Deliverable D-JRP21-WP 3.7

Workpackage 3

Responsible Partner: RKI

Contributing partners: NVI, APHA

GENERAL INFORMATION

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BIOPIGEE

Assessed methods for testing disinfectants on *Salmonella* in biofilm

Objective
The objective was to establish and assess biofilm models from three different laboratories for future use in disinfectant testing on *Salmonella* biofilms.

Participating countries
Germany (RKI), Norway (NVI), UK (APHA)

Background
Currently, the efficacy testing of disinfectants is based on laboratory tests using planktonic bacteria. However, like many other microorganisms, *Salmonella* is able to persist in the environment by forming biofilm communities, which can provide effective protection against disinfectants and other stressors. This can cause severe hygiene problems in swine farm environments and in the food production industry, where *Salmonella* embedded in biofilms could withstand routine cleaning and disinfection protocols. It is therefore important to establish and standardize a test method for assessing the efficacy of disinfectants to inactivate *Salmonella* biofilms. This can result in changes in recommended concentrations and/or contact times for disinfectants in practical settings, e.g. in swine production industry.

Methods
For the assessment of different biofilm models for efficacy testing of disinfectants, the participating laboratories used two commercially available *Salmonella* Typhimurium strains, ATCC 14028 and ATCC 13311, as reference strains. The following disinfectants in different concentrations were tested on 2-day-old biofilms [cultured in Luria Bertani broth (LB) without NaCl] at 20°C:
A) peracetic acid (Lerasept® Spezial), contact time 10 min, neutralization with 1.65 % sodium sulfite in PBS (0.1 M, pH 7) and
B) glutaraldehyde (Protectol®GA50), contact time 30 min, neutralzation with 20 g/L glycine, 10 g/L tween 80 in 0.25 M PBS.
The efficacy of a disinfection was measured by assessing the recoverable viable colony forming units (CFU). Disinfection was defined as ≥ 5 $\log_{10}$ reduction in recoverable mean CFU in accordance with European standards.

The following disinfection testing methods on biofilms were applied:

1) **Bead-based biofilm model by RKI**
   Biofilms were cultivated on 4 mm porous glass beads (Sinterglas Pellets, ROBU Glasfilter-Geräte GmbH) in 24-well microplates (one bead per well), each well containing 1 mL of 10^5 CFU/mL bacterial inoculum. Plates were incubated on an orbital shaker at 100 rpm at 20 °C for 48 h for biofilm cultivation. Thereafter, each bead was carefully dipped in 2 mL sterile H_2O and placed in a 2 mL microcentrifuge tube containing 0.2 mL disinfectant. After incubation for the defined contact time (see above), 1.8 mL of the neutralizing agent was added to each microcentrifuge tube. Subsequently, bacteria were sonicated in an ultrasonic bath for 10 min to detach the biofilm from the bead surface and quantified by serial dilution. All dilution steps were done in neutralizing agent. The controls were treated with hard water instead of disinfectant. All experiments were performed three times with three technical replicates each.

2) **Biofilm model by NVI**
   To create a working culture in broth bacterial cultures on blood agar were transferred to 5 mL LB and the optical density (OD) was measured and adjusted to 1. Then 0.5 mL of each bacterial suspension was added to 10 mL of LB without NaCl together with an autoclaved stainless-steel

coupon of 75x24x1 mm (Stainless steel AISI304, 2B Olaf Johansens Eftf. A/S, Oslo, Norway) and incubated at 20 °C for 48 h. Thereafter, the coupon was rinsed 3x in 40 mL sterile saline and transferred to a tube with 10 mL of disinfectant (or saline for controls) for the applicable contact time (see above). The coupon was then moved to a tube with appropriate neutralization broth before it was rinsed 3x again and added to a tube containing glass beads and 5 mL saline. Here, visible biofilm was scraped off both sides of the coupon by using an 18 cm long cell scraper with a blade of 1.8 cm. The coupon was discarded before the tube was vortexed for one minute. An aliquot of 0.2 mL from each tube was added to wells in a microtiter plate. Serial dilutions were performed before plating 5 µL on blood agar and incubation at 37 °C for 24 h to determine the viable cfu count. All experiments were performed three times with two technical replicates each.

3) **Biofilm model by APHA**
Salmonella isolates were inoculated into LB without NaCl and the bacterial suspension adjusted to 1 McFarland. To each well in a 12-well microplate 1.5 mL of this bacterial suspension was added along with one sterile Polyvinyl carbonate (PVC) coupon (10x20x0.6 mm; Pegen industries Inc, Ontario, Canada). Coupons were placed in the well so that only the bottom half of the coupon was submerged. Microtiter plates underwent static incubation for 48 h at 20 °C. After incubation, each coupon was washed three times, with light agitation, in 9 mL sterile saline and left to dry at room temperature. Coupons were submerged in either 10 mL disinfectant or 10 mL hard water for the specified contact time (see above), before being transferred to 10 mL of the appropriate neutralizer broth. After a minimum of 5 min neutralization time, coupons were transferred to 5 mL sterile saline with glass beads and shaken at the lowest speed on a vortex-mixer for 2 min, before a further 5 mL of saline was added. Serial dilutions were performed spread-plated on blood agar plates to allow for enumeration of bacteria after overnight incubation at 37°C. All experiments were performed three times with two technical replicates each.

