Use of different methods for the evaluation of teat disinfectant products

Sarah Rose Fitzpatrick, Mary Garvey, Jim Flynn, Bernadette O’Brien and David Gleeson

ABSTRACT

There are many test methods used to determine the effectiveness of teat disinfectants. The objective of this study was to determine the outcome from the use of three different test methods (BS EN 1656 [laboratory], disc diffusion [laboratory] and an experimental challenge teat swabbing method [in-field]) when evaluating the effectiveness of ten teat disinfectant products. Products were tested against three bacterial strains [Escherichia [E.] coli [ATCC® 10536™], Staphylococcus [Staph.] aureus [ATCC® 6538™] and Streptococcus [Str.] uberis [ATCC® 19436™]]. All products satisfied the requirements of the BS EN 1656 method achieving >10⁵ log reductions. Two laboratory methods were consistent in identifying effective products against Staph. aureus and E. coli, whereas, all three methods were consistent in identifying effective products against Str. uberis. Within the two laboratory methods, products containing 2% lactic acid combined with 0.3% chlorhexidine gluconate and 5% lactic acid combined with 0.3% chlorhexidine were within the top four most effective products against Staph. aureus. For Str. uberis, products containing 2.4% lactic acid, 0.29% iodine combined with 0.8% lactic acid and 0.5% iodine (control) were within the top four most effective products. This study has established that effectiveness of teat disinfectant products can be determined by different test methods.

Introduction

The teat orifice is the first line of defence against the invasion of mastitis pathogens into the cow’s udder. Teat disinfection is an important tool in the reduction of intramammary infections (IMIs) and mastitis cases as pre- and post-milking teat disinfection has been shown to reduce bacterial numbers on teat skin (Gleeson et al. 2009; Mišiškiné et al. 2015; Baumberger et al. 2016; NMC 2017; Gleeson et al. 2018). Reducing bacterial numbers on teat skin can have a positive impact on minimizing new infection rates. Post-milking disinfection has been demonstrated to reduce subclinical mastitis caused by Staph. aureus and Str. uberis by 60% and clinical mastitis cases by 50% (Williamson and Malcolm, 2012). Regulations for the sale of teat disinfectant products and approval requirements can depend on the country of distribution. At present, teat disinfectant products are viewed as over-the-counter products in the United States. Therefore, these products are subject to the laws outlined by the Food and Drug Administration (FDA). Alternatively, in Europe, teat disinfectant products are regulated by the European Medicines Evaluation Agency (EMEA) or European Medicines Agency (EMA) (NMC 2017; EMA 2019), and the Health Products Regulatory Authority (HPRA) and the Department of Agriculture, Food and Marine (DAFM) in Ireland.

Teat disinfectant products can be evaluated using in field [in vivo] and laboratory [in vitro] methods. The main disadvantage of laboratory testing is the inability to identify the true potential of the teat disinfectant products in reducing new IMIs. Laboratory methods also do not account for contact on the teat skin and the bacterial load on the teat skin surface and the presence of contamination. While it is more beneficial to test the teat disinfectant products in the environment they are to be used in and over full lactation studies, it is necessary to screen products before embarking on expensive in-field studies. Screening of products allows for effective and ineffective products to be identified and to identify susceptible bacterial groups. One of the main laboratory test standard recognized to test the effectiveness of teat disinfectant products is the BS EN 1656 (MSG 2020). This is an European standard that requires a minimum bacterial log reduction of 5 (10⁵) within 5 minutes and can be used to compare the effectiveness of a range of disinfectants. A modified version of the BS EN 1656 was used in a study by Lopez-Benavides et al. (2012) to test the effectiveness of four teat disinfectant products (two 0.5% hydrogen peroxide products and two chlorine dioxide generating products) against the bacteria required by the standard (E. coli [ATCC® 10536™], Staph. aureus [ATCC® 6538™] and Str. uberis [ATCC® 19436™]). All four products satisfied the standards by achieving a bacterial log reduction greater than 5.

