factor of 16.

The advantage of the local treatment compared with the systemic treatment is that the antibiotic compound will reach a higher concentration in the affected tissue more quickly, and less antibiotic compound will therefore be required to achieve the same effect (Pyörälä, 2009). However, Ehinger et al. (2004) studied isolated perfused bovine udders and found that the distribution of IMM-administered benzylpenicillin was impeded in acute mastitis; they therefore recommended combined systemic and local administration. However, systemic administration has been associated with the development of antimicrobial resistance related to bovine mastitis, and local administration is therefore preferable from a resistance perspective (Nobrega et al., 2018).

To our knowledge, no previous study has investigated the effects of local treatment compared with combination treatment with penicillin products only. However, Kalmus et al. (2014) reported no difference in bacteriological cure rates of clinical mastitis when comparing local and systemic administration with procaine benzylpenicillin. Even though a combination treatment may lead to higher cure rates compared with local treatment alone, we assumed that a moderately lower cure rate for the local treatment would be acceptable when taking into account the fact that less antibiotic active compound could be used. We therefore carried out a noninferiority trial. The noninferiority margin was set following guidelines from the European Medicines Agency (EMEA CHMP, 2005). The size of an acceptable noninferiority margin was discussed with other mastitis researchers and veterinary practitioners and selected before undertaking the study. We considered that the effect of local treatment should be higher than spontaneous cure rates despite being less effective than the combined treatment. In a previous study, Pinzón-Sánchez et al. (2011) estimated the spontaneous cure of gram-positive bacteria to be 0 to 5% for *Staph. aureus*, 25 to 30% for environmental streptococci, and 55 to 60% for NAS, whereas the estimated cure rates after 5 d of local treatment (not using penicillin) were estimated to be 20 to 25%, 65 to 70%, and 75 to 80% for *Staph. aureus*, environmental streptococci, and NAS, respectively (Pinzón-Sánchez et al., 2011).

If the cure rates of the local treatment alone are similar to those of the combination treatment, potential exists to reduce antimicrobial use associated with mastitis treatment. In the present study, we therefore aimed to evaluate whether local (IMM) administration of penicillin is noninferior at a 15% level relative to a combination of local (IMM) and systemic (i.m.) administration of penicillin in terms of the bacteriological cure of mild and moderate clinical mastitis cases caused by gram-positive bacteria.

MATERIALS AND METHODS

The authors obtained ethical approval from the Animal Ethics Institutional Review Board (AEIRB) at the Department of Veterinary and Animal Sciences,

Faculty of Health and Medical Sciences, University of Copenhagen (Frederiksberg, Denmark). The study was assigned AEIRB number 2021-08-AWD-009A.

Study Design and Overview

The study was a longitudinal randomized clinical noninferiority trial of the effect of local (IMM) penicillin treatment and combination (IMM and i.m.) penicillin treatment on bacteriological cure of naturally occurring clinical mastitis cases. The study was carried out as a per-protocol analysis (Piaggio et al., 2012).

The margin of noninferiority was set to 15% before the study, and our null hypothesis was therefore that the bacteriological cure rate of clinical mastitis was more than 15% lower with local treatment than with combination treatment. The corresponding alternative hypothesis was that the bacteriological cure rate of local treatment was equivalent to that of combination treatment, given the noninferiority margin of 15%.

In short, data collection proceeded as follows. Farm personnel detected clinical mastitis cases during forestripping and collected quarter foremilk samples from mild to moderate cases. An on-farm test was carried out on the milk sample immediately afterward, and the same sample was shipped for subsequent laboratory analysis. If the on-farm test indicated gram-positive growth in the sample, the case was treated according to protocol, and 2 follow-up milk samples were collected at approximately 2 and 3 wk after treatment ended, respectively.

The inclusion criteria for mastitis cases were (1) mild to moderate clinical severity, (2) treatment according to local or combination treatment protocol, (3) verification as culture-positive, with at least 1 of 2 identified species being gram-positive in the microbiological analysis, and (4) at least 1 of 2 follow-up samples being useful for evaluating bacteriological cure.

Study Herds

The study was carried out in 12 Danish dairy herds (**H1**–**H12**) from May 2020 to June 2021. Herds were included based on geographical location (regions of Central and Southern Denmark) and the willingness of the farmers and herd veterinarians to participate (convenience). Further inclusion criteria were that cows were milked in a parlor or rotary (to ensure human visual inspection of milk at each milking), that DHI testing was performed 11 times per year, and that it was a conventional herd with at least 200 cows. Herd characteristics are shown in Table 1.

Training of Farm Personnel

As clinical mastitis can occur at any time, the farm personnel on each farm were carefully introduced to the study protocol and instructed by author LS before the study. The milking personnel were trained in how to recognize, register, and classify the severity of clinical mastitis cases. Furthermore, LS trained the relevant personnel in the aseptic collection of quarter milk samples and in the use of the on-farm test (described later).

Selection of Cases

At each milking, all lactating cows were pre-milked before the milking cluster was attached (screening; Figure 1). Cases of clinical mastitis detected during screening were classified according to the International Dairy Federation guidelines (IDF, 2011) as follows. Mild mastitis (grade 1) was characterized by visible abnormalities in the milk (flakes, clots, watery). Moderate mastitis (grade 2) was characterized by visible abnormalities in the milk and signs of inflammation in the udder (warm, swollen, reddened, painful udder quarter). Severe mastitis (grade 3) was characterized by systemic illness such as fever in addition to grade 1 and 2 symptoms. Each new case of clinical mastitis (i.e., those that were not already undergoing antibiotic treatment) was registered by noting the cow identification number, affected quarter, date, time, and mastitis grade using a form provided by LS. The farm personnel collected milk samples from clinical mastitis cases classified as grades 1 and 2, and initiated treatment with a nonsteroidal anti-inflammatory drug (**NSAID**). In parallel with the initiation of NSAID treatment, on-farm selection based on the Gram stain status of the infection was carried out before antibiotic treatment was administered. If the on-farm selection test showed that the milk sample contained gram-positive bacteria, farmers were advised to initiate 1 of the 2 study treatments, allocated by ear tag number. Only cases that followed this protocol were included in the microbiological analysis. As mentioned earlier, cases that were already undergoing antibiotic treatment at the time of detection of the clinical mastitis case were excluded from the study by farm personnel. In addition, we also excluded cases that were later found to have been enrolled during the withdrawal time of a previous antibiotic treatment.

Farmers were advised to follow the study protocol but could at any time treat or withhold antibiotic treatment for various reasons, thus excluding the case from the study. Severe mastitis (grade 3) cases were not included in the study and were handled by farmers

Table 1. Herd characteristics for the 12 study herds: herd size, milk production, SCC, and breed are given as the mean over the 12 mo (June 2020 to May 2021) that covered the sampling period for most herds; treatment rate is calculated for the same period, as well as for the previous 12 mo

Herd	Herd size[1]	Milk production[2]	SCC[3]	Treatment rate[4] (previous year)	Breed[5] (%)	Milking system	No. of daily milkings	Bedding[6]	Sampling period[7]	Length of study period, mo (rounded)
H1	254	11,496	184	0.39 (0.42)	Jersey (100)	Parlor	2	Straw DB + lime	2020-08-20 to 2021-05-23	9
H2	1,384	9,664	229	0.43 (0.43)	Jersey (92)	Rotary	2	Sand DB	2020-10-07 to 2021-05-09	7
H3	427	11,660	225	0.55 (0.49)	Jersey (100)	Parlor	2	Straw DB	2020-09-23 to 2021-04-18	7
H4	921	11,296	164	0.56 (0.40)	DH (94)	Parlor	2	Sand DB	2020-10-03 to 2021-06-07	8
H5	241	12,199	204	0.54 (0.30)	DH (100)	Parlor	2	Straw	2020-09-18 to 2021-06-04	9
H6	333	10,886	159	0.36 (0.29)	DH (94)	Rotary	2/3[8]	Wood shavings	2020-09-04 to 2021-05-26	9
H7	886	12,288	275	0.42 (0.34)	DH (97)	Parlor	2/3[9]	Straw/sand DB	2020-06-09 to 2021-05-17	11
H8[10]	1,009	11,321	163	0.47 (0.55)	DH (91)	Parlor	2	Straw + hydrated lime	2020-12-07 to 2021-05-31	6
H9	800	12,962	169	0.14 (0.12)	DH (98)	Parlor	3	Straw + hydrated lime	2020-05-13 to 2021-05-28	12
H10	639	12,654	243	0.24 (0.19)	DH (95)	Parlor	3	Straw + hydrated lime	2020-05-14 to 2021-06-02	13
H11	489	11,213	271	0.75 (0.72)	DH (83)	Parlor	2	Straw + lime	2020-11-24 to 2021-05-29	6
H12	481	13,478	258	0.23 (0.28)	DH (98)	Parlor	2	Sand DB	2020-09-21 to 2021-06-12	9

[1]Herd size presented in cow-years. A cow-year corresponds to 365 feeding days.

[2]Estimated kg of ECM/cow per year based on monthly records from DHI testing.

