A review of test protocols for the evaluation of teat disinfectants

SARAH FITZPATRICK, 1,2 MARY GARVEY2 and DAVID GLEESON1,*

1Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, County Cork, Ireland, and
2Cellular Health and Toxicology Research Group, Institute of Technology Sligo, County Sligo, Ireland

The prevention of mastitis is the most important part of a mastitis control programme. Postmilking disinfection is considered the most effective procedure for preventing new intramammary infections in dairy herds. This article reviews the different protocols used to evaluate the efficacy of teat disinfectant products. These protocols include experimental challenge, natural exposure and excised teat, along with agar diffusion assays and teat swabbing. The experimental designs, limitations, positive and negative aspects of each protocol were compared and discussed throughout this article.

Keywords Teat disinfectants, Product efficacy, Experimental challenge, Natural exposure, Excised teat, Mastitis.

INTRODUCTION

Bovine mastitis is an inflammation of the mammary glands within the udder and classified as intramammary infections (IMIs) (Bradley 2002). Mastitis can be further classified as contagious or environmental. Contagious bacteria can adapt to survive within the host (mammary glands of the udder), establish subclinical infections and can be spread from cow to cow during milking (Radostits et al. 1994). Environmental bacteria are opportunistic invaders of the udder but cannot adapt to live within the host. They usually cause an immune response and are quickly removed (Bradley 2002).

Teat disinfectants have been used to reduce the number of new IMIs caused by mastitis-causing pathogens (Schmidt et al. 1984; Foret et al. 2005). This practice is widely accepted as a fundamental part of a successful mastitis control programme (Oliver et al. 1991; Oura et al. 2002; Leslie et al. 2006). Teat disinfectants may be used pre- and/or postmilking. Premilking disinfection involves application by dipping or spraying teats in disinfectant, followed by wiping off the disinfectant solution approximately 30 s after application and prior to milking (Gleeson et al. 2009). Premilking teat disinfection has been found to be more effective in controlling environmental bacteria such as Escherichia coli and Streptococcus uberis (Enger et al. 2015, 2016) with new IMIs 48% lower compared to postmilking disinfection only (Oliver et al. 1993). Postmilking disinfection involves applying a disinfectant to the teat directly after the milking clusters have been removed, and has been found to assist in the reduction in mastitis caused by environmental and contagious bacteria, such as Streptococcus agalactiae and Staphylococcus aureus, by 53%, compared to undisinfected teats (Wesen and Schultz 1970).

There are several different active ingredients found in products used for teat disinfection. The main disinfectant ingredients incorporated in products are iodine, chlorhexidine gluconate, chlorine dioxide and lactic acid, with many combinations of these ingredients. There are many different types of teat disinfectant products such as barrier teat disinfectants, powders, teat foams, high viscosity liquids, liquid concentrates and ready-to-use liquid disinfectants. Teat disinfectant products must meet many different criteria to serve their purpose in modern milking parlours (Godden et al. 2016). They must be persistent, have proven germicidal efficacy, prevent new IMIs, maintain optimal teat condition and aid in the healing of teat skin as this is the main, and natural, defence barrier against infection (Lago et al. 2016). Cracked skin is more
likely to harbour mastitis-causing bacteria (Blowey and Edmondson 2010; Lago et al. 2016). Disinfectants must not leave any harmful residues in milk that could affect public health (Godden et al. 2016). For example, the application of an iodine as a premilking disinfectant can have implications for processors as unacceptable levels of iodine can occur in the milk (O’Brien et al. 2013).

For products to be considered satisfactory, they must be compliant with at least the minimum standards of efficacy (Boddie et al. 2004). The European standards (such as EN 1656 and EN 1276) are generally used to determine the threshold of efficacy for different disinfectants. The purpose of these tests is to evaluate the bactericidal, fungicidal, yeasticidal, basic sporicidal or mycobactericidal activity of products used under various conditions (ATS, 2014). Many tests are suspension based and used to support general antimicrobial claims, while other tests are carrier based and used to support the antimicrobial activity of products on surfaces for devices. Any disinfectant or antisepetic chemicals intended for registration within Europe are tested using a variety of methods developed by the CEN (European Committee for Standardisation; Ats 2014). Interpretation of the results of these methods is very important. According to Boddie et al. (2002), an effective teat disinfectant should give a minimum yield of 3 log reduction, with 4–5 log reduction being preferable, for treated teats, when 6 log units are recovered from untreated control teats, all within 10 min of application. This reduction has been considered an acceptable estimate of germicidal activity when using the excised teat protocol (Schmidt et al. 1984; Watts et al. 1984). In comparison, EN 1276 requires a 5 log reduction within 60 min of application.

Protocols determining the effectiveness of teat disinfection products in controlling mastitis

The National Mastitis Council (NMC), based in Minnesota, USA, monitors the development of protocols that determine the effectiveness of mastitis control products such as teat disinfectant solutions (Hogan et al. 1990). Due to the lack of uniformity between governmental standards of teat disinfectant products (Schukken et al. 2013), the NMC research committee and the Teat Dip committee reviewed and updated guidelines and protocols in an effort to standardise procedures to ensure uniform and accurate comparison of studies (Schukken et al. 2013). These guidelines include (1) challenge organisms for experimental exposure studies; (2) procedures for statistical analyses; (3) appropriate sample size to detect a difference between treatments; (4) milking systems operations and udder preparation procedures; and (5) removal of the requirements for at least two herds and a study length of at least 12 months from the natural exposure protocol (Nickerson et al. 2004).