The disc diffusion method has previously been used in laboratories for the evaluation of antimicrobial sensitivity within both the medical and veterinary fields (Balouiri et al. 2016; Hardefeldt et al. 2018). Some studies have slightly modified the method to allow for the testing of teat disinfectants (Garvey et al. 2017; Haggag et al. 2018; Fitzpatrick et al. 2019a). These studies evaluated the sensitivity of a range of bacteria (isolated from quarter foremilk samples and teat skin...
swabs) against teat disinfectants. Garvey et al. (2017) and Fitzpatrick et al. (2019a) evaluated the effectiveness of products which contained various concentrations of various ingredients using this method. The study by Garvey et al. (2017) compared five different teat disinfectant formulations (three lactic acid, one chlorhexidine and one iodine [control]). That study demonstrated that the lactic acid formulations provided greater zones of bacterial inhibition compared to chlorhexidine and provided comparable level of bacterial inhibition to a 0.5% iodine disinfectant. These studies demonstrate that the disc diffusion method can screen and evaluate the disinfectant. The study by Garvey et al. (2017) compared which contained various concentrations of various ingredients to teat disinfectant products against specific bacterial strains. The teat swabbing method involves the application of a teat treatment to cows teats followed by swabbing with a cotton swab before and after its application, to measure reductions in bacterial numbers naturally present on the teat skin. This method has been used in many studies to evaluate pre-milking teat preparation treatments (Galton et al. 1986; Gleeson et al. 2009; Miseikienė et al. 2015; Baumberger et al. 2016; Fitzpatrick et al. 2019b). Dependence on naturally present bacteria on teats is a limitation, in that bacterial levels can vary from teat to teat and can complicate analysis. The use of experimentally challenged teat skin will allow for the evaluation of teat disinfectant products against specific purchased strains of known levels of bacteria (Barkema et al. 2009; Fitzpatrick et al. 2019b) and against new IMIs over a fixed time period (Boddie et al. 2000; Oura et al. 2002; Schukken et al. 2013). The experimental challenge method is recommended by the National Mastitis Council (NMC). The NMC is a professional organization which promote research and provides information to the dairy industry on topics such as milk quality, udder health and milking management (NMC 2020). The NMC also monitor the development of protocols that determine the effectiveness of mastitis control products (Hogan et al. 1990). Limiting the time period of exposure of a known amount of bacteria on teats may reduce the likelihood of new IMIs, while allowing for the measurement of bacterial reduction due to the application of different teat disinfectants. Limitations, challenges and advantages may be associated with each of the three methods described above. Therefore, the objective of this study was to investigate the outcome of three different test methods; BS EN 1656, disc diffusion, and an adapted experimental challenge teat swab method, when measuring the effectiveness of 10 commercially available teat disinfectant products of varying ingredients and concentrations.

Materials and methods

Three test methods were applied to evaluate a range of teat disinfectant products. The BS EN 1656 and disc diffusion methods allowed for the evaluation of products in the laboratory while the experimental challenge teat swab method allowed evaluation in-field.

Teat disinfectant information

Ten commercially available teat disinfectant products, with different active ingredients of varying concentrations (i.e. lactic acid, chlorhexidine), were tested for their effectiveness against the following strains of bacteria: Staph. aureus (ATCC® 10536™), E. coli (ATCC® 6538™) and Str. uberis (ATCC® 19436™). All teat disinfectant products used, except one, were ready-to-use (RTU) products and commercially available (Table 1). One product (product 7) was mixed with an activator before use, according to manufacturer’s recommendations. The disinfectant products used were generally suitable for both pre- and post-milking teat disinfection, with the exception of two products (products 7 and 10), which were recommended only for post-milking disinfection. Product 10 was considered as a control as it contained 0.5% w/w iodine and such products have previously been shown to effectively inhibit mastitis-related bacteria (Boddie et al. 2000; Boddie et al. 2002; Enger et al. 2015; Baumberger et al. 2016; Garvey et al. 2017).

### BS EN 1656 method

This method is commonly used to test chemical disinfectants used in the veterinary area. To meet the requirements of this test, the teat disinfectant product must demonstrate a bacterial log reduction of at least 10^5 within 5 minutes. The method specifies that the test is performed using the following bacterial strains; E. coli (ATCC® 10536™), Staph. aureus (ATCC® 6538™) and Str. uberis (ATCC® 19436™). Bacterial test suspensions containing bacterial numbers ranging from 1.5 × 10^8 to 5 × 10^8 cfu/mL were prepared as recommended in the method. Product test solutions were prepared as recommended by the manufacturer. Before testing, all reagents were equilibrated to the test temperature of 30 °C using a water bath. (BSI 2009).