[3]SCC values ×1,000 cells/mL: mean based on monthly cow-level records from DHI testing.

[4]Treatments per cow-year (365 feeding days).

[5]DH = Danish Holstein.

[6]Top layer on mattress if not stated that it is deep bedding (DB).

[7]Sampling period dates are presented as year-month-day.

[8]Switched from 3 to 2 milkings/d during the sampling period.

[9]Cows in their first lactation and low-yielding cows were milked 2 times/d.

[10]First-lactation cows not included.

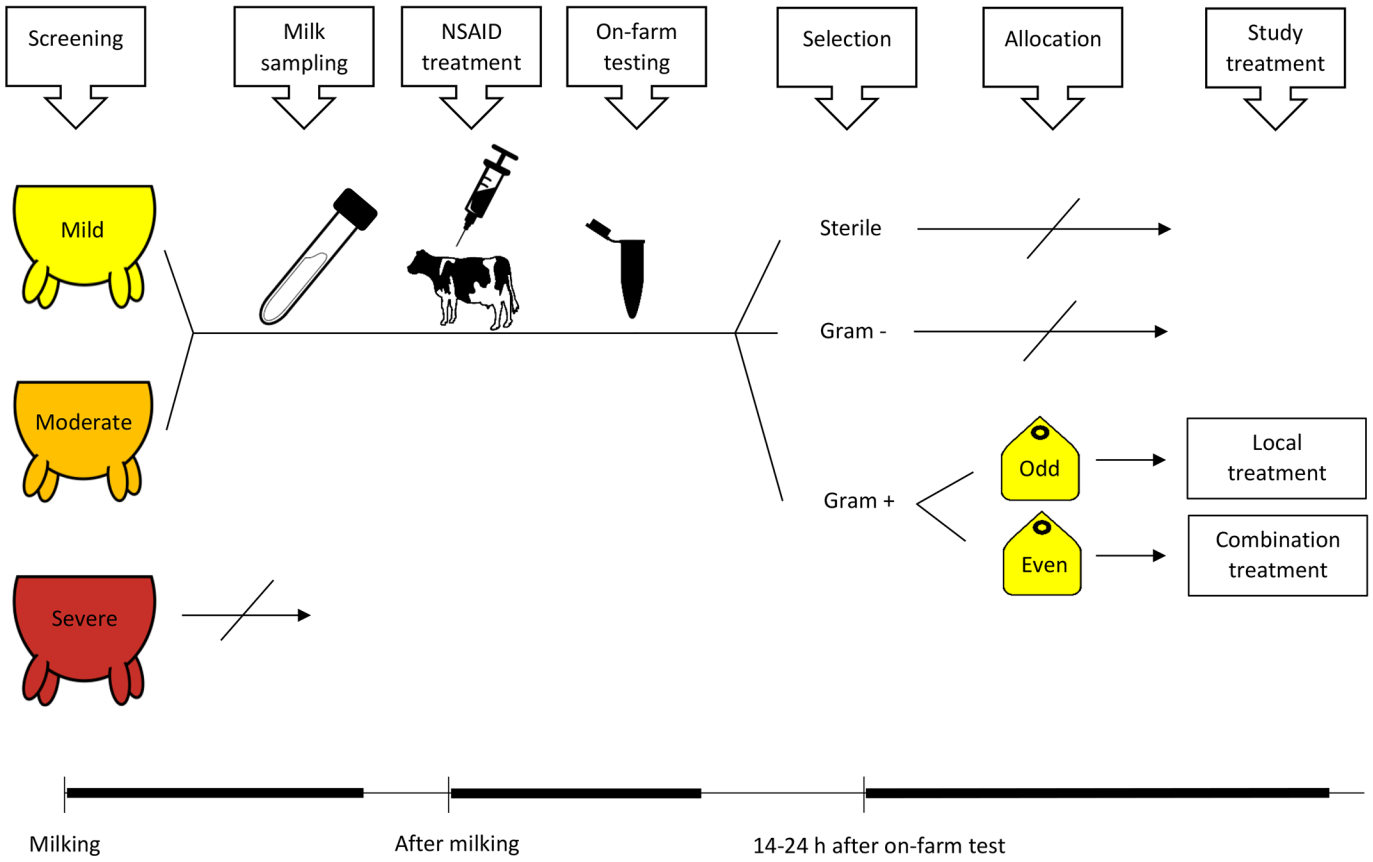

Figure 1. Flowchart showing how cases were selected for the study treatments (local [intramammary] and combination of local and systemic [intramuscular injection] antibiotic administration). At each milking, cows with clinical mastitis were registered and graded. Mild to moderate cases were included for milk sampling, and an on-farm test was used to select cases caused by gram-positive bacteria. Selected cases were allocated according to ear-tag numbers (odd vs. even) before receiving one of the study treatments.

according to the plan given by the herd veterinarian. Figure 1 presents an overview of the selection of cases.

Collection of Milk Samples From Clinical Mastitis Cases

All milk samples from the clinical cases were collected by farm staff during milking. Foremilk samples were collected aseptically according to standards from the National Mastitis Council (Hogan et al., 1999) with a few modifications. Teats were cleaned using the procedure normally used in each farm before attachment of the cluster (premilking soap or teat disinfectant and wet or dry cloth). New gloves were worn for each milk sample collected. Teat ends were cleaned with cotton moistened in 70% alcohol, and 3 streams of milk were discarded before a minimum of 5 mL of milk was collected in a 15-mL sterile screw-cap tube. The milk samples were cooled to 5°C if they were not tested immediately using the on-farm test. Following on-farm testing, samples were frozen at −18°C. Later, samples were transported on ice to the laboratory for microbiological analysis after a maximum freezing time of 6 wk.

On-Farm Selection Based on Gram Status

Of the 12 farms, 11 were provided with the MastDecide on-farm test (Quidee), validated to sensitivity and specificity of 84 and 94%, respectively, for the detection of gram-positive cocci (Leimbach and Krömker, 2018). The on-farm test kit includes a 0.1-mL disposable pipette and 2 Eppendorf tubes containing a substrate. The test was carried out by adding 0.1 mL of milk to both of the tubes and incubating at 37°C for 12 to 24 h before reading the result according to the manufacturer's instructions. The substrate color change indicated whether the milk contained gram-positive, gram-negative, or no bacteria. The farms were provided with a small incubator, an illustrated protocol for the on-farm test, and registration forms. The time of incubation varied according to the working routines on each farm.

Bacteriological culture was used on 1 farm (H3, Table 1) for on-farm selection of gram-positive cases. This farm had an on-farm veterinarian who provided a bacteriological culture result within 24 h. The bacterial culture was carried out by spreading 0.01 mL of milk with a disposable loop onto an esculin blood agar plate and a chrome agar plate, and bacteria were identified based on biochemical tests (NMC, 2004).

Allocation to Treatment Groups

Cases were pseudorandomly allocated to a treatment protocol based on ear-tag number: Cows with odd ear-tag numbers were allocated to local treatment, and cows with even ear-tag numbers were allocated to combination treatment. However, at one farm (H11), only even-numbered ear-tags were assigned to heifers; thus the majority of the cows had even numbers. Therefore, the end digit of the ear-tag number was used for the treatment allocation instead: end digits below 5 were allocated to local treatment, and end digits above 5 were allocated to combination treatment.

Treatment Protocols

Two treatment protocols were evaluated for the treatment of gram-positive bacteria. The local treatment included NSAID treatment on d 1 and IMM treatment on d 2 to 4 (3 d in total). The combination treatment included NSAID treatment on d 1, IMM treatment on d 2 to 4 (3 d in total), and i.m. treatment on d 2 to 4 (3 d in total). All products were used according to the summaries of product characteristics. The NSAID used was ketoprofen, administered i.m. at a dose of 3 mg/kg of BW (Rifen, 100 mg/mL, Salfarm Denmark A/S; Dinalgen, 150 mg/mL, Scanvet A/S, Denmark; or Coxofen, 100 mg/mL, Biovet Aps). These products required no withdrawal period for milk. The IMM treatment comprised 600 mg (600,000 IU) of procaine benzylpenicillin administered in the affected quarter once per day (Carepen, 600 mg, Boehringer Ingelheim Animal Health Nordics A/S), with a 6-d withdrawal period for milk. The i.m. treatment comprised 15 mg (15,000 IU) of penethamate hydroiodide/kg of BW, administered in the muscle once per day (Mamyzin Vet., 10,000,000 IU, Boehringer Ingelheim Animal Health Nordics A/S; or Penethaone Vet., 250,000 IU/mL, Scanvet A/S), and the withdrawal periods for milk were 96 and 60 h for the 2 products, respectively.

An "escape therapy" was established in case clinical symptoms developed from mild or moderate to severe. The farmers would typically contact their herd veterinarian, and the case was excluded from the study.

It was not possible to blind the farm personnel to the treatment groups, as they administered the antibiotics. Furthermore, authors LS and APS were not blinded, but information about treatment was not provided to the laboratory personnel before microbiological analyses were complete.

