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Randomized noninferiority field trial evaluating a postmilking teat dip for the prevention of naturally occurring intramammary infections

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ABSTRACT

The aim of this study was to perform a positive-controlled field study under natural exposure conditions to test the efficacy of a newly developed chlorine dioxide-based postmilking teat disinfectant (experimental product, EX) for noninferiority compared with an already established chlorine dioxide-based teat disinfectant (positive control product, PC). After blocking by parity, approximately 200 Holstein cows in early to mid-lactation stages from a dairy farm near Padua, Italy, were randomly assigned to one of 2 groups. Over a 13-wk period between September and December 2021, the teats of cows were dipped with the EX or the PC after each milking. Milk samples were collected from individual quarters of enrolled cows for 13 wk to determine infection status. Teat condition was assessed at wk 1, 5, and 9. Mixed logistic regression was used to analyze the effect of treatment on the incidence of new intramammary infections. For the noninferiority analysis, the upper limit of the 95% confidence interval for the difference in new intramammary infection (NIMI) rate between the 2 treatments (EX − PC) had to be to the left of the critical value d (0.035) to conclude that EX was noninferior to PC in terms of the risk of NIMI. The results showed that the incidence of new infections in the quarters treated with EX (3.1%) was not different from that in the udder quarters treated with PC (2.6%). No overall difference was found between the treatments in terms of teat condition. As the upper limit of the 95% confidence interval of the NIMI rate difference was smaller than the predefined noninferiority limit, we concluded that the EX was noninferior compared with the PC.

Key words: teat disinfectant, chlorine dioxide, intramammary infection, noninferiority

INTRODUCTION

Although treatment and prevention of dairy cow mastitis has improved, mastitis continues to be a major cause of profit reduction for dairy farmers due to decreased milk quality and production as well as loss of animals (Ruegg, 2012). Requirements for a teat disinfectant for dairies include new IMI (**NIMI**) prevention, nonirritation for animals and humans, proven germicidal efficacy, promotion of lesion healing and teat condition, and no disinfectant residue that may affect human health.

Today, teat disinfectant products are considered as over-the-counter products in the United States. These products are subject to the laws outlined by the Food and Drug Administration, whereas in Europe, teat disinfectants are regulated by the European Medicines Evaluation Agency or European Medicines Agency (NMC, 2017; EMA, 2019).

Teat disinfectant products can be evaluated using in field and laboratory methods. Although standard in vitro tests (e.g., EN 1656; EN 1657) can be used to assess germicidal efficacy, on-farm studies provide further proof of product safety and postmilking teat disinfectant efficacy to control mastitis. Verification of mastitis prevention can be confirmed when disinfectant products are field tested against a product of proven efficacy. The NMC (2022) protocols are available to produce these comparisons. Bacterial colonization and possibly IMI may be reduced by the maintenance of healthy teat skin. The NMC (2022) has published guidelines for teat end scoring.

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Worldwide, more regulations on raw materials used for teat disinfectant manufacturing are being implemented. For example, EU countries are required to conduct strict controls for any product that may come into contact with an animal used in food production (e.g., European Parliament, 2012). These regulations require that new products are reviewed and authorized before being sold and their active substances must have already been approved. Teat disinfectants for dairy animals are included under these regulations.

An excellent disinfection effect is achieved when using a chlorine dioxide-based disinfectant (Noszticzius et al., 2013; Jefri et al., 2022). Chlorine dioxide used for postmilking applications has been proven successful over time to reduced *Staphylococcus aureus* infections for 67.4%, *Streptococcus dysgalactiae* for 63.8%, and *Streptococcus uberis* for 27.8% (Oliver et al.,1989). A pH shift in the disinfectant solution begins the disinfecting process and this process allows for maximum disinfection performance at the targeted site. The pH shift is achieved by combining 2 solutions immediately before use.

The primary aim of this study was to demonstrate noninferiority of the experimental test product (**EX**) when compared with an already established chlorine dioxide-based teat disinfectant product (positive control, **PC**) with regard to the incidence of NIMI that occurred under natural challenge conditions on a commercial dairy farm. Secondary objectives included describing the effect of treatment on the prevalence of IMI and teat condition throughout the trial period.

MATERIALS AND METHODS

Ethical Statement

This study was approved by the Animal Welfare Organization (OPBA) of the University of Milano and the Italian Health Ministry (Authorization no. 68/2016-PR) and also approved by Dipartimento di Medicina Veterinaria e Scienze Animali with protocol number CS-NAZPR22PMORO_01.

All cows were randomly assigned to one of 2 groups, after clustering by parity, and were not subjected to any invasive procedures. Milk samples used for the culture were collected weekly by trained staff from the University of Milano (AM and DM), with training and supervision conducted by the principal investigator (NR).

