Veterinary Microbiology 136 (2009) 403–407

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

In vitro assessment of the effectiveness of powder disinfectant (Stalosan® F) against *Lawsonia intracellularis* using two different assays

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ARTICLE INFO

Article history:
Received 30 August 2008
Received in revised form 1 December 2008
Accepted 2 December 2008

Keywords:
Proliferative enteropathy
Lawsonia intracellularis
Disinfectant effectiveness

ABSTRACT

The objective of this study was to determine the *in vitro* efficacy of Stalosan® F, a mixed chemical and heavy metal disinfectant, against two strains of *Lawsonia intracellularis* using both a modified tissue culture and a direct count method. For testing as a powder, 1 g, 0.5 g, or 0.25 g of Stalosan F was applied to bacterial solutions spread into sterile dishes. For use as an aqueous suspension, Stalosan F was prepared to final concentrations of 1%, 4%, 8%, 16%, and 32%. In both applications, *L. intracellularis* was exposed to Stalosan F for 0.5 h, 1 h, 2 h, and 4 h. The results showed that both strains were similar in their susceptibilities to Stalosan F. The modified tissue culture assay showed no detectable *L. intracellularis* in cell culture after exposure to all levels of Stalosan F powder for 0.5 h. Furthermore, the number of viable bacteria was markedly reduced in the aqueous concentration of 4% and no *L. intracellularis* was detected at concentrations of ≥8% for 0.5 h. Using the direct count method, detection of live bacteria was less than 1% after exposure to the powder for 0.5 h. After exposure to the aqueous form, the number of viable bacteria killed was over 99% in concentrations of ≥16% compared to controls. Our results indicate that Stalosan F in both powder and suspension forms is able to inactivate over 99% of *L. intracellularis* after 30 min of exposure. Furthermore, both laboratory methods can be used to determine the effect of disinfectants on *L. intracellularis* viability.

1. Introduction

There are limited data on the effectiveness of disinfectants against *Lawsonia intracellularis*, a Gram-negative obligately intracellular bacterium that causes proliferative enteropathy (PE) (Lawson and Gebhart, 2000). This is mainly due to the difficulty of finding good methods to measure the efficacy of disinfectants against an obligately intracellular bacterium. One study used a conventional tissue culture method to measure the viability status of *L. intracellularis* after exposure to some disinfectants (Collins et al., 2000). That assay was time-consuming and incapable of distinguishing between proportions of viable or non-viable bacteria. Recently, a modified tissue culture method that cultured bacteria in 96-well tissue culture plates has been developed for use in serological diagnosis (Guedes et al., 2002) and for the determination of the *in vitro* antimicrobial activity (Wattanaphansak et al., 2009) of *L. intracellularis*. This method allows the testing of a large number of compounds over various concentrations at the same time. Additionally, the viability of *L. intracellularis* in a mixed bacterial population can be directly measured using a direct count method with specific fluorescence staining

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doi:10.1016/j.vetmic.2008.12.002

(Wattanaphansak et al., 2005). Both assays make it possible to evaluate the effectiveness of disinfectants against *L. intracellularis* using bacterial viability as an indicator.

Stalosan® F (Stormollen, Tureby, Storstrom, Denmark), a powder disinfectant mainly composed of phosphate compounds (85%), copper sulfate (2.5%), ferrous sulfate (2.1%), active chlorine (0.25%), perica oil (0.05%), and al-silicate (10.1%), is indicated for use in livestock farms for reducing the number of microorganisms in the environment, absorption of moisture, and reduction of ammonia production. The susceptibility of *L. intracellularis* to Stalosan F has not been reported. Therefore, the objective of this study was to evaluate both the modified tissue culture method and the direct count method for testing the efficacy of disinfectants against *L. intracellularis* and to determine the bacteriocidal activity of Stalosan F against *L. intracellularis* using both methods.

2. Materials and methods

2.1. Microorganism strains and preparations

L. intracellularis strains VPB4 and PHE/MN-01, which were isolated from proliferative hemorrhagic enteropathy infected pigs in the United States in 1991 and 2000, respectively (Guedes and Gebhart, 2003a), were used throughout this study, prepared independently and tested twice. Both were grown, maintained, and harvested as described previously (Guedes and Gebhart, 2003b; Wattanaphansak et al., 2005). The final concentration of *L. intracellularis* was determined using a direct count staining procedure as described previously (Guedes and Gebhart, 2003b).

