



The development of nylon membrane-based filter technique in the evaluation of bactericidal effectiveness of mastitis control

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Abstract

A nylon membrane-based filter technique was developed to evaluate the bactericidal efficacy of mastitis control teat dip products. Using this method, we were able to determine that this protocol was both a rapid and effective method for evaluating the efficacy of our iodine- and bronopol-containing teat dips against a variety of mastitis-causing strains of bacteria such as *E. coli*, *S. aureus*, *P. aeruginosa*, *S. uberis*, *E. faecalis*, and *S. agalactiae*, *S. dysgalactiae*, as well as the fungal strain *C. albicans*. The data presented in this report suggests that this method is useful in the initial assessment of newly developed bovine teat dips, as well as other medical formulations.

Keywords: Bactericide, bovine mastitis, disinfectant teat dip

INTRODUCTION

Control of mastitis in dairy cows is essential for the production of high quality milk. Because this was recognized very early on, the use of teat dips as a means of disinfection can be traced back to the early 1900's (Moak, 1916).

Iodine has been widely used as the active ingredient in the majority of mastitis control dips, with concentrations of iodine ranging from 0.10% to 1.0 (Boddie et al., 2000; Leslie et al., 2005). Other disinfectants commonly used include bronopol (Boddie and Nickerson, 2002), chlorhexidine (Boddie et al., 1997), sodium chlorite (Boddie et al., 1994; Hillerton et al., 2007; Oura et al., 2002), hypochlorous acid (Boddie et al., 1998), hydrogen peroxide (Leslie et al., 2006), phenol (Oliver et al., 1999), and quaternary amines (Pankey and Watts, 1983; Stewart and Philpot, 1982).

The most common bacteria pathogens leading to the development of mastitis are *Staphylococcus aureus* and *Streptococcus agalactiae*. Milk samples collected from over 100,000 dairy cows in New York and Pennsylvania between 1991 and 1995 showed that the incidence of mastitis was ~49%, with *Staphylococcus* spp. and *Streptococcus* spp. accounting for about 38% of these infections (Wilson et al., 1997). In a similar study in Wisconsin

(Makovec and Ruegg, 2003), seasonal trends in the major mastitis-causing pathogens between 1994 and 2001 were found to exist. Other pathogens, alone or in combination with *Staphylococcus* spp. and *Streptococcus* spp. such as *Escherichia coli* and *Klebsiella* spp. account for a smaller number of mastitis-causing pathogens.

The recommended protocols for evaluating the efficacy of teat dips are published by the National Mastitis Council (2004) and describe methods for evaluating germicides under field conditions. These are expensive, time-consuming, and require a large number of cows. This necessitated the development of other methods so that the efficacy of teat dips could be quickly evaluated. In one of these methods, excised teats are cleaned and stored frozen for future use (Philpot et al., 1978). Attempts to properly prepare these samples for analysis, however, were shown to be ineffective (Hall and Yordy, 1980). A more recent paper describes an efficient excised teat model which was used to evaluate fifteen post-milking teat antiseptics (Watts et al., 1988).

In this paper, we developed and utilized an *in-vitro* method which is simple, yet reliable, for evaluating the efficacy of teat dip formulations. In this manner, we were able to demonstrate efficacy of our teat dip formulations against strains of microbes commonly encountered in bo-

vine mastitis.

MATERIALS AND METHODS

Materials

Nylon mesh filters (20 μm pore size, 25 mm diameter) were purchased from Millipore Corp. (Billerica, MA). Bronopol was purchased from BASF (Florham Park, NJ) and povidone iodine was purchased from either ISP Technologies (Wayne, NJ) or Spectrum (New Brunswick, NJ). LB media and LB agar plates were purchased from Fisher (Boston, MA).

Teat dip preparations

T-HEXX[®] is a registered trademark of Hydromer, Inc. (Branchburg, NJ). T-HEXX ORO dip contains 0.2% bronopol and T-HEXX ONE dip contains 1% iodine. Both are used according to the instructions provided. Hydromer's new experimental teat dip formulations 2298-059 and 2298-182 contain 0.20% bronopol and 0.22% iodine, respectively. Bronopol concentrations were determined by HPLC. Available iodine was confirmed analytically (titrimetric) using the method for povidone-iodine published in U. S. Pharmacopeia National Formulary, 2nd edition (p1119, 1989).