The methods discussed by the NMC include the natural exposure, the experimental challenge and the excised teat protocols (Nickerson 2001; Enger et al. 2015). Agar diffusion (disc diffusion or well diffusion) assays have been used in many clinical microbiological laboratories for antimicrobial susceptibility testing (Balouiri et al. 2015). This assay has not been as extensively used as the other protocols, but it could be adapted to determine the efficacy of teat disinfectant products.

The objective of this review is to evaluate the various methodologies used to determine the efficacy of teat disinfectants. These methods can include in vitro (standard laboratory) and in vivo (in-field) tests (Godden et al. 2016). In vivo testing refers to experimentation using a whole, living organism (i.e. herd of cows) within its natural environment as opposed to a living or dead organism (i.e. excised teat) in a laboratory. These tests can include teat swabbing, an experimental challenge and natural exposure protocols. This review also discusses the experimental design such as sampling procedures, split herd and udder designs between different studies. In vitro testing refers to performing a procedure in a controlled environment (laboratory), outside of a living organism. These tests can include excised teat protocol and agar diffusion assay. A number of reviewed studies were compared to determine differences between the experimental design applied and the results obtained (Table 1). The limitations and positive and negative aspects of each protocol have also been discussed in this review.

IN VIVO TESTING

In vivo testing has benefits over laboratory tests as it observes the overall effects of a disinfectant product within the environment. This can help to determine the efficacy of products against new IMIs in actual dairy herds as opposed to identifying the efficacy of a disinfectant product within a laboratory situation.

Experimental challenge protocol

The experimental challenge protocol is commonly applied on a research farm and is the industry standard for manufacturers of agricultural disinfectants for determining the efficacy of new and existing teat disinfectant products. The aim is to reduce the incidence of new IMIs compared with non-disinfected controls when teats are challenged with mastitis-causing bacteria to increase the infection rate (Nickerson 2001). Directly after milking, cows’ teats are dipped with a suspension of bacteria, and immediately afterwards, two diagonally (left front, right rear) or horizontally (front left, front right) opposite teats are disinfected in the test product and the remaining teats are either left not disinfected to serve as negative controls or disinfected with a product of known efficacy to serve as a positive control (Nickerson et al. 2004). Milk samples may be taken weekly for a number of weeks from each intramammary quarter and tested to determine the number of new infections arising from the bacterial suspension. The disinfected and control quarters are then compared, and efficacy is expressed as the
percentage reduction in new infections in disinfected quarters (Nickerson 2001).

**Bacteriological status of herd**

Bacteriological status of the herd refers to the determination of the infection status of each cow involved in the study. This status gives a baseline for determining the rate of new IMIs during the study. Studies reviewed in this article carried out an initial bacteriological infection status for the herd before the start of each study. Of the five studies reviewed, Boddie et al. (2004) and Leslie et al. (2005) have longer periods of bacteriological records for their herds. Leslie

<table>
<thead>
<tr>
<th>Study</th>
<th>Teat swabbing</th>
<th>Excised teat</th>
<th>Natural exposure</th>
<th>Experimental challenge</th>
<th>Split udder</th>
<th>Split herd</th>
<th>Control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enger et al. (2015)</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>Negative</td>
<td>% Reduction: Contagious: 88%, Enviro.: 82%, CNS: 80.0%</td>
</tr>
<tr>
<td>Boddie et al. (2002)</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>Negative</td>
<td>LR of 6.29 (M. bovigenitalium), 5.41 (M. bovis) &amp; 5.70 (M. californicum)</td>
</tr>
<tr>
<td>Schmidt et al. (1984)</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg. &amp; Pos.</td>
<td>LR of 78.5–100% for Gram-negative bacteria</td>
</tr>
<tr>
<td>Philpot et al. (1978)</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg. &amp; Pos.</td>
<td>Idophor, sodium hypochlorite &amp; sodium dichloro-s-triazinetrione: effective against all strains</td>
</tr>
<tr>
<td>Watts et al. (1984)</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg. &amp; Pos.</td>
<td>LR of 3–4 for Staph. aureus &amp; Str. agalactiae</td>
</tr>
<tr>
<td>Lago et al. (2016)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>Positive</td>
<td>EX was 17% more effective than the PC</td>
</tr>
<tr>
<td>Godden et al. (2016)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Positive</td>
<td>Product found to be noninferior</td>
</tr>
<tr>
<td>Foret et al. (2005)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Positive</td>
<td>Major pathogens reduced by 58% &amp; minor pathogens by 54%</td>
</tr>
<tr>
<td>Ceballos-Marquez et al. (2013)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Positive</td>
<td>EX was 19% more effective than the PC</td>
</tr>
<tr>
<td>Oliver et al. (1991)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Negative</td>
<td>Efficacy against minor pathogens (35%), CNS (33.1%) &amp; C. bovis (37.8%)</td>
</tr>
<tr>
<td>Boddie et al. (2004)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>8 weeks</td>
<td>✓</td>
<td>Negative</td>
<td>% reduction: 88% (Staph. aureus) &amp; 67% (Str. agalactiae)</td>
</tr>
<tr>
<td>Leslie et al. (2006)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>10 weeks</td>
<td>✓</td>
<td>Positive</td>
<td>No differences found between products</td>
</tr>
<tr>
<td>Oura et al. (2002)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>12–14 weeks</td>
<td>✓</td>
<td>Negative</td>
<td>Reduction of 72% &amp; 75% and 100% &amp; 88% for Staph. aureus &amp; Str. agalactiae, respectively</td>
</tr>
<tr>
<td>Foret et al. (2003)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>7 weeks</td>
<td>✓</td>
<td>Positive</td>
<td>% reduction: 90% (Staph. aureus) &amp; 73% (Str. agalactiae)</td>
</tr>
<tr>
<td>Leslie et al. (2005)</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>10 weeks</td>
<td>✓</td>
<td>Positive</td>
<td>No differences found between EX &amp; PC</td>
</tr>
<tr>
<td>Gleeson et al. (2009)</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Teat disinfectants had different efficacies when used on cows indoors or outdoors</td>
</tr>
<tr>
<td>Gibson et al. (2008)</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>No significant difference between treatments in the reduction in TVC</td>
</tr>
<tr>
<td>Baumberger et al. (2016)</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>No differences in reduction between treatment for most bacterial counts</td>
</tr>
<tr>
<td>Mileikiené et al. (2015)</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Lactic acid and iodine had highest probability of reducing TBC</td>
</tr>
</tbody>
</table>