Collection of Follow-Up Milk Samples

In addition to the initial quarter milk sample from the clinical mastitis case, 2 follow-up milk samples were collected from all cases after treatment to assess bacteriological cure. Follow-up milk samples were collected by LS, APS, or the farm manager, following the same procedure as for the milk samples from the clinical cases, with the exception that samples were not always collected during milking. Therefore, in some cases cleaning of the teat before disinfection with alcohol was performed using wipes only. We aimed to collect the follow-up samples on d 14 and 21 after the treatment ended, but for practical reasons and with one visit per herd per week, the first follow-up sample was collected between d 14 and 20, and the second follow-up sample was collected at least 7 d after the first, in line with guidance from the European Medicines Agency (EMA CVMP, 2017).

Follow-up samples were either frozen immediately on the farm or transported on ice and frozen within 5 h. The samples were later transported on ice to the laboratory where microbiological analyses were carried out after a maximum freezing time of 6 wk.

Microbiological Analysis

All milk samples were analyzed at the Veterinary Bacteriology Laboratory of the Technical University of Denmark (**DTU-Vet**), Lyngby, Denmark. Milk samples were thawed and homogenized, and 0.01 mL of milk was streaked on a blood agar plate (5% calf blood, SSI Diagnostica A/S) using a disposable loop. Plates were incubated at 37°C and read after 24 and 48 h. If no growth occurred after 48 h, the milk sample was recultured and incubated in CO_2 and read after 24 and 48 h. If there was still no growth, the sample was considered culture negative (no growth). Growth was defined as at least 1 colony corresponding to a cut-off of 100 cfu/mL of milk. Samples that included growth of 3 or more phenotypically different colony types were discarded as visually contaminated. Samples with 1 or 2 phenotypically different colony types had a representative colony of each type subcultivated on blood agar, incubated at 37°C, and read after 24 h. All subcultures that seemed pure cultures were identified by MALDI-TOF (Bruker

Biotyper software system, Microflex LT, Bruker Daltonics GmbH) in triplicate. Colonies were identified by direct deposition on the target plate according to Bizzini et al. (2010), combined with overlay of 70% formic acid before matrix deposition using the BDAL (Bruker Daltronics) database combined with the DTU-Vet database for veterinary spectra and staphylococci (Mahmmod et al., 2018; Nonnemann et al., 2019). The MALDI-TOF results were considered valid according to the criteria proposed by the manufacturer (Bizzini et al., 2010). Following MALDI-TOF analysis, contamination was defined as more than 2 different pathogens at species level. Thus, a positive sample included at least 1 colony of each 1 or 2 species. In addition, for the milk samples from the clinical cases, at least 1 species had to be gram-positive. In cases where MALDI-TOF only reported at genus level, we included the pathogen at genus level and considered the sample contaminated if more than 1 other species was present (i.e., more than 2 species but with 1 identified only at genus level).

Assessment of Treatment Outcome

The success of an antibiotic treatment was evaluated based on microbiological analysis of the milk sample from the clinical case and the follow-up samples (bacteriological cure). If clinical mastitis symptoms were present at the first follow-up, the case was excluded as "not clinically cured." If clinical mastitis symptoms were only present at the second follow-up, the case was included, but only using the first follow-up sample, and the symptoms were considered to represent a new case of clinical mastitis.

Clinical cases with contaminated samples were excluded. The pathogens detected in samples that were not contaminated were considered to be the cause of infection. Likewise, contaminated follow-up samples were not used to assess bacteriological cure.

A case was considered bacteriologically cured when none of the bacterial species detected in the clinical sample were detected in any of the follow-up samples. In cases where it was only possible to identify a bacterial colony at genus level using MALDI-TOF, we considered the case to be cured if the follow-up samples did not contain bacteria that could only be assigned at the same genus level. For example, a *Staphylococcus* sp. in the clinical sample could be considered cured even though NAS were identified at species level at follow-up. This interpretation of MALDI-TOF results is based on the application of strict inclusion criteria for valid MALDI-TOF results given by the manufacturer, which are further enforced by the added DTU-Vet database for veterinary spectra and staphylococci (Bizzini et al., 2010; Nonnemann et al., 2019). Hence, species reported only at genus level would likely be rare species other than those identified at species level from the same genus.

In cases where it was not possible to obtain both follow-up samples, cure was evaluated based on a single follow-up sample.

Sample Size

We calculated the sample size for a noninferiority trial with a binary outcome using the chosen noninferiority margin of 15%. Based on previous knowledge from the included herds, we expected environmental streptococci to be the most frequent cause of clinical mastitis in our study and the pathogen distribution to be similar to that reported in the study by Kalmus et al. (2014), and we therefore set the expected success of the local treatment to 56% based on their results. We set the expected effect of the combination treatment to 58%, which is higher than the 54% reported for systemic treatment by Kalmus et al. (2014). To obtain a power of 80% and $\alpha = 5\%$ in a one-sided test, 180 cases were required in each treatment group (Sealed Envelope, 2020).

Statistical Analysis and Data Management

Microsoft Excel (2016; Microsoft Corp.) was used to record the on-farm registrations and for the daily management and planning of follow-up sample collection. Data management and statistical analysis were performed using R version 4.2.0 (Apr. 22, 2022, "Vigorous Calisthenics"; R Core Team, 2022). Information about herds, cows, and treatments and results from the monthly DHI tests were extracted from the central Danish cattle database (SEGES Livestock Innovation, Aarhus, Denmark).

Cow and case characteristics were cross-tabulated by treatment group to assess the allocation procedure. Differences between groups were assessed by the *P*-value from univariable logistic regression using the *glm()* function in R for categorical variables and the *ks.test()* function in R for the continuous variables. We investigated clinical score (mild or moderate), DIM (4 levels: 1–30, 31–100, 101–200, and >200), parity (3 levels: 1, 2, 3+), prior treatment within the same lactation (yes or no), quarter affected (front or rear), and SCC at last DHI test before the clinical case (<200,000 cells/mL or >200,000 cells/mL) as categorical variables that we believed a priori to be potentially biologically meaningful in relation to known effects on mastitis cure (Ziesch and Krömker, 2016). Pathogens were initially categorized into 8 groups (*Streptococcus uberis*, *Streptococcus dysgalactiae*, other streptococci, *Staph. aureus*, NAS,

Enterococcus spp., other gram-positive, and mixed infections). The ECM at last DHI test before the clinical case was assessed as a continuous variable. The number of days between the last DHI test and the clinical case was investigated as a categorical variable (3 levels: 0–14, 15–28, and >28) to assess the temporal relevance of the DHI measures.

The DHI testing before the clinical case within the same lactation was not set as an inclusion criterion, resulting in missing values for SCC and ECM, primarily from cases treated early in lactation. Therefore, a subset of data including only complete registrations was created (hereafter referred to as subset data). Univariable analyses were carried out on both data sets with bacteriological cure (dichotomous) as the outcome, using the *glm()* function in R for the categorical variables and the *ks.test()* for the continuous variables.

We performed a noninferiority trial on the full data and the subset data using a one-sided test at the 5% level, which corresponds to the upper 90% confidence level. This was obtained using the *prop.test()* function in R. We set the noninferiority limit to 15% as mentioned previously. We also performed a two-sided test at the 95% confidence level for comparison.

We analyzed the effect of treatment on bacteriological cure in a multivariable mixed logistic regression model on the subset data. We tested variables with $P < 0.2$ in the univariable analyses and the 2-way interactions between these variables in the multivariable model, according to McDougall (2003) and others. Herd was included as a random effect. Cases from the same cow were assumed to be independent, as information was at quarter level and there was a minimum of a withdrawal period (9 d) between cases, in accordance with the suggested time of at least 8 d between cases, as defined by the International Dairy Federation (IDF, 2011). The model was reduced using backward elimination based on the Akaike information criterion (Akaike, 1973), although treatment was forced into the model to assess the primary outcome of the study. For the multivariable model based on the subset data we further categorized the pathogens into 5 groups (from the 8 groups used in the full data): *Streptococcus* spp. (including *Strep. uberis*, *Strep. dysgalactiae*, and other streptococci), *Staph. aureus,* NAS (including NAS species and *Staphylococcus* spp.), other (including *Enterococcus* spp., *Lactococcus* spp., and others), and mixed infections (2 pathogens). This was necessary to ensure a sufficient size of each pathogen group for the analysis. We fit the model in R using the *glmer* function in the lme4 package (Bates et al., 2015) and validated it through a visual assessment of plots using the DHARMa package (Hartig, 2022) in R.

The 95% confidence limits for the difference between treatments were bootstrapped using the *bootMer* function in the lme4 package (Bates et al., 2015), resampling the model for 1,000 iterations.

We performed a visual examination of the distribution of case characteristics from treated cows, treated but wrongly allocated cows, and cows that were not treated (at the farmer's discretion) even though they met the inclusion criteria, to assess for signs of systematic bias. We did the same for cases that were lost to follow-up or excluded for other reasons (e.g., contaminated milk sample from a clinical case). Furthermore, we carried out a robustness analysis of the multivariable model without wrongly allocated cows, to assess whether this bias had an effect on the results, and we performed a visual examination of the effect of time, to follow up on the probability of being cured.