Study Farm and Study Pens

This randomized, noninferiority, positive-controlled field study was conducted on a commercial Holstein dairy farm near Padua in Northeast Italy. The farm milks approximately 987 lactating cows 2 times daily in an 80-stall rotary parlor with herd average milk production of 35 kg/d, SCC of 229,000 cells/mL, butterfat at 3.7%, and protein at 3.4%. Lactating cows were housed in 1 of 8 freestall pens using recycled manure bedding from a biodigestor. After calving, cows spent approximately 30 d in a fresh cow pen, after which they are moved into early- or mid-lactation pens. The 4 pens were selected because they were completely identical in design, located under the same barn roof on a common feeding axis, and offered the highest probability that the animals could remain in their respective pens for the entire experimental procedure. General information on farm management practices was collected at the beginning of the study using a brief questionnaire. In the period before the start of the trial, findings of *Staph. aureus* had occurred several times in clinical mastitis cases. The herd was also prescreened using bulk tank PCR (Cremonesi et al., 2007) to determine that it was negative for *Mycoplasma* spp.

Cow Enrollment

Two weeks before the study, cows were initially identified as a subset of 600 Italian Holstein cow located in 4 pens of 150 each (2 with first lactation cows and 2 with older cows). The study animals were randomly chosen from these 4 pens (~55 animals per pen). Only those clinically healthy animals not having received antibiotics or anti-inflammatory products within 14 d before enrollment and without a diagnosed IMI within the past 30 d before enrollment and expected to remain in their respective barns for the duration of the experiment were included in the study (e.g., no dry-off in the study period). In the wk 0 sampling, 14 animals (6 for EX, 8 for PC) of the preselected animals with blind quarters were excluded and 17 (8 for EX, 9 for PC) were excluded due to contaminated samples. Animals with blind quarters were excluded to avoid bias in the results, and animals with contaminated quarters in wk 0 were excluded because it was not possible to confidently classify new infections of individual quarters in wk 1.

No other inclusion or exclusion criteria were applied for enrollment eligibility. After blocking by parity (lactation 1 vs. lactation $\geq$2), cows were assigned to treatment groups using randomly generated numbers (Excel, Microsoft Corp.). A cow was identified by a unique ear tag and colored chalk paint (EX blue, PC red). After this initial treatment group assignment, new cows, which did not enter the experiment, could be assigned to the 4 study pens by the producer during the 13-wk study period [with the same randomization

scheme (random number list) but without following any cow-dependent criteria], after leaving the fresh cow pen approximately 30 d after calving.

Teat Disinfectant Activation and Identification

Throughout the 13-wk study, the same commercially available ready-to-use premilking teat disinfectant with chlorhexidine, glycerin, D-glucitol, and lanolin (Clorex Foam, 2,000 mg/L; by Klareco S.r.l) was used on both EX and PC animals. Cows in both groups were marked before start of the trial with 2 lines of paint (authorized for animal use) on the back of each rear leg under the hocks (for EX cows blue, PC cows red). Cows in EX group were treated after each milking using a chlorine dioxide-based postmilking teat disinfectant product (Bioxy Shield, Baxter Post; by Klareco S.r.l.). Cows in PC group were treated postmilking using a chlorine dioxide generated from sodium chlorite by acidification (Alcide Uddergold Platinum Ecolab S.r.l.).

The premilking product was placed at the cow entrance of an 80-unit rotary parlor. The 2 postmilking products (EX and PC) were placed at the exit of the parlor in 3 separate cans: EX product in a 10-L mixing can, PC product inside its commercial package, without original branding labels, consisting of 2 cans of 20 L each, one labeled "Prodotto B base," the second labeled "Prodotto B attivatore." The EX product mixture was made by pouring 5 L from a 25-L can labeled "Prodotto A base" and 5 L from a 25-L can labeled "Prodotto A attivatore," turned upside-down 10 times to mix. The 10-L can was filled with the EX product mixture on average once every 3 d, under the supervision of the barn manager. The PC product was instead mixed in equal parts inside a nonreturn dipping cup. Mixing was done by farm personnel at each milking time. Nonreturn dip cups of different colors were used (EX = orange; PC = yellow) and hung in separate areas. Farm employees filled color-coded dip cups with the corresponding product. Cans of each product were replaced as needed by the study monitor or the herd manager. Because EX and PC products could be identified visually, it was not possible to blind the on-farm personnel involved in the study. Farm personnel were not aware of the specific details regarding the composition and brand of the products tested.

Application of Teat Disinfectants by Parlor Staff

During the 13-wk study, teats of cows entering the milking parlor were fore-stripped onto the floor (3 squirts/each) and at least two-thirds of the length of each teat was dipped with the premilking teat disinfectant by means of a foaming dip cup. After a period of 30 to 45 s, the premilking teat dip was removed by a second milker using a single-use paper towel; a third milker attached the units. After milking, at least two-thirds of the length of the teat was dipped by a fourth milker using one of the 2 postmilking teat disinfectants included in this trial (either EX or PC) by means of a color-coded nonreturn dip cup.

