Short communication: Barrier characteristics of 3 external teat sealants to prevent bacterial penetration under in vitro conditions using rubber calf-feeding nipples

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ABSTRACT

The main objective of this study was to evaluate the barrier characteristics of 3 external teat sealants for dry cows in preventing bacterial penetration by 3 common major mastitis pathogens (*Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus uberis*) via a novel in vitro simulation model using rubber calf-feeding nipples. All feeding nipples were filled with a sterile cotton plug soaked in sterile broth heart infusion medium and were treated as follows: rubber teats 1 and 5 were sealed with Ubera Dry (Inovet, Arendonk, Belgium); rubber teats 2 and 6 were sealed with T-Hexx Dry (Huvepharma Livestock, St. Louis, MO); rubber teats 3 and 7 were sealed with Uddergold Dry (Ecolab Food and Beverage Division, St. Paul, MN); and rubber teats 4 and 8 remained unsealed and served as positive and negative controls, respectively. After the dips had dried, rubber teats 1 to 4 were immersed in a suspension of *E. coli* (experiment 1), *Staph. aureus* (experiment 2), or *Strep. uberis* (experiment 3; $\geq 1.5 \times 10^8$ cfu/mL) for 24 h, whereas rubber teats 5 to 8 were not exposed to the bacterial suspensions. All external teat sealants adhered well to the rubber teats. All cotton plugs collected from the teats that were not exposed to *E. coli*, *Staph. aureus*, or *Strep. uberis* (rubber teats 5 to 8) remained culture-negative, except for 1 (due to contamination). Of the rubber teats that were exposed to the major mastitis pathogens, all cotton plugs collected from the teats dipped with Ubera Dry and T-Hexx Dry remained culture-negative for the mastitis pathogen they were exposed to. The cotton plugs of the rubber teats that were sealed with Uddergold Dry and exposed to *E. coli* and *Strep. uberis* showed positive cultures for the respective bacteria, as demonstrated using strain-typing. The cotton plugs collected from the rubber

teats that were not sealed with an external teat sealant and that served as positive controls became culture-positive for the mastitis pathogens they were exposed to. We conclude that Ubera Dry showed comparable and superior barrier performance against penetration of *E. coli*, *Staph. aureus*, and *Strep. uberis* compared with T-Hexx Dry and Uddergold Dry, respectively, under in vitro conditions using a novel in vitro simulation model. Although one should be aware that the method has not yet been validated to predict risk of intramammary infections, the proposed technique can be a meaningful starting point to evaluate and compare the barrier characteristics of external teat sealants in preventing bacterial penetration. A large-scale clinical trial is needed before any definite conclusions can be drawn as to the adherence, duration of adherence, barrier performance, and efficacy in protection against intramammary infections of the 3 external teat sealants under field conditions.

Key words: external teat sealant, in vitro, dry period

Short Communication

The dry period is known as a most crucial yet ambivalent period for udder health in a cow's lactation cycle. The steady-state phase offers an excellent opportunity for curing existing IMI, whereas the involution and colostrogenesis phases are, conversely, periods of high risk for contracting new IMI (Bradley and Green, 2010). The risk of new IMI during the dry period is up to 10 times higher than the rate of new IMI during lactation, with a peak around involution of the mammary gland just after drying off and during the transition from pregnancy to lactation (Smith et al., 1985; Bradley et al., 2015). Internal teat sealants are well introduced on modern dairy farms. They are used as standalone products in noninfected cows or quarters in cases of selective dry-cow therapy or in combination with long-acting antibiotics to provide protection beyond that achieved through antibiotics alone (Halasa et al., 2009). Internal teat sealants function as inert

Received September 11, 2019.

Accepted February 10, 2020.

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physical barriers in the teat cistern, with the goal of preventing penetration of bacteria from the environment into the udder during the entire dry period. Cows can also be protected against new IMI during the dry period through use of external teat sealants (Lim et al., 2007a). When an external teat sealant is used, an artificial polymer or latex skin is put on the teat, usually via teat-dipping. In contrast to internal teat sealants, external teat sealants tend not to persist on the teat for more than a few days or weeks. Still, this might be enough to protect the mammary gland in the first days after dry-off, a high-risk period for new IMI (Lim et al., 2007a), but not later. A major advantage of external versus internal teat sealants is that use of an external teat sealant will reduce the risk of damage to the teat canal or accidental introduction of pathogens (Godden et al., 2003). Remarkably, except for the study of Lim et al. (2007a), no reports on the use and effects of external teat sealants during the dry period are available in peer-reviewed literature to our knowledge.

