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Improved enzyme protection assay to study Staphylococcus aureus internalization and intracellular efficacy of antimicrobial compounds

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TITLE:

Improved Enzyme Protection Assay to Study *Staphylococcus aureus* Internalization and Intracellular Efficacy of Antimicrobial Compounds

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SUMMARY:

This protocol aims to describe how to study the extent of *Staphylococcus aureus* internalization and its ability to survive inside the human host cell, as well as the intracellular efficacy of antimicrobial compounds.

ABSTRACT:

Staphylococcus aureus expresses virulence factors to trigger its internalization into eukaryote cells and to survive inside different subcellular compartments. This paper describes an enzyme protection assay to study the extent of *S. aureus* internalization and its intracellular survival in adherent non-professional phagocytic cells (NPPCs) as well as the intracellular efficacy of antimicrobial compounds. NPPCs are grown in a multi-well plate until they reach 100% confluence. *S. aureus* cultures are grown overnight in cell culture medium. The bacterial suspension is diluted according to the number of cells per well to inoculate the cells at a controlled multiplicity of infection. Inoculated cells are incubated for 2 h to allow the bacteria to be internalized by the NPPCs, following which lysostaphin is added to the culture medium to selectively kill extracellular bacteria. Lysostaphin is present in the culture medium for the

rest of the experiment.

At this point, the infected cells could be incubated with antimicrobial compounds to assess their intracellular activities against *S. aureus*. Next, the cells are washed three times to remove the drugs, and intracellular *S. aureus* load is then quantified by culturing on agar plates. Alternatively, for studying staphylococcal virulence factors involved in intracellular survival and cell toxicity, lysostaphin could be inactivated with proteinase K to eliminate the need for washing steps. This tip improves the reliability of the intracellular bacterial load quantification, especially if cells tend to detach from the culture plate when they become heavily infected because of the multiplication of intracellular *S. aureus*. These protocols can be used with virtually all types of adherent NPPCs and with 3D cell culture models such as organoids.

INTRODUCTION:

Staphylococcus aureus is both a life-threatening pathogen and a commensal bacterium of the skin and the mucosa that colonizes two billion individuals around the world[1]. In humans, nasal carriers of *S. aureus* have an increased risk of infection with their own strain of carriage; however, the multifactorial determinants of *S. aureus* mucosal carriage are still unclear[1,2]. In addition to acute infections, patients can also develop chronic *S. aureus* infections that are often challenging to cure[3]. A better understanding of host-pathogen interactions during colonization and infection is crucial for developing novel therapeutic strategies and improving patient management.

In vitro, *S. aureus* can trigger its internalization into host cells expressing the α5β1 integrin[4]. The tripartite interaction between the staphylococcal fibronectin-binding proteins anchored to the cell wall of *S. aureus*, the fibronectin, and the β1 integrin expressed at the host cell surface is well known as the main pathway of *S. aureus* internalization in NPPCs such as keratinocytes, osteoblasts, fibroblasts, and epithelial and endothelial cells[4]. Recent studies show that *S. aureus* can be found inside human cells during nasal colonization[5,6] and infection[7]. However, the role of the intracellular reservoir in the pathogenesis of *S. aureus* infection remains unclear. The host cells could act as a shelter for *S. aureus,* which is protected from both the immune system[8] and most antimicrobial compounds[6,9].

The lysostaphin protection assay, described by Proctor[10] earlier in the 1980s, enables the study of bacterial and host factors involved in the internalization of *S. aureus* isolates. Lysostaphin is a bacteriocin produced by *Staphylococcus simulans*, which exhibits potent activity against almost all *S. aureus* isolates, including antibiotic-resistant strains[11]. Lysostaphin has been used to destroy only extracellular *S. aureus* to enable the counting of only viable intracellular bacteria[12]. This technique has been widely used and has contributed to the discovery of several virulence factors of *S. aureus*. Gentamycin, alone and combined with lysostaphin, is also widely used to study intracellular bacteria.

However, a recent study showed that gentamycin enters eukaryotic cells and reaches internalized bacteria in a time- and concentration-dependent manner[13]. This study also demonstrated that lysostaphin does not enter eukaryotic cells, confirming that a lysostaphin-based enzyme protection assay (EPA) is the most accurate assay for quantifying intracellular *S. aureus* load by culture[13]. Regardless of which compound is used to destroy extracellular bacteria (e.g., lysostaphin or gentamycin), it should be removed by washing the cells before

plating intracellular *S. aureus* on agar plates. Successive washes may result in the detachment of cells, especially poorly adherent cells (e.g., heavily infected cells), which would lead to an underestimation of the intracellular *S. aureus* load. This paper describes in detail how EPA can be used to quantify the intracellular *S. aureus* load and to measure the intracellular efficacy of antimicrobials compounds using an *in vitro* model. Of note, a simple method has been proposed to improve the reliability of intracellular load quantification by avoiding intensive washes.

PROTOCOL:

1. Culture of human epithelial cells

1.1. Prepare complete culture medium with Dulbecco's modified Eagle medium (DMEM) high glucose with phenol red, supplemented with 10% fetal bovine serum (FBS) without antibiotics.

1.2. Grow A549 epithelial cells in complete culture medium at 36 ± 1 °C in 5% CO_2. Ensure the use of an appropriately sized culture vessel to have enough cells for subsequent steps (see step 1.10).

NOTE: One 75 cm² (T-75) flask is sufficient to seed two 24-well plates and subculture the cells.

1.3. **Two days before infection**, prepare a single 24-well plate.

1.4. Remove and discard the spent culture medium from the T-75 flask and wash the cells once with 10 mL of Dulbecco's phosphate-buffered saline (DPBS).

1.5. Add 5 mL of trypsin-EDTA and incubate the cells for 5 min at 36 ± 1 °C in 5% CO_2.

1.6. Add 5 mL of complete culture medium and transfer the cells into a tube.

1.7. Centrifuge the cells for 5 min at 300 × *g*.

1.8. Discard the supernatant and resuspend the cells in 10 mL of fresh complete culture medium.

1.9. Count the cells with an automatic cell counter (or a counting chamber).

1.10. Dilute the cells in complete culture medium to prepare 30 mL of cell suspension at a concentration of 2.0×10^5 cells/mL.