### Disc diffusion method

This test may be used to determine the ability of the teat disinfectant products to inhibit bacterial growth. The disc diffusion laboratory test method was carried out using the bacterial strains recommended for the BS EN 1656 standard (E. coli

| Code | Product | Ingredient | Manufacturer/Supplier |
|------|---------|------------|-----------------------|
| 1    | Lacto-cel® | 2.4% w/w lactic acid | Biocel Ltd. |
| 2    | Duogold® | 2% w/w lactic acid and 0.3% w/w chlorhexidine gluconate | Gold Assure |
| 3    | Arkshield® | 5% w/w lactic acid and 0.3% w/w chlorhexidine | Ark Farm Innovations Ltd. |
| 4    | Super Cow Teat Foam® | 0.6% w/w diamine | Milk Solutions Ltd. |
| 5    | Sensodip 50® | 0.5% w/w chlorhexidine | GEA Farm Technologies Ltd |
| 6    | PureChem Chlorhexidine® | 1.49% w/w chlorhexidine | Central Chemical Supplies Ltd. |
| 7    | Kenonix® | 0.0157% w/w chlorhexidine | CID Lines N.V. |
| 8    | Lanodip Pre-Post® | 0.29% w/w iodine and 0.8% w/w lactic acid | Kilco International Ltd. |
| 9    | Hyped Quick Spray® | 2% w/w lactic and 0.1% w/w salicylic acid | Grassland Agro Ltd. |
| Control Maxidine RTU® | 0.5% w/w iodine | Biocel Ltd. |

*RTU.

*Pre- and post-milking application.

*Post-milking application only.
[ATCC® 10536™], Staph. aureus [ATCC® 6538™] and Str. uberis [ATCC® 19436™]). The method was carried out as described by Fitzpatrick et al. (2019a). Individual suspensions of the test bacterial strains were made (approx. $10^8$ cfu/mL) and 100 μL of the suspensions was overlaid onto separate Mueller Hinton agar (sigma-Aldrich, Dublin, Ireland) plates. Following this, three filter discs (6 mm) (Cruinn, Dublin, Ireland) were soaked in the different teat disinfectant products, the excess allowed to drip off and placed onto the agar plate. These plates were then incubated at 37°C for 24 hr. The zones of inhibition were then measured using a digital calliper (Mitutoyo Digital, RS digital caliper 600/880) in millimetres (mm).

**Experimental challenge teat swabbing**

The experimental challenge teat swabbing method used in this study is a modification of the experimental challenge method which is recommended by the NMC for determining the effectiveness of teat disinfectant products against new IMIs (Nickerson et al. 2004). This modification represented a change in the time period that the bacterial suspension was applied to the teat skin of cows. Rather than applying a bacterial suspension, of a desired concentration, to the teat skin, for a varying time period of several weeks, the bacterial suspension was applied once a day before milking in the morning for a short time period (approx. 1 min before being removed). The same Staph. aureus and Str. uberis bacterial strains, as used with the other test methods, were used. The E. coli strain was excluded from the teat swabbing method due to the risk of severe mammary gland infection.

**Preparation of the challenge suspension**

Suspensions of Staph. aureus (ATCC® 6538™) were prepared as described by Schukken et al. (2013). One 6 mL tube of sterile tryptic soy broth (TSB) was inoculated from two to three colonies of plated stock culture and incubated for 5–7 h at 37°C. This culture was streaked onto a blood agar plate containing 5% sheep blood to ensure purity. Following this, a 500 mL duran bottle of sterile TSB was inoculated with the entire contents of the 6 mL culture tube. This was incubated for 16-18 h at 37°C on a gyratory shaker. After incubation, the bacterial cells were pelleted by centrifugation (3000 $\times$ g for approx. 15 min), washed twice with 0.1% proteose peptone and resuspended in 50 mL of 0.1% proteose peptone. A plate count was conducted on the stock culture. This plate count was used to determine the dilution required to prepare an initial bacterial suspension containing $5 \times 10^7$ cfu/mL in TSB. The stock culture was stored at 4°C and was used daily for 3 days to prepare challenge suspensions of Staph. aureus (ATCC® 6538™).