RESULTS

Included Cases

The study period for the respective herds ranged from 6 to 13 mo (Table 1). Figure 2 shows the full case enrollment process for the analysis. A total of 1,972 cases of clinical mastitis were registered, and 828 (42%) of these were mild to moderate cases assumed to be caused by gram-positive bacteria based on the initial on-farm selection. Of the 828 mild to moderate gram-positive cases, 575 (69%) received one of the treatment protocols in our study (local: n = 268, combination: n = 307). Reasons for the farmer not to treat the remaining 31% (133 and 120 cases allocated to local and combination treatments, respectively) included the cow being close to dry-off, or the clinical symptoms improving before the result of the on-farm test was available. Of the 575 treated cases, 14% received a different treatment than the one to which they should have been allocated: 32 cases wrongly received local treatment, whereas 49 cases wrongly received combination treatment. According to the farmers, the incorrect allocation of cows primarily occurred by accident, and the results of the multivariable analysis on a subset of the data without the wrongly allocated cows did not indicate any change (Supplemental Table S1, https://doi.org/10.6084/m9.figshare.21915444.v1; Svennesen, 2023). Therefore, we included the wrongly allocated cases in the study. Furthermore, visual inspection revealed that the distributions for parity, DIM, severity grade, SCC, and previous treatment status were the same for cases that received the allocated treatment, were wrongly allocated, or were not treated (Supplemental Figure S1, https://doi.org/10.6084/m9.figshare.21915444.v1; Svennesen, 2023).

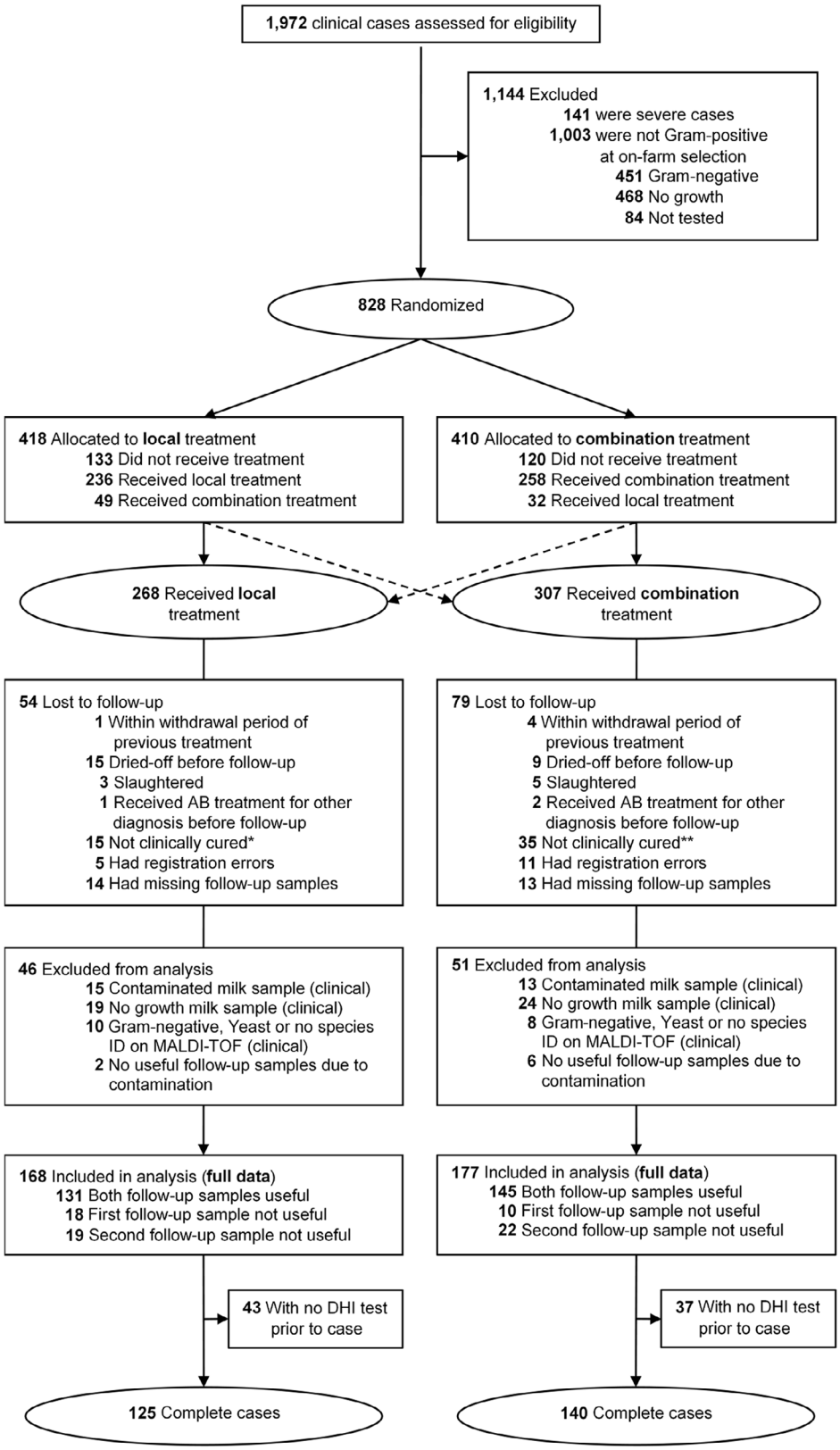

Figure 2. CONSORT (Piaggio et al., 2012) flow diagram showing the enrollment of clinical mastitis cases for analysis. *Of these 15 that were not clinically cured, 13 quarters were retreated and 1 was dried off. **Of these 35 that were not clinically cured, 20 quarters were retreated and 9 were dried off. AB = antibiotic.

Table 2. Cross-tabulation and univariable statistics of cow and case characteristics of clinical mastitis cases included in the final data set (full data); numbers (%) are shown per category and treatment group

		Treatment		
Variable and level	Total, n (%)	Local, n (%)	Combination, n (%)	*P*-value
Total cases included	345 (100)	168 (48.7)	177 (51.3)	—
Cure				
Cured	276 (80)	129 (76.8)	147 (83.1)	0.4666
Not cured	69 (20)	39 (23.2)	30 (16.9)	
Herd				
H1	24 (7)	13 (7.7)	11 (6.2)	0.2634
H2	72 (20.9)	31 (18.5)	41 (23.2)	
H3	65 (18.8)	35 (20.8)	30 (16.9)	
H4	21 (6.1)	5 (3)	16 (9)	
H5	13 (3.8)	8 (4.8)	5 (2.8)	
H6	14 (4.1)	7 (4.2)	7 (4)	
H7	36 (10.4)	21 (12.5)	15 (8.5)	
H8	20 (5.8)	8 (4.8)	12 (6.8)	
H9	18 (5.2)	9 (5.4)	9 (5.1)	
H10	26 (7.5)	14 (8.3)	12 (6.8)	
H11	26 (7.5)	10 (6)	16 (9)	
H12	10 (2.9)	7 (4.2)	3 (1.7)	
Clinical score				
Mild	154 (44.6)	72 (42.9)	82 (46.3)	0.5168
Moderate	191 (55.4)	96 (57.1)	95 (53.7)	
DIM				
0–30	80 (23.2)	40 (23.8)	40 (22.6)	0.4888
31–100	100 (29)	46 (27.4)	54 (30.5)	
101–200	108 (31.3)	58 (34.5)	50 (28.2)	
>200	57 (16.5)	24 (14.3)	33 (18.6)	
Parity				
1	81 (23.5)	34 (20.2)	47 (26.6)	0.2688
2	73 (21.2)	34 (20.2)	39 (22)	
3+	191 (55.4)	100 (59.5)	91 (51.4)	
Pathogen				
Streptococcus uberis[1]	138 (40)	72 (42.9)	66 (37.3)	0.4332
Streptococcus dysgalactiae[1]	21 (6.1)	11 (6.5)	10 (5.6)	
Other streptococci[1,2]	7 (2)	3 (1.8)	4 (2.3)	
Staphylococcus aureus[1]	23 (6.7)	11 (6.5)	12 (6.8)	
NAS[1,3]	48 (13.9)	21 (12.5)	27 (15.3)	
Enterococcus spp.[1,4]	7 (2)	4 (2.4)	3 (1.7)	
Other gram-positive[1,5]	4 (1.2)	0	4 (2.3)	
Mixed infections[6]	97 (28.1)	46 (27.4)	51 (28.8)	
Treated previously				
No	301 (87.2)	150 (89.3)	151 (85.3)	0.2672
Yes	44 (12.8)	18 (10.7)	26 (14.7)	
Quarter				
Front	163 (47.2)	76 (45.2)	87 (49.2)	0.4666
Rear	182 (52.8)	92 (54.8)	90 (50.8)	
Number of cases with previous test day available	265 (100)	125 (47.2)	140 (52.8)	—
SCC at last DHI test before clinical case				
<200,000 cells/mL	147 (55.5)	68 (54.4)	79 (56.4)	0.7401
>200,000 cells/mL	118 (44.5)	57 (45.6)	61 (43.6)	
Days between last DHI test and clinical case				
0–14	122 (46.0)	57 (45.6)	65 (46.4)	0.6226
15–28	100 (37.7)	48 (38.4)	52 (37.1)	
>28	43 (16.2)	20 (16.0)	23 (16.4)	
ECM at last test day before clinical case (continuous)				
ECM in kg, mean (SE)	39.70 (0.64)	40.87 (0.84)	38.65 (0.95)	0.5092

[1]Isolated in pure culture.