2.2. Disinfectants and test procedures

Two forms of Stalosan F preparations, a powder disinfectant and an aqueous suspension, were used for testing. For use as a powder, Stalosan F was tested at three final concentrations which were 2*X*, 1*X*, and 0.5*X* of recommended dosages (*X* = dose recommended on label). Three hundred microliters of bacterial solution containing approximately 10^8 *L. intracellularis*/ml were added in duplicate and spread onto 10 cm × 10 cm square sterile Petri dishes. Then, 1 g, 0.5 g, or 0.25 g of Stalosan F powder was applied evenly to cover the entire surface of the Petri dish. These yielded final concentrations of Stalosan F equivalent to 100 g/m^2, 50 g/m^2, and 25 g/m^2, respectively.

For testing as an aqueous suspension, Stalosan F was prepared to final concentrations of 1%, 4%, 8%, 16%, and 32% in Dulbecco's modified Eagle medium (DMEM). The suspension was mixed and 8 ml of each concentration was aliquoted in duplicate, with the addition of 300 μl of 10^8 *L. intracellularis*/ml to each.

In both applications, *L. intracellularis* was exposed to Stalosan F for 0.5 h, 1 h, 2 h, and 4 h at room temperature. The controls for each time point were live *L. intracellularis* in DMEM without exposure to Stalosan F and dead *L. intracellularis* in which the bacteria were exposed to isopropyl alcohol for 30 min. After incubation, the powder in the Petri dishes was washed with 8 ml DMEM and the suspension was immediately transferred to 15 ml tubes. The bacteria in both applications were separated from the powder by passing the suspension through 5 μm filters into microcentrifuge tubes and centrifuged at 10,000 rpm for 3 min. The pellet was washed twice with sterile distilled water and half was re-suspended with 2 ml sterile distilled water for enumeration by the direct count method. The other half was re-suspended with 2 ml of *L. intracellularis* culture media and used to infect 1-day-old McCoy cells in the modified tissue culture method.

2.3. Bacterial survival assay

The percentage of *L. intracellularis* surviving after exposure to the disinfectant was assessed using both direct count and the modified tissue methods. The direct count method was conducted using Live/Dead® BacLight™ staining as described in a previous study (Wattanaphansak et al., 2005). In this study, only green fluorescent cells of live bacteria that stained with SYTO-9 were counted. The modified tissue culture method was performed as described previously (Wattanaphansak et al., 2009) to determine *L. intracellularis* viability. The effectiveness of Stalosan F was determined by evaluating the number of heavily infected cells (HICs), which were defined as the relative number of cells that were infected with the surviving *L. intracellularis* after exposure to the Stalosan F. These numbers were used as a live *L. intracellularis* indicator.

2.4. Scanning electron microscopy (SEM)

For SEM observations, *L. intracellularis* was exposed to 0.5 g of Stalosan F powder and 16% of Stalosan F suspension for 30 min. The bacteria were then filtered through a 5 μm filter and washed with phosphate buffer saline (PBS) twice. The samples were fixed with 2.5% glutaraldehyde in PBS for 1 h at room temperature. After three washes with PBS, the bacterial cells were fixed with 1% osmium tetroxide in PBS and washed three more times with PBS. The bacterial cells were dehydrated with increasing concentrations of ethanol (25%, 50%, 75%, 95%, and 100%) and dried in a Balzer Critical Point Dryer 010® unit. The fixed bacteria were coated with a thin film of gold–palladium and examined using a VPSEM-Hitachi S-3500N scanning electron microscope.

2.5. Data analysis

The number of HIC and the number of green fluorescent bacteria in each treatment of Stalosan F were expressed as percentages as compared to the controls. The correlation between the modified tissue culture and direct count methods was estimated with Spearman's coefficient of rank correlation using the MedCalc® version 9.1.0.1 software.

