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Date: 06.10.2021

Reviewer's Report on the Doctoral Thesis

High-throughput and precise methods for bacteria counting, identification and antibiotic susceptibility testing

by Natalia Pacocha

The miniaturization of analytical systems is a very active area in diagnostics in order to achieve fast and reliable detection for the purpose of applying those methods to the point-of-care/need. In this context, Natalia Pacocha's dissertation project aimed to develop droplet-based assay for miniaturised total analysis systems, in which she detected and enumerated bacteria cells, and tested the antibiotic susceptibility in single and mixed bacterial cultures in a high-throughput format. Since there are currently no miniaturised methods for reliable, sensitive and less laborious detection of heteroresistance, the premise of the thesis work is to demonstrate that microfluidics can be employed at the single-cell analysis level in high-throughput systems to detect heteroresistance to antibiotics.

To describe the background of the work, the state-of-the-art in antibiotics, laboratory testing, droplet microfluidics, and the capabilities of microfluidic miniaturisation for advancing point-of-care diagnostics is comprehensively summarized.

Subsequently, the thesis reports on Natalia Pacocha's experimental work to optimize droplet digital CFU and species quantification by using direct droplet digital PCR of various species. Next, label-free high-throughput quantification of single and mixed bacteria cultures is documented, as well as the detection of mixed bacterial populations. All of these methods are subsequently applied for antibiotic susceptibility testing under various conditions.

An impressive result is that the newly introduced method of droplet digital CFU counting reduces the time required for detection by a factor of three; a dramatic reduction compared to the tedious classical plate counting method. Droplet digital CFU counting is based on the use of a fluorescent reporter. In another outstanding development in Natalia Pacocha's work, this limitation is circumvented by using scattered light and autofluorescence detection for label-free detection of bacterial cells in droplets with remarkable resolution at a frequency of 1,200 droplets/second.

The results obtained are interpreted and discussed in light of previously published findings. In particular, the potential use of direct digital droplet PCR to discriminate between closely related species and heteroresistance in polymicrobial infections of patient samples is considered.

I do have several questions:

pg. 43 "Droplet microfluidics allows for high-throughput and faster antibiotic susceptibility testing comparing to standard AST methods. Also, as described in the previous chapter it brings essential innovations in the testing of bacteria behavior in the presence of antibiotics at the single-cell level."

- What do you consider the innovation of droplet microfluidics that your approach brings to the table to detect heterogeneous resistance?
- How do you think it can be integrated into either (a) clinical workflow OR (b) point-of-care diagnostics?
- How do you think does the inoculum effect play a role in single cell susceptibility testing? What can you conclude if you know that a single cell is susceptible to an antibiotic?

pp. 65 "Direct digital droplet PCR technology"

- Why not using isothermal DNA amplification techniques, such as recombinase polymerase amplification (RPA) or Loop-mediated isothermal amplification (LAMP)?
- What is the sensitivity and the specificity in your dddPCR technique? Can you distinguish between different concentrations?
- What is a **“very”** fresh bacterial culture (pp. 66)? What is your concern in detecting dead bacteria (metabolically?)

- *sodA* can be used similar to 16S rRNA gene for genotyping. What is the reason to choose *sodA* instead of using primers for 16S rRNA?
- Maybe I missed this point: Did the fluorescent probes have different signals for *S. aureus*, *S. capitis* and *S. epidermidis*, so you could use them to detect various species in parallel?

pp. 70 "Label-free High-Throughput density measurements in nanoliter droplets"

- You used high-density overnight cultures of bacteria, why weren't you concerned with dead cells here?
- pg. 76: "*S. aureus* SH 1000 tends to form clumps", I would argue that most non-flagellated bacteria do form clumps while dividing, especially while being captured in a droplet?
- How would phenotypic changes in bacteria morphology be distinguished in your label-free approach? What is the accuracy of selecting for example rod from spherical shape and how about a mixture of say *E. coli* and *Vibrio cholerae* OR *Caulobacter crescentus*?
- I do understand that you would want to measure the presence of bacteria using light scattering without a label. But in your results you describe that you need at least 400 CFU/droplet to get reliable readouts. With this in mind, would you prefer to measure dddPCR **OR** HT label-free detection?
- Where is the autofluorescence coming from? And why not using *P. aeruginosa* which is really autofluorescent (*P. aeruginosa* expresses a fluorescent siderophore: pyoverdine).
- Why do you think, you weren't able to detect most of the selected Gram-positive bacteria by autofluorescence? Do you think that morphology/geometry of rod-shaped Gram-negative vs. spherical/coccus-shape Gram-positive (and their size difference) is the difference for detecting them? Did you try *B. subtilis*

In summary, the dissertation project is impressively distinguished by its level of technical and scientific innovation. The concepts studied have been very carefully elaborated, and the reported results are supported by state-of-the-art analytical experiments. The data obtained are critically discussed and interpreted, demonstrating a sound knowledge of the technical and theoretical background. The conclusions drawn are scientifically sound, and the dissertation is well written and thoughtfully organized.

In my opinion, the examined work meets all the requirements for a doctoral thesis. The thesis is ready to be defended orally before the relevant committee.

Dr. Lars Renner