Training and Monitoring. Before initiating the study, all milkers from the 2 milking shifts were trained in teat disinfectant product management. Study technicians were trained in milk sample collection and management, and one (lead) study technician was trained in completion of teat condition scoring (skin and sphincter, performed at wk 1, 5, and 9). One of the study investigators (NR) visited the dairy to oversee milking procedures and activities at sampling events every week. Another study investigator (REE) visited the herd weekly to check the milking procedure, product availability, product activation, and product application. To ensure that the milking parlor was not a variable, technicians from Associazione Provinciale Allevatori evaluated the milking parlor before the project began according to the guidelines outlined by the National Mastitis Council (NMC, 2012).

The study monitor (NR) visited the herd at weekly intervals to collect milk samples, evaluate management and application of the teat disinfectants (dip cup identification and cleanliness, product application, and activation procedures), monitoring of product usage volumes (measured daily by the herd manager using an electronic scale), and ensuring correct identification and application of leg paint.

Milk Sample Collection and Teat Condition Scoring. Starting at wk 0 (baseline sample), sampling of all quarters of all study cows was conducted every week for a total of 13 wk between September 21 and December 14, 2021. Sampling took place during the afternoon milking according to NMC (2017) recommendations for aseptic collection of milk samples. The procedure was performed as follows: fore-stripping, premilking disinfection, wiping dry after 30 to 45 s of contact time, scrubbing the teat end with an alcohol scrub, discarding 3 or 4 squirts of foremilk onto the floor, and sample collection. Approximately 10 mL of milk was collected with a sterile technique from each teat into sterile vials. After collection, milk samples were delivered at 4°C to the Dipartimento di Medicina Veterinaria e Scienze Animali laboratory and bacteriological assays were performed the day after.

Teat hardness, color, and degree of hyperkeratosis at the teat orifice were evaluated by the same lead study technician at wk 1, 5, and 9 of the study. This evaluation was performed after the milking according to Falkenberg et al. (2003).

Teat hardness was scored on a scale of 0 to 1, where 0 = normal (soft and supple) and 1 = firm, swollen or hard, or severely wedged.

During teat color evaluation, black teats were excluded from any color-based evaluation. Changes were examined within 1 min of cluster removal and classified according to the proportion of light-colored teats, as follows: normal (pink), reddened (part of or all the teat end or barrel may be discolored), and blue-colored (part of or all the teat surface appears to be tinged with blue or purple). A further simplification was to combine the red and blue categories into a single category for analysis of normal (pink) versus discolored (red or blue-colored). Teat color was scored on a scale of 0 to 1, where 0 = normal (pink) and 1 = discolored (red or blue-colored).

Hyperkeratosis of the teat orifice was scored on a scale of 1 to 4, with 1 = no ring, smooth teat end and sphincter with no evidence of roughness, 2 = smooth or slightly rough ring, slight irregularities or fringes of roughness near orifice, 3 = rough, teat end sphincter is moderately roughened with radial cracks, 4 = very rough, teat orifice is significantly roughened with pronounced cracking (Mein et al., 2001). To simplify the presentation, the degree of hyperkeratosis score was then merged to normal (no relevant hyperkeratosis; includes score 1 and 2) and to abnormal (relevant hyperkeratosis, score 3 and 4).

Bacteriological Analysis. The samples were processed the day after retrieval and bacteriological milk culturing was performed at the University of Milano Dipartimento di Medicina Veterinaria e Scienze Animali, which followed published procedures recognized by the National Mastitis Council for bovine mastitis (NMC, 2017). Laboratory technicians were blinded to treatment and cow. Ten microliters of each milk sample were spread on blood agar plates (5% defibrinated sheep blood; Microbiol). Plates were incubated aerobically at 37°C and examined after 24 and 48 h. In the case of bacterial growth, a representative colony was submitted for MALDI-TOF analysis as described by Randall et al. (2015). The instrument reports a logarithmic score between 0 and 3 quantifying similarities to known database entries. A log (score) $\geq$2.0 was the threshold for the species level identification. Microorganisms other than bacteria were confirmed by microscopic appearance. Samples with the growth of 3 or more pathogens were considered contaminated.

Definition of Infection Status. An IMI was defined as 1 or more colonies isolated from the 10-μL milk sample for all pathogens except for non-*aureus* Staphylococci (**NAS**). For NAS, 2 or more colonies isolated from the 10-μL milk sample were needed to establish presence of an IMI (Dohoo et al., 2011).

The initial bacteriological status of each quarter was established at the beginning of the trial using a single sample (wk 0). Once a quarter was identified as being infected with a particular organism, any repeat infection of the same quarter with the initially identified pathogen was not considered an NIMI (Ceballos-Marquez et al., 2013). An NIMI was defined as a quarter wherein the organism isolated had not been present in any previous bacteriological sample. In the case of a mixed infection where both organisms present were new, the NIMI was only counted once at the quarter level. Only one NIMI per pathogen species was allowed per quarter during the 13-wk trial period, meaning that a quarter could have NIMI caused by several pathogen species.

