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Mechanical damage to Gram-negative bacteria by surface plating with the Drigalski-spatula technique

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Abstract

Colony counting by spreading bacterial suspensions on plating media by various techniques is of general concern. Comparative studies between hand plating (Drigalski-spatula technique) for different time intervals and spiral plating resulted in significant differences in colony counts. Lower counts of Gram-negative bacteria were obtained by using hand plating for more than 10 s, compared with short time hand plating (5 s) or spiral plating. Colony counting of Gram-positive bacteria showed no differences between both techniques. Further characterisation of Escherichia coli cells spread with the Drigalski-spatula technique by electron microscopy revealed a large number of damaged cells compared to control samples. The data clearly shows that the mechanical forces during hand plating are sufficient to damage E. coli cells.

Research Highlights

- Different surface plating techniques result in significant differences in the recovery of Gram-negative bacteria.
- Mechanical forces during hand plating are sufficient to damage cells of Gram-negative bacteria.
- Efflux of cell content is observed by EM after mechanical damage of *E. coli* cells.

1. Introduction

Common methods for the estimation of viable microorganisms involve the pour plate or surface plate techniques ([European Pharmacopoeia 6.0, 2008] and [ISO/TS 11133–2, 2003]). For surface plating cells can be either dispensed on the agar surface with a glass or plastic-spatula (Drigalski-spatula) or alternatively with a spiral plater. The spiral plater is a dispenser that deposits a liquid in a spiral pattern on the surface of a rotating agar plate (Gilchrist et al., 1973). Previous comparative studies of viable counting methods reveal comparable results for the spiral plating method with other techniques ([Donnelly et al., 1976] and [Greenwood et al., 1984]). Preliminary data from our labs has revealed significant differences in the recovery of Gram-negative bacteria when bacterial suspensions were spread by hand plating as compared to spiral plating. This prompted us to investigate this observation in more detail.

2. Materials and methods

2.1. Bacterial strains, culture conditions and strain-characterization

Escherichia coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 were freshly cultivated on nutrient agar, ready-to-use plates (lot CHB 844070, Oxoid, Basingstoke, UK) or on brain heart infusion broth (heipha Dr. Müller GmbH, Eppelheim, Germany) at 36±1 °C overnight. Suspensions of ca. 10¹⁰ CFU ml⁻¹ in saline were streaked onto Mueller Hinton agar with 5% sheep blood (Oxoid, ready-to-use plates) and onto Bile-Chrysoidin-Glycerol-Agar (SIFIN, Berlin, Germany). Pure colonies grew typically on the plating media.

2.2. Plating experiments

Diluted suspensions (40 µl) of *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *B. subtilis* ATCC 6633 and of *S. aureus* ATCC 6538 containing 10–100 CFU were plated on tryptic soy agar (ready to use plates of suppliers A, B and C) by hand plating (Drigalski-spatula) for 5, 10 and 20 s or by spiral plating using the uniform mode (Autoplate 4000, Spiral Biotech, Norwood, USA). Plates were subsequently incubated at 36±1 °C overnight. The grown colonies were counted.

2.3. Spreading cells on agar plates and cell preparation for electronmicroscopy

0.1 ml of the suspensions (ca. 10^{10} CFU ml⁻¹) described above were separately plated on nutrient agar (see above) by hand plating (Drigalski-spatula) until dry (ca. 15 s). 0.4 ml of saline solution was added immediately and the cells were gently resuspended using a Drigalski-spatula. To obtain sufficient cells, suspensions of at least four of such experiments were pooled and cells were pelleted at 2500 × *g* for 10 min. In parallel, 0.1 ml of the same bacterial suspensions was pipetted on nutrient agar plates without the spatula technique, washed away with 0.4 ml saline solution and pelleted as described above.

Cell pellets were resuspended in 0.2 ml fixation solution consisting of 20 g paraformaldehyde in 0.05 M HEPES, pH 7.2. Before use, the fixation solution was heated at 60 °C for 30 min for activation and subsequently diluted 1:10.

As a control the original cell suspensions (ca. 10^{10} cfu ml⁻¹) were fixed as described above.