1.11. Add 1 mL of the cell suspension to each well of a 24-well plate, which corresponds to a cell density of approximately 1.0×10^5 cell/cm² for a well area of 2 cm².

1.12. Incubate the cells for 48 h at 36 ± 1 °C in 5% CO_2 until they reach 100% confluence.

NOTE: In addition to the conditions to be tested, three wells should be reserved for cell counting on the day of infection (see step 3.1.4). According to the number of conditions to be

tested, Up to two 24-well plates can be prepared simultaneously. Volumes indicated in the protocol should be increased accordingly.

2. Culture of *S. aureus* strains

2.1. **Two days before infection**, prepare complete infection medium with DMEM high glucose without phenol red, supplemented with 10% FBS without antibiotics.

2.2. Thaw *S. aureus* strains to be tested on agar plates.

2.3. Incubate the agar plates for 18–24 h at 36 ± 1 °C.

2.4. **The day before inoculation**, inoculate one colony of the *S. aureus* strain to be tested in 10 mL of complete infection medium.

2.5. Incubate the bacteria for 18–24 h at 36 ± 1 °C with shaking at 160 rpm. Use 50 mL tubes held at 45° to avoid the bacteria settling.

NOTE: Before starting with a new strain, it is recommended to verify its lysostaphin susceptibility in the same conditions of culture that will be used for further experiments (media, bacterial loads, and lysostaphin concentration and incubation time). It is also important to determine the bacterial load corresponding to an OD_{600nm} of 0.5 because it could vary slightly from one strain to another. Culture conditions of bacterial strains could be adapted according to the experimental aim.

3. Infection assay with *S. aureus*

3.1. Determination of cell density and viability

3.1.1. Remove and discard the spent culture medium from the three wells dedicated for counting A549 cells.

3.1.2. Add 1 mL of complete infection medium containing 5 µg/mL of Hoechst 33342 and 1 µg/mL of propidium iodide.

NOTE: Hoechst 33342 is a known mutagen and should be handled with care. Propidium iodide, a potential mutagen, must be handled with care and disposed of safely according to applicable regulations.

3.1.3. Incubate the cells for 30 min at 36 ± 1 °C in 5% CO_2.

3.1.4. Count the cell number and calculate the cell viability using a wield-field fluorescence microscope.

NOTE: If a fluorescence microscope is not available, the cell density and viability can be calculated with trypan blue staining by using a cell counting chamber.

3.2. Preparation of the bacterial suspension

3.2.1. Dispense 25 mL of complete infection medium in a tube and pre-warm at 36 ± 1 °C.

3.2.2. Adjust the *S. aureus* suspension to anOD_{600nm} of 0.5 in complete infection medium using a cell density meter.

3.2.3. Prepare 20 mL of bacterial suspension for cell inoculation by diluting the 0.5 OD_{600nm} in complete infection medium to achieve a multiplicity of infection (MOI) of 1 according to the number of cells per well.

NOTE: The MOI corresponds to the number of bacteria added per cell in each well. For example, to achieve an MOI of 1 with 1.0×10^6 cells per well, prepare a bacterial suspension at 2.0×10^6 CFU/mL so that 10^6 CFU can be added in a volume of 500 µL (see step 3.3.3). The MOI can be adjusted according to the cell types and bacterial strains to be tested.

3.2.4. Use an automatic spiral plater to determine the *S. aureus* load of the diluted bacterial suspension to be used for the cell inoculation step.

3.2.5. Incubate the agar plates for 18–24 h at 36 ± 1 °C.

3.2.6. The next day, count the number of colonies with a colony counter to calculate the accurate MOI for each strain tested.

NOTE: If no automatic spiral plater is available, the bacterial load could be determined by serial dilution on an agar plate. See the bacteriological analytical manual for details[14].

3.3. Cell inoculation

3.3.1. Observe every well of the 24-well plate by low magnification microscopy to ensure that the cells are healthy and growing as expected.

3.3.2. Remove and discard the spent cell culture medium from the 24-well plate.

3.3.3. Add 500 µL of the bacterial suspension for inoculation to each well with 100% confluent cells.

3.3.4. Incubate the cells for 2 h at 36 ± 1 °C and 5% CO_2.

NOTE: it is recommended to use three wells of the plate for each condition to be tested (triplicate) and to perform at least three independent experiments. The delay of incubation can be adapted according to the experimental aim.

3.4. Quantification of intracellular bacteria with improved enzyme protection assay (iEPA)

3.4.1. Prepare 7 mL of 4x lysis buffer with 3.5 mL of 2% Triton X-100 in sterile water and 3.5 mL of trypsin-EDTA.

3.4.2. Prepare a lysostaphin stock solution at 10 mg/mL in acetate buffer and aliquot 25 µL into cryovials. Store at -80 °C for up to 6 months.

3.4.3. Prepare 250 µL of a fresh lysostaphin working solution at 1 mg/mL by mixing 25 µL of the lysostaphin stock solution (10 mg/mL) and 225 µL of 0.1 M Tris-HCl. Store at 4 °C for up to 48 h.

3.4.4. Prepare 6.25 mL of complete infection medium supplemented with lysostaphin by adding 6 mL of complete infection medium to 250 µL of the lysostaphin working solution.

3.4.5. Add 250 µL of complete infection medium supplemented with lysostaphin into each well and gently agitate the plate by swiveling the plate by hand.

3.4.6. Incubate the cells for 1 h at 36 ± 1 °C in 5% CO_2 to let the lysostaphin kill the extracellular bacteria.

3.4.7. At the end of the incubation time, add 10 µL of proteinase K at 20 mg/mL into each well to inactivate the lysostaphin.

