

## Validation of drop plate technique for bacterial enumeration by parametric and nonparametric tests

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### Abstract

Drop plate technique has a priority and preference compared with the spread plate procedure, because of less time, quantity of media, effort requirement, little incubator space, and less labor intensive. The objective of this research was to compare the accuracy and fidelity of drop plate method vs. spread plate method by parametric and nonparametric statistical tests. For bacterial enumeration by drop and spread plate methods, successive dilutions of second subculture of *Lactobacillus casei* and *Salmonella* Typhimurium were transferred to selective agar. The correlation of agreement between both methods was evaluated by using statistical proofs. Results showed that mean value (parametric unpaired *t*-test) comparison at 95 percent confidence level did not reject null hypothesis, which it meant that the equality of the mean data could not be ruled out. Nonparametric method was used because of approximately Gaussian pattern of data distribution. For this purpose, Mann-Whitney test (equivalent nonparametric *t*-test) was used. It meant that the equality of medians obtained from two methods were similar. Spearman's rho correlation coefficient (*r*) via both methods due to data distribution patterns for enumeration of *S. Typhimurium* and *L. casei* were 0.62 and 0.87, respectively; which represented moderately strong and strong relationship between two methods, respectively. Besides, there was a significant and strong positive correlation ( $p < 0.001$ ) between spread and drop plate procedures. Because of aforementioned reasons, the spread plate method can be replaced by drop plate method.

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### اعتبار سنجی روش قطره ای برای شمارش باکتریها با آزمونهای پارامتریک و غیر پارامتریک

#### چکیده

روش قطره‌ای، به علت صرفه‌جویی در زمان، مقدار محیط کشت مورد استفاده، سهولت انجام، نیاز کم به فضای انکوباتور در مقایسه با روش کشت سطحی دارای اولویت و رجحان می‌باشد. هدف از این تحقیق، مقایسه دقت و صحت روش قطره‌ای در مقایسه با روش کشت سطحی بوسیله آزمونهای آماری پارامتریک و غیر پارامتریک می‌باشد. برای شمارش باکتری‌ها با استفاده از روش قطره‌ای و کشت سطحی، از رقت‌های تهیه شده کشت دوم لاکتوباسیلوس کازنی و سالمونلا تیفی موریوم به محیط آگار انتخابی منتقل شد. همبستگی بین دو روش با استفاده از اثبات آماری مورد بررسی قرار گرفت. نتایج نشان داد که مقایسه میانگین با استفاده از آزمون *t* جفت نشده پارامتریک در سطح معنی‌داری ۹۵ درصد، فرضیه صفر را رد نمی‌کند، این بدان معنی است که برابری میانگین داده‌ها را نمی‌توان رد کرد. در این مطالعه، توزیع داده‌ها الگوی تقریباً نرمالی را نشان می‌داد، پس بهتر است از روش‌های غیر پارامتریک بهره جست. برای این منظور، از آزمون Mann-Whitney، معادل غیر پارامتریک آزمون *t* استفاده گردید. آتالیز داده‌ها حاکی از این بود که برابری میانه به دست آمده از دو روش باز اختلاف معنی‌داری با هم نداشتند. ضریب همبستگی Spearman "رو" برای هر دو روش با توجه به الگوی توزیع داده‌های شمارش باکتری سالمونلا تیفی موریوم و لاکتوباسیلوس کازنی به ترتیب ۰/۶۲ و ۰/۸۷ بود که بازگوکننده ارتباط نسبتاً قوی و قوی بین دو روش بود ( $p < ۰/۰۵$ ). علاوه بر این، یک همبستگی مثبت قوی و معنی‌دار بین روش کشت سطحی و قطره‌ای وجود داشت ( $p < ۰/۰۰۱$ ). بنابراین، با توجه به دلایل فوق، روش قطره‌ای را می‌توان جایگزین روش کشت سطحی نمود.

**واژه‌های کلیدی:** آزمونهای پارامتریک و غیر پارامتریک، روش کشت سطحی، روش کشت قطره‌ای، سالمونلا تیفی موریوم، لاکتوباسیلوس کازنی

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## Introduction

Some methods are found application in bacterial enumeration of milk, foods and cosmetics like microscopic scanning of stained organisms, measurement of various enzyme concentrations, spiral plate, quantitative real-time PCR, etc. Among conventional methods (pour plate, spread plate method) drop plate technique (DP) has been utilized routinely because of their less expenditure and equipment.

