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KSA-SM-09

Importance of checking whether the harvesting and disaggregating steps bias the results of a surface disinfectant test

[*Key Words*: declumping, dispersion, log reduction, recovery, removal, resuspension, validity]

The log reduction (LR; KSA-SM-07 The log reduction measure of disinfectant efficacy) can be biased if there are different harvesting efficiencies or different disaggregation efficiencies for treated carriers compared to control carriers. It is important to check for such bias. A recent publication by the Standardized Biofilm Methods Laboratory reviewed laboratory methods for conducting bias checks (Hamilton et al. 2009). The publication explains why it is necessary to measure the efficiency of the harvesting and disaggregating steps for treated carriers as well as for control carriers. It provides a formula for calculating a quantitative estimate of bias and describes how to use microscopy for qualitative bias checks.

Goals: Provide some background and motivation for our concerns about bias

The goals of this article are to provide some background and motivation for our concerns about bias and to recommend that disinfectant efficacy test results include data demonstrating that the harvesting and disaggregating steps do not bias the LR.

Harvesting: Purposely separating surface-associated bacteria from the carrier, usually by mechanical means

Harvesting

The term harvesting indicates the laboratory manipulation that purposely separates surface-associated bacteria from the carrier, usually by mechanical means. This is different from chemical cleaning of bacteria from the carrier during the disinfection step. The harvesting step also has been called removal or recovery. Harvesting techniques include scraping, vortexing (with or without beads), sonicating, stomaching, swabbing, and washing, where each technique can be applied with or without the addition of chemical agents such as surfactants or enzymes (Donlan *et al.*, 1999; Gagnon & Slawson, 1999; Lindsay & von Holy, 1997; Morris *et al.*, 1998; Simoes *et al.*, 2005; Sreenivasan & Chorny, 2005; Zelver *et al.*, 2001).

Disaggregating: Breaking up clumps to create a suspension of single cells

Disaggregating

The disaggregation step breaks up clumps of bacteria, creating a suspension of randomly distributed single cells. Disaggregation is conducted immediately prior to making a dilution series and plating for viable cell counts. The disaggregation step also has been called destabilization, dispersion, resuspension, declumping, or disintegration. Commonly-used disaggregation methods include vortexing (with or without beads), local agitation by repeated fill/expel pipetting when forming the dilution series, sonicating, and homogenizing, where each technique can be applied

with or without the addition of chemical agents such as surfactants or enzymes (Camper *et al.*, 1985; Morris *et al.*, 1998; Salhani & Uelker-Deffur, 1998; Joyce *et al.*, 2003; Sreenivasan & Chorny, 2005).

Harvesting and disaggregating in one step

The harvesting and disaggregating are not easily separated in some applications; e.g., disinfectant tests in which the bacteria are associated with surfaces that are soft, porous, granular, or fragile. For preparing food samples, soil samples, fabric samples, or samples of packing material (e.g., sand, peat, or granular activated carbon), various combinations of sonicating, stomaching, vortexing, and homogenizing, with and without the addition of glass beads or of chemicals such as enzymes, surfactants, or chelating agents, have proven effective at simultaneously harvesting and disaggregating surface-associated bacteria (Andrews *et al.*, 1978; Cody *et al.*, 1984; Camper *et al.*, 1985; Mermillod-Blondin *et al.*, 2001; Donlan *et al.*, 2001; Bockelmann *et al.*, 2003; Khammar *et al.*, 2004). These combination techniques are used also for carriers that are made of nonporous, hard materials such as polycarbonate, steel, or glass (Gagnon & Slawson, 1999; Oulahal *et al.*, 2004). By simultaneously harvesting and disaggregating, one minimizes sample manipulations and thereby potentially reduces both the risk of contamination and the cost of experimentation.

Potential effects on the validity of the disinfectant test

Examples: Bias has led to consequential, misleading efficacy results

The harvesting or disaggregating steps can affect the validity of the log reduction (*LR*) measure of disinfectant efficacy. Important consequences attributable to harvesting and disaggregation bias has been observed in practical circumstances. One case pertains to an antimicrobial-coated sewing cuff on an artificial heart valve. Laboratory antibacterial tests of the cuff showed good efficacy at resisting bacterial colonization. However, subsequent investigation utilizing confocal scanning laser microscopy showed that, in fact, the cuff was not effective at preventing bacterial colonization (Cook *et al.*, 2000). Apparently, the initial laboratory tests produced a biased log reduction because, unknown to the investigators but subsequently observed using microscopy, the harvesting efficiency for the antimicrobial coated cuff was lower than for the control cuff. Consequently, the viable cells on coated surfaces were undercounted and the efficacy result was systematically too high.

In a different application, Midelet & Carpentier (2004) discussed the potential for bias after observing that a glutaraldehyde formulation had the fixative effect of increasing both attachment strength and micro-colony cohesion in a biofilm. They cautioned that conventional harvesting techniques were unlikely to remove the viable cells from treated carriers, thereby undercounting the viable cells and biasing the LR.