2.4. Electronmicroscopy

For negative staining EM the fixed bacterial pellets were centrifuged again and adsorbed on Pioloform-F coated, carbon stabilised and glow-discharged copper grids. The adherent bacteria were washed three times with distilled water. After negative staining with 0.5% uranyl acetate (pH 4.0–4.5) the samples were analyzed using an electronmicroscope TEM 902 (Zeiss, Oberkochen, Germany) at 80 kV and their images digitised using a slow scan CCD-camera (Proscan, Scheuring, Germany).

2.5. Statistical analysis

Results shown in Table 1 and Table 2 are expressed as mean values with standard deviations. Pairwise comparisons were performed with Student's *t*-test (SPSS 13.0, Chicago, USA). Multiple group comparisons were performed with one-way ANOVA, followed by Dunnett-T3 test (SPSS 13.0, Chicago, USA) to compare multiple experimental groups pairwise. Significant levels were set at *P* values < 0.05.

3. Results and discussion

Table 1 shows the colony counts of *E. coli* ATCC 8739 obtained after hand plating (Drigalski-spatula technique) with different time intervals compared to spiral plating. Using the hand plating technique, an increasing plating time resulted in a decrease in plate counts. Maximal counts were obtained by hand plating for 5 s and by spiral plating. Hand plating for 20 s resulted in strongly reduced plate counts (Table 1). This was observed on ready to use plates from different suppliers (Table 2). Comparable data were obtained for *P. aeruginosa* ATCC 9027 (Table 1). In contrast, for the Gram-positive bacteria *B. subtilis* ATCC 6633 and *S. aureus* ATCC 6538, colony counts were in the same range regardless of the plating technique (Table 1). For *S. aureus* hand plating for 10 s and 20 s even resulted in a slight increase in colony counts. This is probably due to a disaggregation of *S. aureus* cell aggregates from the mechanical treatment.

It was hypothesized that Gram-negative bacteria are mechanically damaged by squeezing during hand plating. To demonstrate such squeezing, *E. coli* and *S. aureus* were dispensed on nutrient agar

with a Drigalski-spatula (for 15 s) or simply pipetted on the plates without further treatment. The cells were recovered from the plates by washing with saline and subsequently characterized by electronmicroscopy. Fig. 1b provides unequivocal evidence for the efflux of *E. coli* cell content when cells were dispensed with a Drigalski-spatula. No efflux of cell content was observed in the *E. coli* control samples (Fig. 1a and c) or with *S. aureus* (Fig. 2).

Obviously, *E. coli* ATCC 8739 cells and *P. aeruginosa* ATCC 9027 cells were squeezed by using the Drigalski-spatula technique. Hence, normal hand-pressure is sufficient to disrupt the cell wall of Gram-negative bacteria. The cell wall of *S. aureus* ATCC 6538 and of *B. subtilis* ATCC 6633 is stable enough to withstand this pressure. Hand plating as described here is a common method for colony counting by ISO-standards, EP and USP, as well as national standards ([European Pharmacopoeia 6.0, 2008] and [ISO/TS 11133–2, 2003]). The data provided here shows that the plating method can largely influence recovery rates. This issue clearly requires more attention and discussion by the committees.

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Tables and Figures

Table 1. Comparison of the recovery (CFU) of selected strains after hand plating and spiral plating on tryptic soy agar plates (mean values $\pm$ standard deviations of ten plates).

	Recovery (CFU)			
	Hand plating (plating time)			Spiral plating
	5 s	10 s	20 s	
<i>E. coli</i> ATCC 8739	97 $\pm$ 11	40 $\pm$ 13 ^a	22 $\pm$ 4 ^a	109 $\pm$ 9
<i>P. aeruginosa</i> ATCC 9027	66 $\pm$ 14	30 $\pm$ 9 ^a	11 $\pm$ 5 ^a	59 $\pm$ 9
<i>B. subtilis</i> ATCC 6633	34 $\pm$ 8	28 $\pm$ 8	29 $\pm$ 9	30 $\pm$ 5
<i>S. aureus</i> ATCC 6538	74 $\pm$ 6	77 $\pm$ 8	85 $\pm$ 7	88 $\pm$ 13

^a Recovery after handplating is significantly different ($P < 0.05$) as compared to spiral plating. Comparable results were obtained in three independent experiments using different batches of tryptic soy agar.