[2]*Streptococcus* sp. (n = 3), *Streptococcus canis* (n = 2), *Streptococcus agalactiae* (n = 1), *Streptococcus gallolyticus* (n = 1).

[3]Species are shown in Table 5.

[4]*Enterococcus faecium* (n = 6), *Enterococcus saccharolyticus* (n = 1).

[5]*Lactococcus garvieae* (n = 2), *Corynebacterium* sp. (n = 1), *Carnobacterium maltaromaticum* (n = 1).

[6]Two pathogens detected in the same sample; species are summarized in Table 4.

Of the treated cases, 54 of 268 cases (20%) and 79 of 307 cases (26%) were lost to follow-up after local and combination treatment, respectively. This is a total of 133 cases, corresponding to 23% of treated cases. Reasons included drying-off or lack of clinical cure. Furthermore, 97 cases (local: n = 46, combination: n = 51), corresponding to 17% of treated cases, were excluded following the microbiological analysis, either because no gram-positive bacteria were identified in the clinical sample or because both follow-up samples were contaminated. Visual inspection of the excluded cases stratified on treatment group (Supplemental Figure S2, https://doi.org/10.6084/m9.figshare.21915444.v1; Svennesen, 2023) revealed that the distributions for parity, DIM, severity grade, SCC, and previous treatment status were the same as for cases that were kept in the data set (Supplemental Figure S1).

The full data set included 345 cases (local: n = 168, combination: n = 177), corresponding to 60% of the treated cases and 18% of the clinical cases initially registered. Of the 345 cases included in the full data, 24 cows each represented more than 1 case. Of these, 12 cows were registered with 2 or 3 quarters affected on the same day (26 cases), and 13 cows had a repeated case, either on the same quarter (6 cases) or on different quarters (8 cases).

Cow and case characteristics for the 345 cases included in the full data are shown in Table 2, distributed by treatment group. The intended random allocation appears to have been successful, as no noticeable difference between the treatment groups was found (Table 2).

The subset of data that included complete cases with DHI measurements before the clinical case and within the same lactation amounted to 265 cases (subset data), 125 and 140 cases from the local and combination treatment groups, respectively (Figure 2). Within the subset data (n = 265), 9 cows were registered with 2 or 3 quarters affected on the same day (19 cases), and 9 cows had a repeated case, either on the same quarter (5 cases) or on different quarters (5 cases).

The results of the univariable statistics for both the full data and subset data are shown in Table 3. This shows that the largest proportion of cases were caused by *Strep. uberis* in pure culture (40.0% of full data), followed by mixed infections (28.1% of full data) and NAS in pure culture (13.9% of full data). The pathogens included in the group of mixed infections, as well as the combinations of those, are specified in Table 4, which shows that NAS and *Strep. uberis* were the predominant pathogens involved in general and as a combination. Species included in the NAS group are specified in Table 5, which shows that *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus* sp., and *Staphylococcus simulans* were mostly found in pure culture, whereas *Staphylococcus* sp., *Staph. chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, and *Staphylococcus sciuri* were most abundant in mixed infections.

Follow-Up Samples

For 276 (80%) of the 345 cases (full data), both of the follow-up samples were available and useful for evaluating bacteriological cure (Figure 2). For the remaining 69 cases (20%), cure was evaluated based on a single follow-up sample [local: n = 37 (22%), combination: n = 32 (18%)]. Of the first follow-up samples, 28 (8.1%; local: n = 18, combination: n = 10) were excluded due to contamination, meaning that cure was evaluated based on the second follow-up sample only. Second follow-up samples were not collected for 17 cases, for reasons including dry-off or other treatment. Furthermore, 24 of the second follow-up samples were excluded due to contamination, so for 41 cases (11.9%; local: n = 19, combination: n = 22) in the full data, cure was evaluated based on the first follow-up sample only.

The intentional time for collecting the first follow-up sample was d 14 after the end of treatment. For practical reasons, we collected the first follow-up samples between d 14 and 20 in connection with our weekly herd visit. The interquartile range of first follow-up was 14 to 18 d, with a median of 16 d. It was practically possible to collect the second follow-up sample between d 21 and 28, but our protocol required at least 7 d between the 2 follow-up samples, so cases that were sampled late for the first follow-up were also delayed for the second follow-up. The interquartile range of the second follow-up was 22 to 26 d, with a median of 24 d. See Supplemental Figure S3 (https://doi.org/10.6084/m9.figshare.21915444.v1; Svennesen, 2023) for the distribution of times for follow-up sample collection for the cured and noncured cases stratified on treatment group.

Effects of Treatment

Based on the full data, the bacteriological cure rates of local and combination treatments were 76.8% (129 of 168 cases) and 83.1% (147 of 177 cases), respectively (Table 3). Based on the subset data with complete DHI registrations, the bacteriological cure rates of local and combination treatments were 78.4% (98 of 125 cases) and 83.6% (117 of 140 cases), respectively. Results of the noninferiority trial on the full data and the subset data (unadjusted) showed that the test statistics were 0.134 and 0.131, respectively, meaning that the local

Table 3. Univariable effects of treatment, cow characteristics, and case characteristics on bacteriological cure from clinical mastitis, based on the full data as well as a subset with complete cases (subset data)

	Full data (n = 345)				Subset data (n = 265)			
Variable and level	Total, n (%)	Cured n (%)	Not cured n (%)	*P*-value	Total n (%)	Cured n (%)	Not cured n (%)	*P*-value
Treatment								
Local	168 (48.7)	129 (76.8)	39 (23.2)	0.1457	125 (47.2)	98 (78.4)	27 (21.6)	0.2831
Combination	177 (51.3)	147 (83.1)	30 (16.9)		140 (52.8)	117 (83.6)	23 (16.4)	
Herd								
H1	24 (7)	18 (75)	6 (25)	0.1536	22 (8.3)	17 (77.3)	5 (22.7)	0.0715
H2	72 (20.9)	62 (86.1)	10 (13.9)		42 (15.8)	36 (85.7)	6 (14.3)	
H3	65 (18.8)	54 (83.1)	11 (16.9)		54 (20.4)	48 (88.9)	6 (11.1)	
H4	21 (6.1)	19 (90.5)	2 (9.5)		18 (6.8)	17 (94.4)	1 (5.6)	
H5	13 (3.8)	6 (46.2)	7 (53.8)		12 (4.5)	6 (50)	6 (50)	
H6	14 (4.1)	9 (64.3)	5 (35.7)		11 (4.2)	7 (63.6)	4 (36.4)	
H7	36 (10.4)	29 (80.6)	7 (19.4)		26 (9.8)	22 (84.6)	4 (15.4)	
H8	20 (5.8)	16 (80)	4 (20)		19 (7.2)	15 (78.9)	4 (21.1)	
H9	18 (5.2)	15 (83.3)	3 (16.7)		12 (4.5)	10 (83.3)	2 (16.7)	
H10	26 (7.5)	21 (80.8)	5 (19.2)		23 (8.7)	19 (82.6)	4 (17.4)	
H11	26 (7.5)	21 (80.8)	5 (19.2)		21 (7.9)	16 (76.2)	5 (23.8)	
H12	10 (2.9)	6 (60)	4 (40)		5 (1.9)	2 (40)	3 (60)	
Clinical score								
Mild	154 (44.6)	123 (79.9)	31 (20.1)	0.9568	105 (39.6)	82 (78.1)	23 (21.9)	0.3088
Moderate	191 (55.4)	153 (80.1)	38 (19.9)		160 (60.4)	133 (83.1)	27 (16.9)	
DIM								
0–30	80 (23.2)	61 (76.2)	19 (23.8)	0.3446	16 (6)	14 (87.5)	2 (12.5)	0.2746
31–100	100 (29)	78 (78)	22 (22)		86 (32.5)	65 (75.6)	21 (24.4)	
101–200	108 (31.3)	87 (80.6)	21 (19.4)		106 (40)	86 (81.1)	20 (18.9)	
>200	57 (16.5)	50 (87.7)	7 (12.3)		57 (21.5)	50 (87.7)	7 (12.3)	
Parity								
1	81 (23.5)	67 (82.7)	14 (17.3)	0.7766	45 (17)	38 (84.4)	7 (15.6)	0.8159
2	73 (21.2)	58 (79.5)	15 (20.5)		57 (21.5)	46 (80.7)	11 (19.3)	
3+	191 (55.4)	151 (79.1)	40 (20.9)		163 (61.5)	131 (80.4)	32 (19.6)	
Pathogen								
Streptococcus uberis[1]	138 (40)	113 (81.9)	25 (18.1)	0.0000	98 (37)[7]	82 (83.7)[7]	16 (16.3)[7]	0.0000
Streptococcus dysgalactiae[1]	21 (6.1)	21 (100)	0		19 (7.2)[7]	19 (100)[7]	0[7]	
Other streptococci[1,2]	7 (2)	7 (100)	0		6 (2.3)[7]	6 (100)[7]	0[7]	
Staphylococcus aureus[1]	23 (6.7)	12 (52.2)	11 (47.8)		17 (6.4)	9 (52.9)	8 (47.1)	
NAS[1,3]	48 (13.9)	43 (89.6)	5 (10.4)		35 (13.2)	32 (91.4)	3 (8.6)	
Enterococcus spp.[1,4]	7 (2)	1 (14.3)	6 (85.7)		6 (2.3)[8]	1 (16.7)[8]	5 (83.3)[8]	
Other gram-positive[1,5]	4 (1.2)[a]	4 (100)	0		3 (1.1)[8]	3 (100)[8]	0[8]	
Mixed infections[6]	97 (28.1)	75 (77.3)	22 (22.7)		81 (30.6)	63 (77.8)	18 (22.2)	
Treated previously								
No	301 (87.2)	238 (79.1)	63 (20.9)	0.2397	222 (83.8)	178 (80.2)	44 (19.8)	0.3535
Yes	44 (12.8)	38 (86.4)	6 (13.6)		43 (16.2)	37 (86)	6 (14)	
Quarter								
Front	163 (47.2)	133 (81.6)	30 (18.4)	0.4827	123 (46.4)	102 (82.9)	21 (17.1)	0.4861
Rear	182 (52.8)	143 (78.6)	39 (21.4)		142 (53.6)	113 (79.6)	29 (20.4)	
SCC at last DHI test before clinical case								
<200,000 cells/mL					147 (55.5)	126 (85.7)	21 (14.3)	0.0338
>200,000 cells/mL					118 (44.5)	89 (75.4)	29 (24.6)	
Days between last DHI test and clinical case								
0–14					122 (46.0)	96 (78.7)	26 (21.3)	0.4465
15–28					100 (37.7)	85 (85)	15 (15)	
>28					43 (16.2)	34 (79.1)	9 (20.9)	
ECM at last test day before clinical case (continuous)								
ECM in kg, mean (SE)					39.70 (0.64)	39.78 (0.73)	39.36 (1.32)	0.6780