The main objective of this study was to evaluate the barrier characteristics of 3 external teat sealants for dry cows in preventing bacterial penetration by 3 common major mastitis pathogens (*Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus uberis*) using a novel in vitro model with rubber calf-feeding nipples.

Three separate in vitro experiments were conducted in the laboratory of the Mastitis and Milk Quality Research Unit of Ghent University (Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Merelbeke, Belgium). The barrier characteristics of a newly developed and available external teat sealant, Ubera Dry (Inovet, Arendonk, Belgium) for preventing bacterial penetration by the major mastitis pathogens *Staph. aureus*, *Strep. uberis*, and *E. coli* were evaluated under in vitro conditions by use of rubber calf-feeding nipples (Yuancheng, YC-P97, Shandong, China; hereafter referred to as “rubber teats”) and compared with 2 other commercially available barrier-type teat dips for dry cows: T-Hexx Dry Teat Sealant (Huvepharma Livestock, St. Louis, MO) and Uddergold Dry (Ecolab Food and Beverage Division, St. Paul, MN). Before the experiments, a hole was drilled in each rubber teat with a diameter of 1.5 mm, to mimic the teat orifice and teat canal, and the 24 rubber teats were subjected to plasma sterilization at 35°C for 52 min.

Separate experiments were conducted for each of the 3 major mastitis pathogens, using 8 rubber teats per mastitis pathogen. In each experiment, all rubber teats were first filled with 1 g of sterile cotton plug soaked in 19 g of sterile broth heart infusion (BHI) medium (Oxoid, Basingstoke, UK). Before use, the cotton plugs

were autoclaved for 4 h at 135°C. The cotton plug soaked in sterile BHI (total weight 20 g) was gently pushed to the bottom of the rubber calf-feeding nipples. Subsequently, another 2 mL of BHI medium was drenched on the cotton plug. This step was repeated 2 more times. Finally, another small (0.25 g) sterile cotton plug was added on top of the first cotton plug, and 1 mL of BHI medium was drenched on top of the second cotton plug 2 times.

The rubber teats were treated as follows: rubber teats 1 and 5 were sealed with Ubera Dry; rubber teats 2 and 6 were sealed with T-Hexx Dry Teat Sealant; rubber teats 3 and 7 were sealed with Uddergold Dry; and rubber teats 4 and 8 remained unsealed, serving as positive and negative controls, respectively (Table 1). When retracting the rubber teats from the teat sealant, each teat was rotated to make sure the orifice was properly sealed, at least visually. In all cases, the teat sealant was air-dried in a laminar flow for 10 min. After the dips had dried, the first 2 cm of rubber teats 1 to 4 were immersed in a bacterial suspension of $\geq 1.5 \times 10^8$ cfu/mL each of *E. coli* (experiment 1), *Staph. aureus* (experiment 2), or *Strep. uberis* (experiment 3; Table 1) for 24 h at 37°C (4 separate plastic jars with 30 mL each of the same bacterial suspension; see further). Rubber teats 5 to 8 were not immersed in bacterial suspensions and served as negative controls.

To verify the concentrations of the bacterial suspensions, 20 μ L of each suspension was used to produce a 10-fold dilution in a 96-well plate (Sigma-Aldrich, Overijse, Belgium). The dilutions were plated on tryptic soy blood agar (Thermo Scientific, Oxoid, Merelbeke, Belgium) and counted after 24 h of incubation at 37°C.

After 24-h immersion of the rubber teats, the cotton plugs were removed with sterile tweezers and placed into 50-mL Falcon tubes (BD Bioscience, Erembodegem, Belgium) filled with 5 mL each of BHI medium, after which each tube was filled with another 25 mL of BHI medium and gently inverted for 1 min. The BHI medium was then plated on tryptic soy blood agar and incubated for 24 h via a 10-fold dilution to detect bacterial growth. Additionally, after the cotton plug was removed, the exterior and interior of each rubber teat was swabbed with sterile cotton swabs to check for contamination with bacteria other than the reference isolates of *E. coli*, *Staph. aureus*, and *Strep. uberis*. These cotton swabs were plated on tryptic soy blood agar plates and incubated for 24 h at 37°C.

