Evaluation of Mycobactericidal and Brucellicidal Efficacy of an Aldehyde and Quaternary Ammonium Solution and a Mixture of Phenolic Compounds

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ABSTRACT

Both tuberculosis (TB), caused by the *Mycobacterium tuberculosis* complex, and brucellosis (BR), caused by several species of *Brucella*, are considered emerging zoonoses. Disinfectants are essential for the control and prevention of TB and BR infections; therefore, their efficacy, either as isolated products or in combination with other disinfectants, must be ensured. The aim of this study was to evaluate the *in vitro* mycobactericidal and brucellicidal activity of AVT-450® and Poly-Phen®. AVT-450® contains a mixture of quaternary ammonium, ethanol aldehyde, and glutaraldehyde. Poly-Phen® is a mixture of phenylphenol, benzyl-para-chlorophenol, and amylphenol. Two virulent laboratory strains, *Mycobacterium tuberculosis* H37Rv (MtbH37Rv) and *Brucella canis* (Brc), were used.in this study To determine their viability, both bacteria were exposed to dilutions of AVT-450® and Poly-Phen® (1:1000, 1:500, and 1:250) for 10, 20, and 60 minutes for MtbH37Rv and 10, 20, and 30 minutes for Brc. The results obtained demonstrated that AVT-450® and Poly-Phen® effectively eliminated MtbH37Rv and Brc in all tested dilutions, even during short incubation periods. These satisfactory results may be attributed to the synergistic effect of the compounds in question. We thus conclude that AVT-450® and Poly-Phen® can be used as field disinfectants for the control of TB and BR, using the dilutions recommended by the manufacturer.

Keywords: Aldehydes; Brucellosis; Disinfectant; Phenols; Quaternary Ammonium; Tuberculosis.

INTRODUCTION

Tuberculosis (TB) is an important infectious diseases worldwide. As more than 1 billion people have died from tuberculosis in the last 200 years, it represents a significant challenge to global health (1, 2). TB is caused by different species of the *Mycobacterium tuberculosis* complex (Mtb), including *Mycobacterium bovis* (3,4). Due to the great impact of this disease on human health, Mtb has been considered one of the most dangerous emerging zoonoses (5).

Brucellosis (BR), on the other hand, is also considered a global zoonosis, since it is widespread in Europe, Western Asia, and some regions of Africa, as well as in several coun-

tries in the Americas (6,7). The causal agent of BR corresponds to different species of *Brucella*, particularly *B. abortus* and occasionally *B. mellitensis*, however, *B. canis* has recently become a noteworthy health risk for companion animals (8). Brucellosis affects a wide variety of animal species, including humans, making it a health risk of particular importance for people who work with or consume unpasteurized animal products (9,10). This disease also affects the dairy industry by causing abortions and considerable loss of livestock, with consequent increases in milk production costs (7,11,12).

Due to the difficulties involved in the treatment of TB and BR, more preventive measures are unquestionably required, specifically using new synthetic molecules that efficiently inactivate *Mycobacterium* spp. and *Brucella* spp. (13). Recently, certain non-quaternary ammonium groups, such as non-quaternary polyelectrolytes composed of diallyl ammonium, trifluoroacetate, and diallylmethylammonium trifluoroacetate, have been found to exert a strong effect against *Mycobacterium smegmatis* and *M. tuberculosis* (5). New preparations of low foam, non-ionic surfactant, chelating agent, and biocide composed of quaternary ammonium components, commonly used in the disinfection of medical instruments, eliminate many different types of bacteria, including *M. bovis* (4,14).

Aldehyde-based disinfectants, such as glutaraldehyde (2%) and orthophtaldehyde (OPA, 0.55%), have long been used to disinfect hospital medical instruments (4,15). Some mycobacteria strains, however, cannot be eliminated by these chemicals, which has been attributed to acquired resistance to glutaraldehyde (16). The resistance mechanism may be due to decreased availability of proteins on the cell surface, which constitute the principal target for aldehydes (17). Despite this situation, other glutaraldehyde-based disinfectants have been shown to inhibit growth of *M. tuberculosis* (4). Similarly, the mycobactericidal activity of several disinfectants and antiseptics with different concentrations of glutaraldehyde have been investigated, revealing mycobactericidal activity against *Mycobacterium avium* and *Mycobacterium terrae* (1). Due to the unsatisfactory effect of glutaraldehyde-based disinfectants in general, additional evaluation of these products is necessary to control those species of mycobacteria that have a notorious impact on animal and human health.