3.4.8. Incubate the cells for 2 min at room temperature.

3.4.9. Add 250 µL of 4x lysis buffer to lyse the cells by osmotic shock.

3.4.10. Incubate the cells for 10 min at 36 ± 1 °C.

3.4.11. Mix thoroughly by pipetting up and down ten times all over the bottom of the well to ensure that the cells are fully lysed and homogenized.

3.4.12. Use an automatic spiral plater to determine the *S. aureus* load of each well.

3.4.13. Incubate the agar plates for 18–24 h at 36 ± 1 °C.

3.4.14. The next day, count the number of colonies with a colony counter to calculate the intracellular *S. aureus* load of each well.

3.5. Measurement of intracellular efficacy of antimicrobial compounds with enzyme protection assay (EPA)

3.5.1. Prepare 25 mL of 1x lysis buffer with 3.125 mL of 2% Triton X-100 in sterile water, 6.25 mL of trypsin-EDTA, and 15.625 mL of sterile water.

3.5.2. Prepare 250 µL of a fresh lysostaphin working solution at 1 mg/mL by mixing 25 µL of a lysostaphin stock solution (10 mg/mL) and 225 µL of 0.1 M Tris-HCl.

3.5.3. Prepare 25 mL of complete infection medium supplemented with lysostaphin by adding 24.75 mL of complete infection medium to 250 µL of the lysostaphin working solution.

3.5.4. For each antimicrobial compound to be tested, prepare 3.1 mL of complete infection medium supplemented with lysostaphin and the antimicrobial compound at the concentration to be studied.

3.5.5. Remove and discard the spent cell culture medium from the 24-well plate.

3.5.6. Add 1 mL of complete infection medium supplemented with lysostaphin.

3.5.7. Incubate the cells for 1 h at 36 ± 1 °C in 5% CO_2 to let the lysostaphin kill the extracellular bacteria.

3.5.8. Remove and discard the medium supplemented with lysostaphin from the 24-well plate.

3.5.9. Fill three wells with 1 mL of medium supplemented with lysostaphin plus the antimicrobial compound to be tested.

3.5.10. Repeat step 3.5.9 for each antimicrobial compound to be tested.

3.5.11. For the control condition, fill three wells with 1 mL of medium supplemented with lysostaphin without any antimicrobial compound.

3.5.12. Incubate the cells for 24 h at 36 ± 1 °C in 5% CO_2.

3.5.13. At the end of the incubation period, remove and discard the spent medium and gently wash each well three times with sterile DPBS with $CaCl_2$ and $MgCl_2$.

3.5.14. Add 1 mL of 1x lysis buffer to each well to detach and lyse the cells by osmotic shock.

3.5.15. Incubate the cells for 10 min at 36 ± 1 °C.

3.5.16. Mix thoroughly by pipetting up and down ten times all over the well to ensure that the cells are fully lysed and homogenized.

3.5.17. Use an automatic spiral plater to determine the *S. aureus* load of each well.

3.5.18. Incubate the agar plates for 18–24 h at 36 ± 1 °C.

3.5.19. The next day, count the number of colonies with a colony counter to calculate the intracellular *S. aureus* load of each well.

NOTE: The intracellular activity of each antimicrobial compound should be calculated according to the bacterial load of the control condition. It is also important to check the cytotoxicity of all antimicrobial compounds to prove that the differences observed between the control and the compounds are not due to cell death.

REPRESENTATIVE RESULTS:

The results of *S. aureus* internalization by A549 epithelial cells are depicted in **Figure 1A**. A549 cells were inoculated with *S. aureus* SF8300 WT and SF8300 Δ*fnb*A/B, which lacks fibronectin-binding proteins A and B, at an MOI of 1 for 2 h. To destroy extracellular *S. aureus*, lysostaphin was added to the culture medium, and the cells were incubated for 1 h. Next, lysostaphin was either removed by washing for EPA or inactivated with proteinase K for iEPA. Then, the cells were disrupted in lysis buffer, and the bacterial load was quantified by culture. By using EPA, the mean intracellular loads were 4.46 and 0.49 Log CFU/mL for SF8300 WT and SF8300 Δ*fnb*A/B, respectively (**Figure 1A**, green bars). Using iEPA, the mean intracellular loads were 4.53 and 0.56 Log CFU/mL for SF8300 WT and SF8300 Δ*fnb*A/B, respectively (**Figure 1A**, red bars). It is interesting to note that both EPA and iEPA showed similar results, which can be explained by the ease of performing the washes when the cells are in good condition and because the *S. aureus*-induced cytotoxicity is very low in these experimental settings (data not shown).

The results of intracellular activity of vancomycin, rifampicin, and levofloxacin against *S. aureus* are depicted in **Figure 1B**. To measure the intracellular activity of these antibiotics, HaCaT cells were inoculated with *S. aureus* ATCC 29213 at an MOI of 1 for 2 h. The cells were incubated with lysostaphin, with or without the antimicrobial compounds to be tested, for 24 h. Next, lysostaphin and the antimicrobial compounds were removed by washing. The cells were disrupted in lysis buffer, and the bacterial load was quantified by culture. The mean intracellular loads were 4.57, 4.51, 3.03, and 2.91 log CFU/mL for control, vancomycin (50 µg/mL), rifampicin (7 µg/mL), and levofloxacin (10 µg/mL), respectively (**Figure 1B**).

FIGURE AND TABLE LEGENDS:

Figure 1: **Intracellular *Staphylococcus aureus* load in epithelial cells**. (**A**) Enzyme protection assay (green bars) and improved enzyme protection assay (red bars) in A549 cells infected with *S. aureus* SF8300 WT and Δ*fnb*A/B. (**B**) Intracellular activity of antimicrobial compounds in HaCaT cells infected with *S. aureus* ATCC 29213. Bars represent the mean values of three independent experiments performed in triplicate. Error bars represent the standard deviations. **** $p < 0.0001$. Abbreviations: Ctrl = control; cfu = colony-forming units.