The DP is being exploited in numerous laboratories across the world. In spite of its widespread applicability, the DP has not been standardized.<sup>1,2</sup> Numerous papers have compared the accuracy of enumeration methods. Whereby there is not a standardized procedure for the size of the drops (10-30  $\mu\text{L}$  per drop), the number of replications, or the number of sectors (dilutions) used per plate.<sup>1</sup>

The DP method is a mixture of microbiological components and design components. The microbiological factors should have been tuned up for different bacteria. They include the bacterial species, strains and growth conditions e.g., media, agar, temperature, time.<sup>3</sup> In fact the DP has a miniaturized version of spread method (SP) method. In this investigation we tried to take advantage of parametric and nonparametric tests to show that SP could be replaced with DP as an alternative method.

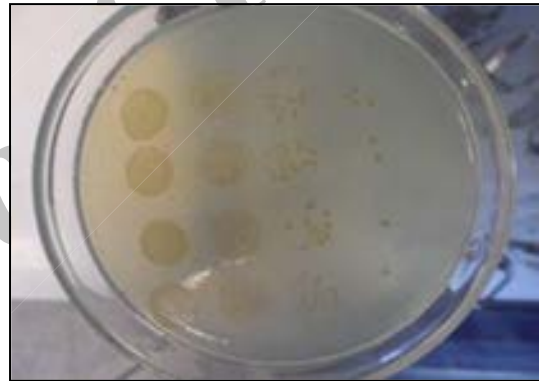
## Materials and Methods

Bacterial strains and culture condition *Lactobacillus casei* (ATCC 39392) and *Salmonella* Typhimurium LT<sub>2</sub> (ATCC 700792) were obtained from the culture collection of the Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

The bacteria were maintained in bead containing cryotubes at  $-70^\circ\text{C}$ . Working inoculums of bacteria were prepared by transferring a bead containing bacteria to the brain heart infusion (BHI) agar (Himedia, Mumbai, India) and/or De Mann, Rogosa and Sharp (MRS) agar (Merck, Darmstadt, Germany) slant tubes for *S. Typhimurium* and *L. casei*, respectively and incubated at  $37 \pm 1^\circ\text{C}$  for 24 hr. Prior to the empirical test, the bacteria were reactivated by two subcultures. For first subculture, three or four well-isolated colonies were touched with a sterile wire loop and suspended into 10 mL of Luria-Bertani (LB) broth (Biomark, Pune, India) and MRS broth (Merck, Darmstadt, Germany), for *S. Typhimurium* and *L. casei* respectively and incubated at  $37 \pm 1^\circ\text{C}$  for 24 hr with continuous agitation at 150 rpm. Subsequently, a second subculture was prepared and incubated for 20 hr at  $37 \pm 1^\circ\text{C}$  as well. Bacterial suspensions adjusted to approximately desired  $\log_{10}$  CFU  $\text{mL}^{-1}$  by ultraviolet-visible spectrophotometry at 600 nm then by using standard serial 10-fold dilution in buffered peptone water (Merck, Germany) and eventually transferred 10 and 100  $\mu\text{L}$  for drop and spread plating on bismuth sulfite agar (BSA) (Merck, Darmstadt, Germany) and MRS agar, for *S.*

*Typhimurium* and *L. casei*, respectively. The drops were absorbed to agar in less than half an hour. After the drops on the agar absorbed, the plates were incubated at inverted positions.<sup>2</sup> Enumeration of *S. Typhimurium* viable cells were done after 17-20 hr at  $30 \pm 1^\circ\text{C}$  in aerobic incubation and for *L. casei* after 48 hr at  $37 \pm 1^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere.<sup>4</sup> At least 3 to 30 colonies grew from 10  $\mu\text{L}$  of drop and 30 to 300 CFU per 100  $\mu\text{L}$  of SP as a confidence technique were chosen to count by using colony counter Funke-Gerber GMBH, Nr. 2774, Berlin/Munchen (Fig. 1). We averaged the total count of CFU over all at least 3 drops at the countable dilution. Finally, the total count was scaled up and the viable cell counts were expressed as CFU  $\text{mL}^{-1}$ .<sup>5</sup>

**Statistical analysis.** For analyses and graphical presentations, Graphpad prism<sup>®</sup> Software (version 5.04, San Diego, CA, USA) and MINITAB (version 16/2/0, Minitab Inc, State College, Pa, USA) were used. Each dilution was plated in duplicate with four drops per plate. A significant difference was considered as  $p < 0.05$ .