CNS, Coagulase Negative Staphylococci; EX, experimental control; M. bovigenitalium, Mycoplasma bovigenitalium; M. bovis, Mycoplasma bovis; M. californicum, Mycoplasma californicum; C. bovis, Corynebacterium bovis; LR, log reduction; PC, positive control; TBC, total bacterial contamination; TVC, total viable count.
et al. (2005) established the bacteriological status by sampling the cows weekly for 3 weeks prior to the study. Boddie et al. (2004) determined the bacteriological status of the herd prior to the study. The herd was sampled monthly throughout the year so that all cows had lifetime cultures for all lactations. When comparing the bacteriological results at the end of the study to the initial herd status, either a 3-week bacteriological status study or a lifetime culture record would give a satisfactory outlook on the actual bacteriological status of the overall herd. This information can be used to resolve discrepancies in infection results, should they occur during the study.

Collecting milk samples and definition of new IMIs

Within the reviewed studies, intramammary quarter milk samples were collected on a weekly basis and each teat forestripped before one sample was collected. Foret et al. (2003) and Boddie et al. (2004) additionally used a cotton pledget moistened with 70% w/v alcohol to wipe each teat, which is somewhat similar to the Animal Health Ireland (AHI) CellCheck recommendations (CellCheck, 2012). Milk samples were then stored at 5 °C (Foret et al. 2003; Boddie et al. 2004) and –20 °C (Oura et al. 2002). When defining a new IMI, Oura et al. (2002), Foret et al. (2003) and Boddie et al. (2004) had similar standards, such as (1) *Staph. aureus* or *Str. agalactiae* was isolated from a clinical intramammary quarter, (2) two consecutive samples yielded >500 cfu/mL of the same pathogen, or (3) three consecutive samples contained 100–400 cfu/mL of the same pathogen. Oura et al. (2002) had an additional fourth standard where five consecutive samples contained >1 cfu/mL of the same pathogen if any of the following criteria were met: (1) signs of clinical mastitis were observed (samples were taken to confirm bacteria present), (2) *Staph. aureus* or *Str. agalactiae* were cultured in high numbers (>500 cfu/mL) in two consecutive weeks, and (3) *Staph. aureus*, *Str. agalactiae*, *Staphylococcus* species or *Corynebacterium bvis* were cultured in low numbers (100–500 cfu/mL) in three consecutive samples. In addition to these criteria, somatic cell count (SCC) data were used to screen culture negative intramammary quarters to or show elevated SCC. Any quarter with a SCC of >300 000 cells/mL were resampled and submitted for culture. These differences in criteria used to define new IMIs make comparing results from different studies challenging. The criteria described by Foret et al. (2003) and Boddie et al. (2004) would be preferred by the authors as the most suitable criteria for defining new IMIs.

Challenge suspension and application

All reviewed studies created stock suspensions with a concentration of $5 \times 10^7$ cfu/mL for each bacterial strain, before application to the teats. Differences in the preparations of stock suspension were observed between the different studies. Leslie et al. (2005, 2006) created *Staph. aureus* and *Str. agalactiae* stock suspensions from culture stocks, three times weekly. Foret et al. (2003) and Boddie et al. (2004) prepared stock suspensions from lyophilised vials, of *Staph. aureus* weekly and suspensions of *Str. agalactiae* were prepared daily. Oura et al. (2002) used isolated colonies of *Staph. aureus* and *Str. agalactiae* and created both stock suspensions daily. The creation of stock solutions daily may help to reduce the chances of the stock solution becoming contaminated. In all studies, the suspensions were applied to all four teats immediately after removing the milking cluster and the suspension left to dry before the application of teat disinfectant. However, differences in application schedules were observed between studies. Oura et al. (2002) applied the suspension for 7 days each week after the morning milking and Leslie et al. (2005, 2006) applied the suspension for 7 days each week, but after morning and afternoon milking. Studies by Foret et al. (2003) and Boddie et al. (2004) applied the suspension for 5 days each week after the afternoon milking. The reduced challenge days may have an impact on the outcome of results as the challenge exposure time was reduced.

Experimental design

Studies undertaken by Oura et al. (2002), Foret et al. (2003), Boddie et al. (2004) and Leslie et al. (2005, 2006), used varying herd numbers ranging from 45 to 165 cows and also varying trial periods from a 7- to 14-week period. All studies were satisfied that the herd numbers enrolled in the studies accounted for possible errors and gave representable results with the exemption of Leslie et al. (2006) who stated that the low animal number used (45 cows) may have limited the possibility of detecting differences between treatment groups. This would indicate that a herd size lower than 90 cows may be insufficient in an experimental challenge study.