Three different major mastitis pathogens were selected from the reference strain collection of the Mastitis and Milk Quality Research Unit, Ghent University, Belgium: *E. coli* ATCC 25922, *Staph. aureus* ATCC 25923, and *Strep. uberis* BAA-854. The *Strep. uberis*

Table 1. Summary of bacteriological culture results from the cotton swabs used to swab the interiors and the exteriors of the rubber teats and the cotton plugs collected from the rubber teats after 24 h of incubation at 37°C for 3 experiments

Experiment	Bacterial suspension	Immersed in bacterial suspension	Teat dip ¹	Teat number per experiment	Sample type		
					Cotton swabs		Cotton plug (cfu/mL)
					Teat interior (cfu)	Teat exterior (cfu)	
Experiment 1	<i>Escherichia coli</i> (ATCC 25922)	Yes	Ubers Dry	1	Negative	>150 ²	Negative
		Yes	T-Hexx	2	Negative	>150 ²	Negative
		Yes	Uddergold Dry	3	>150 ²	>150 ²	8.0 × 10 ^{8.2}
		Yes	Control	4	>150 ²	>150 ²	1.0 × 10 ^{9.2}
	—	No	Ubers Dry	5	Negative	Negative	Negative
		No	T-Hexx	6	>150 ³	Negative	1.5 × 10 ^{6.4}
		No	Uddergold Dry	7	Negative	Negative	Negative
		No	Control	8	Negative	Negative	Negative
Experiment 2	<i>Staphylococcus aureus</i> (ATCC 25923)	Yes	Ubers Dry	1	Negative	>1505	Negative
		Yes	T-Hexx	2	105 ⁵	>1505	Negative
		Yes	Uddergold Dry	3	>150 ⁵	>150 ⁵	7.50 × 10 ^{4.5}
		Yes	Control	4	>150 ⁵	>150 ⁵	Negative
	—	No	Ubers Dry	5	Negative	Negative	Negative
		No	T-Hexx	6	Negative	Negative	Negative
		No	Uddergold Dry	7	Negative	Negative	Negative
		No	Control	8	Negative	Negative	Negative
Experiment 3	<i>Streptococcus uberis</i> (BAA-854)	Yes	Ubers Dry	1	Negative	>150 ⁶	2.4 × 10 ^{5.7}
		Yes	T-Hexx	2	Negative	>150 ⁶	Negative
		Yes	Uddergold Dry	3	>150 ⁶	>150 ⁶	1.6 × 10 ^{6.6}
		Yes	Control	4	>150 ⁶	>150 ⁶	2.0 × 10 ^{5.8}
	—	No	Ubers Dry	5	Negative	Negative	Negative
		No	T-Hexx	6	Negative	Negative	Negative
		No	Uddergold Dry	7	Negative	Negative	Negative
		No	Control	8	Negative	Negative	Negative

¹Ubers Dry, Inovet, Arendonk, Belgium; T-Hexx Dry Teat Sealant, Huvepharma Livestock, St. Louis, MO; Uddergold Dry, Ecolab Food and Beverage Division, St. Paul, MN.²Pure culture of the original *E. coli* reference strain, as confirmed by random amplification of polymorphic DNA (RAPD) strain typing (see Figure 1A).³Pure culture of *Staphylococcus hominis*, as confirmed by MALDI-TOF MS.⁴Mixed culture of *Staph. hominis*, *Staphylococcus warneri*, and *Micrococcus luteus*, as confirmed by MALDI-TOF MS.⁵Pure culture of the original *Staph. aureus* reference strain, as confirmed by RAPD strain typing (see Figure 1B).⁶Pure culture of the original *Strep. uberis* reference strain, as confirmed by testing via bile esculin agar and antimicrobial sensitivity pattern.⁷Mixed culture of *Staph. hominis* and *M. luteus*, as confirmed by MALDI-TOF MS.⁸Mixed culture of the original *Strep. uberis* reference strain with *Staph. hominis*, *Staph. warneri*, and *M. luteus*, as confirmed by MALDI-TOF MS.

reference strain atypically hydrolyses esculin and forms thereby a dark brown or black complex on bile esculin agar (Thermo Scientific).

The mastitis isolates were initially stored at -80°C (Microbank, Pro-Lab Diagnostics, Toronto, Canada). A growth curve was set up for each isolate by culturing the selected isolates on Columbia blood agar plates (Thermo Scientific), which were incubated for 24 h at 37°C . A few colonies of the selected isolates were transferred with sterile cotton swabs into 2 mL of 0.85% NaCl tubes (bioMérieux, Brussels, Belgium) up to a concentration of 0.5 McFarland. Then, 10 μL of the 0.5-McFarland solution was transferred to 5 mL of BHI medium in 10-mL Falcon tubes with screw caps. In total, for each isolate, 24 Falcon tubes were prepared. The Falcon tubes were incubated at 37°C for 6 h. An additional Falcon tube with 5 mL of sterile BHI medium was incubated and served as negative control. Based on the growth curve, 6 h of incubation at 37°C allowed a bacterial concentration of $>1.5 \times 10^8$ cfu/mL to be reached for each isolate while isolates were still in their late log phase. After incubation, all 24 Falcon tubes of each isolate were mixed into 1 sterile jar (total volume: 120 mL) and then again divided into 4 small plastic jars, each containing 30 mL of bacterial suspension, as mentioned before. The final concentrations of the bacterial suspensions after 6 h of incubation at 37°C were 1.7×10^8 cfu/mL for *E. coli*, 1.4×10^9 cfu/mL for *Staph. aureus*, and 9×10^9 cfu/mL for *Strep. uberis*.