[1]Isolated in pure culture.

[2]*Streptococcus* sp. (n = 3), *Streptococcus canis* (n = 2), *Streptococcus agalactiae* (n = 1), *Streptococcus gallolyticus* (n = 1, n = 0 in subset data).

[3]Species are shown in Table 5.

[4]*Enterococcus faecium* (n = 6, n = 5 in subset data), *Enterococcus saccharolyticus* (n = 1).

[5]*Lactococcus garvieae* (n = 2), *Corynebacterium* sp. (n = 1), *Carnobacterium maltaromaticum* (n = 1, n = 0 in subset data).

[6]Two pathogens detected in the same sample; species are summarized in Table 4.

[7]*Streptococcus uberis*, *Streptococcus dysgalactiae*, and other streptococci were grouped together as streptococci in the multivariable analysis.

[8]*Enterococcus* spp. and other gram-positive were grouped together as other for the multivariable analysis.

Table 4. Numbers and proportions of pathogens and combinations of pathogens in mixed infections (samples with 2 pathogens detected) in full data and in a subset with complete cases (subset data)

	Full data (n = 97 cases)		Subset data (n = 81 cases)	
Item	n	%	n	%
Pathogen				
Total	194	100	162	100
Streptococcus uberis	44	22.7	34	21.0
Streptococcus dysgalactiae	9	4.6	8	4.9
Other streptococci[1]	6	3.1	6	3.7
Staphylococcus aureus	10	5.2	10	6.2
NAS[2]	80	41.2	67	41.4
Enterococcus spp.[3]	6	3.1	6	3.7
Other gram-positive[4]	20	10.3	17	10.5
Not gram-positive[5]	19	9.8	14	8.6
Combination of pathogens in mixed infection				
Total	97	100	81	100
Streptococci and streptococci[6]	2	2.1	2	2.5
Streptococci and *Staphylococcus aureus*	5	5.2	5	6.2
Streptococci and NAS	33	34.0	26	32.1
Streptococci and other gram-positive[7]	8	8.2	7	8.6
Streptococci and not gram-positive	9	9.3	6	7.4
Staphylococcus aureus and NAS	3	3.1	3	3.7
Staphylococcus aureus and other gram-positive[7]	1	1.0	1	1.2
Staphylococcus aureus and not gram-positive	1	1.0	1	1.2
NAS and NAS	11	11.3	10	12.3
NAS and other gram-positive[7]	15	15.5	13	16.0
NAS and not gram-positive	7	7.2	5	6.2
Other gram-positive[7] and not gram-positive	2	2.1	2	2.5

[1]*Streptococcus* sp. (n = 4), *Streptococcus gallolyticus* (n = 1), *Streptococcus parauberis* (n = 1).

[2]Species are shown in Table 5.

[3]*Enterococcus faecium* (n = 2), *Enterococcus saccharolyticus* (n = 1), *Enterococcus hirae* (n = 1), *Enterococcus malodoratus* (n = 1), *Enterococcus* sp. (n = 1).

[4]*Aerococcus viridans* (n = 4), *Bacillus* sp. (n = 1), *Bacillus pumilus* (n = 1) *Corynebacterium* sp. (n = 2, n = 1 in subset data), *Corynebacterium amycolatum* (n = 1), *Corynebacterium ulcerans* (n = 1), *Corynebacterium xerosis* (n = 1), *Lactococcus garvieae* (n = 4), *Lactococcus* sp. (n = 2), *Micrococcus luteus* (n = 2, n = 1 in subset data), *Micrococcus* sp. (n = 1, n = 0 in subset data).

[5]*Acinetobacter lwoffii* (n = 1, n = 0 in subset data), *Escherichia coli* (n = 10, n = 7 in subset data), *Klebsiella oxytoca* (n = 1), No ID (n = 1), *Pantoea agglomerans* (n = 1), *Pasteurella multocida* (n = 1), *Proteus* sp. (n = 1), *Pseudomonas stutzeri* (n = 1), *Serratia liquefaciens* (n = 1, n = 0 in subset data).

[6]Includes *Streptococcus uberis*, *Streptococcus dysgalactiae*, and other streptococci.

[7]Includes *Enterococcus* spp. and other gram-positive described in footnote 4.

treatment was noninferior to the combination treatment at the 15% margin, because the test statistic was below 0.15. The estimated differences between treatments, based on a two-sided test of the full data with 95% confidence levels, are shown in Figure 3.

In the backward elimination procedure for the multivariable model on the subset data, we retained only pathogen type and SCC, and treatment was forced in (Table 6). The cure rate for local treatment was not significantly different ($P = 0.197$) from that for combination treatment. Regarding pathogens, the only significantly differences to the cure rate of streptococci were cure rates of *Staph. aureus* ($P = 0.001$) and other pathogens ($P = 0.004$), with odds ratios of 0.128 [95% CI: 0.039;0.413] and 0.108 [95% CI: 0.022;0.480], respectively. The odds of cure were lower (0.414 [95% CI: 0.196;0.828]) for cows with SCC >200,000 cells/mL compared with SCC <200,000 cells/mL ($P = 0.016$). The effects of pathogen and SCC on cure rates were independent of whether treatment was local or combined. The predicted values from the multivariable model are plotted in Figure 4. The figure shows that the highest cure rates were found for streptococci, NAS, and mixed infections across both treatment groups. The same pattern was found for cows with high SCC (>200,000 cells/mL) but with overall lower cure rates.

A test statistic of 0.083 (i.e., below 0.15) was obtained from the noninferiority trial on the modeled subset data (adjusted). Thus, the model results also show that the local treatment was noninferior to the combination treatment using the 15% noninferiority margin (Figure 3).