3. Results

The results (Figs. 1 and 2) show that both strains of *L. intracellularis* were similar in their susceptibilities to both

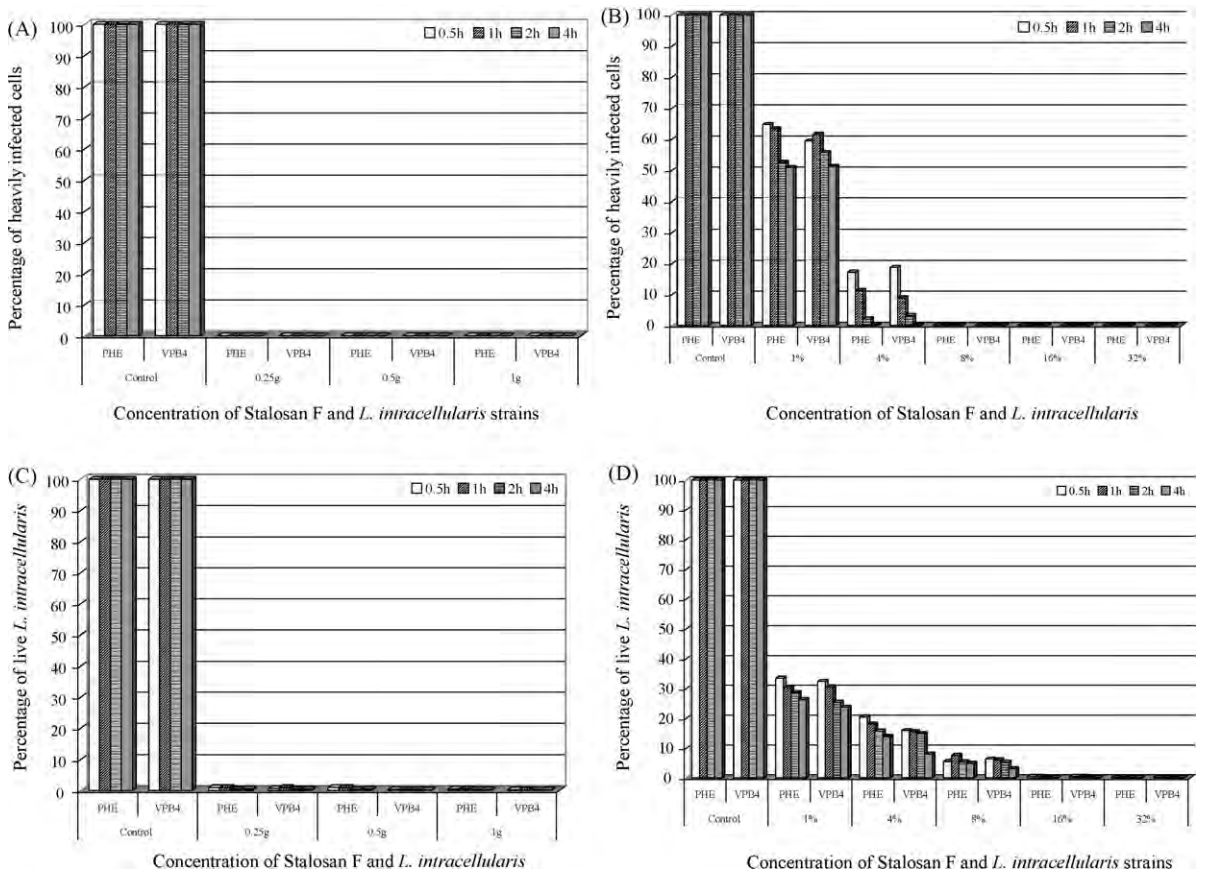

Fig. 1. The effectiveness of Stalosan F used as a powder (A) and as an aqueous suspension (B) against *Lawsonia intracellularis* measured with the modified tissue culture method. The effectiveness of Stalosan F used as a powder (C) and as an aqueous suspension (D) against *L. intracellularis* measured with the direct count method.

powder and aqueous suspensions of Stalosan F. The modified tissue culture method was used to determine the surviving population and most of the McCoy cells in live control wells were heavily infected with *L. intracellularis*, indicating that the percentage of *L. intracellularis* infection was close to 100%. In contrast, no HICs were detected after exposure to 0.25 g/cm^2, 0.5 g/cm^2, and 1 g/cm^2 of Stalosan F for 30 min, indicating 100% inactivation compared to the live control (Figs. 1A and 2B).

