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Technical Note

Identification and elimination of errors in the drop plate counts

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Abstract: The experimental details which enable accurate and reproducible drop plate counts to be performed are described. By following the procedure described, microbiologists can obtain accurate triplicate plate counts of 3 to 4 dilutions or duplicate plate counts of 6 dilutions of a sample by using only one agar plate.

Keywords: drop plate method, dropping technique, dilution technique, diluent

INTRODUCTION

The enumeration of viable bacterial cells is essential for qualification studies such as food and water, bio-contaminations, agricultural inoculants, and bacterial populations in environmental monitoring. There are 3 plate count methods for counting colony-forming units (CFU) of bacteria and yeasts, namely the pour plate (PP), spread plate (SP) and drop plate (DP) methods. While the PP and SP methods are conventional and more widely used than the DP method, some microbiologists have become increasingly interested in the DP method by virtue of its convenience in the inoculation step and the economy of the method. The DP method was invented in 1938 by Miles and Misra [1] who described how ten-fold serially diluted samples can be inoculated on the pre-dried agar plate by using a calibrated Pasteur pipette having a dropping tip with 0.96-mm outside diameter. This dropping pipette was invented by Fildes and Smart in 1926 [2]. The drop size of this calibrated dropping

pipette is 0.02 mL (20 μL). The agar plate should be pre-dried to the degree that it will absorb the drops in about 8-15 min. Each drop will spread to form a circle about 1 cm in diameter. One agar plate can conveniently accommodate 12 drops. After being incubated for 12-24 hr depending on the species, the drops that have about 25-250 CFU, which is the most suitable number for colony counting [3], can be counted by using a stereo dissecting microscope and a dissecting needle.

Many researchers have compared the DP, PP and SP methods to determine which one demonstrates the highest accuracy in data interpretation and reproducibility of counting common and specific species of bacteria [4-10]. While the PP method generates error through the death of bacteria by melted agar medium at 50°C during the PP procedure [6, 8], the SP method is affected by inadequate distribution of bacteria due to the formation of bacterial clusters, possibly through adherence of the bacteria to the glass spreader during plating [10]. In comparison, the DP method shows a slightly greater accuracy than both the PP and SP methods [4-10] but still displays some errors arising from various factors. Since then, there have been many efforts to validate the accuracy and eliminate the errors in the DP method using statistical analysis [11-14]. However, to our knowledge the accuracy and reproducibility of the DP method have not been systematically studied and reported.

Although the DP method was improved to achieve high accuracy and reproducibility by Supanwong [15], precise details of the technique are not well known. This article explains why the authors choose to improve the DP method and how it is achieved. The improved DP method is so accurate that it can be performed by counting the CFU of one drop of sample to obtain the same accurate count as from 5 drops [16]. For statistical analysis, triplicate counts of 3 dilutions were performed in order to increase the level of confidence.

MATERIALS AND METHODS

Microorganisms, Culture Media and Serial Dilution Preparation

E. coli was used in the tests. The bacteria were grown in lactose broth overnight at 37ºC and used as the original suspension. Before preparing the dilutions, the original suspension must be well mixed by shaking with elbow-action for 10, 15 or 20 times depending on the preference of the operator. The serial ten-fold dilutions were prepared by transferring 1 mL of the culture sample into 9 mL of 0.1% peptone solution using a blow-out pipette. For each transfer, after blowing out the content in the pipette into the next dilution bottle, the same pipette was used to suck up the liquid to the 1-mL mark and the liquid blown out. This was repeated 6 times. For preparing the next dilution, the dilution bottle was shaken by elbow-action 6 times. A new pipette was used to transfer 1 mL into the next dilution bottle and the suck-up and blow-out process repeated as in the first dilution. Three microbiologists performed the SP and DP tests. For the reproducibility test of the DP method, each operator performed the DP of a sample by preparing 2 sets of dilutions with 5 replicates for each dilution. For comparison of the DP and SP methods, each operator used the same dilution sets and performed the SP and DP methods with 10 replicates.

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Method of Counting

Both the SP and DP methods were employed using the same serial dilutions of the test material. The DP method was performed using the method described by Miles and Misra [1] with some modifications. The ten-fold serially diluted samples were inoculated on a pre-dried agar plate using a calibrated Pasteur pipette having a dropping tip with 0.96-mm outside diameter. The drop size of this calibrated dropping pipette was 0.02 mL (20 μL). The agar plate was pre-dried to the extent that it could absorb the drops in about 8-15 min. Each drop spread to form a circle about 1 cm in diameter. After being incubated for 12-24 hr depending on the species, the drops that had about 30-300 CFU could be counted using a stereo dissecting microscope and a dissecting needle. For the SP method, 100 μL of the dilution of *E. coli* were spread over the surface of the plate with a glass spreader. The distribution of bacteria on the plate by the SP and DP methods were observed using a Nikon Eclipse LV100 DAU microscope.

