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Short communication: Minimum bactericidal concentration of disinfectants evaluated for bovine digital dermatitis-associated *Treponema phagedenis*-like spirochetes

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ABSTRACT

The bacterial spirochetes, *Treponema* spp., are thought to be a major contributor to the etiology of bovine digital dermatitis (DD), a skin disease with worldwide economic impact. Hoofbath strategies are commonly used in an attempt to control and prevent the development of DD and continuing research has been done to develop an optimal hoofbath strategy for this purpose. The aim of this study was to develop a protocol that can be used as part of the screening process for candidate hoofbath disinfectants. This protocol allows an accurate determination of the in vitro minimum inhibitory concentration and minimum bactericidal concentration of a series of disinfectants for *Treponema* microorganisms. Assays were performed in triplicate for each of the disinfectants at 30-s and 10-min exposure times and exposed to 10 and 20% manure (vol/vol). The results of this study can be used to categorize disinfectants based on the effect of exposure and manure concentration regarding their ability to inhibit *Treponema* growth. This information can then aid in optimizing strategies for hoofbath-based control of DD development and spread.
Key words: digital dermatitis, *Treponema*, hoofbath, disinfectant

Short Communication

Bovine digital dermatitis (**DD**) is an infectious skin disease that affects cattle worldwide (Walker et al., 1997; Read and Walker, 1998; Rodriguez-Lainz et al., 1999; Barker et al., 2009; Yano et al., 2010a). The painful skin lesions associated with DD lead to lameness, declining body condition, decreased reproductive performance, and decreased milk production, resulting in a significant economic loss each year for the dairy industry (Argáez-Rodríguez et al., 1997; Read and Walker, 1998; Hernandez et al., 2001; Losinger, 2006; Ettema et al., 2010). Although a specific causative agent has not yet been identified, *Treponema* bacteria are consistently found to be associated with the lesions and are thought to be major contributors to the disease (Evans et al., 2009; Yano et al., 2010b; Gomez et al., 2012).

Hoofbathing is a commonly used strategy to prevent the development and spread of DD (Laven and Logue, 2006; Teixeira et al., 2010). Such a strategy is convenient because a disinfectant can be applied to a large number of cows with minimal time and labor. To identify chemicals that are effective hoofbath agents, it is necessary to address the efficacy of chemical compounds at inhibiting or killing treponemes in vitro before testing the chemicals in the field.

The aim of this study was to develop a protocol that can be used as a screening test for potential hoofbath disinfectants and to use this protocol to establish the in vitro minimum bactericidal concentration (**MBC**) and MIC of various disinfectants for a *Treponema phagedenis*-like microorganism (**MO**).

A *Treponema phagedenis*-like isolate obtained from a biopsy of an active DD lesion (red ulcer of more than 2 cm diameter) from a dairy cow in southern Wisconsin was stored at −80°C in a solution of 10% (vol/vol) glycerol, 9% (vol/vol) fetal bovine serum (**FBS**; Lonza, Walkersville, MD) and 81% (vol/vol) oral treponeme enrichment broth (**OTEB**; Anaerobe Systems, Morgan Hill, CA). One week before each assay, a stock culture was prepared by inoculating 2 mL of OTEB and 10% (vol/vol) FBS by volume with the *Treponema* culture and incubated at 37°C in an anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR) in a 70% N_2:25% CO_2:5% H_2 atmosphere (vol/vol/vol). Three days before each assay, 15 mL of OTEB and 10% (vol/vol) FBS were added to the culture, allowing it to be in the log phase of growth for the assays (1.2×10^4 to 1.6×10^7 cfu).

Manure, collected immediately after defecation from piles deposited on the ground at a dairy farm, was au-

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toclaved at 134°C and 232.42 Pa for 45 min, then frozen at −20°C until needed. To prepare for the assays, the manure was thawed to room temperature, homogenized with an overhead mixer, divided into 5-g aliquots, and then autoclaved and frozen as before. Individual aliquots were thawed to room temperature and combined with equal parts of sterilized PBS before use.

Minimum inhibitory concentration and MBC assays were performed in triplicate for copper sulfate, zinc sulfate, glutaraldehyde, formaldehyde, peroxyacetic acid, sodium omadine, Double-Action, 4Hooves, salicylic acid, a phenolic compound, and a series of test disinfectants referred to as A through E (all from DeLaval, Kansas City, MO) at 2 exposure times [**ET**; 30 s (the shortest reasonable ET) and 10 min] and at 2 manure concentrations [10 and 20% (vol/vol)].