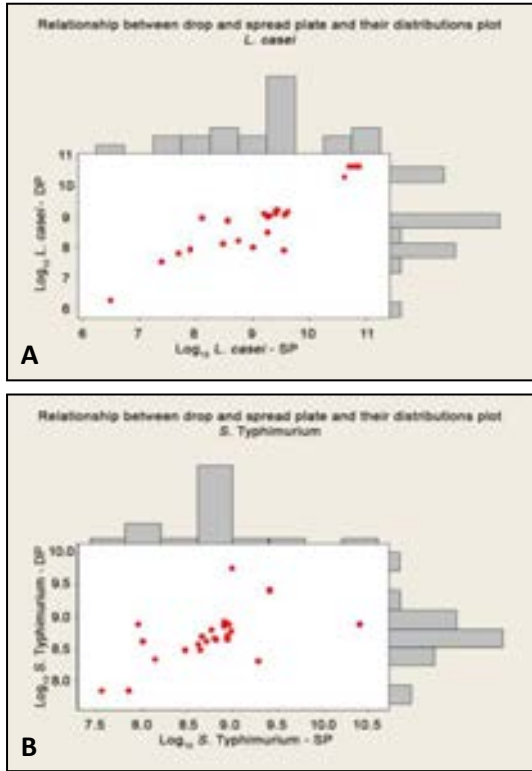
**Fig. 1.** The DP method for enumeration of *S. Typhimurium*. The dose of spots from left to right corresponded to 4, 5, 6, 7  $\log_{10}$  *S. Typhimurium*.

## Results

The distribution histogram of data represent approximate a Gaussian distribution. Relationship between DP and SP methods and their distributions among aforementioned bacteria were shown in marginal plots (Fig. 2).

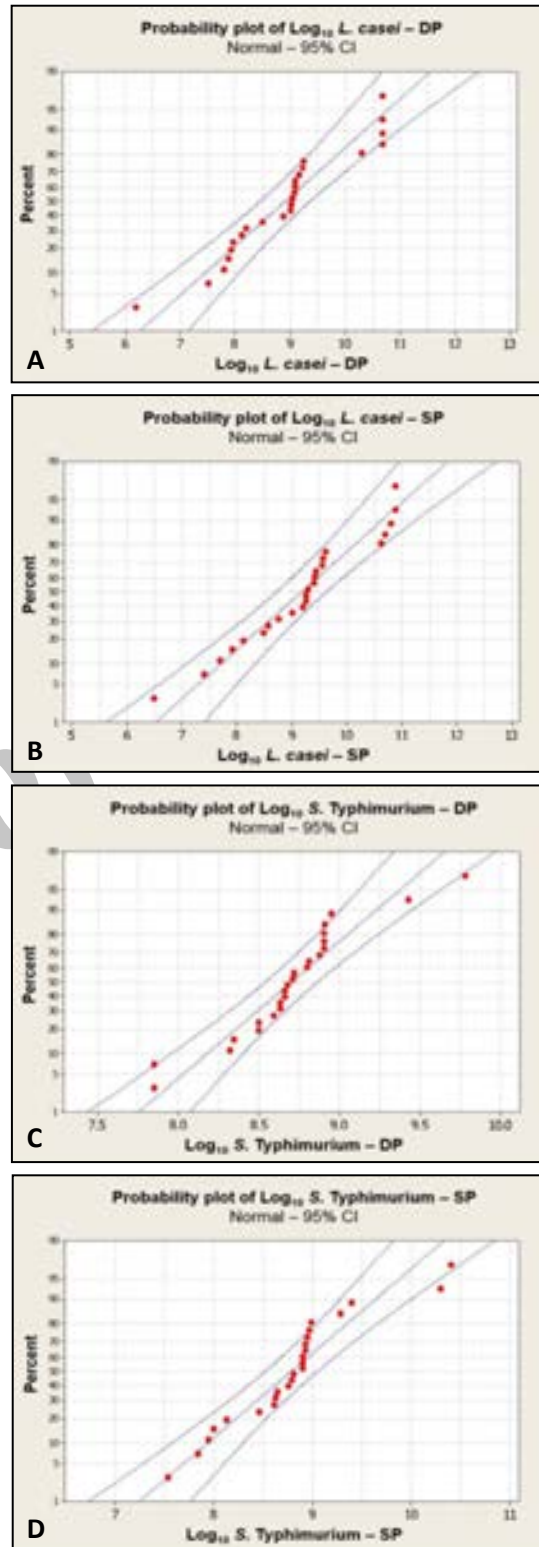
**Parametric analysis.** Although the parametric test is based on the assumption that the data are normally distributed, this assumption is not important when the sample sizes are at least 15 or 20. It showed that the difference of *L. casei* and *S. Typhimurium* countable means between DP and SP methods by unpaired *t*-test (parametric) were 0.4256 and 0.8867, respectively. The *p*-values (0.4256 and 0.8867) for counting indicated that there was sufficient evidence that all the means were equal when alpha is set at 0.05. Therefore the null hypothesis (equality of means) cannot be denied. The two aforementioned methods showed no significant difference. On the other hand, in two procedures the comparison of variability of variances by *F* test was not significant for both bacteria.