Suspensions of Str. uberis (ATCC® 19436™) were prepared as described by Galton et al. (1988) and Klostermann et al. (2009). One 2 mL tube of TSB was inoculated from 2–3 colonies of plated stock culture. This was incubated for 75 min at 37°C. The contents of the 2 mL tube was then transferred to a 200 mL duran bottle of sterile TSB and incubated for 16–18 h at 37°C until a stock culture with a concentration of $5 \times 10^7$ cfu/mL was obtained. The stock culture was dispensed in 50 mL aliquots and stored at -20°C. An aliquot was defrosted overnight for use in each daily challenge.

**Teat swabbing procedure**

The Teagasc Animal Ethics Committee (TAEC) granted ethical approval for the experimental challenge teat swabbing study to be carried out (ref. TAEC 199-2018). The Health Products Regulatory Authority (HPRA) Ireland provided project authorization (ref. AE19132/ P092) as required under statutory instrument no. 543 of 2012 for the ‘Protection of animals used for scientific purposes’. The trial was undertaken using five cows from the Teagasc Moorepark research farm, County Cork, Ireland. Two different teat disinfectant products were applied to each cow, with different products applied over a period of 6 days at each morning milking.

Before treatments were applied, all four teats of each cow were thoroughly cleaned using cotton wool soaked in methylated spirits. An internal teat sealer was administered to each quarter to prevent bacterial colonization of the cow teat canal (and the possible development of an IMI) by the bacterial suspension. Following this, the teats were challenged by immersion (25 mm) in a teat dip cup of prepared suspension ($5 \times 10^7$ cfu/mL) of Staph. aureus. Swabs (Copan Italia S.p.A Via F. Perotti, 10 25125 Brescia – Italy) were moistened in sterile TSB (Merck Millipore, Ireland) to aid in the recovery of bacteria from the teat skin. The swab was then drawn over the teat surface approximately 1 min after application of the bacterial suspension (PRE). Swabs were drawn across the teat orifice and down the side of each teat avoiding contact with the udder hair or cows flank at all times. Teat swabbing was carried out by the same two operators for all treatments. Two separate swabs were used to collect a composite sample from the left teats (left front [LF] and left hind [LH]) and from the right teats (right front [RF] and right hind [RH]). Following this, two different teat disinfectant products were applied. One product was applied to the left teats of the cow and the other product was applied to the right teats. Teat disinfectant products were applied using non-return teat dip cups and the teats were immersed to a depth higher than the bacterial challenge applied (30 mm). The teat disinfectant products were left on the teat skin for approximately 1 min, after which teats were wiped with an individual disposable paper towel. Swabbing was again carried out to collect two composite samples from the left and right teats (POST). Teats were then washed thoroughly with water and disinfected with cotton wool soaked in methylated spirits and the teat sealer was removed. Subsequently, all cows teats were thoroughly cleaned (washed with water, forestripped and dried), cows were milked but their milk was excluded from the bulk milk tank. At the evening milking, the cows on the trial were prepared and milked as normal, with no disinfection. There were 120 swabs collected in total during the trial period; 60 associated with each bacterial strain Staph. aureus and Str. uberis (20 × 3 days; 10 swabs PRE and 10 swabs POST teat disinfection application).

Immediately after the teat swabbing procedure, swabs were placed into individual sterile bottles containing 10 mL of sterile TSB and neutralizer (30 g/L polysorbate 80 & 3 g/L l-a-phosphatidylcholine from egg yolk). The TSB and neutralizer were prepared in 250 mL lots and autoclaved at 121°C for 15 mins, and then distributed into 10 mL aliquots in a laminar flow
cabinet. The sterile bottles containing the swabs were placed in storage at −20°C until analysed. Before analysis, the swab samples were defrosted, vigorously shaken on a vortex and the swab squeezed. All PRE and POST swab samples were plated in triplicate. Maximum Recovery Diluent (Sigma-Aldrich, Ireland) was used to make 1:100 dilutions in sterile tubes for the PRE swabs. The POST sample was used undiluted. The samples were subsequently plated onto blood agar plates for* Staph. aureus* (ATCC® 6538™) and *Str. uberis* (ATCC® 19436™). Following incubation at 37°C for 24 hrs, microbial counts for each pathogen type were manually determined.

**Statistical analysis**

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) and all statistical significance was defined at P≤.05. Within the study, each experiment was repeated three times, with three replicates each.

**BS EN 1656 method**

The bacterial log reductions of the 10 teat disinfectant products for the different bacterial strains were compared to the control and to other teat disinfectant products using PROC GLIMMIX. Pair-wise comparisons were adjusted for multiplicity effect using simulation procedures to adjust P-values. Residual checks were made to ensure assumptions of analysis were met.