In an anaerobic chamber, a colony-forming unit plate count was performed for each *Treponema* culture at the start of the assays by serial dilution in 180 μL of OTEB, plated onto Fastidious Anaerobe Agar (**FAA**) plates (Lab M Ltd., Bury, Lancashire, UK) and incubated at 37°C for 7 d.

For each 96-well treatment plate, dilution assays were run in triplicate for 2 disinfectants that included a negative control column (without *Treponema* culture) and a positive control column (with *Treponema* but without disinfectant). For the manure treatments, a 10 or 20% manure *Treponema* culture was prepared by adding 1 mL of sterilized PBS/1 g of sterilized manure slurry to the culture. One hundred-microliter *Treponema* cultures (with or without manure) were added to all wells except for the negative controls, to which 50 μL of OTEB was added instead.

At this stage, the plates were removed from the anaerobic chamber and transferred to a fume hood. To complete the negative control preparation, 50 μL of each disinfectant was added to the negative control column. For the treatments, dilution series were performed by adding 100 μL of each disinfectant at working concentration [5% copper sulfate, 3% formaldehyde, 5% zinc sulfate, 3% glutaraldehyde, 0.4% A (proprietary), 0.27% B (proprietary), 0.5% C (organic acid blend), 0.1% peroxyacetic acid, 0.2% phenolic compound, 0.12% sodium omadine, 1% Double-Action, 0.5% 4Hooves, 0.25% D (proprietary), 1% E (organic acid blend), and 0.1% salicylic acid (all percentages are vol/vol)] to one column and performing a serial 2-fold dilution. The 10-min ET plates were allowed to sit for 10 min after the dilution series had been performed.

The microplates were covered with Parafilm and centrifuged (Eppendorf tabletop centrifuge; Eppendorf AG, Hamburg, Germany) at 1,811 × *g* for 5 min at room temperature; then, a sterile aspirator was used to evacuate the supernatant from each well. The contents of each well were resuspended with OTEB and vortexed (Thermal Fisher Scientific, Madison, WI) until all material was in suspension (maximum of 5 min) and transferred back to the anaerobic chamber.

Approximately 3 μL from each well was spot plated onto FAA plates (36 wells/plate in a 6 × 6 grid) and incubated in an anaerobic chamber at 37°C for 10 to 21 d. Spirochete growth was confirmed with dark-field microscopy (Nikon 80i; Nikon, Chicago, IL).

The MIC and MBC for each disinfectant were determined as the disinfectant dilution at which decreased or no bacterial growth was noted on the FAA plates compared with the controls, respectively. An average of the 3 replicates was used to calculate the MIC for each disinfectant.

The in vitro MIC and MBC for each disinfectant are shown in Figures 1 and 2. The MIC and MBC for zinc sulfate were much higher than most of the other results and were not included in the figures [MIC/MBC at 30 s = 0.6250%/1.2500%; MIC/MBC at 10 min = 0.1563%/0.3125%; MIC/MBC in 10% manure = 1.25%/2.5%; MIC/MBC in 20% manure = 2.5%/2.500% (all percentages are vol/vol)]. Many of the disinfectants were equally as effective for killing bacteria (MBC) at the 30-s and 10-min ET (glutaraldehyde, A, B, C, the phenolic compound, Double-Action, 4Hooves, D, and E). Other disinfectants were more effective at killing bacteria over a longer period of time (at the 10-min ET; copper sulfate, formaldehyde, zinc sulfate, and sodium omadine). In the case of peroxyacetic acid, the calculated MBC at 30 s (0.025%) was less than the MBC at 10 min (0.050%; vol/vol).

When manure was not present in the assay, copper sulfate was the most effective against *Treponema* MO in vitro (lowest MIC and MBC; Figure 1). However, the results of this study also indicate that the effectiveness of copper sulfate may be severely hampered when manure is present in the solution (Figure 2). It is possible that manure and OM bind to the Cu^{2+} ions, preventing their disinfecting action or they may inhibit the ability of the copper sulfate salts to dissociate (Ippolito and Barbarick, 2008). Several disinfectants (A, B, and C) performed better than copper sulfate in 10 and 20% manure. In 20% manure, the effectiveness of copper sulfate was greatly diminished and only (zinc sulfate) had a higher MBC, whereas formaldehyde was as effective as copper sulfate in 20% manure (Figure 2).