All reviewed studies are in agreement with using the split udder design when applying the teat disinfectant, with Foret et al. (2003) and Leslie et al. (2005) also implementing a split herd design. Oura et al. (2002), Foret et al. (2003) and Boddie et al. (2004) used negative controls by leaving the other half of the udder as undisinfected controls. This is less likely to be carried out in future studies as it can be seen as unethical due to leaving teats exposed to an increased level of IMIs (Schukken et al. 2013). Studies by Leslie et al. (2005, 2006) used a positive control teat disinfectant, which may be more favourable in terms of animal welfare as the teats are still given protection against the introduced bacteria.

Evaluation of disinfectant products using 'experimental challenge protocol'

In a number of challenge studies, different bacterial reductions were observed when different disinfectant products
were evaluated. Studies by Foret et al. (2003), Boddie et al. (2004) and Leslie et al. (2005) evaluated iodine disinfectant products. Boddie et al. (2004) found that a 0.1% w/w iodine teat disinfectant reduced the infection rate for *Staph. aureus* by 88% and 67% for *Str. agalactiae*, whereas Foret et al. (2003) found that a 1% w/w iodine concentration reduced the infection rate by 90% for *Staph. aureus* and 73% for *Str. agalactiae*. Leslie et al. (2005) compared a 1% w/w iodophor product against a commercially available product and found there were no significant differences in new IMIs caused by *Staph. aureus* and *Str. agalactiae*. Leslie et al. (2006) also evaluated disinfectants containing hydrogen peroxide (H$_2$O$_2$) and dodecylbenzene sulphonate acid (DBSA) against a commercially available product. The authors concluded that there was no significant difference between the two products in new IMIs caused by *Staph. aureus* and *Str. agalactiae*. A further study by Oura et al. (2002) compared two different concentrations of dodecylbenzene sulphonate acid (DBSA) (0.53% w/w vs 0.27% w/w). They found new IMIs were reduced by 72% (*Staph. aureus*) and 75% (*Str. agalactiae*) and 100% (*Staph. aureus*) and 88% (*Str. agalactiae*), respectively.

**Summary**

Use of the experimental challenge protocol allows the tester to evaluate the effectiveness of the teat disinfectant but may not represent the efficacy of the product in the chosen environment as only the bacterial suspension bacteria are of interest, while the new IMI could be caused by any other mastitis-causing bacteria. The design of the experiment must be efficiently planned to avoid any animal health welfare issues (Schukken et al. 2013) and, as stated by Nickerson et al. (2004), the experimental challenge protocol must be carried out in research farms. The increased infection status of the herd as a result of challenge application would prevent the use of this protocol on commercial farms. The experimental designs used in experimental challenge studies can be compared to other studies to enable comparison of results. In comparison with a natural exposure protocol, a challenge study can be carried out in a shorter time period but involves a higher workload in the preparation of the challenge suspensions, such as ensuring suspensions are free from contamination, the application of the challenge suspension, drying and then the application of the teat disinfectant.

As the teats of the cow are challenged with bacteria, an increased rate of new IMIs would be expected as compared to natural exposure studies. Therefore, the use of controls is needed within the protocol to ensure correct reductions are determined.

Although the experimental challenge protocol evaluates the product within the environment, it may not accurately simulate the efficacy of a product as the infection is not naturally occurring. Furthermore, the increased infection status and rate of new IMIs within the herd, due to the introduction of challenge bacteria, would not be representative of the situation on commercial farms.

**Natural exposure protocol**

The natural exposure protocol is suitable for application on commercial herds and can be used to evaluate the efficacy of a teat disinfectant product in reducing the incidence of new IMIs, without the requirement of challenging teats with mastitis-causing bacteria. The new infection rate is dependent on natural exposure to mastitis-causing bacteria in the farm environment of the cow. In natural exposure studies, other protocols such as noninferiority studies, split herd and split udder models have been used (Nickerson 2001). Non-inferiority studies compare a teat disinfectant of unknown efficacy to a teat disinfectant of known efficacy (also referred to as positive control studies; Godden et al. 2016). Intramammary quarter milk samples are taken from nondisinfected and disinfected teats of each cow and results compared against each other to determine the rate of reduction in IMIs (Nickerson 2001). A split herd refers to disinfecting one half of the herd and leaving the other half of the herd without disinfection or a known disinfectant used as a control. Somatic cell count results are compared between herds to determine the rate of reduction in IMIs (Nickerson 2001). A split udder model refers to half the teats of the cow being disinfected, with the other half left undisinfected (negative control) or a control disinfectant is applied (positive control). A split udder protocol is commonly used in natural exposure studies (Nickerson et al. 2004).

There were many differences between the numbers of cows enrolled in the reviewed studies and in the length of the study periods. Oliver et al. (1991) had the highest number of cows enrolled in their study with 374 cows, followed by Lago et al. (2016) and Godden et al. (2016) with 299 and 317 cows, respectively. Foret et al. (2005) and Ceballos-Marquez et al. (2013) used 90 and 199 cows, respectively. Oliver et al. (1991) and Foret et al. (2005) undertook their studies for the longest period of 9–12 months. In comparison, Ceballos-Marquez et al. (2013), Godden et al. (2016) and Lago et al. (2016) carried out their studies within a 10- to 12-week period. The sample size and study length used in the studies gave adequate statistical power when analysing results.