**Disc diffusion method**

Different sized zones of inhibition occurred on the agar plates depending on the susceptibility or resistance of the different bacterial strains to the different teat disinfectants. Diameters of the zones of inhibition were compared to the control and other teat disinfectant products using PROC GLIMMIX. Pair-wise comparisons were adjusted for multiplicity effect using simulation procedures to adjust P-values. Residual checks were made to ensure assumptions of analysis were met.

**Experimental challenge teat swabbing**

Bacterial counts (cfu/mL) of the teat swab samples were transformed to base-10 logarithm for analysis. Reduction in experimentally challenged bacterial strains was calculated as the difference between the Log_{10} values of PRE and POST swabs. The reductions for the bacterial strains tested were analysed using two models (one for each bacterial strain). This model included the reduction as a dependent variable and product and day as independent variables. The equation for all models was: y_{ijkm} = μ + Pi + Dj + (P × Dj)f + e_{ijkm} where, y_{ijkm} = the base-10 logarithm reduction of the cfu/mL unit of interest + 1μ = the overall mean, Pi = the fixed effect of the product (i = 1,2,3,4,5,6,7,8 or 9), Dj = the fixed effect of the day of the experiment (j = 1,2 or 3), (P × Dj) = the interaction of product and day, f = the random effect of cow, and e_{ijkm} = the residual random error.

Within these models, product was a fixed effect with cow as a random effect. Non-significant variables were not retained in the models. Log reduction percentage was defined from the PRE swab samples. To determine log reduction, the following equation was used: \( \frac{100(\log_{10} \text{PRE swab} - \log_{10} \text{reduction})}{\log_{10} \text{PRE swab}} \) Log_{10} reduction is the difference between bacterial counts for PRE and POST swabs. This equation was adapted from studies by Schmidt et al. (1984) and Enger et al. (2015). PROC GLIMMIX was used to perform multiple pair-wise comparisons. The LSMEANS statement in PROC GLIMMIX was used to differentiate statistical differences between the control and other teat disinfectant products. Residual checks were made to ensure assumptions of analysis were met.

**Results**

**BS EN 1656**

BS EN 1656 details quantitative suspension tests for the evaluation of bactericidal activity of chemical disinfectants and anti-septics used in the veterinary area. The log reduction in viable cell number for the test strains conducted in accordance with the guidelines of BS EN 1656 is shown in Table 2. All 10 teat disinfectant products tested achieved a log reduction of 5 (10^5) or higher within 5 min of treatment time for all three bacterial strains. This indicates that all of the teat disinfectant products meet the requirements of the BS EN 1656.

For *E. coli*, all products resulted in log reductions comparable to the control (P = .05). However, product 2 achieved the significantly larger log reduction of 5.69 ± 0.175, compared to products 3 (5% w/w lactic acid combined with 0.3% w/w chlorhexidine; 5.13 ± 0.128) and 4 (0.6% w/w diamine; 5.11 ± 0.014) (P < .05). For *Str. uberis*, eight products were similar to the control. However, product 4 resulting in a significantly lower log reduction (5.39 ± 0.093) compared to the control (5.76 ± 0.048) and product 8 (0.29% w/w iodine combined with 0.8% w/w lactic acid; 5.77 ± 0.046) (P < .05).

**Disc diffusion method**

Significant differences in bacterial inhibition for different bacterial strains were observed for the different teat disinfectant products when the disc diffusion method was applied. Diameters of the zones of inhibition, in millimetres (mm), generated by the 10 teat disinfectants tested, are presented in Table 3. All of the teat disinfectants showed varying levels of antibacterial activity against the three bacterial strains tested, with zones of significant inhibition found at different sized zones of inhibition on the agar plates for each bacterial strain.