Preparation of bacteria stocks and solutions

Bacterial and fungal cultures used in these studies were obtained from MicroBioLogics, Inc. (St. Cloud, MN) and include *E. coli* (ATCC # 25922), *S. aureus* (ATCC# 29213), *Pseudomonas aeruginosa* (ATCC# 27853), *Streptococcus uberis* (ATCC# 9927), *Enterococcus faecalis* (ATCC# 292121), *S. agalactiae* (ATCC# 27956), *Streptococcus dysgalactiae* (ATCC# 27957) and the fungus *Candida albicans* (ATCC# 14053). Individual bacterial and fungal cultures were grown overnight in Luria-Bertani broth (LB) or Sabouraud Dextrose broth (SD), respectively. The cultures were then diluted to 0.50 McFarland Units ($A_{600\text{ nm}} = \sim 0.132$, $\sim 10^8$ CFU/ml). Just before use, the suspensions are diluted 1:1000 to yield $\sim 1 \times 10^5$ CFU/ml.

Protocol for testing teat dips formulations

Millipore filters were initially sterilized for 15 min using a model GB-518 UV sanitizer (Infra Corp., Waterford, MI) and then submerged into the various teat dip materials until completely saturated. The filters were then hung in a chemical fume hood to air dry. The filters were then carefully placed onto the LB agar surface. Twenty micro-liters of a bacterial suspension ($\sim 1 \times 10^5$ CFU/ml) was pipetted onto the center of the filter. The plates were then typically incubated for 1 - 5 h at 23°C, depending on the designated use of the experimental teat dip. The filters were removed and the plates incubated overnight at 37°C. The absence or presence of growth was examined and assessed visually the next morning. Positive controls consisted of filters which were treated in an identical fashion except that the teat dip formulation did not contain any active antimicrobial agent. Negative controls (LB broth alone) were also always run concurrently.

RESULTS AND DISCUSSION

Determining the efficacy of teat dips by their ability to reduce the incidence of infection in the field is not only ex-

pensive, but requires a great deal of time. An excised teat dip model has been described (Hall and Yordy, 1980) which attempts to overcome these difficulties but requires that the teats, obtained from slaughter houses, be cleaned prior to use because of the extensive contamination present and both physical and chemical means of decontamination have been shown to be ineffective. These treatments can also result in extensive tissue damage and in doing so render the teats unusable in the assay. Therefore, we were motivated to develop an alternate *in-vitro* method which we could use to pre-evaluate the developmental progress of our teat dip formulations in both a rapid and cost-effective manner. In this regard, we now use nylon mesh membranes which we can coat with our formulations and then test for the inhibition of growth of various bacterial and/or fungal strains. The absence of microbial growth below the filter on the LB agar surface would serve as an indication of the antimicrobial efficacy of the teat dips under laboratory conditions. Our initial studies using uncoated filters confirmed that a 20 micron pore size would freely allow the passage of microbes through the filter (data unpublished). In these studies we found that *E. coli* could penetrate the filter to the underlying agar plate within 5 min after application and was nearly maximal after 30 min and maximal at 60 min. These results were typical for all other strains of bacteria that we examined.

In order to validate the method, membrane filters were then coated with T-HEXX ORO and T-HEXX ONE, two products currently used on the market. Following a short drying period, a suspension of *S. aureus* was applied to the center of each filter and incubated for 60 minutes at room temperature, after which time the filters are removed and the plates incubated at 37°C overnight.

Figure 1 shows no bacterial growth associated with filters coated with these formulations and indicated a complete inhibition of *S. aureus* on the underlying agar, compared to a control (base formulation-coated) membrane. These data indicated that the method could be used to evaluate other teat dips currently under preparation.

We then evaluated two new teat dip formulations, designated 2298-182 and 2298-059, against *S. aureus*. In a similar manner, membrane filters coated with these new teat dip formulations were found to be fully effective in preventing any bacterial growth due to *S. aureus* that could have resulted during this 60 minute exposure of the bacteria to the coated membrane surface (Figure 2).

Incubations for as long as 5 h yielded similar results (data not shown). As shown in Table 1, our teat dip formulations were very effective against additional strains of bacteria implicated in bovine mastitis such as *E. coli*, *S. uberis*, *E. faecalis*, *P. aeruginosa*, *S. agalactiae*, and *S. dysgalactiae*, as well as the fungal strain *C. albicans*. These data also indicated that bronopol at the concentration used was not sufficient to completely eliminate fungal growth

Table 1. Efficacies of hydromer's teat dip formations on other microbial species.