The bactericidal effect of other commonly used chemical disinfectants, such as sodium hypochlorite, trichloroisocyanuric acid, benzalkonium chloride, Lysol®, and sodium hydroxide, has been evaluated against *Brucella melitensis*, with differing levels of effectiveness depending on the amount used and exposure time (10).

Another interesting option has been the use of phenolic compounds with a variety of biological activities, among which antimicrobial activity is prominent (18,19). After being trapped by membrane phospholipids, these phenolic compounds act on the cell membrane, in addition to inactivating intracytoplasmic enzymes by forming unstable complexes. In low concentrations, nucleic acids or glutamic acid are released, while in high concentrations, phenolic compounds inhibit permeases, provoking denaturation of bacterial proteins resulting in cell membrane lysis (20). Many of these compounds are commonly employed as active ingredients in an assortment of agricultural, medical, and veterinary disinfectants (21).

The central goal of this study was to evaluate the *in vitro* mycobactericidal and brucellicidal activity of two commercial mixtures of disinfectants. The first disinfectant was enhansed with a glutaraldehyde base and quaternary ammonium, whereas the second contained phenolic groups.

MATERIAL AND METHODS

Evaluated disinfectants

Two commercial products were used in this study, each with a distinct mixture of active ingredients. The first product was AVT-450®, which contains quaternary ammonium, ethanol aldehyde, and glutaraldehyde; whereas the second corresponded to Poly-Phen®, a mixture of phenylphenol, benzyl-para-chlorophenol, and amylphenol. Both products were provided by Impexvet S.A de C.V (Mexico).

Bacterial cultures

Mycobacterium tuberculosis H37Rv (MtbH37Rv) used in this study was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). This mycobacterium was cultured in Middlebrook-7H9 broth (BD-DIFCO, USA), supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC, BD-DIFCO, USA), and incubated at a temperature of 37°C under steady agitation at 150rpm, until reaching the exponential growth phase. Mycobacterial inocula were prepared in a Glutamax RPMI-1640 medium (Invitrogen™, Carlsbad, California, USA) and supplemented with 10% fetal bovine serum (SFB, Life Technologies,

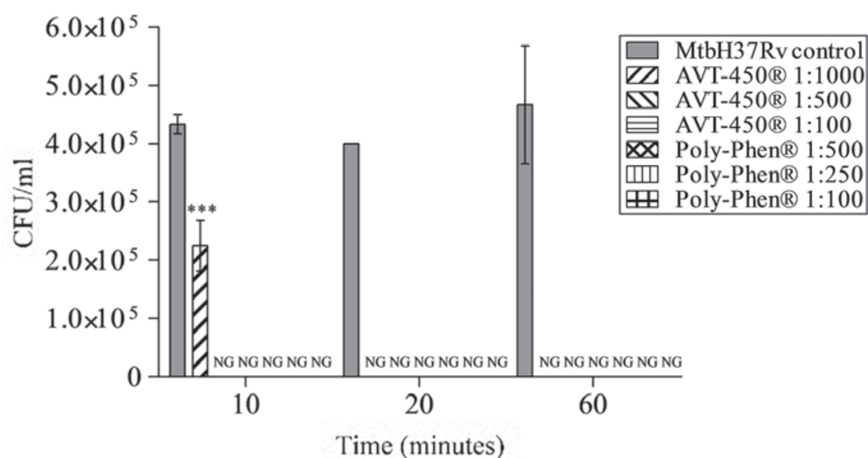

Figure 1: Incubation of Mycobacterium H37Rv (MtbH37Rv) for 10, 20, and 60 minutes without ATV-450® or Poly-Phen® (MtbH37Rv control) and ATV-450® or Poly-Phen® dilutions. Distilled water was used make dilutions of 1:1000, 1:500, and 1:250, as indicated by the manufacturer. In all cases, dilutions presented significant statistical differences with their respective control group (P<0.05), one-way ANOVA, Tukey.

Abbreviation: NG = No Growth MtbH37Rv.

USA). Mycobacterial suspensions were adjusted to $3x10^8$ mycobacteria/mL using a 1.0 nephelometer, according to the McFarland standard. After performing serial dilutions seeded on plates containing Middlebrook 7H10-OADC agar bacterial viability, colony forming units per milliliter (CFU/mL) were determined and calculated.