### Table 2. The log reduction (average ± SE) of test bacterial strains as per BS EN 1656 testing guidelines for ten teat disinfectants.

| Product       | Staph. aureus (ATCC® 6538™) | E. coli (ATCC® 10536™) | Str. uberis (ATCC® 19436™) |
|---------------|------------------------------|------------------------|-----------------------------|
| 1             | 5.71 ± 0.097 ab              | 5.24 ± 0.214 ab        | 5.70 ± 0.078 ab             |
| 2             | 5.73 ± 0.089 a              | 5.69 ± 0.175 a         | 5.69 ± 0.062 a              |
| 3             | 5.62 ± 0.198 b              | 5.13 ± 0.128 b         | 5.67 ± 0.109 b              |
| 4             | 5.44 ± 0.203 a              | 5.11 ± 0.014 b         | 5.39 ± 0.093 b              |
| 5             | 5.43 ± 0.167 a              | 5.31 ± 0.057 ab        | 5.66 ± 0.032 ab             |
| 6             | 5.54 ± 0.108 ab             | 5.26 ± 0.099 ab        | 5.59 ± 0.094 ab             |
| 7             | 5.38 ± 0.164 a              | 5.42 ± 0.032 ab        | 5.70 ± 0.077 ab             |
| 8             | 5.50 ± 0.127 a              | 5.42 ± 0.032 ab        | 5.77 ± 0.046 ab             |
| 9             | 5.68 ± 0.093 a              | 5.40 ± 0.034 ab        | 5.74 ± 0.041 ab             |
| Control       | 5.43 ± 0.187 a              | 5.42 ± 0.032 ab        | 5.76 ± 0.048 a              |

*ab* denote significant difference (P < .05) between individual bacterial strain sensitivity levels to each teat disinfectant product within columns.

**Product**: 1 = Lacto-cel, 2 = Duogold, 3 = Arkshield, 4 = Super Cow Teat Foam, 5 = Sensodip 50, 6 = PureChem Chlorhexidine, 7 = Kenomix, 8 = Landopid Pre-Post, 9 = Hypred Quick Spray, Control = Maxidine RTU.
Product: 1 = Lacto-cel, 2 = Duogold, 3 = Arkshield, 4 = Super Cow Teat Foam, 5 = Sensodip 50, 6 = PureChem Chlorhexidine, 7 = Kenomix, 8 = Lanodip Pre-Post, 9 = Hypquick Wash, 10 = Maxidine RTU.

**Table 4.** Estimates (mean ± SE) of teat disinfectant products on the bacterial log reduction on teats challenged with Staphylococcus aureus (ATCC® 6538™) and Streptococcus uberis (ATCC® 19436™).

| Product | Staphylococcus aureus (ATCC® 6538™) | Streptococcus uberis (ATCC® 19436™) |
|---------|------------------------------------|-------------------------------------|
|         | PRE cfu/mL | POST cfu/mL | Log Reduction | % Log Reduction | PRE cfu/mL | POST cfu/mL | Log Reduction | % Log Reduction |
| 1       | 16800 ± 1116 | 27 ± 4 | 3.83 ± 0.069 | 73.5% | 7122 ± 19546 | 18 ± 3 | 3.30 ± 0.241 | 72.9% |
| 2       | 212333 ± 17319 | 539 ± 124 | 2.81 ± 0.189 | 53.2% | 7122 ± 20522 | 28 ± 5 | 3.25 ± 0.149 | 70.7% |
| 3       | 199500 ± 29683 | 349 ± 74 | 3.81 ± 0.086 | 54.1% | 10431 ± 4117 | 18 ± 4 | 3.53 ± 0.230 | 74.7% |
| 4       | 29800 ± 38669 | 964 ± 376 | 3.09 ± 0.312 | 56.8% | 9378 ± 3751 | 33 ± 8 | 3.20 ± 0.127 | 69.6% |
| 5       | 96222 ± 23704 | 118 ± 30 | 2.90 ± 0.168 | 59.5% | 8856 ± 2356 | 48 ± 11 | 3.09 ± 0.158 | 67.1% |
| 6       | 209111 ± 56079 | 607 ± 149 | 2.65 ± 0.155 | 51.8% | 8008 ± 309 | 32 ± 10 | 3.23 ± 0.134 | 70.8% |
| 7       | 170889 ± 28156 | 211 ± 90 | 3.34 ± 0.272 | 63.3% | 7800 ± 2196 | 51 ± 11 | 3.12 ± 0.099 | 66.1% |
| 8       | 142667 ± 27828 | 169 ± 74 | 3.37 ± 0.309 | 65.4% | 8383 ± 1986 | 21 ± 4 | 3.54 ± 0.092 | 73.6% |
| 9       | 181556 ± 21208 | 574 ± 258 | 3.09 ± 0.314 | 58.4% | 3433 ± 737 | 26 ± 3 | 3.02 ± 0.183 | 68.5% |
| Control | 175333 ± 10661 | 13 ± 2 | 4.15 ± 0.042 | 79.3% | 5122 ± 790 | 27 ± 6 | 3.31 ± 0.093 | 71.3% |

Product: 1 = Lacto-cel, 2 = Duogold, 3 = Arkshield, 4 = Super Cow Teat Foam, 5 = Sensodip 50, 6 = PureChem Chlorhexidine, 7 = Kenomix, 8 = Lanodip Pre-Post, 9 = Hypquick Wash, 10 = Maxidine RTU.