DISCUSSION:

The assays described here are valuable for studying the extent of internalization and the intracellular survival of *S. aureus* in NPPCs, as well as the intracellular efficacy of antimicrobial compounds[6,15,16]. Some steps in both assay protocols can be critical. The health condition and the density of the cells must be perfectly controlled and consistent between independent experiments. The bacterial inoculum must be carefully standardized to obtain a real MOI close to the targeted theoretical MOI. In general, care must be taken not to detach any of the cells while pipetting. The washes to remove lysostaphin and antibiotics are critical steps in the EPA. The use of proteinase K has been found to improve this step when no antibiotic is used (see below). Last but not least, the cells should be fully detached in each well and thoroughly homogenized after the incubation with the lysis buffer to reliably quantify the *S. aureus* intracellular load.

In some instances, issues may be encountered, and several points must be checked first. In case of a lack of reproducibility, it must be kept in mind that *S. aureus* can form clumps, making

quantification by absorbance inaccurate. The clumping of bacteria can be increased by centrifugation and washing steps if the culture medium is to be replaced (e.g., for eliminating a secreted protein). The bacterial suspension should be used rapidly because bacteria continue to grow at room temperature. The lysostaphin efficacy could decrease because of incorrect storage conditions, suboptimal pH for enzyme activity in the culture media, variability in the enzymatic activity between batches and providers, and lack of lysostaphin sensitivity of some strains in specific growth conditions. Phenol red could have a slight bacteriostatic effect, especially when the culture medium is relatively poor in nutrients compared to the typical broths used for growing bacteria. Thus, it is advisable to use a cell culture medium without phenol red, which also improves fluorescence microscopic observations by reducing the background noise.

Although this method is a valuable tool to study the intracellular fate of different strains, some limits of the method should be considered. The use of a very high MOI can overload the capability of internalization by NPPCs and level out the differences between the different strains tested. The extent of internalization of the most cytotoxic strains may be underestimated because lysostaphin (or antibiotics) rapidly destroys *S. aureus* that is released by damaged cells. Thus, experiments with extended durations (i.e., to study intracellular survival or intracellular activity of antibiotics) are easier to set up with strains with low cytotoxicity. Therefore, the incubation time and the MOI should be accurately adjusted according to the strain virulence, the cell type, and the experimental aim.

The method described here with the use of lysostaphin is more reliable than those based on gentamicin because, unlike lysostaphin, gentamicin tends to be internalized by host cells[13]. The other advantage is the possibility to inactivate the lysostaphin. Inhibition of lysostaphin activity was reported by Kim et al.[13] with the use of EDTA to chelate zinc ions or 1,10-phenanthroline; however, intensive washes are still required to remove the enzyme before plating of the bacteria. Here, proteinase K enables rapid inactivation of lysostaphin. We observed that cells tend to detach from the culture plate when they become heavily infected because of the multiplication of intracellular *S. aureus*. By skipping the final washing step, the iEPA method greatly simplified technical handling and enabled the recovery of the internalized bacteria in loosely adherent or already detached cells.

The more concentrated reagents and buffers used in iEPA also helped reduce pipetting effort and minimize the loss of cells. In addition, iEPA can be used with cells in suspension, as well as with organoids that are difficult to wash. In conclusion, enzyme protection assays enable the study of the extent of internalization and the intracellular fate of *S. aureus*, as well as the intracellular activity of antimicrobials drugs with different *in vitro* models. Improvements should be made to better characterize the relationship between internalization and cytotoxicity to better appreciate the importance of developing drugs capable of reaching *S. aureus* inside the cell.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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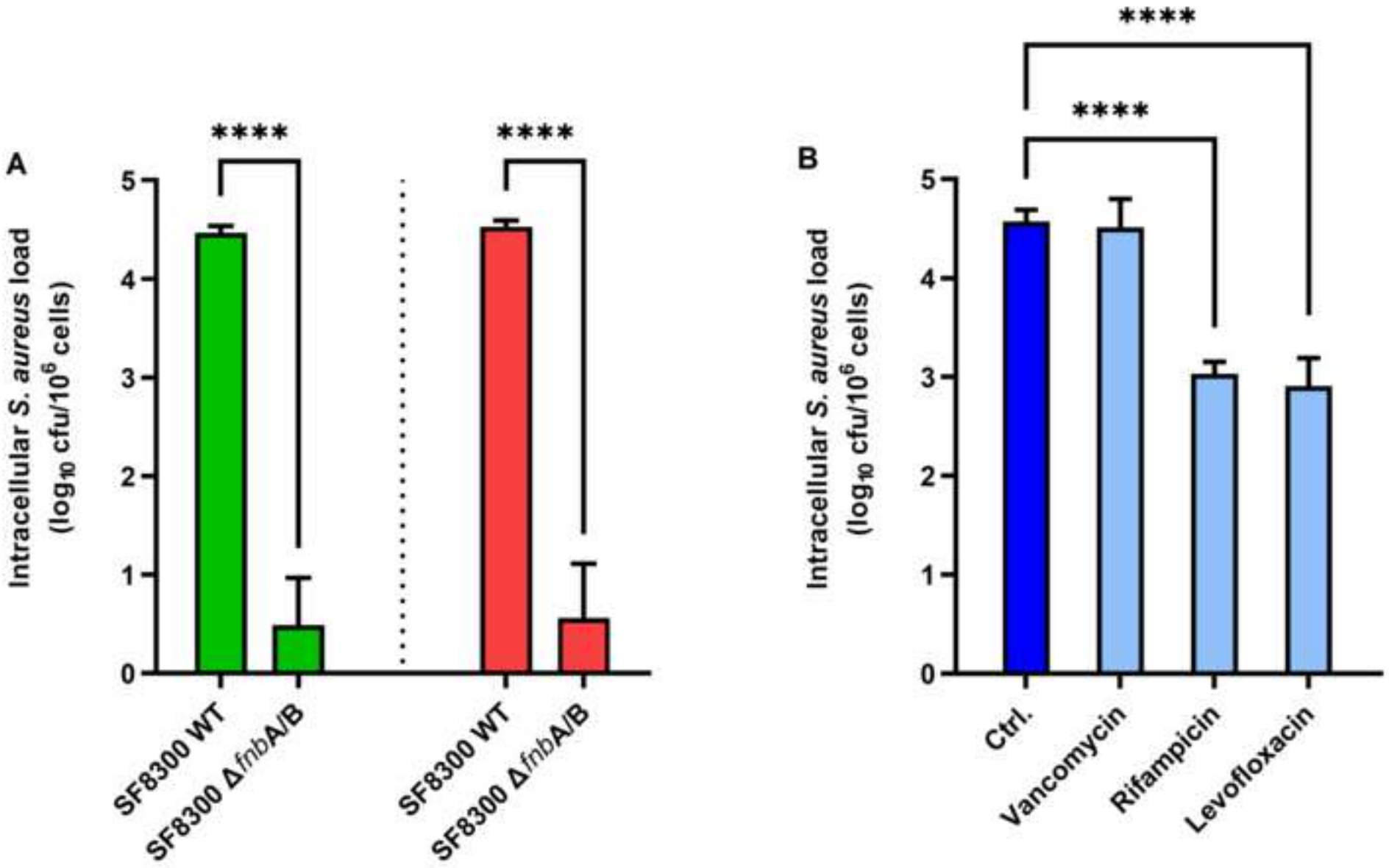
Figure 1