Brucella canis (Brc) strain, obtained from the Brucellosis Laboratory of the National Autonomous University of Mexico School of Veterinary Medicine and Animal Science, was also used. A pure culture was processed in soy trypsin broth (TSB, BD) incubated at 37°C with 5% CO_2 under steady agitation at 150 rpm, until reaching the exponential growth phase. Bacterial suspensions were prepared in a sterile phosphate solution (PBS) and adjusted to $1.5x10^8$ bacteria/mL using a 0.5 nephelometer (10). The number of viable microorganisms in the initial suspension was determined by conducting decimal dilutions in sterile PBS and by plating 100µl of each solution on trypticase soy agar plates (TSA). Next, the plates were incubated for 48 to 72h at 37°C, and colony forming units (CFU) were subsequently counted in order to obtain the total number of CFU/mL.

Evaluation of disinfectant activity

AVT-450® and Poly-Phen® disinfectants were placed per triplicate in a 96-well plate, and distilled water was used make dilutions of 1:1000, 1:500, and 1:250, as indicated by the manufacturer, resulting in a final volume of 200µl per well. Control groups were also included using only distilled water. Approximately, 5 x 10^5 of MtbH37Rv and $7x10^6$ of Brc were added to each corresponding well and incubated at 37°C for different periods (10, 20, and 60 minutes for MtbH37Rv and 10, 20, and 30 minutes for Brc). Samples of each incubation period and corresponding control groups were collected. Finally, we performed serial dilutions seeded in distinct plates containing Middlebrook agar and trypticase soy agar (TSA, BD) to determine the viability of MtbH37Rv and Brc per CFU/mL, respectively.

Statistics

Results were evaluated using a one-way ANOVA and groups compared using the Tukey test. All statistically significant differences were set at P<0.05. Analyses were performed with the IBM SPSSVR Statistics 20 software package.

RESULTS

After incubating MtbH37Rv for 10, 20, and 60 minutes with the 1:250 and 1:500 AVT-450® dilutions, this mycobacterium did not show any growth. In the case of the 1:1000

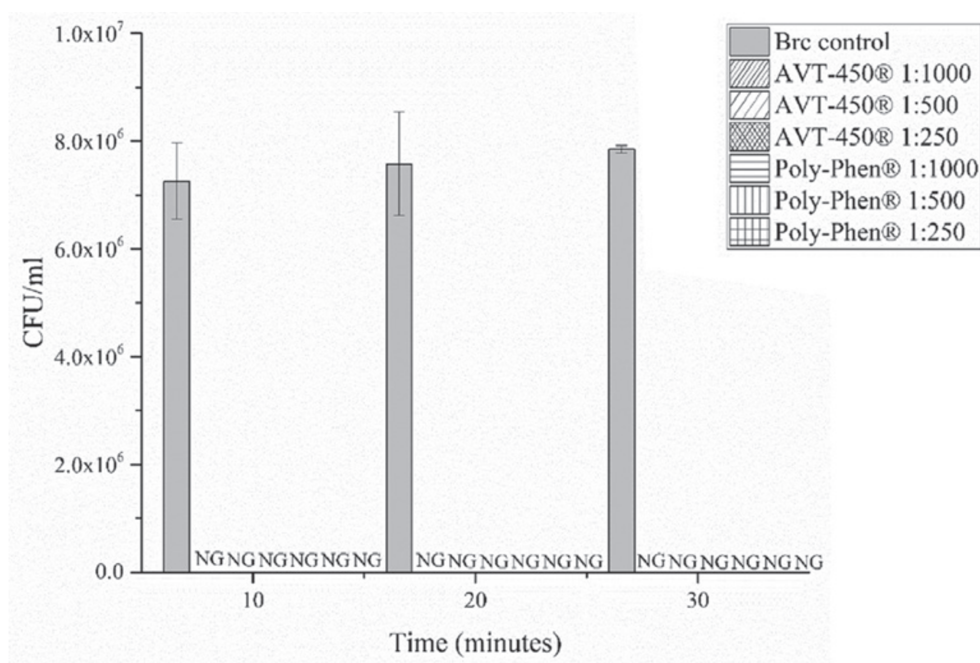

Figure 2: Incubation of *Brucella canis* (Brc) for 10, 20, and 30 minutes without ATV-450® or Poly-Phen® (Brc control) and ATV-450® or Poly-Phen® dilutions. Distilled water was used make dilutions of 1:1000, 1:500, and 1:250, as indicated by the manufacturer. In all cases, dilutions presented significant statistical differences with their respective control group (P<0.05), one-way ANOVA, Tukey NG: No Growth *Brucella canis.*