Statistical Analysis

Establishing a Margin of Inferiority. We followed the same approach as previously described by Ceballos-Marquez et al. (2013) and Godden et al. (2016), and therefore proposed a noninferiority margin, d, of 0.035 (3.5%). The selected noninferiority margin is consistent with the recommendations of the National Mastitis Council that the noninferiority margin should not be less than 0.3.

Our null hypothesis was that treatment with EX was inferior to treatment with PC, or $P(NIMI)_{Test} - P(NIMI)_{Control} \geq \Delta$, where Δ = the prespecified margin of inferiority. Our alternative hypothesis, if we rejected the null, was that treatment with EX was not inferior compared with treatment with PC, or $P(NIMI)_{Test} - P(NIMI)_{Control} < \Delta$.

Sample Size Calculations. Having established the margin of inferiority (d = 0.035) and assuming a 3.5% NIMI rate for the PC group, it was estimated that at least 342 quarters (approximately 86 cows) per treatment group were required to provide the desired 80% power and 95% confidence ($\alpha = 0.05$) to detect a treatment difference, if one was truly present (noninferiority tests for two proportions, Sealed Envelope Ltd. 2012). This value was then inflated to a target of 400 quarters (100 cows) per treatment group due to the need to control for within-cow and within-quarter correlations or clustering in the statistical analysis (Dohoo et al., 2009). This also provided a margin of safety against expected losses (e.g., contaminated samples) and in case a lower than expected incidence of NIMI was encountered within the study herd. In addition, we performed a midterm evaluation to ensure that no adjustment of the study duration was necessary to ensure the minimum power requirements.

Effect of Treatment on Udder Health Measures. We followed statistical plan described by God-

den et al. (2016). Descriptive statistics were first generated by treatment group to describe baseline measures at wk 0 including parity, DIM, teat hardness scores, teat color scores, teat end hyperkeratosis scores, and baseline bacteriology results. Descriptive statistics were also generated by treatment group to describe at the quarter level, by week of study (13 wk, baseline wk 0, 1–12), and overall, the crude incidence of NIMI (and bacteriology for NIMI), and the crude level prevalence of IMI (and bacteriology for IMI). Crude teat condition scores were described by treatment group for wk 1, 5, 9, and overall. Crude incidence of NIMI (at wk 1–12) was calculated as the number of quarters developing a NIMI between successive weekly sampling events divided by the number of quarters at risk for NIMI during that wk interval. All udder quarters sampled on a given study day that had a valid test result (no missing sample and not contaminated) on the last sampling date were considered at risk for NIMI on the next weekly sampling date. The prevalence of IMI during the study period was calculated as the number of IMI present divided by the number of udder quarters sampled at each time point and also for the entire study period as period prevalence. The effect of treatment on quarter level risk for NIMI for each week sampling interval (primary outcome variable) was modeled as a function of treatment group and other variables using mixed logistic regression (generalized linear mixed model of SPSS version 28.0; IBM). The final mixed model was

$$f(Y_{ijk}) = \text{intcpt} + \beta_1 \times \text{treatment} + \beta_k \times \text{covariates}_k + \text{week} + \text{treatment} \times \text{week} + \text{cow}_j(\text{random}) + \text{quarter}_i\ (\text{cow}_j)(\text{random}) + \text{error}_{ijk},$$

where f(Y) is the logit link function, Y_{ijk} is the occurrence of a NIMI (yes/no) in quarter i, intcpt is the intercept, treatment is the variable indicating whether a quarter is in the PC or EX group, and β_1 refers to the regression coefficient for treatment. Other covariates (β_k) investigated included quarter location (LF = left front, RF = right front, LH = left hind, RH = right hind), lactation, and DIM. Because quarters are known to be interdependent within a cow and a pen (Barkema et al., 1997), we included, as in Godden et al. (2016), a variable to account for the specific risk of infection in a cow and a pen. This was done by adding a continuous variable that included the total number of infected quarters in a pen at the time of each observation. It was forced into the model to correct for contagiousness of infections between quarters in a cow and in a pen.

Time of sample collection (wk 1 to 12) was forced as a fixed effect into the model. Cow is an indicator for cow_j that was used as a random effect to model the within-cow correlation of quarters in the same cow. $\text{Quarter}_i(\text{cow}_j)$ was used as a random effect to model the longitudinal correlation of observations within the same quarter.