**Bacteriological status of the herd and sampling schedule**

A review of studies by Oliver et al. (1991), Foret et al. (2005), Ceballos-Marquez et al. (2013) and Godden et al. (2016) showed that the bacteriological status of their herds was determined by collecting milk samples before the commencement of the study. These samples were used to determine the infection status and create a baseline of each individual cow. Within the reviewed studies, intramammary quarter milk samples were collected. Oliver et al. (1991), Foret et al. (2005), Godden et al. (2016) and Lago et al.
(2016) collected milk samples every 2 weeks with Foret et al. (2005) collecting a monthly sample, whereas Ceballos-Marquez et al. (2013) collected milk samples bimonthly but only cultured them depending on SCC analysis.

**Analysis of milk samples and definition of new IMIs**

A review of studies conducted by Ceballos-Marquez et al. (2013), Godden et al. (2016) and Lago et al. (2016) used SCC analysis to decide whether a sample should be cultured and considered samples from clinical cases as eligible for identifying new IMIs. Godden et al. (2016) and Lago et al. (2016) determined subclinical mastitis using SCC levels above a defined threshold and an IMI as being present when a single colony from a sample was isolated from any bacterial species with the exception of coagulase-negative *Staphylococcus* (CNS) and *Bacillus* species. A single IMI was defined as the presence of only one pathogen type in the sample, whereas mixed infections corresponded to the presence of two different bacterial species. In comparison, Oliver et al. (1991), Foret et al. (2005) and Ceballos-Marquez et al. (2013) considered an IMI as a new infection based on the presence of a microorganism that was not identified in that quarter previously. Ceballos-Marquez et al. (2013) used single intramammary quarter samples to determine new IMIs while Oliver et al. (1991) used duplicate individual quarter milk samples and Foret et al. (2005) used two consecutive quarter milk samples to determine new IMIs.

To standardise the routine identification of new IMI from quarters not showing signs of clinical infection, quarters with a SCC >300 000 cells/mL and with a pathogen present on one occasion could be considered a new IMI.

**Split herd vs split udder**

When considering whether to use split herd or split udder sampling, split herd appears to give a more representative result of product efficacy (Enger et al. 2016; Lago et al. 2016). Although, with this in mind, Enger et al. (2016) state that future natural exposure studies using a nondisinfected negative control treatment should employ a split udder design rather than a split herd design to help to reduce the effect of a teat disinfectant efficacy study on herd udder health. Furthermore, Lago et al. (2016) state there are some advantages to using a split herd design rather than a split udder design. This is due to the split herd design remaining more realistic when using a large number of animals. It is also unlikely to affect the prevention and treatment methods used at cow level due to the interdependence between intramammary quarters.

**Evaluation of disinfectant products using ‘natural exposure protocol’**

Many studies have evaluated the efficacy of a range of different iodine concentrations in disinfectants. A study by Oliver et al. (1991) evaluated a postmilking teat disinfectant that contained 0.25% w/w available iodine, glycerine and water. In a herd with a high prevalence of contagious mastitis, the overall efficacy of the experimental product against mastitis pathogens was 62%. In a herd with a high prevalence of environmental pathogens, the overall efficacy of the product against major mastitis pathogens was 24%. Similarly, Foret et al. (2005) evaluated the application of 0.25% w/w iodine postmilking teat disinfectant. The product reduced the infection rate for major pathogens by 58% and minor pathogens by 54%. Similarly, Ceballos-Marquez et al. (2013) found that the application of a premilking 0.5% w/w iodine teat disinfectant was 19% more effective compared to the positive control. While Foret et al. (2005) used a low concentration (0.25% w/w) iodine-based product as a postmilking disinfectant, it was more effective than a higher concentration (0.5% w/w) iodine-based product used by Ceballos-Marquez et al. (2013) as a premilking disinfectant.

The evaluation of a new range of active ingredients in teat disinfectant products in comparison with iodine-based products is beneficial due to the potential iodine residues in milk which could possibly affect human health (O’Brien et al. 2013). Studies by Godden et al. (2016) and Lago et al. (2016) evaluated a glycolic acid-based postmilking teat disinfectant. Both studies concluded that the experimental product was more effective at reducing IMIs than a positive control.

**Summary**

The natural exposure protocol can give a better indication of how the teat disinfectant would work within the environment. A limitation of the natural exposure protocol is that it can be time-consuming with studies normally being conducted during a full lactation to get a complete comparison between disinfected and nondisinfected controls. However, when testing the efficacy of teat disinfectants, the number of diagnostics needed to determine a new IMI, along with a large number of bacteriological culturing can raise costs and leave the protocol unreasonably expensive (Ceballos-Marquez et al. 2013). Ceballos-Marquez et al. (2013) addressed this limitation by evaluating the use of a novel two-step diagnostic process to evaluate a premilking disinfectant. Rather than conducting the study over a period of 10–12 months, they used a 10-week period. In this study, authors used SCC analysis to determine whether samples should be cultured. This resulted in a more economical and efficient way to identify new IMIs. This process was also used by Godden et al. (2016) and Lago et al. (2016) during a postmilking efficacy trial. Another possible limitation is the use of study herds with different levels and types of infection. As stated by Oliver et al. (1991), many studies have been conducted on herds with a high prevalence of contagious mastitis which can make the evaluation of teat disinfectant efficacy against environmental pathogens difficult. Therefore, efficacy studies should be conducted on two separate herds with one having a high prevalence of
contagious and the other with a high prevalence of environmental pathogens to generate the number of IMI necessary for determining the efficacy of the disinfectant.