RESULTS AND DISCUSSION

Analysis of Plate Count Methods

Of the three plate count methods, viz. the PP method, the SP method and the DP method [1], microbiologists have accepted that none of these methods give accurate and reproducible results. The PP and SP methods cannot give accurate results since each of them has errors inherent in the inoculation technique. The main source of error in the PP method is the death of some microbial cells when exposed to the hot melted agar medium at about 50^{o}C, while in the SP method it is the attachment of various amounts of liquid sample to the glass spreader after spreading. Furthermore, the spreading of microbial cells on the sticky surface of the agar medium causes clumping which can greatly reduce the CFU count of the sample (Figure 1).

Since the DP method has no inherent errors in the inoculation step, it can give accurate and reproducible results, but only if the operator can apply each drop with equal weight (or volume). If each drop has an equal volume, the CFU counts should be statistically equal. In earlier work the DP method was improved to give a high degree of accuracy [15] compared with the method of Miles and Misra [1] using the same calibrated Pasteur pipette having a 0.96-mm external diameter. This calibrated Pasteur pipette gives a drop size of 20 mg or 20 μL (0.02 mL) with a variation in drop size of as low as 1.5% [2]. This article is based on the earlier work of Supanwong [15] plus a succession of refinements gained from the experience of using the method during the intervening years.

Dropping Technique for Accurate DP Counts

The only conceivable error in the DP method is the inability to control the calibrated pipette to give drops of equal volume so that the CFU counts of each drop are statistically equal. There are three factors controlling the drop size of liquid emerging from the tip of the calibrated pipette:

1) External diameter of the dropping pipette. Since each pipette is calibrated to the same size, there is no error in this factor.

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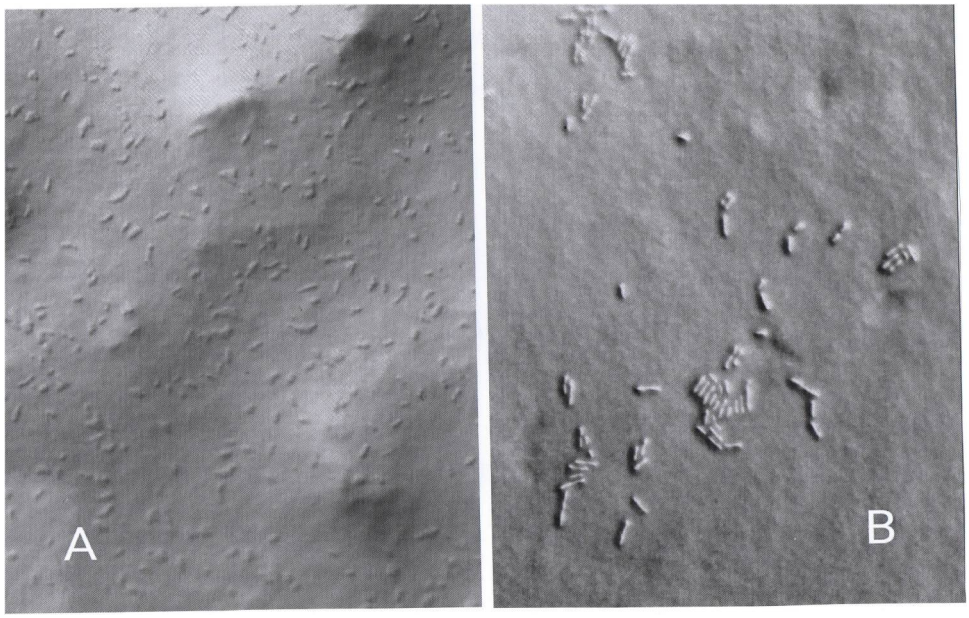

Figure 1. Arrangement of *E. coli* cells on an agar surface: (A) DP method, (B) SP method. (Specimens were prepared and photographed using a Nikon Eclipse LV100 DAU microscope.)

2) Steadiness in handling the silicone rubber bulb (SRB). When the SRB is squeezed any lack of steadiness can result in drops of different sizes. This factor can be corrected by holding the pipette as shown in the illustration (Figure 2). By holding the pipette at two points, the pipette remains steady with no shaking down of the drop, thereby eliminating any error.

3) Squeezing force on the SRB. The force with which different operators squeeze the SRB can vary significantly, resulting in drops of different sizes. Even the same operator may produce drops of different sizes. This error can be eliminated by developing a consistent squeezing technique in which the operator controls the take-up and dispensing of a very small volume of liquid in the range of microlitres.