Dear Editor,

We would like to thank you for the editorial comments. We also thank the reviewers for the quality of their work which helped us to improve the manuscript.
We took into account all the editorial comments. We provide below a point-by-point reply to the reviewer's comments. As requested, additional experiments were performed to answer some specific questions.
A revised version of the manuscript is also provided. The modifications made in the text appears in blue. The script for the video is highlighted in yellow.

Best wishes,

On behalf all the authors,

Paul Verhoeven

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have double-checked all abbreviation add we added the definition of PBS and MOI. The manuscript has been carefully proofread.

2. Please provide an email address for each author.

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In the introduction, please mention the advantages over alternative techniques with applicable references to previous studies. In particular, please discuss (briefly) the lysostaphin protection assay so that you can then present your improvement. Please also consider including a description of the context of the technique in the wider body of literature.

Response: As suggested, a paragraph was added in the introduction.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: OK. Done.

4. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: OK. Done.

5. Don’t include multiple notes between steps.

Response: OK. Done.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: OK. Details have been added throughout the protocol. Paragraph 3.1 to 3.4 included will be used to generate the script of the video.

7. 3.1.2 and 3.2.3: Please mention what MOI was used in this paper. It will also help in filming.

Response: OK. A MOI of 1 was chosen.

8. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed. Please do not highlight notes.

Response: OK. Paragraph 3.1 to 3.4 included are highlighted.

9. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. The legends should appear only in the Figure and Table Legends section after the Representative Results.

Response: OK. Done.

10. Please sort the Materials Table alphabetically by the name of the material.

Response: OK. Done.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript by Rigaill et al. describes an improved protocol to quantify *S. aureus* internalization within non-phagocytic cells and the intracellular efficiency of antimicrobial compounds. The key finding resides in the usage of lysostaphin, resulting in the elimination of the washing steps. This provides a crucial improvement of the protocol since heavily infected cells easily detach from their support when washed. This protocol will be of great interest for the microbiology and cell biology community since it will be readily applicable to a large number of bacterial pathogens and cell types.

Major Concerns:

none

Minor Concerns:

a few typos and grammatical errors.

Response: OK. We have carefully corrected the manuscript.

Reviewer #2:

Manuscript Summary:

The manuscript by Rigaill et. al. entitled "Improved lysostaphin protection assay to study Staphylococcus aureus internalization and intracellular efficacy of antimicrobial compounds" describes the existing protocol Enzyme protection assay (EPA), and an improvement in published EPA in Infection and immunity (IAI, 2019) (doi: 10.1128/IAI.00119-19, reference 12 of the manuscript). This comprehensive IAI paper raised the longstanding drawbacks associated with gentamicin protection assay (GPA), and advocated the use of lysostaphin in Enzyme protection assay (EPA) for the precise enumeration of intracellular Staphylococcus. During GPA, the gentamicin entered inside the host cells, and kills the intracellular bacteria, while IAI paper conclusively proved that lysostaphin (EPA) does not enter the host cells; therefore, EPA could precisely determine the intracellular bacteria. Rigaill et al. rename the same protocol as lysostaphin protection assay (LPA), and improved lysostaphin protection assay (ILPA) which will be somewhat confusing for the readers to follow. I, therefore, suggest the authors to keep the same name Enzyme protection assay (EPA), and add their precise improvement to the IAI method published as EPA. The improvement to add of Proteinase K to deactivate the lysostaphin is a welcome step suggested in this manuscript. In addition, Rigaill et. al. also determined the efficacy of 3 different antibiotics to kill the intracellular bacteria. The protocol needs to provide several specific details and the background of the w steps before publication. Please see the minor/major comments below:

Major/minor Concerns:
1. In title of the paper, the lysostaphin protection assay (LPA) or improved lysostaphin protection assay (ILPA) should be renamed as improved enzyme protection assay (iEPA) for the readers to easily follow the one additional improvement (deactivation of lysostaphin using proteinase K instead of EDTA or 1,10-phenanthroline, Fig S12 of Kim et al. 2019, IAI) in the existing EPA published in IAI (doi: 10.1128/IAI.00119-19, reference 12 of the manuscript). Therefore, please replace the LPA or ILPA to iEPA throughout the manuscript.

Response: We agree with this comment. Corrections were done.

2. In abstract, line no. 31 "NPPCs are grown in multi-well plate until confluence" What was the level of confluency because the level of confluency of mammalian cells changes the property of the cells as the high-cell density/confluency leads to contact inhibition of proliferation (CIP), a fundamental property whereby normal cells cease proliferation and cell division when they occupy all the space allocated to them upon reaching confluence (https://doi.org/10.1038/2131102a0). Does the cell confluency was maintained through in all the experiments? If not, please specify the level of confluency maintained for specific experiments.