While the natural exposure protocol involves a longer trial period than experimental challenge protocol, it has a lower workload and potentially a lower incidence of new IMIs. It also has a higher potential to be used within commercial farms as infections are naturally occurring.

**Teat swabbing protocol**

Swabbing teats before and after teat disinfection may be useful to determine the efficacy of teat disinfectant products. Studies by Gibson et al. (2008), Gleeson et al. (2009), Mišeikienė et al. (2015) and Baumberger et al. (2016) used a teat swabbing protocol to determine the efficacy of a range of premilking teat disinfectants. Swab samples of teats were taken using swabs moistened using buffered peptone water (Gibson et al. 2008; Baumberger et al. 2016). The actual sample can be collected in different ways such as wiping one side of the teat barrel from top to bottom, passing over the teat end and wiping the other side of the teat barrel from top to bottom (Baumberger et al. 2016), rubbing across the teat orifice and down the side of each teat avoiding contact with the udder hair or cows' flank (Gleeson et al. 2009) or performed by triple rotary motions around the surface of the teat close to the tip from the front teat only (Mišeikienė et al. 2015). Different types of media have been used to store the sample swabs: 4 mL of buffered peptone water (Baumberger et al. 2016), 5 mL of sterile recovery medium 1.0 g/L protease peptone, 8.5 g/L sodium chloride and 2.0 g/L sodium thiosulphate (Gibson et al. 2008) or 5 mL of sterile tryptic soy broth (TSB) (Gleeson et al. 2009) with samples being kept at low temperatures (4 °C) (Gibson et al. 2008; Mišeikienė et al. 2015; Baumberger et al. 2016) or frozen (Gleeson et al. 2009; Baumberger et al. 2016; Separate to the reference (Gleeson et al. 2009; Baumberger et al. 2016) depending on time allowed between sampling and culturing of samples. The samples can be vortexed or agitated for 5–10 s (Gibson et al. 2008; Mišeikienė et al. 2015) or left unmixed (Gleeson et al. 2009; Baumberger et al. 2016). Swab samples are usually diluted to $10^{-2}$ to $10^{-3}$ for postdisinfectant swab samples (Mišeikienė et al. 2015; Baumberger et al. 2016) and $10^{-4}$ for predisinfectant swab samples (Baumberger et al. 2016).

**Evaluation of disinfectant products using ‘teat swabbing protocol’**

In four reviewed studies, teat swabbing was used to compare the difference in bacterial counts on cows’ teat skin after the implementation of a premilking hygiene regime. A study by Gleeson et al. (2009) used the teat swabbing protocol to evaluate a wide range of premilking teat disinfectants. In this study, disinfectant treatments showed different efficacies depending on whether cows were housed indoors or outdoors on pastures. Chlorine, iodine and wipes had a higher reduction of staphylococcal counts while cows were outdoors, with iodine also achieving a high reduction of streptococcal counts when used on cows indoors. Furthermore, Gibson et al. (2008) and Baumberger et al. (2016) identified a significant treatment by farm interaction for the reduction in all bacterial counts. This may be due to factors such as herd size, milk yield, field conditions, management techniques and housing (Gibson et al. 2008). Mišeikienė et al. (2015) used the teat swabbing protocol to evaluate a 0.2% w/w iodine disinfectant, 0.5% w/w aminopropyl-laurylamine disinfectant and a lactic acid-based disinfectant. The authors concluded that lactic acid and iodine had the highest probability of reducing total bacterial contamination, compared to 0.5% w/w aminopropyl-laurylamine. Baumberger et al. (2016) used the teat swabbing protocol to compare a 0.5% w/w iodine premilking disinfectant to a teat scrubber system which used chlorine dioxide. They found no differences in reduction between the treatments for most bacterial counts on teat skin.

**Summary**

The teat swabbing protocol can determine the efficacy of different premilking disinfectant treatments, by measuring the bacterial counts on the teat skin surface before and after treatment. A limitation of this method would be the high degree of variability in the numbers of recovered bacteria depending on the pressure applied to the teat from the swab, the choice of area on the teat, as the whole teat cannot be practically swabbed, and the variability in the surface area of the teat swabbed (Bade et al. 2008). In comparison with the natural exposure and experimental challenge protocols, the protocol does not give information on the reduction in new IMIs.

**IN VITRO TESTING**

There are a variety of laboratory test methods available to evaluate the efficacy or the antimicrobial activity of teat disinfectants against mastitis-causing bacteria. These methods include the excised teat protocol and agar diffusion assay (Balouiri et al. 2015).

**Excised teat protocol**

The excised teat protocol was used as a screening test to assess the teat disinfectants ability to reduce viable bacteria on teat skin surfaces (Nickerson et al. 2004), but in 1989, the membership of NMC decided to omit the excised teat model (Hogan et al. 1990). It was considered less time-consuming and less expensive than the other protocols described but was mainly used to determine whether an experimental product had the potential to be further evaluated as a teat disinfectant for cows. It involved using...
Excised teat preparation
To be accurate and reproducible, teat skin condition must be recorded and the teat prepared by trimming excess skin, washing in warm detergent, rinsed in sterile water, dried (Philpot et al. 1978; Schmidt et al. 1984; Boddie et al. 2002; Enger et al. 2015) and disinfected in 70% w/v ethyl alcohol (Boddie et al. 2002), 70% w/v isopropyl alcohol (Enger et al. 2015) or 70% w/v alcohol (type not stated) (Philpot et al. 1978; Schmidt et al. 1984). Teats can be placed in plastic bags in a glycerine and water solution and frozen at −20 °C until further use (Boddie et al. 2002; Enger et al. 2015). A range of 20–30 teats was used within the studies reviewed, with 10 teats used as negative control, 10 teats for the evaluation of the experimental product and, in some cases, 10 positive control teats. Teats were prepared by thawing firstly in warm water, dipping them in 70% w/v ethyl alcohol, dried with a paper towel and suspended by metal clips from a glass rod (Boddie et al. 2002).