DISCUSSION

To our knowledge, this is the first study to compare the bacteriological cure rates for local and combined treatment with penicillin only. We found that local

Table 5. Numbers and proportions of NAS species and isolates identified as *Staphylococcus* sp. (via MALDI-TOF) in pure-culture and mixed infections; the numbers are shown for the full data and for a subset with complete cases (subset data)

	Full data		Subset data	
Species	n	%	n	%
Pure				
Total	48	100	35	100
Staphylococcus chromogenes	13	27.1	9	25.7
Staphylococcus epidermidis	2	4.2	2	5.7
Staphylococcus equorum	2	4.2	2	5.7
Staphylococcus gallinarum	2	4.2	2	5.7
Staphylococcus haemolyticus	10	20.8	5	14.3
Staphylococcus hyicus	1	2.1	1	2.9
Staphylococcus sciuri	3	6.3	2	5.7
Staphylococcus simulans	5	10.4	4	11.4
Staphylococcus sp.	8	16.7	6	17.1
Staphylococcus xylosus	2	4.2	2	5.7
Mixed				
Total	80	100	67	100
Staphylococcus capitis	1	1.3	1	1.5
Staphylococcus chromogenes	17	21.3	12	17.9
Staphylococcus epidermidis	10	12.5	9	13.4
Staphylococcus equorum	10	12.5	9	13.4
Staphylococcus gallinarum	1	1.3	1	1.5
Staphylococcus haemolyticus	5	6.3	4	6.0
Staphylococcus hominis	1	1.3	1	1.5
Staphylococcus hyicus	1	1.3	1	1.5
Staphylococcus sciuri	10	12.5	7	10.4
Staphylococcus sp.	17	21.3	15	22.4
Staphylococcus succinus	4	5.0	4	6.0
Staphylococcus xylosus	3	3.8	3	4.5

treatment of nonsevere clinical mastitis cases with penicillin was noninferior to the combination of local and systemic treatment with penicillin, with a noninferiority margin of 15%. Adjusting for SCC category and pathogen type further reduced the difference between the 2 treatments, because some of the variation was absorbed by the additional explanatory factors. Accordingly, potential exists to reduce the amount of antibiotic active compound used for a single mastitis treatment of a 600-kg cow by a factor of 16, thereby reducing the selection pressure for antimicrobial resistance (Nobrega et al., 2018).

It has previously been suggested that local treatment could replace systemic or combined treatment to reduce the use of antimicrobials in the treatment of *Strep. uberis* mastitis (Hillerton and Kliem, 2002). By comparing combined, local, and systemic treatments containing penicillin and dihydrostreptomycin, Hillerton and Kliem (2002) showed no significant difference in the bacteriological cure rates of experimentally infected *Strep. uberis* mastitis cases in 54 cows. The reported cure rates after treatment were 70 to 80%, which is in line with the 82% overall cure rate (unadjusted, full data) for *Strep. uberis* found in the current study (Table 3). This cure rate may actually be even higher because we did not have strain information available, and some noncured cases may therefore be reinfections with another strain of the same species, which appears to be common for streptococci (Wente et al., 2020).

In contrast, the cure rate of *Staph. aureus* was low (52%, Table 3) compared with results reported in previous studies (Taponen et al., 2003; Kalmus et al., 2014), and significantly lower compared with the cure rate of streptococci (Table 6). A positive effect of systemic treatment on *Staph. aureus* has been suggested (Taponen et al., 2003; Barkema et al., 2006), as this species penetrates udder tissue and causes deep infection, meaning it is unlikely that antibiotics administered IMM will reach an effective concentration at the location of the *Staph. aureus* infection (Pyörälä, 2009). However, our model results do not suggest that the efficacy difference between local and combination treatment was pathogen-dependent, as the interaction between pathogen and treatment was excluded from the model based on model fit. This could, however, be due to the limited number of *Staph. aureus* cases observed. Taponen et al. (2003) reported a significant difference between the bacteriological cure rate of β-lactamase-negative *Staph. aureus* cases receiving 5 d of systemic penicillin G treatment and that of a combination treatment also including 4 d of local treatment with penicillin and neomycin. The cure rate was 79% for the combination treatment, which is higher than the estimated 52% cure rate for *Staph. aureus* in the current study (Table 3). Furthermore, Kalmus et al. (2014) reported 6 of 8 cases cured, but they found no difference in the effect of local treatment compared with systemic treatment. The lower cure rates of *Staph. aureus* found in the current study may be explained by a lack of knowledge about β-lactamase susceptibility, the small number of *Staph. aureus* cases (which was also the situation in the study by Kalmus et al., 2014) resulting in wide confidence intervals (Figure 4), and the lack of neomycin (Mehta and Champney, 2003) in the combination treatment used by Taponen et al. (2003). By contrast, Waage (1997) reported a cure rate of 52% for *Staph. aureus* and found no difference between treatment effect of adding either 1 or 3 d procaine benzylpenicillin i.m. to 5 d of IMM administration of dihydrostreptomycin sulfate and procaine benzylpenicillin.

We found a clear difference in the probability of cure depending on the pathogen group (Table 6), where the lowest cure rates were found for *Staph. aureus* and other pathogens (see Table 3 for specification of "other"), and the highest probability of cure was found for streptococci and NAS (Figure 4). Furthermore, we found that the probability of cure was smaller if the SCC at the last DHI test before the clinical case was >200,000 cells/mL, compared with <200,000 cells/mL (Table 6 and Figure 4). Surprisingly, we found a signifi-

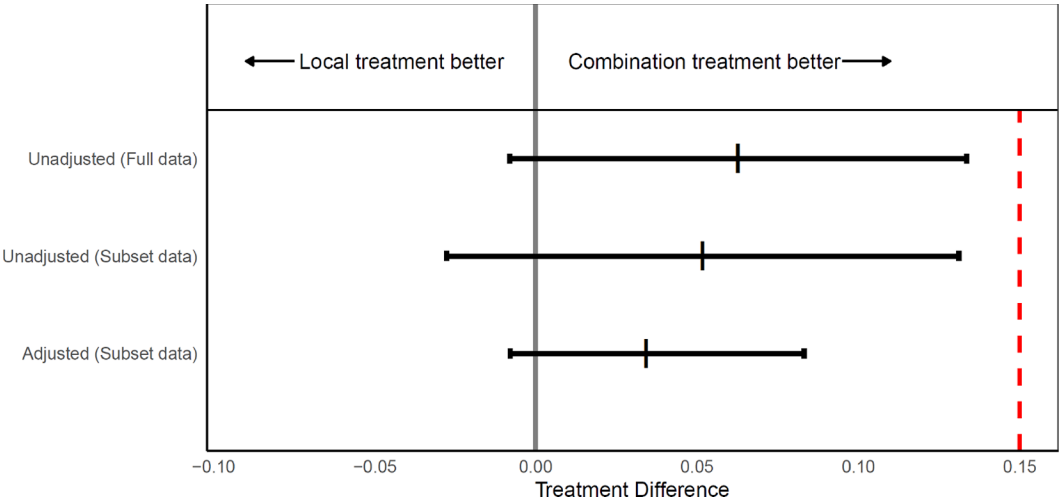

Figure 3. Mean estimated difference and 95% confidence intervals in proportions of cure between the 2 treatments. The figure shows the estimated difference from the full data (upper bar) with no adjustment (n = 345 cases), as well as from the subset data (middle bar) with no adjustment (n = 265 cases) and the final model (lower bar) with adjustment for pathogen and SCC group in the subset data (n = 265 cases). The red dotted line shows the chosen noninferiority margin at 15%. The figure shows that the local treatment is noninferior to the combined treatment, for both the unadjusted and the modeled (adjusted) estimates.

cant association ($P = 0.016$) between the probability of cure and SCC even though the DHI measurement was at cow level and about half of the cases had a DHI test more than 14 d before the clinical case (Table 3). This could be explained by more chronic infections being harder to cure (Ziesch and Krömker, 2016), in line with recent findings by Williamson et al. (2022), who reported that bacteriological cure rates after antibiotic treatment were negatively associated with SCC at the time of the clinical case.

The pathogen-dependent cure rates are in line with previous estimations based on local treatment, including treatments with different antibiotics (Pinzón-Sánchez et al., 2011; Ruegg, 2018). Furthermore, the cure rates are higher than the expected spontaneous cure rates given by Pinzón-Sánchez et al. (2011) at 0 to 5%, 25 to 30%, and 55 to 60% for *Staph. aureus*, streptococci and NAS, respectively, suggesting that local treatment is more effective than no treatment. Compared with these low cure rates, a 15% noninferiority limit seems very acceptable (EMEA, 1999).

The distribution of pathogens in this study, with *Strep. uberis* being the most prevalent of the gram-positive bacteria detected by the on-farm test (Table

Table 6. Multivariable model estimates of the effect of treatment, pathogen, and SCC at the last DHI test before the clinical case on bacteriological cure from mild to moderate clinical mastitis cases; the multivariable logistic regression analysis was carried out on subset data

Variable and level	Estimate	95% CI estimate	Odds ratio	95% CI odds ratio	*P*-value
Treatment					
Combination	Reference				
Local	−0.441	[−1.124; 0.226]	0.643	[0.325; 1.253]	0.197
Pathogen					
Streptococci[1,2]	Reference				
Staphylococcus aureus[1]	−2.052	[−3.248; −0.884]	0.128	[0.039; 0.413]	0.001
NAS[1,3]	0.469	[−0.772; 2.008]	1.599	[0.462; 7.447]	0.492
Other[1,4]	−2.229	[−3.809; −0.733]	0.108	[0.022; 0.480]	0.004
Mixed infections[5]	−0.762	[−1.551; 0.006]	0.467	[0.212; 1.006]	0.053
SCC at last DHI test before clinical case					
<200,000 cells/mL	Reference				
>200,000 cells/mL	−0.882	[−1.631; −0.188]	0.414	[0.196; 0.828]	0.016

[1]Isolated in pure culture.

[2]Including *Streptococcus uberis* (n = 98), *Streptococcus dysgalactiae* (n = 19), other streptococci (n = 6); see Table 3 (subset data).

[3]Species are shown in Table 5 (pure culture, subset data).