RESULTS AND DISCUSSION

Study Strengths and Limitations

The present study is one of the few commercial noninferiority studies evaluating a new postmilking teat disinfectant according to the protocols of Ceballos-Marquez et al. (2013), Schukken et al. (2013), and Godden et al. (2016). While the conditions of natural exposure over a 13-week period of typically high risk for NIMI (temperature, humidity, seasonality), sufficient power, and successful randomization of cows to treatment (comparable groups of animals based on similar baseline characteristics) are among the strengths of the study, the generalizability of the results is limited by implementation in only one dairy herd. All activities related to milk sample collection and teat health scoring, sample analysis, data analysis, and manuscript preparation were performed by professional personnel to better ensure objectivity.

In considering the generalizability of study results, the study was conducted in one large, modern commercial Holstein dairy farm in Northeast Italy. It was conducted in 4 pens, with all treatment groups housed under the same barn shed and identical pen design and divided only by a simple partition control for pen effect and to prevent confounding the effect of treatment.

Similar to previous published studies using a similar design (Ceballos-Marquez et al., 2013; Godden et al., 2016), every effort was made to ensure that trial design was unlikely to have a confounding effect in this study.

Cow numbers (stocking density) within the 4 pens remained equal throughout the 13-wk study period (e.g., no overcrowding of one pen as compared with the other). All pens used were identical in their size, design, layout of cubicles, feeding management, bedding management, and manure management. Although we cannot prove with absolute certainty that the physicality of the pen did not confound our assessment of the effect of treatment on the various outcomes measured in this study, we are very comfortable that this risk was very low. We attempted to control for the interdependence of quarters within cow and within pen by controlling for random effects of cow, random effects of quarter (cow).

A limitation of this study, when considering teat skin conditioning properties, is that the study was not con-

Table 1. Descriptive results of pretreatment cow- and quarter-level characteristics (mean; SD in parentheses) according to treatment group (EX = experimental teat dip; PC = positive control)

	Treatment		
Item	EX (n= 96)	PC (n = 93)	*P*-value
Parity	1.65 (1.10)	1.70 (1.16)	0.242
DIM[1]	78 (43)	86 (36)	0.106
LSSCC[2]	1.89 (0.37)	1.86 (0.34)	0.538
Milk production[3] (kg/d)	37.27 (9.23)	38.12 (7.65)	0.464
Teat end score (% abnormal)	17.78	21.87	0.309
Teat hardness (% firm, swollen, hard)	10.50	7.29	0.136
Teat color (% discolored)	0.55	0	0.168
Reproductive status (n)			0.322
Fresh	3	6	
Open	47	44	
Inseminated	31	30	
Pregnant	15	13	

[1]Days in milk at the day of trial start.

[2]Linear score of SCC: LSSCC = $\log_2$(SCC/100) + 3 (Schukken et al., 2003).

[3]Milk production of cows enrolled in the study based on the 7-d mean before the start of the trial.

ducted in winter months when it can be more challenging to maintain healthy skin condition, but a period was chosen with significant temperature and humidity differences that stress the teat skin. The aim of this study was to compare the reduction of NIMI with an established and effective teat disinfectant.

Descriptive Data and Baseline Characteristics for Cows, Quarters, and Weather Conditions

A total of 220 cows were enrolled into the study on September 21, 2022 (wk 0), with 110 and 110 cows assigned to the PC and EX group, respectively. Fourteen animals (EX 6; PC 8) were excluded from further testing because they had blind quarters and 17 other animals (EX 8; PC 9) were excluded because of contamination on individual quarter samples in the wk 0 sampling. The total number of cows that finished the trial were 96 (EX) and 93 (PC) on wk 13. Baseline characteristics of cows and quarters, including parity, DIM, linear score of SCC, milk production (kg of ECM/d), reproduction status, prevalence of IMI, and teat skin condition measures were not different between treatment groups on the start date (Table 1; wk 0; $P >$

Table 2. Baseline bacteriology results at enrollment (wk 0) for quarters assigned to the positive control (PC) and experimental (EX) post-milking teat disinfectant treatment groups

	Treatment group			
	PC		EX	
Quarter sample status	n	%	n	%
Total quarters	440	100	440	100
Blind quarters	6	1.4	8	1.8
Samples cultured	434		432	
Contaminated quarters	14		13	
Total quarters for analysis	384		372	
Samples with growth from animals not excluded (contamination)	52	13.5	43	11.6
NAS	27	7.03	22	5.91
Enterococcus spp.	7	1.82	4	1.08
Escherichia coli	1	0.26	0	0
Corynebacterium spp.	2	0.52	9	2.42
Raoultella spp.	1	0.26	3	0.81
Staphylococcus aureus	2	0.52	0	0
Serratia spp.	3	0.78	1	0.27
Streptococcus uberis	1	0.26	2	0.54
Candida rugosa	1	0.26	0	0
Other gram-positive	2	0.52	2	0.54
Other gram-negative	5	1.30	0	0