For determining the bacterial numbers in the effluent of bioreactors by plate counts, Salhani & Uelker-Deffur (1998) found that the main problem was neither non-viable cells nor physiological specialists, but aggregates of bacteria. They noted that many techniques for converting aggregates into isolated colony-forming units also killed part of the bacterial community. One could reasonably expect that a viable, but injured, subpopulation within the treated cell suspension would be especially sensitive to disaggregation trauma. The result would be an artificially low viable cell count for treated carriers and an exaggerated LR.

Current status:
Disinfectant tests seldom include appropriate checks for harvesting and disaggregating biases

Important facts:

1. A convincing check must measure the harvesting and disaggregation efficiencies for treated carriers as well as for control carriers

2. Bias occurs if, and only if, the harvesting or disinfecting steps produce different efficiency fractions for disinfected carriers than for control carriers.

3. Laboratory methods are available for determining harvesting and disaggregating efficiency fractions. The methods can be used to assess bias.

Status of the field: convincing checks for bias are not being done

Although the preceding examples point to an obvious need for laboratory methods for checking the validity of the harvesting and disaggregation steps, the development of check methods has lagged behind. A few methods have been devised for comparing the efficiencies of alternative harvesting or disaggregation techniques. Published evaluations show that the success of a harvesting or disaggregation method often depends on application-specific factors such as surface material and bacterial species. Almost all published evaluations have focused on control carriers only, treated carriers were not considered. The usual goal was to determine the technique that achieved the highest viable cell counts in the suspension. Such check methods are incomplete. A convincing bias check must consider treated carriers as well as control carriers. Validity is not assured by high control carrier viable cell counts. For example, a method that harvests almost all bacteria from the surface of a control carrier will produce a biased LR whenever the fraction harvested from treated carriers is consistently smaller. On the other hand, even though a harvesting technique consistently removes just 0.1% of the bacteria from all treated and control carriers, it is satisfactory for disinfectant testing. The reason is that a 0.1% sample of the bacteria is counted on every carrier, control and treated, and the average calculated LR will be exactly the same numerical value as when 100% of the viable bacteria are counted on each carrier. The harvesting and disaggregating steps will not bias the LR if the efficiencies of those steps are the same for treated carriers as for control carriers. But, if an efficiency differs between the treated and control carriers, the technique does bias the LR. The paper by Hamilton et al., (2009) provides formulas for assessing the bias and presents laboratory bias checking results.

Laboratory check methods are available

Among the published techniques for checking the validity of the harvesting step are methods for counting the cells on carriers (Morris *et al.*, 1998; Donlan *et al.*, 1999; Mermillod-Blondin *et al.*, 2001; Simoes *et al.*, 2005) and techniques for measuring the aggregate amount of biofilm on carriers (Kirchman & Mitchell, 1982; Mermillod-Blondin *et al.*, 2001; Staudt *et al.*, 2004; Pitts *et al.*, 2003; Parini *et al.*, 2005).

The literature also contains methods for comparing the efficiencies of alternative disaggregation techniques. Among the published evaluations are viable cell counts before and after disaggregation (Salhani & Uelker-Deffur, 1998; Morris *et al.*, 1998; Joyce *et al.*, 2003) and quantitative microscopic assessment of filtered samples of the suspension before and after disaggregation (Morris *et al.*, 1998; Wilson *et al.*, 2004).

Published laboratory evaluations of combined harvesting and disaggregating techniques include viable cell counts, total cell counts, functionally active cell counts, and counts in electron microscope images (Bockelmann *et al.*, 2003; Cody *et al.*, 1984; Lindsay & von Holy, 1997; Khammar *et al.*, 2004; Mermillod-Blondin *et al.*, 2001). Because of the mechanical energy required to accomplish both harvesting and disaggregating, many studies checked whether the techniques caused cell injury or death. Some studies uncovered the lethal effects of a chemical

additive, such as an enzyme or a chelating agent (Bockelmann *et al.*, 2003; Oulahal *et al.*, 2004). Because of the tradeoff between the beneficial effect of separating aggregates into single cells and the detrimental effect of damaging or inactivating cells, it is especially challenging to identify optimum specifications when applying a sonication method (Camper *et al.*, 1985; Scherba *et al.*, 1991; Mermillod-Blondin *et al.*, 2001; Joyce *et al.*, 2003; Oulahal *et al.*, 2004).

Conclusions: Bias checks are feasible and should be conducted routinely. Visual confirmation via microscopy is recommended.

Conclusions

Bias checks are feasible and should be conducted for each combination of carrier material, microbial species, and disinfectant formulation (Hamilton et al. 2009; Coenye and Nelis 2010). It is prudent to support the checks with qualitative, visual confirmation, such as microscopic examination of carrier surfaces.

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