The MBC identified for copper sulfate were all well below the commonly used field concentrations of copper sulfate, which range from 2 to 10% (Laven and Hunt, 2002; Jorritsma et al., 2007; Teixeira et al., 2010), indicating that concentrations of less than 2% copper sulfate may still be effective at harming and killing *Treponema* MO (Gomez et al., 2012; Santos et al.,

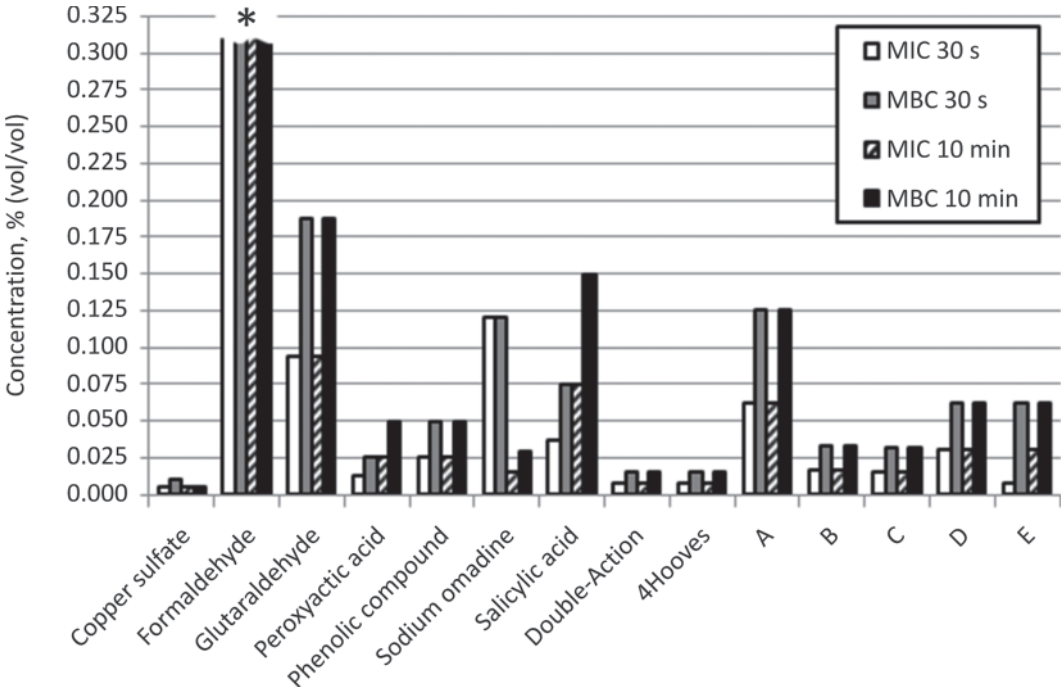

Figure 1. Minimum inhibitory concentrations and minimum bactericidal concentrations (MBC) for various disinfectants (all from DeLaval, Kansas City, MO) at 30-s and 10-min exposure times. *The MIC and MBC for formaldehyde were larger than the readable scale of this figure and the values are as follows: MIC/MBC at 30 s = 0.75%/1.5% (vol/vol) and MIC/MBC at 10 min = 0.375%/1.5% (vol/vol).