0.05). The mean (SD; range) parity, DIM for all cows at wk 0 was 1.68 (1.14; 1–9) lactations, and 83.2 (38.5; 6–201) d, respectively. No difference was found between groups in the proportion of teats with abnormal skin condition measures; 80.3% had a normal teat end hyperkeratosis score (score 1 and 2), 91.2% had a normal teat hardness score, and 99.7% had a normal color score. For teats with an abnormal hyperkeratosis score, most of lesions were mild with 18.3%, and 1.5% of all teats evaluated scoring 3, and 4, respectively. Similar baseline characteristics found between groups indicated that the randomization scheme was successful. For milk samples submitted for culture at wk 0, 24 different species were recovered. Predominant organisms recovered were *Corynebacterium bovis*, NAS, and *Enterococcus saccharolyticus* (Table 2). No animal was removed over the 13-wk study period from September 21 to December 14, 2021, due to nonudder diseases or deaths.

In each of the 2 experimental groups, 5 clinical mastitis episodes occurred during the course of the experiment, which led to treatment of the animals (EX: 1 heifer and 4 multiparous cows; PC: 5 multiparous cows). A total of 9,952 quarter milk samples (PC = 5,048; EX = 4,904) were collected during the weekly study sampling dates (wk 0 to 12 for a total of 13 weeks). Data collected from a nearby weather station (Airport Venezia, Tessera) indicated that individual daytime minimum and maximum temperatures ranged between 31.3°C and −1°C during the 13-wk study, and total monthly precipitation ranged between 65 mm (September) and 40 mm (December).

Effect of Treatment on the Incidence of NIMI

The overall crude incidence of NIMI for a 1-wk period at risk was 4.86 and 4.04% for the EX and PC groups, respectively (Table 3). The predominant organisms recovered from quarters with NIMI were NAS, *Corynebacterium* spp., and *Enterococcus* spp. (Table 4). The final logistic regression model showed that the overall adjusted proportion of quarters experiencing a NIMI per weekly period was not different for the EX group (3.1%) as compared with the PC group (2.6%; $P = 0.169$; Table 5, estimated means not shown).

During the study we recognized a reduction of new infections in both groups (Table 3), suggesting that the changing climate from fall to winter influenced the incidence of IMI. We observed many NIMI caused by minor pathogens (*Corynebacterium* spp. and NAS), and a relatively low incidence of NIMI caused by major pathogens (Table 4). One of the reasons for selecting this herd for the trial was a recent history with *Staph. aureus* IMI in a relatively well managed large herd. Few infections with major pathogens occurred within

Table 3. Crude quarter-level incidence of new IMI by 1-wk interval and overall for quarters assigned to the experimental (EX) and positive control (PC) post-milking teat disinfectant treatment groups

Treatment group and parameter	Weekly interval at risk for new infection												Overall
	1	2	3	4	5	6	7	8	9	10	11	12	
EX													
At-risk quarters (n)	362	364	382	370	382	374	376	374	375	353	370	3,380	4,462
New IMI (n)	65	19	16	20	23	7	19	9	26	8	1	4	217
New IMI incidence (%)	17.96	5.22	4.19	5.41	6.02	1.87	5.05	2.41	6.93	2.27	0.27	1.05	4.86
PC													
At-risk quarters (n)	343	362	370	352	369	370	362	359	362	357	358	368	4332
New IMI (n)	37	15	15	17	38	10	14	11	10	5	1	2	175
New IMI incidence (%)	10.79	4.14	4.05	4.83	10.30	2.70	3.87	3.06	2.76	1.40	0.28	0.54	4.04

Table 4. Etiology of new intramammary infections (NIMI) acquired during the 12-wk study for quarters assigned to the positive control (PC) and experimental (EX) post-milking teat disinfectant treatment groups

	Treatment group			
	EX		PC	
Sample status	n	%	n	%
Quarter at risk of NIMI	4,462	100	4,332	100
Quarter with NIMI	217	4.86	175	4.04
Isolates recovered				
Acinetobacter spp.	9	0.20	8	0.18
Corynebacterium spp.	80	1.79	87	2.00
Enterococcus spp.	31	0.69	16	0.37
NAS	63	1.41	50	1.15
Aerococcus spp.	4	0.090	0	0
Escherichia coli	2	0.045	2	0.046
Streptococcus uberis	7	0.16	2	0.046
Staphylococcus aureus	4	0.090		0
Streptococcus dysgalactiae	1	0.022		0
Trueperella pyogenes	0	0	2	0.046
Other	16	0.36	8	0.018

our experimental groups; rather many infections with minor pathogens were identified.

A temporal effect (week) was observed, with significantly higher risks for NIMI at baseline in September weeks and lower risks during sampling weeks in November and December. Several studies have reported a seasonal effect, with SCC and risk of clinical mastitis often highest in the summer months (Erskine et al., 1988; Hogan et al., 1989; Makovec and Ruegg, 2003; Bertocchi et al., 2014). Significant differences in NIMI based on lactation number (age) or stage of lactation were not observed. It must be noted that the animals were all in the range between 30 and 100 lactation days at the beginning of the experiment. Differences in age of the animals were found in some papers. In our work, 75% of the animals were in lactation 1 and 2. Other studies have also reported an effect of age, with older cows being more likely to acquire IMI (Miltenburg et al., 1996; Barkema et al., 1997; Bertocchi et al., 2014).