Challenging teats and sample collection
Within four reviewed studies (Philpot et al. 1978; Schmidt et al. 1984; Boddie et al. 2002; Enger et al. 2015), test teats were diped to a depth of 15 mm in the bacterial challenge, allowed 5 min to dry and then disinfected to a depth of 30 mm in the test product and left to dry for 10 min. Nondisinfectected control teats were left to dry for 15 min. The preparation of the challenge suspension is very similar to challenge preparations outlined for the experimental challenge protocol. Within an excised teat protocol, a quench solution is used to recover bacteria from the teats to allow for culturing. Letheen broth is used within a quench solution to inactivate the disinfectant residue on the teat surface. Philpot et al. (1978) used sets of teats for a series of successive trials on the same day. Between trials, teats were rinsed in warm water for 2 min; dried and rinsed in 0.05% w/v sodium thiosulphate solution for 1 min; dried and rinsed in a 0.05% w/v lecithin and 0.05% w/v Tween 80 solution for 1 min; dried and rinsed in warm water for 1 min; dried and dipped in 70% w/v alcohol; and dried and resuspended. This was done to prevent any bacteria from the previous trial affecting results of successive trials. They concluded that the number of organisms recovered from teats in successive trials on the same day decreased. These reductions were not sufficient to effect log reductions but suggested that negative controls should be included for each successive trial.

When culturing the collected rinse samples, all reviewed studies diluted negative control samples to 10⁻⁴ and plated the treated teats undiluted when culturing samples, with Enger et al. (2015) also plating a dilution factor of 10⁻³ for negative controls.

Evaluation of disinfectant products using the ‘excised teat protocol’
All studies reviewed evaluated a large range of products within their individual studies. This is beneficial as it allows comparison between products and aids in the selection of effective biocidal products. Watts et al. (1984) evaluated 38 different disinfectant products, which included 27 iodophors, one chlorhexidine and two quaternary ammonium compounds (QAC). Twenty-six of twenty-seven iodophor products achieved reductions >log 3 for Staph. aureus. Thirteen of the twenty-seven products, which contained 1% w/w iodophor, reduced Str. agalactiae by log 3 or greater. The QAC products yielded reductions >log 4 against Staph. aureus and Str. agalactiae. A 0.5% w/w cetylpyridinium chloride product had a log reduction of 4.64 and 4.71 for Staph. aureus and Str. agalactiae, respectively, and 1% w/w benzyl alcohol had a log reduction of 3.83 and 4.3 for Staph. aureus and Str. agalactiae, respectively.

Similarly, Philpot et al. (1978) carried out an evaluation of 13 teat disinfectants. Results show that the iodophors, sodium hypochlorite and sodium dichloro-s-triazinetrione were effective against a number of strains (Staph. aureus, Str. agalactiae, E. coli and Pseudomonas aeruginosa), with iodophor and chlorine products yielding the highest reductions. In comparison, QAC, chlorhexidine and cetylpyridinium chloride products were only found to be effective against Staph. aureus and Str. agalactiae and ineffective against E. coli and P. aeruginosa. Sodium hypochlorite was the most effective of all products. Sodium dichloro-s-triazinetrione was as effective as other disinfectants achieving log reductions between 4.92 and 5.31 for strains used. Similarly to Philpot et al. (1978) and Watts et al. (1984), Enger et al. (2015) looked at four different disinfectants (disinfectant A: 1% w/w H₂O₂, disinfectant B: 1% w/w chlorine dioxide, disinfectant C: 1% w/w iodophor and disinfectant D: 0.5% w/w iodophor) against Staph. aureus, Str. agalactiae, Mycoplasma bovis, E. coli, Streptococcus dysgalactiae, Str. uberis, Staphylococcus xylosus, Staphylococcus epidermidis, Staphylococcus chromogenes, Staphylococcus haemolyticus and Staphylococcus hycicus. All four disinfectants yielded log reductions between 0.23 and 2.26. Rather than evaluating the efficacy of a teat disinfectant against Staph. aureus and Str. agalactiae, Boddie et al. (2002) used six different products: (0.5% w/w iodine teat disinfectant, 0.5% w/w chlorhexidine teat disinfectant, two different chlorine teat disinfectants, a 0.5% w/w H₂O₂ mixed with 1.7% w/w lactic acid peroxygen teat disinfectant and QAC teat disinfectant) against Mycoplasma species. In this study, the average log reduction for all products was 6.29 for Mycoplasma bovigenitalium, 5.41 for M. bovis and 5.70 for Mycoplasma californicum. This result differed to
Enger et al. (2015), who found no significant difference between disinfectants used against M. bovis. A study by Boddie et al. (2002) found an average log reduction of 5.41 for M. bovis when products containing iodine, chlorhexidine, chlorine, 0.5% w/w H2O2 mixed with 1.7% w/w lactic acid peroxygen and quaternary ammonium were used. Unlike other studies, Schmidt et al. (1984) evaluated a sodium chlorite and lactic acid disinfectant diluted in water. The disinfectant yielded a high per cent log reduction for 14 of 21 strains tested (which included Str. agalactiae, E. coli, Str. dysgalactiae, Str. uberis, Klebsiella oxytoca, Klebsiella pneumonia and P. aeruginosa). The test disinfectant was least effective against strains of Staphylococcus aureus, as was the positive control (1% w/w iodophor).