[4]Including *Enterococcus* spp. (n = 6), *Lactococcus garvieae* (n = 2), *Corynebacterium* sp. (n = 1); see Table 3 (subset data).

[5]Two pathogens detected in the same sample; species are summarized in Table 4 (subset data).

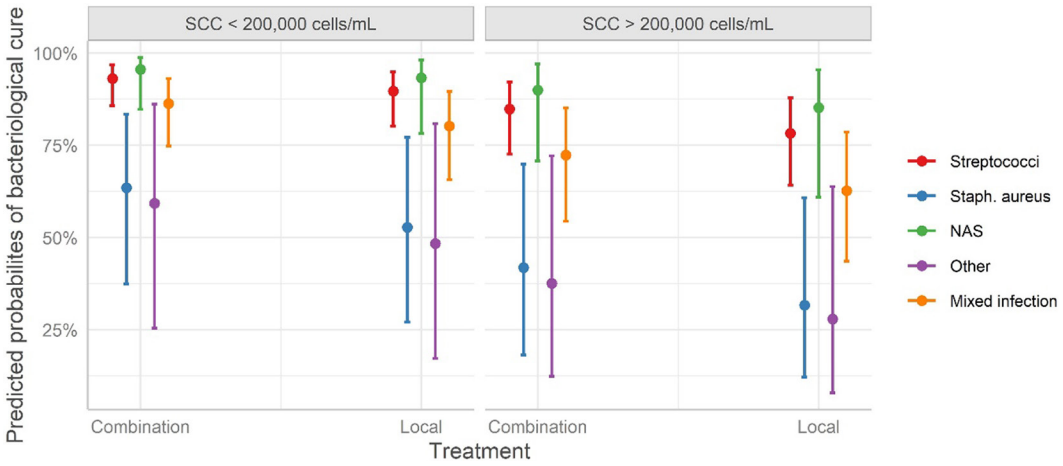

Figure 4. Predicted probabilities of bacteriological cure rates of clinical mastitis cases stratified by SCC (last DHI test before clinical case), pathogen (isolated from the clinical case), and treatment. The plot shows the marginal effects from the multivariable logistic regression analysis carried out on subset data. Whiskers show the 95% confidence intervals.

3), corresponds to the distribution of gram-positive pathogens reported from other countries with larger intensively managed farms (Ruegg, 2018). In that study, the most prevalent reported findings were no growth and gram-negative bacteria, which is in accordance with our study, where more than half of the cases tested were excluded as being gram-negative or showing no growth samples (Figure 2). A recent study in Denmark, however, reported gram-positive bacteria as the most common cause of clinical mastitis, with *Staph. aureus* and *Strep. uberis* as the gram-positive pathogens with the highest prevalence in pure cultures (Astrup et al., 2022).

Even though the distribution of pathogens and the product used for local treatment were similar to those used in the study by Kalmus et al. (2014), and they applied treatment over the course of 5 d, we obtained a higher unadjusted overall bacteriological cure rate (76.8%, Table 3) compared with the 56% they reported. This was likely affected by their choice of PCR testing to assess cure, whereas we used bacterial culture and MALDI-TOF. A higher test sensitivity or the detection of nonviable bacteria by PCR may have resulted in noncured cases that would have been categorized as cured by bacterial culture with a cut-off of 100 cfu/mL, as used in the present study. Furthermore, the PCR test used by Kalmus et al. (2014) detected NAS species as a group; thus bacteriological cure of NAS was assessed at group level, which likely decreased the cure rate compared with our study, where cure was assessed at species level. However, we only assessed bacteriological cure, which can be different than clinical and cytological cures, also important measures, especially in terms of animal welfare and the farm's financial situation (Ruegg, 2021).

In general, high bacteriological cure rates from penicillin treatment in the current study could be a result of the generally low antimicrobial resistance reported in Denmark (Chehabi et al., 2019; DANMAP, 2020). Furthermore, we only estimated bacteriological cure for cows that were clinically cured and for cases selected via on-farm testing. It is therefore possible that the included cases represent those that are most likely to be bacteriologically cured. Moreover, all treatments included ketoprofen (NSAID), which could have positively affected cure rates, especially clinical cure (Petersson-Wolfe et al., 2018). Our main focus, however, was the effect of the systemic treatment added to the local treatment (combination treatment), and it was most important that the treatment groups were comparable. Including cases with lack of clinical cure as treatment failures would have decreased cure rates in general. Noteworthy, we found fewer cases with lack of clinical cure (Figure 2) after local treatment (15 of 268) compared with after combination treatment (35 of 307). Including those cases as treatment failures would have decreased the cure rate of the combination treatment to a higher extent than the cure rate of the local treatment; thus, the conclusion would likely stay the same.

The initial on-farm selection of cases before treatment may be an important first step if antimicrobial consumption should be minimized, ensuring that only cases with a high probability of cure are treated (Ruegg, 2018). Several studies have shown that selective antimicrobial treatment of mild to moderate clinical mastitis has no negative consequences in terms of, for example, cure rate, milk yield, SCC, or risk of recurrence (Lago et al., 2011a,b; Vasquez et al., 2017; de Jong et al., 2023). However, the effects have not really been stud-

ied in conditions where staphylococci and streptococci dominate, and assessing this was outside the scope of this study. Schmenger et al. (2020), however, found no adverse effects of delayed treatment in pathogen distributions relatively similar to that of the current study.

The NSAID was included in both study treatments and therefore did not affect the comparison. In addition, it is common to treat mastitis with NSAID in Denmark (Wilm et al., 2021), and this is recommended in targeted mastitis treatment (Schmenger et al., 2020) where an initial on-farm selection of mastitis cases for treatment is implemented.

We lost a considerable number of cases due to a lack of follow-up samples. These cases were excluded for several reasons (Figure 2), including a lack of clinical cure, missing samples, and registration errors, and we have no reason to believe that this caused any significant selection bias (Supplemental Figure S2). The allocation of cows to treatment group by farm staff may have introduced bias to the comparison of treatment. As treatments were not blinded, there is a risk that deviations from the protocol were not random, although all deviations were discussed with the farm personnel weekly to ensure high compliance. We examined covariates for wrongly allocated and untreated cows and found no noticeable differences compared with the group of cows treated correctly according to the protocol (Supplemental Figure S1). We decided not to conduct the noninferiority test on a subset of the data without these wrongly allocated cows because this would result in a substantially reduced sample size. Furthermore, we assessed the relationship between cure and times for follow-up sample collection, because later follow-up could have increased chance of cure but, on the other hand, increased the risk of new infections. Due to the randomized nature of the study the times for follow-up cannot be systematically biased with respect to treatment group, and we saw no relationship between follow-up times and cure (Supplemental Figure S3). Cure evaluated based on a single follow-up sample has decreased sensitivity compared with evaluation based on 2 follow-up samples. The implication of including cases with a single follow-up sample is, thus, that we are overestimating cure rates. We expect that the impact is limited, as the cure rates are in line with or lower than those observed in previous studies. However, the proportions of cases with a single follow-up sample were 22% and 18% for local and combination treatment, respectively; thus the treatment difference could be slightly increased if we had both follow-up samples available for all cases.

We conducted this study as a field trial in commercial farms to reflect the current situation. Consequently, the cure rates obtained were highly dependent on the included cases, which represented the normal variation in commercial dairy herds, including the farm personnel's identification of clinical mastitis, time before reacting to clinical symptoms, and criteria for treatment. Moreover, the on-farm test was used under field conditions and therefore includes a potential observer effect (Sipka et al., 2021), which may result in lower test performance compared with laboratory test evaluation. For example, variation in incubation time due to differing working routines among farms would affect test characteristics because a longer time for incubation leads to a longer period for bacteria to grow, which will increase the sensitivity but decrease the specificity of the test (Leimbach and Krömker, 2018). Due to these factors, we believe that the results closely reflect what can be expected from other Danish dairy herds. We also found a significant association between the probability of cure and pathogen and SCC. Cows with SCC >200,000 cells/mL had a remarkably reduced probability of cure compared with cows with SCC <200,000 cells/mL. This highlights the importance of taking more information about the individual cow into consideration, rather than just the bacteriological test result. Moreover, it is important to use further diagnostics to identify the causative pathogen and thereby estimate the probability of cure more precisely. It is also important to identify the pathogens circulating at herd level in order to be able to pinpoint the most prevalent among them, which will reflect the general probability of cure in the herd, and ideally to implement preventive measures before clinical mastitis is detected.

Our results reveal that readily accessible knowledge of microbial characteristics (e.g., through on-farm testing) and the reduced use of antibiotics when using local compared with combined treatment have the potential to drastically reduce the overall use of antibiotics in dairy herds.

CONCLUSIONS

Based on our data, we found that bacteriological cure rate associated with using local penicillin treatment for mild and moderate clinical mastitis cases was noninferior to the combination of local and systemic treatment using a 15% noninferiority margin. We therefore find potential to reduce antimicrobial use dramatically without substantially compromising the efficacy of mastitis treatment, although factors influencing the

individual cases should be taken into account when making treatment-related decisions.

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