In addition, to compare the results of biofilm testing with those of planktonic bacteria, the efficacy of the above mentioned disinfectants on planktonic *Salmonella* was assessed by one laboratory using the quantitative suspension test according to the EN 1656 standard.

Results
In order to test for **repeatability** (variation due to procedure) each laboratory reported CFU counts of untreated control biofilms (for both reference strains) of different experiments performed by the same technician and the data was assessed for mean, standard deviation, minimum and maximum variation.

Efficacy testing results. For the reference strain ATCC 14028, all laboratories achieved results for the disinfection of biofilms (≥ 5 $\log_{10}$ reduction in CFU) with peracetic acid and glutaraldehyde. Among all three laboratories the concentrations to achieve full disinfection with peracetic acid ranged between 0.005 and 0.1 % (see Fig. 1 for exemplary results of RKI). The concentrations to achieve disinfection of biofilms with glutaraldehyde ranged between 0.1 and 1 %.

For the reference strain ATCC 13311, only two laboratories were able to achieve results for the disinfection of biofilms with both disinfectants. The concentrations to achieve full disinfection with peracetic acid ranged between 0.04 and 0.1 %, and with glutaraldehyde between 0.1 and 1 %. With the method of the third laboratory, sufficient biofilm could not be obtained to allow for disinfectant testing to be performed.

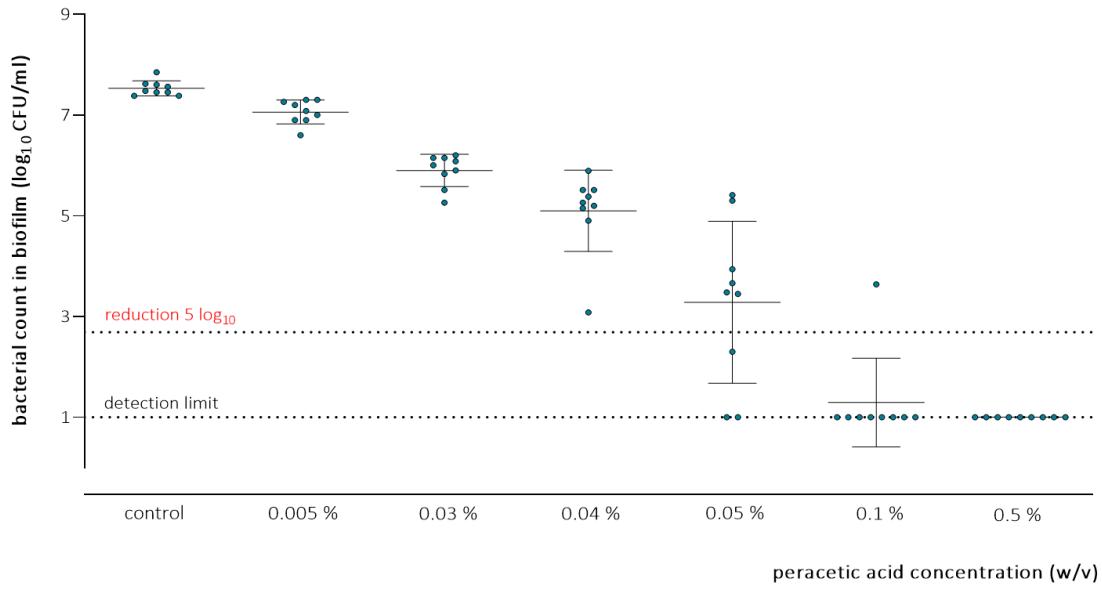

Fig. 1: Disinfectant testing with peracetic acid for ATCC 14028 performed by RKI. Full disinfection was achieved with 0.1 % (w/v) peracetic acid.

In order to test for **reproducibility** (variation due to operators) two technicians of two laboratories performed the same experiment with different concentrations of one disinfectant for one reference strain.

In addition to the biofilm assay, one laboratory also tested the efficacy of disinfectants on planktonic *Salmonella* reference strains. The concentration needed for full disinfection of planktonic bacteria with peracetic acid was 0.002 % for ATCC 14028 and 0.001 % for ATCC 13311 (compared to 0.1 % that was required for disinfection of biofilm of both strains). The concentration needed for full disinfection of planktonic bacteria with glutaraldehyde was 0.03 % for both strains (compared to 0.5 % that was required for disinfection of ATCC 14028 biofilms and 0.1 % for disinfection of ATCC 13311 biofilms).

Conclusions

Results for disinfectant efficacy testing were obtained for the reference strain ATCC 14028 (strong biofilm producer) from all three participating laboratories, whereas one laboratory could not achieve results for the reference strain ATCC 13311 (weak biofilm producer) because of insufficient amounts of biofilm in their setting. Therefore, differences in the suitability of biofilm methods for the use in disinfection testings were observed among the laboratories.
Furthermore, results concerning the required concentrations for full disinfection varied between the laboratories.

According to results obtained by RKI, the concentration of disinfectant required for the disinfection of biofilms was markedly higher than the concentration required for the disinfection of planktonic *Salmonella*.

Further experiments are ongoing for the selection of a biofilm model for future use in disinfectant testing on *Salmonella* biofilms.

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