The technique for delivering accurate drops is as follows:

- Touch the open end of the SRB with a little water.
- Insert the glass pipette into the SRB to about 1.5-2-cm depth.
- Hold the dropping pipette as shown in Figure 2, with the thumb and forefinger holding the upper end of the dropping pipette to control the take-up and dispensing of the liquid. It should be noticed that the main body of the SRB is fully exposed. With the thumb and forefinger pressing on that part of the glass pipette which is inside the SRB, the neck of the SRB can be pressed without creating any deflation of the SRB. Alternatively, a slight deflation can be created by moving the two fingers slightly upwards to sense that the SRB is deflated a little. In this deflated state, put the tip of the dropping pipette in the liquid sample and release the squeezing pressure on the SRB completely. The dropping pipette will then take up some liquid into the dropping tip without any air bubbles. Take up the liquid to about ½ - ¾ of the length of the dropping tip or more but avoid touching the tip of the glass pipette in order to be able to use one dropping tip for all dilutions of that sample.

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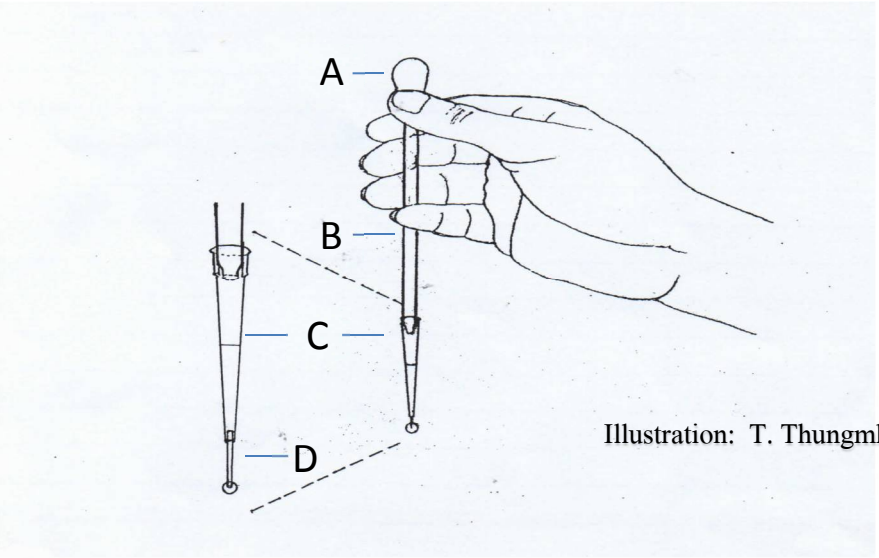

Figure 2. The dropping pipette: (A) narrow-neck SRB, 1-mL capacity; (B) glass pipette, about 12 cm long; (C) dropping tip body, autoclavable plastic, 200-µL capacity; (D) dropping glass tip, 10-12 mm long, 0.96-mm external diameter, giving 0.02-mL drop size. The dropping tip (C+D) was developed for use with semi-automatic machine for the preparation of drop plates [17].

The key to success in getting drops of equal size is the ability of the operator to dispense the drops very slowly by holding the pipette as previously described. Furthermore, the dropping glass tip must be scrupulously clean and free from grease. When performing the dropping, the liquid must creep up smoothly around the external surface of the glass tip. At first, the drop will be oval in shape but as it becomes bigger it will become more rounded, which means that it is going to drop. At this stage the operator must squeeze the SRB as slowly as possible so that the drop will drop under its own weight or with the application of minimal pressure from squeezing the SRB.

Before attempting to perform the DP method, the operator should practice dropping about 5 to 10 plates on a plastic template from the height of about 5 cm. After successfully practicing how to control the drop size to give an equal volume, the DP counts are performed in order to check if 5 drops of the countable dilution give accurate results and if two sets of dilutions of the same sample also give the same or reproducible results.

Now even though the counts of 5 drops in the countable dilution of the first set may give accurate results such as 190, 193, 193, 195, 199, and the counts of the second set are also accurate, such as 95, 97, 98, 101, 105, it is clear that there still are errors in the dilution steps. It is observed that after blowing out the 1 mL of liquid sample into the dilution bottle, there are still some droplets attached to the internal wall of the blown-out pipette no matter how strong the blow-out force is. This dilution error can be eliminated by using the same pipette to suck up liquid from the bottle up to the 1-mL mark and blowing out the liquid back into the bottle. This suck-up and blow-out action should be repeated 6 times to ensure that the liquid that is attached to the internal wall of the pipette would not cause any error since it now has the same CFU per mL as the liquid in the bottle. To perform the next dilution, a new pipette is used employing the same procedure. Before taking any sample, the sample is mixed

thoroughly by shaking with elbow action 6 times since it is considered to be more effective than using a vortex mixer.