	Uncoated Filter	T-HEXX ORO	T-HEXX ONE	2298-182	2298-059
<i>E. coli</i>	Growth	NG	NG	NG	NG
<i>P. aeruginosa</i>	Growth	NG	NG	SG	NG
<i>S. uberis</i>	Growth	NG	NG	NG	NG
<i>E. faecalis</i>	Growth	NG	NG	NG	NG
<i>S. agalactiae</i>	Growth	NG	NG	NG	NG
<i>S. dysgalactiae</i>	Growth	ND	ND	NG	NG
<i>C. albicans</i>	Growth	SG	NG	NG	SG

Efficacy was determined as described under "Material and Methods" and in the legends to Figures 1 and 2. "No Growth (NG)" indicates 100% inhibition of growth of the microbial species indicated. Partial inhibition is indicated by "some growth (SG)". ND refers to not done. These data are derived from duplicate experiments, each done with duplicate filters.

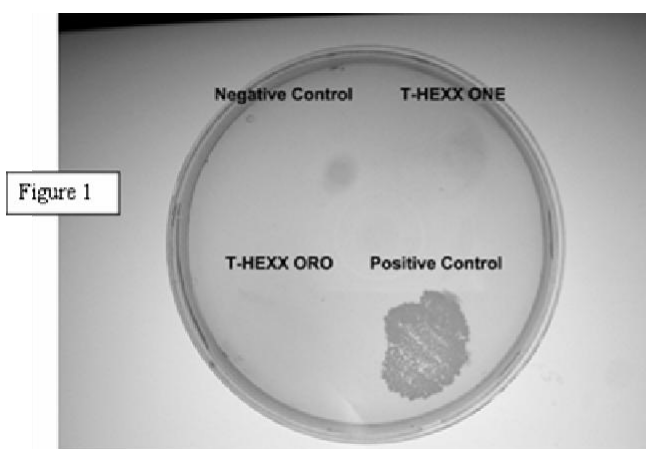


Figure 1. Efficacy of T-HEXX ORO and T-HEXX ONE against *Staphylococcus aureus* after application onto nylon membranes. Nylon membranes were dip-coated with T-HEXX ORO or T-HEXX ONE, air dried for 1 h, and placed onto the surface of an LB agar plate. The analysis of bacterial growth was performed as described under "Materials and Methods". These data are representative of at least two separate experiments.

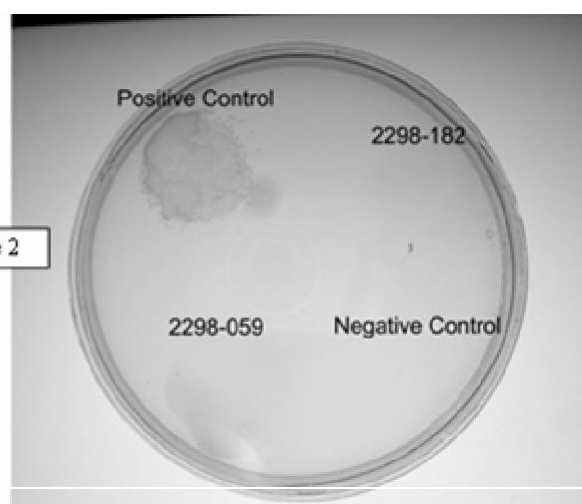


Figure 2. Efficacy of 2298-059 and 2298-182 against *Staphylococcus aureus* after application onto nylon membranes. Nylon membranes were dip-coated with 2298-059 or 2298-182, air dried for 1 h, and placed onto the surface of an LB agar plate. The analysis of bacterial growth was performed as described under "Materials and Methods". These data are representative of at least two separate experiments.

It is also likely that the physical characteristics of the dip play a significant role in the efficacy of the teat dip after its application to the nylon membrane. For example, the viscosity and/or adherent properties of the formulation likely have an influence on the efficacy of these products. Indeed, during formulation development, we observed varying degrees of coating quality after application onto the nylon membranes and during their subsequent drying. In summary, a simple, yet effective, technique has been designed and used in our laboratory to determine the relative disinfectant efficacy of teat dips. Despite differences in the physical characteristics between bovine teats and nylon membranes, we believe that this method is both a reliable and rapid laboratory method for the preliminary evaluation of the bactericidal efficacy of teat dip formulations prior to field testing.

The authors do not in any way purport to underestimate

the value of efficacy testing on excised teats and only suggest that this method is a rapid and valuable adjunct to existing testing protocols.

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