The log reduction means of Staph. aureus and Str. uberis with different letters are statistically significant (P < .05). PRE, Teat skin swab sample after bacterial suspension application and before teat disinfectant application; POST, Teat skin swab sample after teat disinfectant application.

% log reduction determined from log_{10} PRE swab values.
experimentally challenge teat swabbing method allowed for the evaluation of teat disinfectant products ability to reduce the numbers of a specific bacterial strain on the teat skin surface. This method could also be used to evaluate teat disinfectants against bacterial strains isolated from the environment. There are some limitations associated with the three test methods used in this study. Both laboratory methods did not account for the reduction of bacterial load on the teat skin surface, the reaction of the teat disinfectant product on the teat skin surface or skin temperature. The BS EN 1656 method requires a pass/fail result, whereas, the disc diffusion method can compare and contrast the bacterial inhibition of each teat disinfectant and can quickly evaluate a large number of products. Alternatively, the experimental challenged teat swab method allowed for contact time, skin temperature and reduction of a known initial bacterial load on the teat skin. However, it resulted in lower log reductions than that observed with the BS EN 1656. This may be due to the contact of the teat disinfectant on the teat skin which would not be accounted for in the BS EN 1656 laboratory method. However, the requirement for animals and the ethical issues around the use of animals for such challenge experiments may make this method unattractive or harder to implement.

A trend in the level of effectiveness against these strains was observed for some teat disinfectant products within the overall study. Two methods (BS EN 1656 and disc diffusion) were consistent in identifying effective products against Staph. aureus and E. coli. In comparison, all three methods consistently identified the same effective products for Str. uberis. There was a larger variation in effective products across the three methods for Staph. aureus. This may indicate that there may be more variation on outcome when testing for Staph. aureus when comparing laboratory and in-field methods. However, a product could be considered more effective against strains of bacteria if it is identified as effective by both a laboratory and in-field tests.

For Staph. aureus product 1, which contained 2.4% lactic acid, was within the top four most effective products in two methods (BS EN 1656 and experimental teat swabbing). A previous study also indicated that a product containing similar levels of lactic acid (2.4%) resulted in significant reductions of naturally occurring staphylococcal isolates when applied to the teat skin (Fitzpatrick et al. 2019b). Additionally, a product containing 2% lactic acid combined with 0.3% chlorhexidine gluconate (product 2) and 5% lactic acid combined with 0.3% chlorhexidine (product 3) were within the top four most effective products within both the BS EN 1656 and disc diffusion method for Staph. aureus. Product 3 was tested in a previous study against naturally present staphylococcal and streptococcal isolates on the teat skin and a large log reduction was observed (Fitzpatrick et al. 2019b). In the experimental challenge teat swab method, the control, which contained 0.5% iodine, resulted in some of the largest log reductions for both Staph. aureus on the teat skin. This agrees with a study where an iodine dipping agent tested in a laboratory on fragments of teat skin resulted in a 99.9% reduction of the Staph. aureus strain tested (Skowron et al. 2019). A further study by Baumberger et al. (2016) showed that a 0.5% iodine used pre-milking had a greater reduction of staphylococcal species on teat skin in comparison to chlorine dioxide.