dilution, MtbH37Rv decreased by 50% after 10 minutes compared to the control group, and complete elimination was achieved after 20 and 60 minutes of incubation. In the AVT-450® control group, MtbH37Rv showed growth higher than $4x10^5$ CFU/mL in all three incubation times. During the evaluation of the Poly-Phen® disinfectant, no growth of MtbH37Rv was detected in any of the dilutions (1:1000, 1:500, and 1:250) tested at 10, 20, and 60 minutes of incubation (Figure 1). In all cases, the dilutions presented significant statistical differences with the control group (P<0.05).

Disinfectant activity of AVT-450® and Poly-Phen® against Brc was also favorable. During evaluation of these disinfectants at different dilutions (1:1000, 1:500, and 1:250), we found that AVT-450® completely inhibited Brc growth in all three incubation periods (10, 20, and 30 minutes). In addition, Brc did not show any growth with the Poly-Phen® disinfectant at 10, 20, and 30 minutes of incubation, compared to the control group (Figure 2). In all cases, dilutions presented significant statistical differences with their respective control group (P<0.05).

This data indicated that AVT-450® and Poly-Phen® disinfectants eliminate mycobacteria and Brucella even after very brief incubation periods.

DISCUSSION

In this study, we evaluated the commercial disinfectants AVT-450® and Poly-Phen®. The first was a solution of quaternary ammonium, ethanol aldehyde, and glutaraldehyde, while the second was composed of a mixture of phenolic derivatives. For both cases, the virulent laboratory strains Mtb H37Rv and Brc were used. Both bacteria were exposed to different dilutions of AVT-450® and Poly-Phen® during distinct incubation times, after which their viability was determined. Results showed that AVT-450® and Poly-Phen® effectively eliminated MtbH37Rv and Brc in all dilutions tested, even after short incubation periods. Although MtbH37Rv only decreased 50% after 10 minutes of incubation in the 1:1000 dilution of AVT-450®, elimination was achieved with increased exposure.

The activity of disinfectants depends on their chemical characteristics, concentration at which the product is being used, and exposure time (1,10). Glutaraldehyde-based dis-

infectants and ammonium quaternary disinfectants have been reported to produce mycobactericidal activity in 15 minutes (4). With this idea in mind, the mycobactericidal activity we observed within ten minutes with AVT-450® and Poly-Phen® disinfectants suggests a synergistic effect between the active ingredients of both products. As the aldehydes of AVT-450® enter through membrane porins, the lack of these proteins in the mycobacteria membrane probably reduced the action of these compounds (4,16). Nevertheless, the AVT-450® aldehydes' attraction to the negative charge of the cell surface allowed this disinfectant to act on membrane lipids and proteins, provoking loss of membrane integrity and function (4). In this way, the aldehydes clould enter the cytoplasm without the need for porins. As shown in our results, a maximum 1:500 dilution of AVT-450® is required for this synergistic effect to occur in the first 10 minutes of contact with MtbH37Rv. On the other hand, for Brc, this synergy also occurs during the first 10 minutes, even at a 1:1000 dilution. The phenolic derivatives of Poly-Phen® act on the cell membrane; due to the presence of multiple phenolic compounds, appropriate concentrations for inhibiting permeases were obtained and protein denaturation, ending with cell membrane lysis of MtbH37Rv and Brc after just 10 minutes of contact (20).

The synergistic effect has great advantages, among which is reduced development of resistance. This is particularly important because mycobacteria can rapidly develop resistance to aldehydes (4).

To obtain the brucelicide and mycobactericidal effect of AVT-450® and POLY-PHEN®, these disinfectants will have to be applied on a surface of equipment in dilution of 1:500 and 1:1000 respectively allowing them to act for 20 minutes followed by the removal the disinfectants with clean water.

CONCLUSIONS

We can conclude that AVT-450® and Poly-Phen® can be used as field disinfectants for the control of TB and BR, preferably at maximum dilutions of 1:500 for AVT-450® and 1:1000 for Poly-Phen® to obtain bactericidal effects in the shortest time. Note that adequate water quality must be maintained, and all organic matter removed, to achieve good results (10).

CONFLICT OF INTEREST

None of the authors are in any form link financially or laboratory linked to the companies that commercialize the disinfectants used in this study. Authors state that they have no conflicts of interest.

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