When Stalosan F was tested as an aqueous suspension, the number of HICs decreased with increased concentrations of Stalosan F and increased exposure time. The viability of *L. intracellularis* decreased to approximately 65% after exposure to 1% of Stalosan F concentration for 30 min. The surviving population of *L. intracellularis* was markedly reduced in the aqueous concentration of 4% and no *L. intracellularis* was detected in the tissue culture at a concentration of $\geq$8% for 30 min (Fig. 1B). Under these conditions, both the powder and suspension forms of Stalosan F have a bactericidal effect on *L. intracellularis*.

When using the direct count method to determine *L. intracellularis* viability, approximately 95% of live control bacteria exhibited a green fluorescence of SYTO-9, indicating live bacteria with an intact cell membrane. In contrast, a small percentage of mixed bacterial populations had a red fluorescence of propidium iodine, indicating dead bacteria with a damaged cell membrane (Fig. 2A). After 30 min of exposure to Stalosan F powder, the number of live bacteria progressively decreased to less than 1% in all tested concentrations of powder Stalosan F (Fig. 1C). In contrast, more red fluorescent bacteria were observed, indicating that the bacterial structure might be damaged or lysed after contact with Stalosan F.

Similar results were found when *L. intracellularis* was exposed to an aqueous suspension of Stalosan F. The viable populations of *L. intracellularis* decreased with increased concentrations of Stalosan F in the suspension. The number of live bacteria was approximately 30% after exposure to 1% of Stalosan F for 30 min and less than 1% in concentrations of $\geq$16%, with more than 99% of *L. intracellularis* killed when compared to the controls (Fig. 1D).

In this study, the results obtained from the modified tissue culture method were similar to the results obtained from the direct count method. There was a significantly positive correlation between both assays ($\rho = 0.76$, $p = 0.001$) indicating a good agreement between both methods.

The morphological appearance of *L. intracellularis* after 30 min of exposure to Stalosan F is shown in Fig. 2C. Electron microscopy showed the presence of flagellar components on untreated *L. intracellularis* cells and the cell

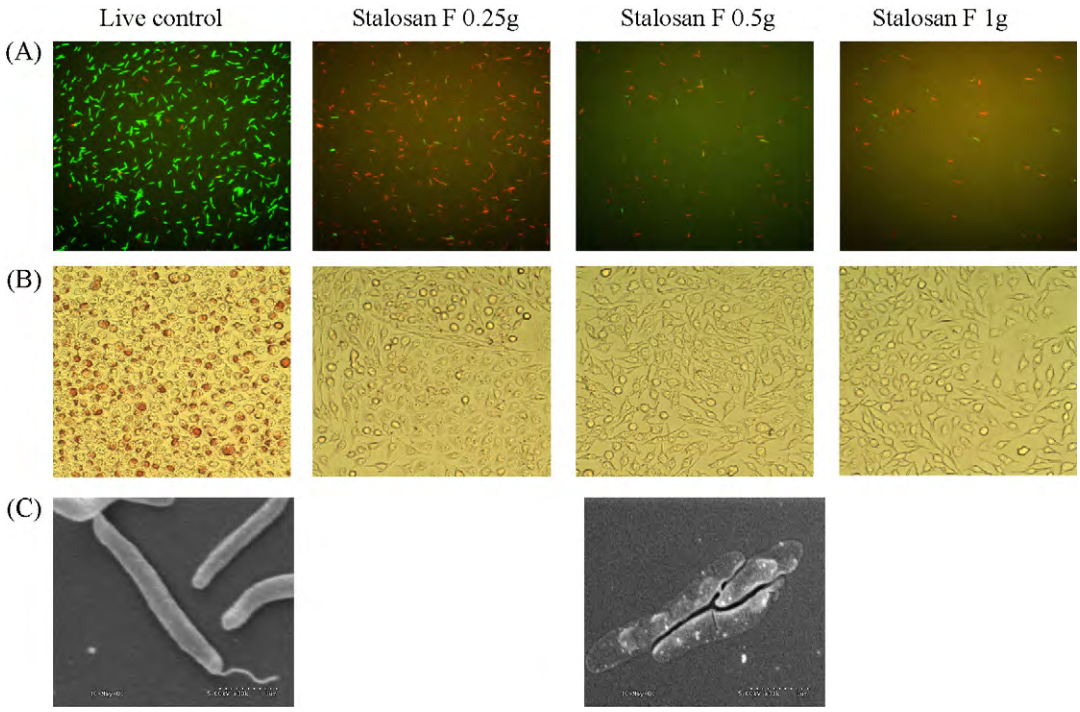

Fig. 2. *L. intracellularis* exposed to Stalosan F powder at 0.25 g, 0.5 g, and 1 g for 0.5 h. The viability of *L. intracellularis* was measured with the direct count method (A) and the modified tissue culture method (B). Scanning electron micrographs of normal *L. intracellularis* and after exposure to 0.5 g of Stalosan F (C).