Table 2. Recovery rates of *E. coli* ATCC 8739 after hand plating with different time intervals on ready to use tryptic soy agar plates from different suppliers (mean values $\pm$ standard deviations of four plates).

Supplier ^a	Recovery rates (%) of <i>E. coli</i> ATCC 8739	
	5 s hand plating	20 s hand plating
Supplier A	93 $\pm$ 15	6 $\pm$ 1 ^b
Supplier B	72 $\pm$ 9	13 $\pm$ 6 ^b
Supplier C	110 $\pm$ 12	6 $\pm$ 2 ^b

^a Ready to use tryptic soy agar plates from different suppliers (A, B and C), packed in cellophane and stored at 8 °C, were tested at the end of shelf life. Columbia blood agar served as a reference medium using 5 s hand plating to spread the bacteria (= 100% value).

^b Recovery rates after 20 s handplating are significantly different ($P < 0.05$) from recovery rates after 5 s handplating.

Figure 1. Electronmicroscopic demonstration of the mechanical damage to *E. coli* ATCC 8739 cells. a) original sample, b) after hand plating, efflux of most cell content, formation of ghosts, c) control sample spotted on agar plates without treatment with a Drigalski-spatula, no efflux of cell content.

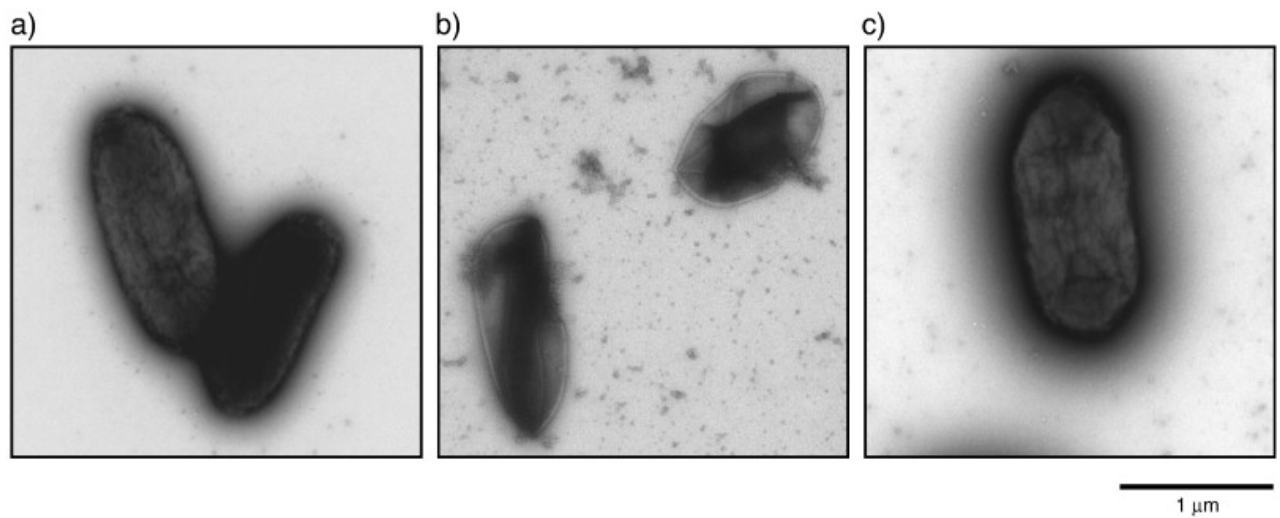


Figure 2. Demonstration of the mechanical stability of *S. aureus* ATCC 6538 cells by electronmicroscopy. a) original sample, b) after hand plating, no efflux of cell content, c) control sample spotted on agar plates without treatment with a Drigalski-spatula, no efflux of cell content.