**Nonparametric analysis.** Most data did not follow the normal distribution. Therefore, nonparametric tests were preferred to exploit. Mann-Whitney is the nonparametric analog of unpaired *t*-test. It is used if the assumptions for the use of the *t*-test are not justified or called into question (e.g. in ordinal data or skewness).



**Fig. 2.** A marginal plot demonstrated a scatterplot with histograms of the x and y variables in the margins. This two-in-one graph compared variables and their distributions at the same time. **A)** *L. casei* **B)** *S. Typhimurium*.

Enumeration of *L. casei* and *S. Typhimurium* data revealed that they had approximately normal distribution. The calculated *p*-value was equivalent to 0.1363 and 0.7799, respectively. They confirmed null hypothesis in which equality of median of two methods were verified (the differences of median between two methods for both bacteria equal zero). Calculated correlation for *L. casei* and *S. Typhimurium* by two methods demonstrated that Spearman's rho correlation coefficient (*r*) were 0.8673 and 0.6199 respectively at 95% significance level.  $H_0: r = 0$  it meant that there is no actual correlation,  $H_A: r \neq 0$  it indicated that this is a correlation.<sup>6</sup> The calculated *p*-values of two methods for both bacteria were dropped below 0.05, which they rejected the  $H_0$ . This elucidates that the null hypothesis were rejected. Therefore, they elucidated a true relationship between two procedures for both bacteria. Figure 3 shows fitted distribution line (middle line) of DP and SP techniques for two bacteria. It is used to represent the trend and distribution pattern of data.



**Fig. 3.** In a fit line, the data points are fitted to a line that usually does not pass through all of the data points. The middle light purple line represents a fitted distribution line that represents the trend of the data. The curved light purple lines display the approximate 95% confidence intervals for the percentiles. **A and B)** related to *L. casei*, **C and D)** related to *S. Typhimurium*.

It is necessary to say that the value of the correlation coefficient alone should never consider important conclusions because the omitting outlier data influenced correlation coefficient, thus examining the respective scatterplot was always recommended. Figure 4 demonstrated the scatter plot of data in both bacteria.