Summary
From reviewing methods, the excised teat protocol is limited to showing the efficacy of the disinfectant and is used as a screening step for new products. The excised teat protocol could potentially give a better indication of efficacy when compared to other laboratory methods as they lack the characteristicts of bovine teat skin (Philpot et al. 1978). The use of negative control teats allows for a comparison with the number of bacteria removed from the treated teats. A potential limitation of excised teat protocol is that it only measures the germicidal activity of teat disinfectants on the skin. The assay is incapable of measuring other important factors of a teat disinfectant such as the promotion of healing lesions, preventing chapping, persistent activity on the skin and formation of a barrier over the teat opening separate to the reference (orifice) (Schmidt et al. 1984). Also, evaluating the effectiveness against new IMIs caused by contagious, environmental and coliform bacteria is less predictable as the teats are not exposed to these organisms, not only during milking but between milkings (Schmidt et al. 1984). Furthermore, on comparing the studies by Schmidt et al. (1984) and Enger et al. (2015), both used the excised protocol as previously described by Philpot et al. (1978). Thus, this protocol has changed very little over the last 30 years which suggests that the protocol is a very effective measure of efficacy; however, few studies have used this method over the years.

Agar diffusion assay
The agar disc and agar well diffusion assays, also known as the Kirby Bauer method, have been mainly used for the detection of antibiotic-resistant bacteria (Biemer 1973). The assay has the potential to be used when determining the efficacy of teat disinfectants against different types of mastitis-causing bacteria and should be considered. Agar disc diffusion was developed in 1940 and is regarded as the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing (Biemer 1973; Balouiri et al. 2015). Agar plates are inoculated with a standardised inoculum of the test microorganism. Then, filter paper discs, which contain the disinfectant being tested, are placed on the surface of the agar (Biemer 1973). The Petri dishes are then incubated under suitable conditions. The disinfectant diffuses into the agar and inhibits the growth of the test microorganism. The diameters of the zones of inhibition are then measured, usually in mm (Biemer 1973; Balouiri et al. 2015). In both agar disc and agar well diffusion assays, a control disinfectant should be used that has a known zone of inhibition towards the test bacteria (Biemer 1973). Agar well diffusion follows the same principles as disc diffusion but instead of using discs containing the test disinfectant, wells of a certain diameter (6–8 mm) are punched into the agar and 10–200 μL of the test substance is put into each well. The experimental disinfectants diffuse into the agar, and inhibition zones are created, just like with the disc diffusion method. The diameter of the well and the zone of inhibition must all be measured to ensure accurate results. Factors such as rate of diffusion must also be taken into account (Balouiri et al. 2015).

Summary
Even though this assay is not a recommended protocol by the NMC, the assay has a high potential to effectively evaluate teat disinfectant products. Results could potentially be obtained quicker with a lower workload than the excised teat protocol.

CONCLUSIONS
Different protocols can be used to evaluate teat disinfectant products, which allow a full evaluation of the product from creation in a laboratory to use on a farm. The protocol chosen depends on the scope requirement of the investigation. The experimental challenge protocol allows for the evaluation of the teat disinfectant against high levels of known bacteria. However, an experimental challenge study is more expensive in terms of an increased number of infected quarters and requires an increased workload as challenge suspensions must be prepared and tested daily. The natural exposure protocol is also time-consuming as it should be conducted over a full lactation to obtain representative results, but it has the potential of giving a better indication of the efficacy of the disinfectant within a farm environment. The use of noninferiority studies within the natural exposure protocol eliminates the use of negative controls (nondisinfected teats), which raise animal welfare and ethical concerns. The use of a split herd vs a split udder protocol may be more beneficial due to the interdependence of intramammary quarters. This could also help to reduce the impact on herd udder health and treatment methods. The teat swabbing protocol is limited due to a high degree of variability of bacteria recovered from teats and it also does not give an indication of the teat disinfectant or regimes’
potential to reduce new cases of IMIs. The excised teat protocol highlights the effectiveness of a disinfectant and indicates if the teat disinfectant should be further tested in an environment similar to where it will be used (i.e. natural exposure study). It is considered a reliable method of screening and evaluating teat disinfectants and is recommended by the NMC. However, the excised teat protocol has become less relevant in recent years, due to possible difficulties of acquiring, handling and disposing of teats. Agar diffusion has been used considerably less when evaluating teat disinfectants. However, agar diffusion is a less costly and time-consuming process as 3–4 teat disinfectants could be tested for each bacterial strain of interest on one agar plate. The inclusion of agar diffusion tests may allow manufacturers to identify efficient formulations at a faster rate.

Although not fully addressed within the review, the effect of each disinfectant product on teat condition and possible residues in the milk from that product must be considered when evaluating any product. Teat condition is mentioned within the NMC recommended protocols but the possibility of residues has not been addressed. Both of these topics require individual reviews of their own to fully ensure that products leave no harmful residues in milk and maintain good teat condition.

REFERENCES