All bacterial isolates that were cultured during the experiments were stored in the freezer at -80°C (Microbank, Pro-Lab Diagnostics) and afterward identified at the species level via MALDI-TOF MS, using the commercially available Bruker MALDI Biotyper system (Biotyper reference database version 4.0.0.1. with 5,627 entries; Bruker Daltonics Inc., Billerica, MA), expanded with a custom reference spectra database for the identification of bovine-associated non-*aureus* staphylococci (Cameron et al., 2018).

Random amplification of polymorphic DNA (RAPD-)PCR was used for strain-typing of *E. coli* ATCC 25922, *Staph. aureus* ATCC 25923, and the isolates collected from the cotton plugs or from the cotton swabs that were used to swab the interiors and exteriors of the rubber teats during the experiments. The RAPD-PCR was performed as described by Gurjar et al. (2012) with some modifications. Isolates of the same experiment were analyzed in the same PCR. For *Strep. uberis* BAA-854 and the *Strep. uberis* isolates recovered during the experiments, no RAPD was performed, in the absence of a functional standard operating procedure. Nonetheless, the isolates were checked for positive results of a bile esculin agar test, a typical trait

of *Strep. uberis* BAA-854. The susceptibility to several antimicrobial compounds was tested using the Kirby-Bauer disk diffusion method (Supré et al., 2014): oxacillin (1 μg), sulfonamide and trimethoprim combination (3.75 and 1.25 μg), cefazolin (30 μg), cefoxitin (30 μg), gentamycin (250 μg), tetracycline (30 μg), tylosin (150 μg), and penicillin (1 IU). After 24 h of incubation at 37°C , the inhibition zone diameters were evaluated according to clinical breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI) to identify antimicrobial resistance, as described by Supré et al. (2014). As a result, the antimicrobial sensitivity patterns of the different *Streptococcus uberis* isolates could be compared.

All 3 external teat sealants adhered well to the rubber teats. However, adherence capacity under in vitro conditions in the current experiments does not necessarily equal adherence capacity in vivo—all the more so because adherence capacity and duration of adherence appear to depend on herd- and cow-management factors such as changing the ration to reduce milk production before drying off, level of milk yield before dry-off, and housing type (Lim et al., 2007b). A field study is needed to evaluate adherence capacity and duration of adherence in vivo. In fact, the only similarity between the rubber calf-feeding nipples used in this study and bovine teats is their conical shape and the constructed opening. Still, the shape of the physical structure is only 1 of many factors—including dermal moisture, pH, and temperature—potentially determining the adherence and persistence capacity of an external teat sealant. Investigation of the adherence and duration of the 3 external teat sealants, and the extent to which the rubber calf-feeding nipples compare with bovine teats for relevant physical traits, fell beyond the scope of this study. The main advantage of the proposed method is that the use of live animals can be avoided while a live bacterial challenge is conducted, representing a meaningful starting point for experimental model research in this area. Still, one should be aware that the proposed method has not yet been validated to predict the risk of new IMI under field conditions.

With 1 exception, all cotton plugs collected from the rubber teats that were not exposed to *E. coli*, *Staph. aureus*, or *Strep. uberis* (rubber teats 5 to 8) remained culture-negative. From the cotton plug collected from rubber teat 6 during the *E. coli* experiment, which was not dipped nor exposed to a bacterial suspension, a mixed culture of *Staphylococcus hominis*, *Staphylococcus warneri*, and *Micrococcus luteus* was isolated (Table 1). Both *Staph. hominis* and *Staph. warneri* are common commensal organisms found among the skin flora of humans and animals (Kloos and Musselwhite, 1975).