wall of the bacterium seemed cloudy and intact. After exposure to Stalosan F powder at 0.5 g for 30 min, the bacterial cell wall became more translucent, indicating damage to the cell wall. The similar appearance of bacteria is found after exposure to aqueous suspension at 4% and 16% of Stalosan F (picture not shown).

4. Discussion

The use of chemical disinfectants in swine facilities is a first line of defense against virus, bacteria, and parasite infection. However, the effect of disinfectants on *L. intracellularis* is very difficult to measure *in vitro*. Unlike other bacteria, *L. intracellularis* is an organism that propagates itself only inside enterocytes. Cell-free culture methods have not been successfully established and so there are no standard *in vitro* assays for assessing the efficacy of disinfectants against *L. intracellularis*. In this study, we compared two systems, a modified tissue culture method and a direct count method, for evaluating the efficacy of Stalosan F in killing *L. intracellularis*.

The results from both methods showed that either the powder or suspension forms of Stalosan F could be used for *L. intracellularis* inactivation. The reduction of *L. intracellularis* viability depended on dose and exposure time of Stalosan F. According to the manufacturer, the compound is to be applied directly on the floor at a concentration of 50 g/m^2, which equaled to 0.5 g/cm^2 in this study, for reducing the number of viable organisms in the environment. This concentration would be able to kill 100% or >99% of *L. intracellularis* after exposure for 30 min, according to the tissue culture and direct count assay, respectively. Although the mode of action of Stalosan F is not fully understood, it has been shown that the viabilities of pathogenic bacteria and viruses were significantly reduced when those organisms were exposed to Stalosan F (Methling et al., 1997).

After exposure to the powder and suspension forms of Stalosan F, a few viable *L. intracellularis* (<1%) were detected in the direct count method, while none of the viable bacteria were found when measured with the modified tissue culture method. In this situation it is possible that the green labeled *L. intracellularis* in the direct count method (which were believed to be live) might have been in the viable but nonculturable state. The bacteria in this state generally remain viable and capable of revival under favorable conditions, but the standard culture methods cannot detect the bacteria. The state of viable but nonculturable has been reported in many Gram-negative bacteria (Xu et al., 1982; Roszak et al., 1984; Gupte et al., 2003; Tholozan et al., 1999). However, this state of viable but nonculturable for *L. intracellularis* has not been described. In addition, Millard and Roth (1997) found that in dead bacteria with minimal or partial membrane damage, propidium iodine showed limited penetration and accumulation in the cytoplasm of the bacteria. Therefore, it is plausible that the green fluorescent bacteria are dead *L. intracellularis* with minimal membrane damage causing a greater accumulation of SYTO-9 than propidium iodine. Clearly, the concentrations of bacteria dramatically decreased after exposure to both forms of Stalosan F. Although the mechanism of action of

Stalosan F remains unclear, it is possible that the membrane of the bacteria was destroyed by Stalosan F. The abnormality of *L. intracellularis* membranes was observed using the SEM, as some parts of the bacterial membrane were clear and transparent after treatment.

5. Conclusion

In summary, we demonstrated that the modified tissue culture and the direct count methods gave similar results for measuring the viability status of *L. intracellularis* after exposure to a powder disinfectant. Our results indicate that Stalosan F in both a powder concentration of $\geq$0.25 g/cm^2 and an aqueous suspension of $\geq$16% concentration are able to inactivate over 99% of both *L. intracellularis* strains after 30 min of exposure as determined by both assays.

Acknowledgements

This study was supported in part by a grant from Phibro Animal Health. The authors would like to thank Benjawan Wijarn and Molly Freese for excellent technical assistance.

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