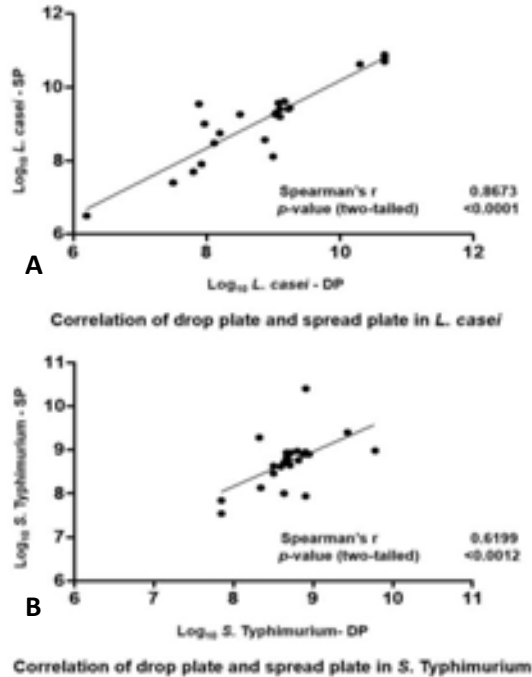


Fig. 4. Scatter plot of DP vs. SP. **A)** *L. casei* and **B)** *S. Typhimurium*.

## Discussion

The most commonly used direct plating method for bacteria is the SP method. Another method, the DP method (also referred to as the spot-titer method) has been used in clinical and other settings for quantifying bacteria for many years. The results of the DP method are interchangeable with those of the SP and double agar layer (DAL) methods by statistical analysis including analysis of variance (ANOVA) and  $\chi^2$ . The DP method has several advantages over the spread-plate and DAL methods: (1) it needs less time to dispense spots than to spread the microbe; (2) it uses fewer materials; (3) it requires less effort and (4) since the sample is distributed in distinct spots, colony/plaque counting is faster and less labor-intensive.<sup>7</sup> The DP method can be successfully used, not only for colony-counting, but also for most probable number (MPN) enumeration.<sup>1</sup> The standardized colony count method is favored over the MPN-method for routine use because of its partly higher productivity and much smaller variation in the results.<sup>3</sup> The three plating methods (pour, SP, and DP techniques) are interchangeable. The DP method has been preferred because of its economy in materials and labor.<sup>8</sup> Three different operators enumerated viable cells of *E. coli* using the DP and the SP methods. Statistical analyses

have showed that counts obtained by the both methods were not significantly different.<sup>9</sup>

The improvement of science associates implying new relations between variables. The ultimate target of every research or scientific analysis is to discover relations between variables. Relations between enumeration of bacterial via DP and SP methods were surveyed by correlational research.

For evaluation of correlation between variables, "magnitude" or "strength" as well as the significance of the correlation are vital issue.

Correlation ( $r^2$ ) between three procedures 6×6 DP colony counts, DP, MPN and spiral plate colony counts- for *E. coli*, *Listeria monocytogenes* and *Campylobacter jejuni* were above 0.95.<sup>1</sup> Spearman's rho correlation coefficient ( $r$ ) via both methods due to data distribution patterns were 0.6199 and 0.8673, respectively; which represented moderately strong and strong relation between two methods for enumeration of *S. Typhimurium* and *L. casei* respectively. Moreover, there was a significant and strong positive correlation between SP and DP procedures.

Uncertainty in repeated bacterial counting is only indirectly affected by the method in use but depends essentially on the number of counted colonies. On the other hand, the inter-laboratory uncertainty is due to fluctuation of enumeration method in use.<sup>10</sup> The estimates of uncertainty are influenced by the test procedure itself, the choice of culture medium and the mathematical procedure used to derive the original 'mean count appraisals'.<sup>11</sup>

Repeatability and reproducibility of every standard method have been estimated by collaborative trials and criticized published methods approve its precision for international use.<sup>12</sup>

For replacement of any alternative method to the corresponding reference method, it is necessary that their performance is similar to each other, without significant differences. In this respect, the ISO 16140 standard represents a key issue in producing such a method based on an inter-laboratory study.<sup>13</sup>

The cost of supplies, the required labor, inoculating, counting the plates, and the disposal of the relatively large volume of biohazardous waste are significant.<sup>14</sup> Like any other method, this method has advantages and disadvantages. For example, for bacteria exhibiting a swarming type of motility; eg, *Proteus mirabilis*, *P. vulgaris*, and *Vibrio parahaemolyticus*, the DP method is not recommended because of the small size of the area covered by the drop.<sup>3</sup> However, the beneficial effects include lesser time consumed, saving expenditure and faster colony counting. Above all, eight- in- one plate could have been gathered with three replications in an eight cm plate.

## Acknowledgment

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