Figure 1. (A) Random amplification of polymorphic DNA (RAPD)-PCR strain typing of *Escherichia coli* isolates. R = original *E. coli* reference strain recovered from the Microbank vial (Pro-Lab Diagnostics, Toronto, Canada); A = original *E. coli* reference strain from the bacterial suspension in which the teats were immersed; B through E = *E. coli* isolates from the exteriors of rubber teats 1 through 4; F and G = *E. coli* isolates from interiors of rubber teats 3 and 4; I and J = *E. coli* isolates from the cotton plugs collected from teats 3 and 4; Cp = 2 positive control strains of *E. coli*, as confirmed via MALDI-TOF MS; and Cn = negative control. (B) RAPD strain-typing of *Staphylococcus aureus* isolates. A = original *Staph. aureus* reference strain from the bacterial suspension in which the teats were immersed; B through E = *Staph. aureus* isolates from the exteriors of rubber teats 1 through 4, respectively, after swabbing; F and G = *Staph. aureus* isolates from the interiors of rubber teats 3 and 4 after swabbing; H = *Staph. aureus* isolates from the cotton plug collected from teat 4; Cp = 2 positive control strains of *Staph. aureus*, as confirmed via MALDI-TOF MS; and Cn = negative control.

In some studies, these bacteria have been isolated from bovine milk (Vanderhaeghen et al., 2015). The isolation of the mixed culture of *Staph. hominis*, *Staph. warneri*, and *M. luteus* is most probably due to contamination from the hands, despite gloves being worn and disinfected throughout the experiments.

Of the rubber teats that were exposed to the major mastitis pathogens, all cotton plugs collected from those dipped with Ubera Dry and T-Hexx Dry remained culture-negative for the mastitis pathogen they were exposed to (Table 1). The cotton plugs collected from the rubber teats that were not sealed with an external teat sealant, which served as positive controls, became culture-positive for the same strain of the mastitis pathogens they were exposed to, as confirmed by strain typing (Figure 1). The culture of the cotton plug collected from rubber teat 4 (positive control) in experiment 3 consisted of a mixed culture of the *Strep. uberis* reference strain (as confirmed by the same antimicrobial resistance patterns) with *Staph. hominis*, *Staph. warneri*, and *M. luteus*, as was the case for rubber teat 6 in experiment 1 (Table 1). Despite the fact that sterile gloves were worn and regularly disinfected during the experiment, contamination cannot be excluded. The cotton plugs of the rubber teats that were sealed with Uddergold Dry and exposed to *E. coli* and *Strep. uberis* became culture-positive for the same *E. coli* and *Strep. uberis* strains, respectively, whereas the cotton plug of the rubber teat that was sealed with Uddergold Dry and exposed to *Staph. aureus* (rubber teat 3) in experiment 2 remained culture-negative.

Similar results to those derived from the cotton plugs were obtained from the cotton swabs used for swabbing the interiors and exteriors of the rubber teats (Table 1). Remarkably, although the cotton plug collected from rubber teat 3 (treated with Uddergold Dry) in experiment 2 remained culture-negative for *Staph. aureus*, the cotton swab used to swab the interior of the rubber teat was culture-positive for the same strain of *Staph. aureus* (Figure 1B).

As mentioned, strain typing confirmed that all recovered *E. coli* isolates belonged to the *E. coli* reference strain (Figure 1A). The same was true for the *Staph. aureus* isolates (Figure 1B). For *Strep. uberis*, all isolates were positive on the bile esculin agar, as was the case for the reference *Strep. uberis* strain. All isolates showed the same antimicrobial sensitivity pattern as that of the reference *Strep. uberis* strain. All isolated *Strep. uberis* strains showed resistance against gentamycin and were intermediately resistant to the combination of trimethoprim and sulfonamide. These results strongly indicate that the isolates collected from

the cotton plugs and those from the cotton swabs were identical to the *Strep. uberis* reference strain.

It is important to note that the proposed method has not yet been validated as a method to predict either the adherence and duration of the external teat sealants or the risk of IMI when these sealants are used under in vivo conditions. However, given the overnight incubation at 37°C in rich BHI medium, the high dose of bacteria the rubber teats were exposed to, the open non-contractile teat end, and the high bacterial growth on the cotton plugs collected from the positive controls, it can be anticipated that the barrier characteristics of Ubera Dry and T-Hexx are stronger in preventing bacterial penetration of *Escherichia coli*, *Staph. aureus*, and *Strep. uberis* than the ones of Uddergold Dry.

In conclusion, Ubera Dry showed comparable and superior barrier performance against the penetration of *E. coli*, *Staph. aureus*, and *Strep. uberis* compared with T-Hexx Dry and Uddergold Dry, respectively, under in vitro conditions using rubber calf-feeding nipples. A large-scale longitudinal clinical trial is needed before any definite conclusions can be drawn as to the adherence, duration of adherence, barrier performance, and efficacy in protection against IMI of the 3 external teat sealants under field conditions.

ACKNOWLEDGMENTS

The research in this study was financially supported by Inovet. The authors have not stated any conflicts of interest.

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