As the trial did not show a significant difference between EX and PC for risk of NIMI, the goal was then to identify whether the conclusion of no difference may result in a noninferiority claim. The claim of noninferiority was based on the right-hand side of the 95%

Table 5. Final multivariable logistic regression model describing the effect of treatment with an experimental post-milking teat disinfectant on odds for acquiring a new IMI per week at risk

			95% CL[1]		
Variable	Coefficient (SE)	Odds ratio	LCL	UCL	Type 3 *P*-value
Intercept	−4.80 (0.65)				<0.0001
Teat dip					
Experimental	0.23 (0.17)	1.26	0.91	1.75	0.169
Positive control	Referent				
Point of time (wk)					<0.0001
1	3.14 (0.46)	23.15	9.41	56.93	
2	2.02 (0.65)	7.53	2.10	26.97	
3	1.76 (0.49)	5.84	2.23	15.26	
4	1.99 (0.49)	7.35	2.76	19.59	
5	2.52 (0.51)	12.38	4.59	33.39	
6	1.29 (0.71)	3.65	0.90	14.79	
7	1.89 (0.53)	6.61	2.32	18.83	
8	1.43 (0.64)	4.18	1.19	14.62	
9	2.02 (0.58)	7.50	2.43	23.16	
10	1.04 (0.68)	2.83	0.75	10.75	
11	−0.94 (0.95)	0.39	0.06	2.52	
12	Referent				
Pen IMI[2]	0.19				0.665

[1]CL = 95% confidence limits; LCL = lower 95% confidence limit; UCL = upper 95% confidence limit.

[2]Pen IMI = total number of infected quarters within the pen.

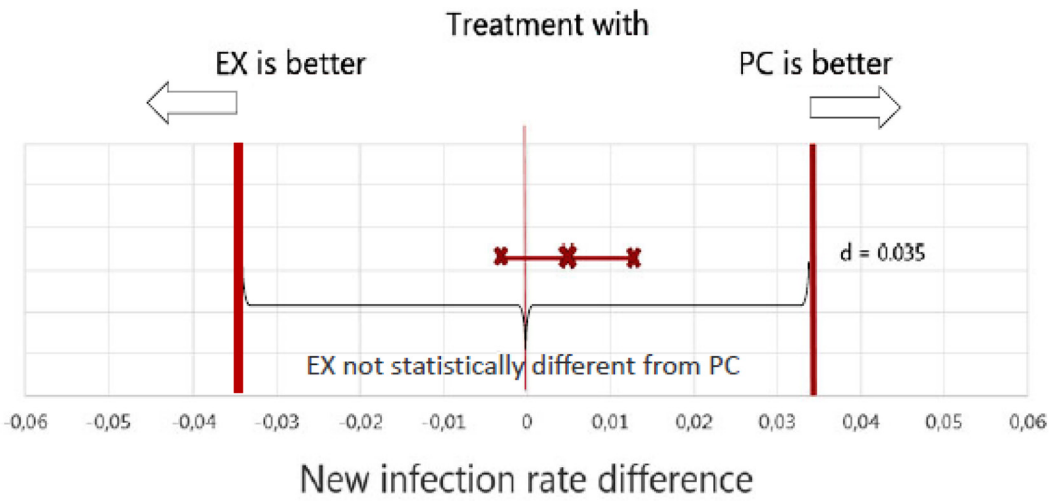

Figure 1. Difference in new IMI rates [observed difference, with 95% confidence interval: 0.005 (−0.00093, 0.0109)] between the experimental dip (EX; 3.10%) and the positive control dip (PC; 2.60%) in the noninferiority trial, where the critical difference (Δ) is shown relative to the observed difference and associated 95% confidence interval, indicated by the horizontal red line with X's. The area to the left of the vertical red bar at 0.035 indicates the zone of noninferiority. d = Δ, the critical value for declaring inferiority.

confidence interval of the rate difference being smaller than the predefined noninferiority limit, d, defined a priori as 3.5% (or 0.035). The NIMI rate estimates (95% CI) for EX [0.031 (0.025, 0.039)] and PC [0.026 (0.020, 0.032) groups were used to calculate the NIMI difference (95% CI) for EX as being 0.005 (−0.00093, 0.0109), (details are given in Godden et al., 2016) using the following steps:

(1) $\text{Diff}(P_{EX} - P_{PC}) = (0.031 - 0.026) = 0.005$
(2) $\text{SD}(P_{EX}) = (0.039 - 0.025)/(2 \times 1.96) = 0.00357 \rightarrow \text{Var}(P_{EX}) = (0.00357)^2 = 0.00000128$
(3) $\text{SD}(P_{PC}) = (0.0032 - 0.02)/(2 \times 1.96) = 0.00306 \rightarrow \text{Var}(P_{PC}) = (0.00281)^2 = 0.000000937$
(4) $\text{Var}[\text{Diff}(P_{EX} - P_{PC})] = (0.00000128 + 0.000000937) = 0.00000221 \rightarrow \text{SD}[\text{Diff}(P_{EX} - P_{PC})] = \sqrt{0.00000221} = 0.00470$
(5) 95% $\text{CI}[\text{Diff}(P_{EX} - P_{PC})] = 0.005 \pm (1.96 \times 0.0047) = (-0.0042, 0.0142)$
(6) NIMI Diff (95% CI) = 0.005 (−0.0042, 0.01422).

Because the upper bound of the CI for the rate difference (0.0142) was to the left of the critical value d (0.035), it can be concluded that EX was noninferiority relative to PC with respect to risk for NIMI (Figure 1).

Effect of Treatment on Prevalence of IMI

The overall crude prevalence of IMI for the entire study period was 11.2% (499/4,462) and 10.0% (432/4,332) for the EX and PC groups, respectively. After adjusting for week, quarter, DIM, and total number of infected quarters in the pen, as well as random effects of cow and quarter (cow), the final mixed logistic regression model estimated the overall adjusted proportion [95% confidence limits (**CL**)] of quarters with IMI present at sampling events every wk to be 14.3% (10.7, 18.9) and 11.5% (8.4, 15.5) for the EX and PC groups, respectively (model not shown; $P = 0.31$). The finding of no treatment difference in prevalence of IMI was consistent with the earlier finding of no significant effect of treatment on NIMI (Table 4). The prevalence of IMI was significantly different between sampling weeks ($P < 0.001$). Although no significant differences were shown in IMI due to quarter position, DIM, and contagiousness within a pen, they were still retained in the model as design variables.

Effect of Treatment on Teat Condition

A total of 2,193 teat skin condition evaluations were performed at wk 1, 5, and 9 (PC = 1,074; EX = 1,119). The crude proportion of teats having normal teat barrel skin condition (teat hardness score; score = 0) was 91.1% (EX = 89.5%, PC = 92.7%). A normal teat skin color was determined in 95.7% of the teats (EX = 92.8%; PC = 98.9%); 77.5% of all teats had no hyperkeratosis (EX = 78.8%; PC = 76.2%).

Multivariable logistic regression found no effect of treatment on odds for teat hardness [score = 0; $\text{Odds}_{\text{PosControl}}$ (95% CL) = 0.624 (0.339, 1.150); $P = 0.131$]. Similarly, the regression model found no effect of treatment on odds for a normal hyperkeratosis score [score = 1+2; $\text{Odds}_{\text{PosControl}}$ (95% CL) = 0.687 (0.229, 2.034);

$P = 0.494$]. Due to the small variation in the color parameter, the final regression model did not converge and is therefore not shown. Risk for a normal degree of hyperkeratosis was associated with week, with the highest proportion of normal scores (estimated means) reported in wk 1 (95.5%) as compared with wk 5 (94.0%) and wk 9 (93.0%; $P < 0.017$).

The role of hyperkeratosis in the development of new infections of bovine mammary glands is unclear. In addition to studies showing an increased risk of severe hyperkeratosis for udder health, there are also studies where this correlation could not be shown (Zadoks et al., 2001; Zoche-Golob et al., 2015). In the current study, both treatment groups had a small increase of teats with hyperkeratosis in the course of the study, which we attribute to the increase in the course of lactation. From wk 1 to 5, the percentage of soft teats (teat hardness score = 0; crude data) decreased to return to approximately baseline levels by wk 9 (wk 1 = 91.1%; wk 5 = 79.6%; wk 9 = 89.1%). From wk 1 to 5, the percentage of normal colored teats decreased, and remained at the level reached to wk 9 (wk 1 = 99.7%; wk 5 = 93.6%; wk 9 = 94.0%). This small decrease can possibly be explained by the aging of the liners used. The milking technique settings and milking routine were not changed during the trial.

CONCLUSIONS

The results of this randomized, positive control, noninferiority study indicate that the chlorine dioxide-based disinfectant EX was noninferior to the previously proven chlorine dioxide-based disinfectant PC for the prevention of naturally occurring intramammary infections. No overall difference was found between the 2 products on the incidence of NIMI, the risk for presence (prevalence) of IMI, or measures of teat skin condition. The EX treatment can be considered an effective teat disinfectant, which was not irritating to teat skin and, overall, did not negatively affect skin condition as compared with the positive control group. Although the results of this study are applicable to herds similar to that used in the current study and during fall and winter months, additional studies to ensure that results are repeatable under different management and seasonal conditions are suggested.

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