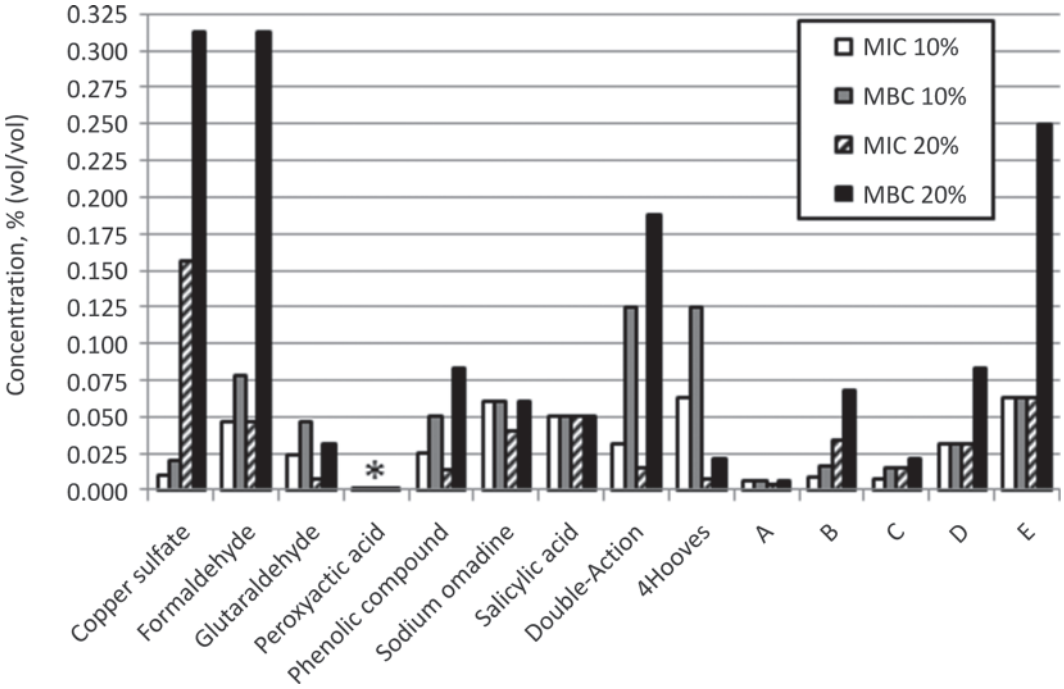

Figure 2. Minimum inhibitory concentration and minimum bactericidal concentration (MBC) of various disinfectants (all from DeLaval, Kansas City, MO) in 10 and 20% manure at a 30-s exposure time. *The MIC and MBC for peroxyacetic acid were smaller than the readable scale of this figure and the values are as follows: MIC/MBC in 10% manure = 0.00020%/0.00039% (vol/vol) and MIC/MBC in 20% manure = 0.00026%/0.00078% (vol/vol).

2012). These results are consistent with other studies that have demonstrated the effectiveness of copper sulfate for preventing DD (Speijers et al., 2010; Teixeira et al., 2010).

Copper sulfate is one of the most commonly used hoofbath agents for the control and prevention of DD but, due to the safety issues and negative environmental impact associated with it, alternatives to this disinfectant as a hoofbath agent are being sought (Laven and Logue, 2006; Teixeira et al., 2010). Chemicals that are less affected by the presence of manure may represent plausible alternatives to copper sulfate. Several of the test disinfectants fit these criteria (sodium omadine, salicylic acid, A, and C; Figure 2).

When challenged with manure, many of the disinfectants required a higher concentration to inhibit or kill the *Treponema* MO (Figure 1). This may be due to interference of the manure with the action of the disinfectants, a protective effect of the disinfectants increasing the adhesion of *Treponema* MO to OM, the formation of a protective barrier of OM around the bacteria, or the triggering of the encystation of *Treponema* with consequent changes in susceptibility to the chemicals under study. It has been shown that *Treponema* can form a cyst (ball up and form a protective mucoid covering) in stressful conditions, such as exposure to drugs or antibodies (Ovčinnikov and Delektorskij, 1971). However, the addition of manure did not increase the MBC for all of the disinfectants as might be expected (Figure 2). For these disinfectants, it can be speculated that the presence of manure may somehow enhance the action of these chemicals on *Treponema* MO.

The organic chemicals included in this study (the organic acids, glutaraldehyde, and formaldehyde) are degraded readily in manure and represent meaningful candidates for new composite hoofbath products that could act as substitutes for environmentally questionable copper sulfate. Other possible alternatives to copper sulfate that should be considered include sodium omadine and phenolic compounds, which are used in hospitals and on farms for other disinfecting purposes.

In this study, an increased ET had little effect on the effectiveness of many of the disinfectants, whereas other disinfectants were more effective at a greater ET (Figure 1). Although controlled in vitro studies are informative to analyze specific aspects of the hoofbath process, one of the limitations of this type of assay is that many other risk factors exist in the field acting upon the cows in addition to the hoofbath agents and these factors are difficult to reproduce in the laboratory. Further research should be done to determine how the presence of manure and other factors associated with hoofbaths affect the effect of ET on the killing and inhibition of *Treponema* MO in vitro.

The in vitro MIC and MBC for these disinfectants provide a stepping stone to the development of an optimal hoofbath strategy for the control and prevention of DD. By comparing the effects of ET and manure concentration on the efficacy of these disinfectants, decisions can be made about the potential for these disinfectants to inhibit *Treponema* growth at specific concentrations in the field. Future research will explore the efficacy of these disinfectants when exposed to other variables associated with hoofbaths, such as number of cow passages, the presence of other bacteria, and varying bath temperatures, among others.

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