Response: Thank you for this comment. We have specified the level of confluency in the protocol.

3. In abstract the line number 31-32, please specify the Staphylococcus culture media instead of "culture broth"

Response: We agree with this comment. The sentence was modified.

4. The last paragraph of introduction needs to be improved in context to methods applied so far to enumerate the intracellular bacteria. Proctor et. al. (1982) used the lysostaphin for the first time to kill the surface bound bacteria. However, the GPA was used for the more than 4 decades until the comprehensive study of the drawback of the GPA was demonstrated for Staphylococcus aureus, and the benefits of EPA employing lysostaphin was elucidated by Kim et al. 2019 (IAI). Therefore, explaining the GPA, and its drawback is necessary for the wide adaptability of the EPA in the field of infectious disease. Thus, the introduction should address these points such as why the most used gentamicin protection assay (GPA) needs to be changed to enzyme protection assay (EPA)? What are the advantages of EPA? What is that precise improvement in this manuscript under review brought to the existing EPA/LPA method to clearly identify the importance of improved enzyme protection assay (iEPA).

Response: As recommended, a paragraph was added in the introduction. LPA/ILPA was replaced throughout the manuscript.

5. In Protocol, section 1, "why only T75 flask not the culture plate?" "what was the level of cell confluency? Please replace the "trypsin cells" with "Trypsinzed cells" "please replace 300g with 300 ×g" "please replace the 1.105 with 1.0×105" and again in 1.7 mention the level of cell confluency

Response: Thank you for these comments.
Sentence about the T-75 was modified (see 1.2).
Few steps were added to the protocol to clarify.
Level of confluency was added (see 1.12)
All other comments have been taken in account.

6. In protocol section 2, why the *S. aureus* needs to be grown in DMEM but not in bacterial media? Please provide the rationale regarding this. The FBS itself hampers the growth, biofilm and adhesion properties of the *S. aureus* (10.1016/j.micpath.2010.07.005), why is the FBS required for the staphylococcal growth? Please justify.

Response: Thank for this comment.
We usually use cell culture media to keep culture conditions stable before and during the infection. We performed additional experiments (see results point 21) and we read carefully the recommended article above (10.1016/j.micpath.2010.07.005). It seems that 10% FBS does not inhibit bacterial growth but have an impact in the biofilm formation.
In our experience, we believe that there is no more reason to use a culture broth than a cell culture media. Moreover, there are probably some advantage to try to mimic conditions that could be encounter by *S. aureus in vivo*, which are probably far from those in a culture broth.
A sentence was added at the end of the note after step 2.5 to give the readers the opportunity to adapt their culture conditions.

7. In section 3.1.1, please define the infection media here with or without FBS.

Response: The composition of the complete infection media was defined at step 2.1.

8. In section 3.2.1, only microscopic observation is not enough, the cell suspension should be seeded in microtitre plate, wherein at least 2 to 3 wells should be a dedicated for the host cell counting to determine the MOI for 100% mammalian cells infection.

Response: Thank you for this comment. A new paragraph was added to specifically describe the cell count procedure (see new 3.1 step).
Before the infection step, we use low magnification microscopy only to verify the cell health and expected growth in every well of the plate.

9. In 3.2.2, "Eliminate the cell medium on 24-well plate" should be rephrased as "Eliminate the spent cell medium on 24-well plate"

Response: We agree with this comment. The sentence was modified (see 3.3.2).

10. In section 3.2.3, Right MOI? Please explain and define the MOI for the 100% host cells to get infected.

Response: Thank you for this comment. The preparation of the bacterial suspension was modified to clarify the protocol (See step 3.2). As suggested by the editor, we used a MOI of 1 in the protocol. In our hands, MOI of 1 is enough to study *S. aureus* internalisation and

intracellular survival with a cytotoxicity below to 5%. Even with a MOI of 100, it is almost impossible to get 100% infected cells in our models of epithelial cell (i.e. A549 or HaCaT cells) without inducing a strong cytotoxicity which lead to under evaluate the *S. aureus* intracellular load.

11. In section 3.2.3, Why is the infection time two 2h? any specific reason? 2 h is too long for infection, because significant number of host cells will start floating due to death, of course, the rate of bacterial internalization as well as their death depends on the type of cells used. Please explain and give the rationale for 2h of infection time.

Response: In our setup, we did not observe a high cytotoxicity by using 2 hours of inoculation but we agree that the cytotoxicity can vary widely according to MOI, strains and cell types. We used 2 hours of incubation with bacteria to minimize the bias due to the delay of handling and pipetting steps between wells. In our hands, it could take up to 15-20 min to inoculate the whole 24 well plate with different strains and conditions.
A sentence was added to note after step 3.3.4 to consider this comment.

12. In section 3.3, ILPA, this is unnecessary and inconsistent acronym (LPA, ILPA for the same method)? As suggested earlier, use iEPA consistently throughout the manuscript.

Response: We agree with this comment. Corrections were done.

13. Line no. 136 and 137 seem unnecessary.

Response: OK. These lines were deleted.

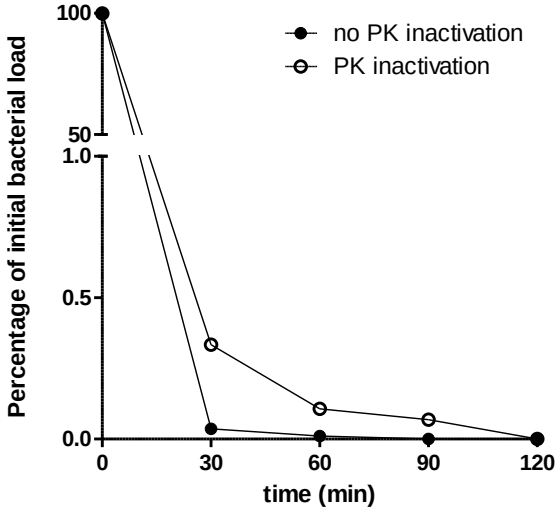
14. Please write the right protocol for the lysostaphin preparation and its long-term storage with glycerol.

