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Simple method for correct enumeration of *Staphylococcus aureus*

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ABSTRACT

Optical density (OD) measurement is applied universally to estimate cell numbers of microorganisms growing in liquid cultures. It is a fast and reliable method but is based on the assumption that the bacteria grow as single cells of equal size and that the cells are dispersed evenly in the liquid culture. When grown in such liquid cultures, the human pathogen *Staphylococcus aureus* is characterized by its aggregation of single cells into clusters of variable size. Here, we show that aggregation during growth in the laboratory standard medium tryptic soy broth (TSB) is common among clinical and laboratory *S. aureus* isolates and that aggregation may introduce significant bias when applying standard enumeration methods on *S. aureus* growing in laboratory batch cultures.
We provide a simple and efficient sonication procedure, which can be applied prior to optical density measurements to give an accurate estimate of cellular numbers in liquid cultures of *S. aureus* regardless of the aggregation level of the given strain. We further show that the sonication procedure is applicable for accurate determination of cell numbers using agar plate counting of aggregating strains.

1. Introduction

The Greek word staphulē can be translated into 'cluster of grapes' and has been used to name the genus of *Staphylococcus* due to its characteristic of growing in clusters composed of aggregated cells (Foster, 1996). The aggregates vary in size from relatively small clusters of 10–20 cells to large millimetre size aggregates visible to the naked eye (Haaber et al., 2012). *Staphylococcus aureus* is a human pathogen that causes a wide range of diseases ranging from benign skin infections to life-threatening conditions such as bacteraemia, infective endocarditis, implant-associated infections and chronic infections (Lowy, 1998). Contributing to *S. aureus* pathogenicity is its ability to form biofilms where single cells are aggregated and embedded in a protective matrix that may or may not be adhered to a surface (Marrie et al., 1982; Bjarnsholt et al., 2013). In fact biofilm formation is very common *in vivo* (Bjarnsholt et al., 2013) and is also observed when *S. aureus* is grown in laboratory vessels (Cassat et al., 2014; Walker and Horswill, 2012). In addition, *S. aureus* efficiently aggregates in human plasma, a condition that is linked to virulence (McAdow et al., 2011) and it may form planktonic aggregates that protect the cells against antibiotics when propagated in laboratory media (Haaber et al., 2012). Aggregation of cells during laboratory growth is not unique for *S. aureus* but has been observed for other bacterial pathogens such as *Pseudomonas aeruginosa* (Alhede et al., 2011; Schleheck et al., 2009), *Listeria monocytogenes* (Travier et al., 2013), *Neisseria gonorrhoeae* (Penn et al., 1980) and *Campylobacter jejuni* (Mahdavi et al., 2014).

Correct enumeration of cell numbers present in a given population at a given time is crucial for most applications related to growth of microorganisms. The two most common methods used for bacterial enumeration are optical density, which measures turbidity of a bacterial suspension as an indirect measure of the cell density of the culture (Monod, 1949; Koch, 1970) and plating of bacterial culture on agar plates as a direct determination of viable colony forming units (CFU) in the culture (Monod, 1949). However, a requirement for both methods is that the cells in the liquid culture are evenly distributed (Monod, 1949). Optical density measurement is based on light scattering by the cells in the liquid and this is correlated to cell numbers under the assumption that the cells are evenly distributed and of equal size. If this assumption is not met for example due to aggregation, optical density measurements will underestimate the actual number of cells in the liquid (Koch, 1970). Other challenges that may potentially bias optical density measurement include viable but non-cultivable cells or non-viable but intact cells present in the culture as well as cells in the process of dividing. Flow cytometry may be used to detect such sub-populations of cells (Lehtinen et al., 2004).

Here we demonstrate how a simple ultrasound sonication protocol applied prior to optical density or CFU determination can be used to disrupt large planktonic aggregates of *S. aureus* otherwise recalcitrant to disruption by shear forces such as whirly mixing and how this method can be used to obtain accurate estimates of cell numbers in laboratory liquid cultures of *S. aureus*.

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Table 1
S. aureus strains used in the study.