For Str. uberis, products containing 2.4% lactic acid (Product 1) or 0.29% iodine combined with 0.8% lactic acid (Product 8) or 0.5% iodine (control) were within the top four most effective products for all three methods. A lactic acid was previously shown to reduce new IMUs caused by Str. uberis by 49.6% in an experimental challenge method (Boddie and Nickerson 1992). Traditionally, iodine has been associated with being the most effective against Str. uberis. A 0.5% iodine teat disinfectant was shown to achieve a high log reduction against Str. uberis when the excised teat method was used (Enger et al. 2015). This method is similar to the challenge method but with the difference being the use of live cows teats within the present method. In both the BS EN 1656 and experimental challenge teat swab test methods, 0.29% iodine combined with 0.8% lactic acid (Product 8), resulted in the highest log reduction for Str. uberis, while 5% lactic acid combined with 0.3% chlorhexidine (Product 3) and 0.5% iodine (the control) resulted in some of the highest reductions for both the disc diffusion and experimental challenge teat swabbing. The same product (0.29% iodine combined with 0.8% lactic acid) was shown in a previous study to reduce naturally present streptococcal species on the teat skin by 83% (Fitzpatrick et al. 2019b).

Similar to Staph. aureus, the disinfectant containing 2% lactic acid combined with 0.3% chlorhexidine gluconate (product 2) also gave consistent results for E. coli across both the disc diffusion and BSEN methods, with this product being in the top four most effective products in each test method. This product was previously shown to achieve large zones of inhibition against an E. coli (20.0 mm) strain isolated from clinical and sub-clinical quarter foremilk samples (Fitzpatrick et al. 2019a).

However, some products may perform differently against different strains or isolates on the teat skin surface. This was seen in in the two laboratory methods, in the present study, where product 4, which contained 0.6% dimine, resulted in some of the lowest log reductions and zones of inhibition. However, when this product was applied to the teat skin, large percentage log reductions were achieved. This may be due to the ingredient, dimine, being stable at a wide range of pH and effective in the present of heavy organic soiling (Slimani et al. 2018) and being less affected by organic material than other ingredients (Blowey and Edmondson 1996). Additionally, a study which evaluated a 0.5% iodine teat disinfectant against naturally present bacteria on the teat skin resulted in an average log reduction of 0.42 and 0.82 staphylococcal and streptococcal isolates on teat skin, respectively.
within 15 s and 30 s. Greater than 5 against a range of mastitis-causing bacteria including Staphylococcus aureus and Streptococcus agalactiae. The experimental challenge teat swab method was used. In this modiﬁcation, a bacterial suspension was applied once a day before milking for approx. 1 min before teat disinfectant application and removal, rather than applying and not removing a suspension after milking over a period of several weeks. A previous study found that initial bacterial levels of naturally present species on the teat skin surface were low which may have impacted results obtained (Fitzpatrick et al. 2019b). Therefore, this modiﬁcation allowed for similar large initial levels of bacterial contamination on the teat skin before teat disinfectant application and tested bacterial strains which are used in the BS EN 1656 method and have also been associated with mastitis in Ireland (Keane et al. 2013).

A contact time of 1 minute was chosen based on previous studies which have tested pre-milking teat disinfectant products on the teat skin surface by allowing a contact time of 15 s (Gibson et al. 2008) or 30 s (Baumberger et al. 2016). A study by Lopez-Benavides et al. (2012), used contact times of 15 s, 30 s and 2 min in a modiﬁed version of the BS EN 1656 method found that teat disinfectant products (hydrogen peroxide and chlorine dioxide; recommended for pre- and post-milking disinfection) achieved the required log reduction of greater than 5 against a range of mastitis-causing bacteria within 15 s and 30 s.

Conclusions
This is the ﬁrst such study to compare the outcome of three test methods when measuring the effectiveness of teat disinfectant products. All disinfectant products tested met the requirements of the BS EN 1656 standard and resulted in adequate levels of bacterial inhibition in the disc diffusion and high log reductions in the experimental challenge teat swabbing method. The disc diffusion method has the ability to quickly screen and compare the bacterial inhibition of teat disinfectant products. This method can differentiate between the different abilities of teat disinfectants to inhibit the growth of different bacterial strains. The experimental challenge teat swab method allowed for the evaluation of teat disinfectant products when applied to teat skin, when bacterial contamination was present. All three test methods identiﬁed a number of similar teat disinfectant products as the most effective against Staph. aureus, whereas, the laboratory methods differed somewhat to the in-ﬁeld method when identifying the most effective teat disinfectant products against Staph. aureus. A limitation of the three test methods used in this study is the inability to determine the efﬁcacy of a teat disinfectant against new intra-mammary infections. This study has demonstrated that some teat disinfectant products react similarly in terms of effectiveness when tested using different test methods against the same bacterial strains.

Disclosure statement
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ORCID
David Gleeson http://orcid.org/0000-0003-3064-3632

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