Response: Thank you for this comment. Protocol of lysostaphin preparation was added to the manuscript (see 3.4.2 and 3.4.3). According to manufacturer's recommendations, stock solution in acetate buffer pH 4.6 can be stored at -20°C up to 6 months. We store stock solution at -80°C to avoid freeze/thaw cycles in -20°C freezer. This information was added to step 3.4.2.
Ambicin is lyophilized recombinant lysostaphin. Freezing at -80°C is recommended for long term storage of the lyophilized form. This information was added in the material table.

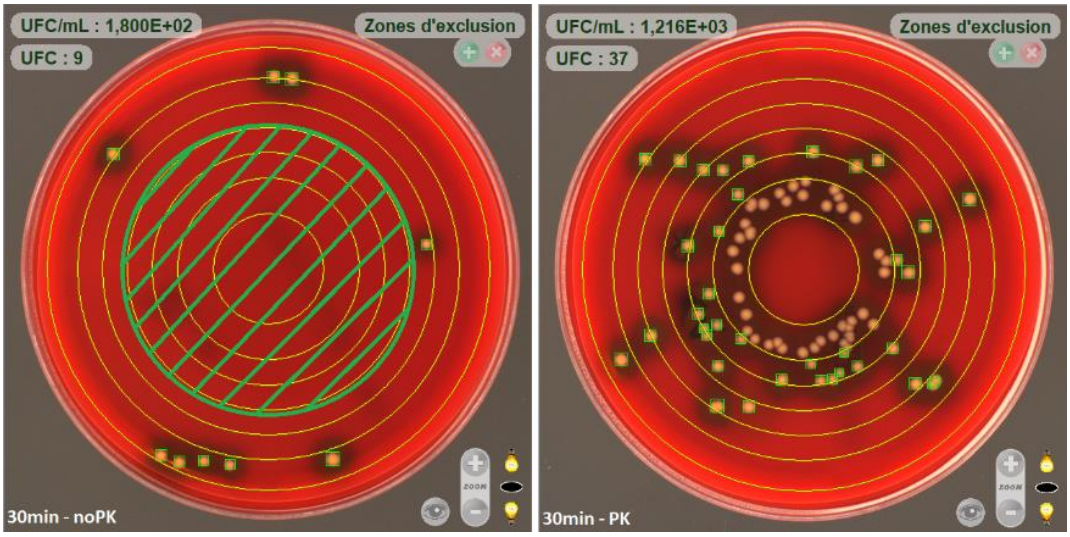
15. One-hour incubation is too long with lysostaphin as the over incubation may allow the entry of lysostaphin in to dead cells during infection. 10-15 min incubation is enough to kill the extracellular *S. aureus*. Please determine the killing-kinetics of lysostaphin in your protocol and provide the data.

Response: Thank you for this comment. We agree we the fact that lysostaphin can enter into dead cells. However, we believe that the situation is very complex. First, in one hand, *S. aureus* can be rapidly relapsed by dead cells and then be destroyed by lysostaphin. In the other hand, we also experienced that *S. aureus* can survive to lysostaphin within cell debris.

Thus, the time of incubation is not the only parameter that can impact the lysostaphin activity.
As requested, we performed killing-kinetics of lysostaphin (see result below):
After 30 minutes of incubation, the initial bacterial load (3,12 x10^5 UFC/ml) decrease by 99.5% and continue to decrease by 99.9% after 60 minutes of incubation, which plaid for an incubation time of 60 minutes. It is important to note that these results were obtained by inactivating the lysostaphin with PK just prior to plate the bacterial suspension on agar plate (open circles). As shown on agar plate, we evidenced that lysostaphin continues to kill *S. aureus* onto agar plate which leads to overestimate the lysostaphin efficacy (close circles). The dashed zone highlights the area where the lysostaphin keep is activity onto agar. Moreover, it was demonstrated by kim *et al.* in 2019 (doi: 10.1128/IAI.00119-19) that "lysostaphin could not be detected inside the host cells" after 30 minutes of incubation in RAW264.7 macrophages. We believe that is unlikely that lysostaphin can enter NPPCs (which have a lower micropinocytosis than macrophages) within 1 hour. Thus, we decided to keep an incubation period of 1 hour for lysostaphin.



S. aureus load according to the incubation period with lysostaphin. Lysostaphine was inactivated with proteinase K (PK) (open circles) or not (close circles).



Spiral plating without lysostaphin inactivation (left) and with lysostaphin inactivation (right).

16. 2% Triton X-100 for the osmotic burst of mammalian cells are too high? Please cite an appropriate paper.

Response: We agree with this comment. We are sorry for the confusion. In fact, the final concentration of Triton X-100 is 0.25% for both protocol:

- For iEPA, the lysis buffer is at 4X concentrated and a 250 µl-volume is added into the well containing 750 µl of media (final concentration 0.25%)
- For EPA, 1 ml of the lysis buffer at 1X concentration is added to the empty well

The preparation of 4X and 1X lysis buffer was clarified in the two parts of the protocol (see 3.4.1 for iEPA and 3.5.1 for EPA)

17. In 3.4, please replace the LPA with iEPA.

Response: OK. Done.

18. In NOTE (line no.182-185), please replace the 3.5.5 with right section as there is no 3.5.5?

Response: Thank you. We made the correction.

19. In discussion, line no 225, "the internalization rate" should be replaced with "internalized bacteria" as there is no time-dependent kinetic study to determine the rate.