	Aggregation level[a]	Other characteristics	Reference
SA564	1	Clinical low passage isolate	Foster (1996)
Newman	1–2	Widely used laboratory strain	Haaber et al. (2012)
15981	3	Clinical isolate	Lowy (1998)
8325-4	4	Widely used laboratory strain	Marrie et al. (1982)
JH628	5	MRSA pig isolate, not typeable by PGFE	Bjarnsholt et al. (2013)

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[a] See Materials and methods for evaluation of aggregation level.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The most relevant *S. aureus* strains used in this study are listed in Table 1. These 5 strains were chosen to represent different levels of aggregation (see below) and to cover both laboratory and recent clinical isolates. An additional 505 strains originating from blood stream infections and MRSA screening of healthy persons were isolated by Statens Serum Institut (Copenhagen, Denmark). These strains were characterized for their ability to aggregate and spa-typing was performed as previously described (Stegger et al., 2012).

All cultures were inoculated with 10^7 cells ml^{-1} to optical density (OD_{600}) of 0.01 in 5 ml tryptic soy broth (TSB; Oxoid) and incubated in 15 ml Falcon tubes in a shaking incubator (200 rpm, 37 °C) for 18 h unless otherwise noted. All cultures containing aggregates were handled using pipette tips with minimum 2.5 mm opening to prevent shearing of the aggregates.

2.2. Enumeration methods

Optical density measurements were performed using a wavelength of 600 nm (OD_{600}) in 1 ml (1 cm) plastic cuvettes using an Eppendorf BioPhotometer plus spectrophotometer. Dense samples were diluted to not exceed the linear range of the spectrophotometer ($OD_{600} < 0.7$). Colony forming units (CFU) per ml was determined by plating 100 μl aliquots of *S. aureus* cultures diluted in 0.9% NaCl on tryptic soy agar (TSA; Oxoid) plates. Dry weight measurements (DW) were determined by adding 1 ml of culture to pre-weighed Eppendorf tubes. Following pelleting (2 min, 13,200 rpm in a tabletop centrifuge (Eppendorf Mini Spin)) supernatant was removed and the tubes were left open in a heating block (50 °C) for 18 h after which period, the dry weight remained constant. The tubes were then weighed again and the dry weight ml^{-1} was calculated.

2.3. Scoring of aggregation level

To evaluate the extent of aggregation among clinical isolates of *S. aureus* we developed a qualitative assay of ranking strains according to their level of aggregation. Strains to be tested were inoculated from a single colony on TSA plates to OD_{600} of 0.01 and incubated as noted above. All tubes were then visually inspected against a source of light for aggregates visible to the naked eye and scored according to the following ranking method: 1) no visible aggregation, 2) tiny aggregates, barely visible to the naked eye, 3) small (up to about 1 mm) but easily visible aggregates, 4) large aggregates (up to 5 mm) sometimes forming strings but also turbid culture supernatant of suspended cells, 5) large aggregates (sometimes larger than 5 mm) and clear supernatant indicating that few cells were un-aggregated in the supernatant. The method was evaluated by comparing the results of 3 persons scoring the same 32 tubes containing cultures covering all 5 aggregation levels and found to be satisfactory for ranking strains according to aggregation level.

2.4. Cryo-SEM

Cryo-SEM was performed as previously described (Alhede et al., 2012). Briefly, aggregates were washed gently in water to avoid

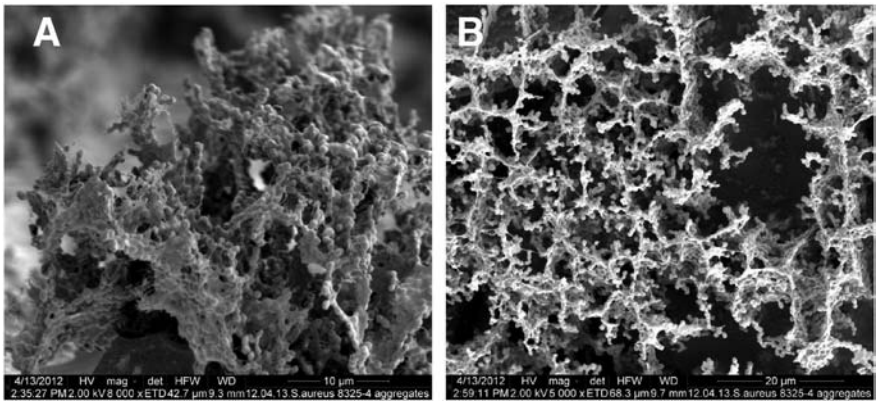

Fig. 1. Cryo-SEM pictures of 8325-4 aggregates. (A) Surface of aggregate showing matrix-embedded cells protruding in branch-like structures (magnification = 8000×). (B) Sponge-like interior structure of aggregates (magnification = 5000×). Scale bars included in lower right corner of pictures.