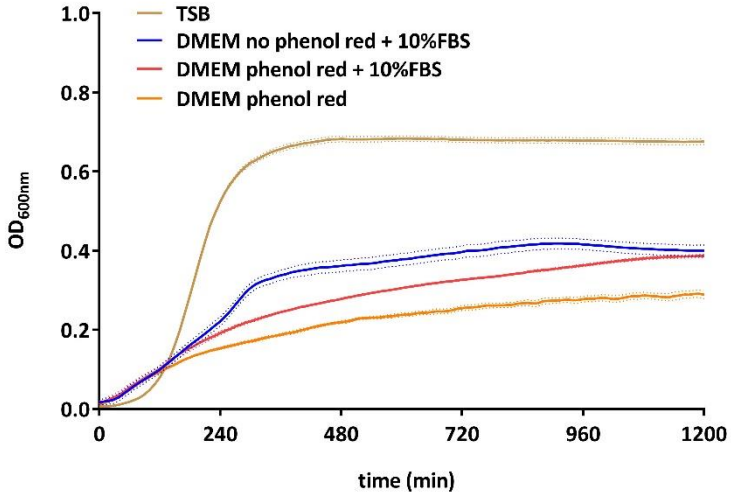
Response: We agree with this comment. The word “rate” was no longer used in the manuscript.

20. In discussion, "Bacterial suspension should be use rapidly and clumps could be dissociated with the help of an ultrasound bath. Alternatively, the inoculum can be prepared in advance and stored at +4°C for 24 hours or frozen at -20°C for several months but the impact on the metabolism of the bacteria must be taken into consideration." Clumping can be resolved by the hard vertexing or by repeated passing the bacteria through syringe, or by ultrasonic wave. However, the alternative method suggested is irrelevant pathophysiological conditions, and thus, should be removed from the manuscript.

Response: OK. The sentence was deleted.

21. In discussion, line no. 246-247, where is data showing phenol red is bacteriostatic? Author should also check and provide the growth inhibitory effect FBS on Staphylococcus aureus.

Response: As requested, we carried out additional experiments. As mentioned in response to point 6, the addition of 10% FBS do not reduce the growth of *S. aureus* SF8300 strain. We have noticed in the past that bacteria grow more slowly in the presence of phenol red using RPMI1640 culture medium. Phenol red seems to have little or no effect on *S. aureus* growth with DMEM.
The sentence in the discussion was modified (see lines 364-365).



Growth curves of *S. aureus* SF8300 (Tecan Infinite M200).

22. In discussion, line no. 255-256, please be careful in using "internalized bacteria" and "internalization rate" interchangeably, as there is no kinetics study of the rate of internalization. Therefore, please use the term "internalized bacteria" than that of "internalization rate".

Response: As mentioned above, the word “rate” was no longer used in the manuscript (see lines 346 and 374). A sentence was deleted (see line 372).

23. In discussion, line no. 263-264, Kim et. al 2019 used EDTA or 1,10-phenanthroline, Fig S12 of Kim et al. 2019, IAI). Please correct.

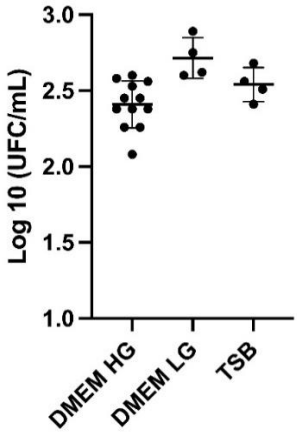
Response: Thank you. The sentence was corrected as proposed (see line 379).

Reviewer #3:

Manuscript Summary:
The authors described a modificiation of lysostaphin protection assay to improve the study of interanlization and intracellular efficacy of antimcirobial compounds. The manuscript is well written and easy to follow. Also the protocol is very clear. However, I have only few questions/comments (see minor concerns)

Minor Concerns:
The authors described the preparation of bacteria in DMEM high glucose withoud phenol red, a bacteriostatic medium. Is it possible the bacterial growth is affected because the microbes are under stress? Can this point affect the expression of adhesins necessary for interanlization?

Response: In our hands, we did not observe a difference of internalization level of *S. aureus* into HaCaT cells according to the media used for growing bacteria.



Intracellular *S. aureus* loads into HaCaT cells at 3 hours post infection according to the culture media used for bacteria (HG: high glucose; LG: low glucose; TSB: Tryptic soy broth).

Why is not possible to culture the bacteria in a growth media and resuspend in DMEM directly before infection?

Response: Theoretically, It is quite possible to grow *S. aureus* in TSB, centrifuge bacteria and resuspend them in DMEM for example. However, in our hand, washing bacteria induces a significant variability between experiment probably because it generates clumps that not always easy to fully dissociate. Thus, we are unwilling to recommend washing bacteria.
A sentence was added in the discussion (see lines 360-361).

Why the points 3.36 and 3.4.5 are different?

Response: We are sorry for the confusion. As mentioned in response to reviewer #2, the final concentration of Triton X-100 is 0.25% for both protocol:

- For iEPA, the lysis buffer is at 4X concentrated and a 250 µl-volume is added into the well containing 750 µl of media (final concentration 0.25%)
- For EPA, 1 ml of the lysis buffer at 1X concentration is added to the empty well

The preparation of 4X and 1X lysis buffer was clarified in both protocol (see 3.4.1 for iEPA and 3.5.1 for EPA)

Are you sure that the long incubation with lysostaphin do not alter the host cells?

Response: Thank you for this interesting comment. To our best knowledge, there is no data in the literature about the effect lysostaphin on mammalian cells. We cannot exclude that lysostaphin could modify (at least slightly) the host cell response. However, in our experience, lysostaphin is not cytotoxic for A549 and HaCaT cells.

The authors claim that this modified protocol improve the intranlization of bacteria. However, the fig. 1a does not show significant differences.

Response: Yes. We decided to show results demonstrating that the EPA and iEPA give similar result when cell health is good. As mentioned in the results section, iEPA is more reliable

when cell are heavily infected. The sentence was modified to clarify what we said (see lines 324-325).

Why the antibiotic treatment was done with other strains and cells?

Response: The journal ask to provide representative results. We work on different research projects using different strains and cell lines in the lab. To be honest, we have used results that are available in our research group.