This dilution technique may sound nonsensical to many microbiologists but in fact it is an effective technique. Without this dilution technique, reproducible CFU counts are impossible. Nowadays, although an automatic pipette is used for dilution, the same procedure as for the blow-out pipette must be used.

Selection of Diluent

However, after eliminating the errors in the dilution steps, the results may still not be sufficiently reproducible, which means that there must be more errors in the dilution process. The only factor left is the diluent. Straka and Stokes [18] studied the toxicity of various diluents and found that 0.1% peptone solution is not toxic to bacteria and yeasts. Indeed, it even supports the growth of some bacteria. Furthermore, the 0.1% peptone solution can also help to form a uniform drop size since it has a uniform surface tension. After we changed the diluent from distilled water to 0.1% peptone solution, the CFU counts of the 2 dilution sets became statistically equal.

Determination of Reproducibility of Modified DP Method

The data in Table 1 compares the results of 3 microbiologists performing reproducibility tests. Each one performed the test at different periods of time using *E. coli* as the test bacteria. The DP method works well with any bacteria that form single colonies. Each microbiologist performed the DP counts of a sample by preparing 2 sets of dilution. The DP of each dilution was performed in 5 replicates. The CFU averages of the two sets were compared by the pair-t test. The results show that the CFU averages in the 2 sets of dilution are not different at 95% confidence level. The results of each dilution by the second and third microbiologists are also statistically equal at 95% confidence level. Table 2 shows the results of 3 microbiologists performing DP counts and SP counts using the same dilution set. The SP counts were included in order to compare the accuracy of the two methods. The DP counts are much more accurate than the SP counts.

Table 1. Determinations of reproducibility of DP method by 3 operators each using different sample

Operator/ Sample	Dilution set	CFU of each drop (0.02 mL) ($1:10^5$)					$\bar{x}$ ± SE	CFU/mL ($x10^8$)
1/A	1	182	180	181	191	180	182.8±2.083	9.14
	2	180	187	190	183	188	185.6±1.805	9.28
2/B	1	69	68	89	80	76	76.4±3.854	3.82
	2	75	74	80	74	66	73.8± 2.244	3.69
3/C	1	93	85	92	97	96	92.6± 2.111	4.63
	2	93	91	95	96	94	93.0± 0.860	4.69

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Table 2. Comparison of plate counts by DP and SP methods performed by three operators using the same dilution set

Operator	Colony count	Plate no. (0.1 mL per plate) / Drop no. (0.02 mL per drop)										C.V.*	CFU/mL ($\times 10^8$)
		1	2	3	4	5	6	7	8	9	10		
1	Spread plate	189	291	300	248	259	327	199	209	117	114	32.51	2.25
	Drop plate	81	85	78	86	86	74	87	86	80	70	7.17	4.06
2	Spread plate	278	283	368	343	359	330	339	324	345	396	10.69	3.37
	Drop plate	80	79	79	75	83	74	82	75	78	80	3.86	3.92
3	Spread plate	283	264	214	200	153	115	264	249	189	117	29.96	2.05
	Drop plate	81	84	87	82	82	93	91	80	72	88	7.23	4.20

* Coefficient of variation

In addition to its accuracy, the DP method is also very convenient to perform with an additional replicate taking less than 10 sec. Furthermore, the DP method is also economical since a CFU determination in triplicate of 3 to 4 dilutions or in duplicate of 6 dilutions requires only one agar plate (Figure 3). Thus, a single agar plate can do the job of 12 plates in the PP and SP methods. A properly dried plate will absorb the drop in about 10 min. (typically 8-15 min.) with the drops of the colonies appearing as perfect circles. When working with a familiar sample, triplicates of 3 dilutions are usually all that is required.

Nowadays more and more microbiologists are using the DP method to determine the CFU of a sample. However, the technique they are using is not really the DP method and should be called a 'spot plate' method, in which the sample is inoculated on the agar surface as a spot using a micropipette tip. Moreover, this tip cannot accurately deliver 0.02 mL. If dropping tips that can accurately deliver 0.02 mL drops were commercially available together with electronic pipettes that can dispense liquid very slowly (5-8 sec./drop of 0.02 mL), the DP method described in "Semi-automatic machine for the preparation of drop plate" [17] would be widely used and would eventually be the most popular plate count method.[#]

[#] Since the dropping pipettes and dropping tips are not commercially available, miro-biologists who would like to use this method can contact the author at LINE ID: kamnird. A reasonable amount of the dropping units can be supplied on a reusable basis.

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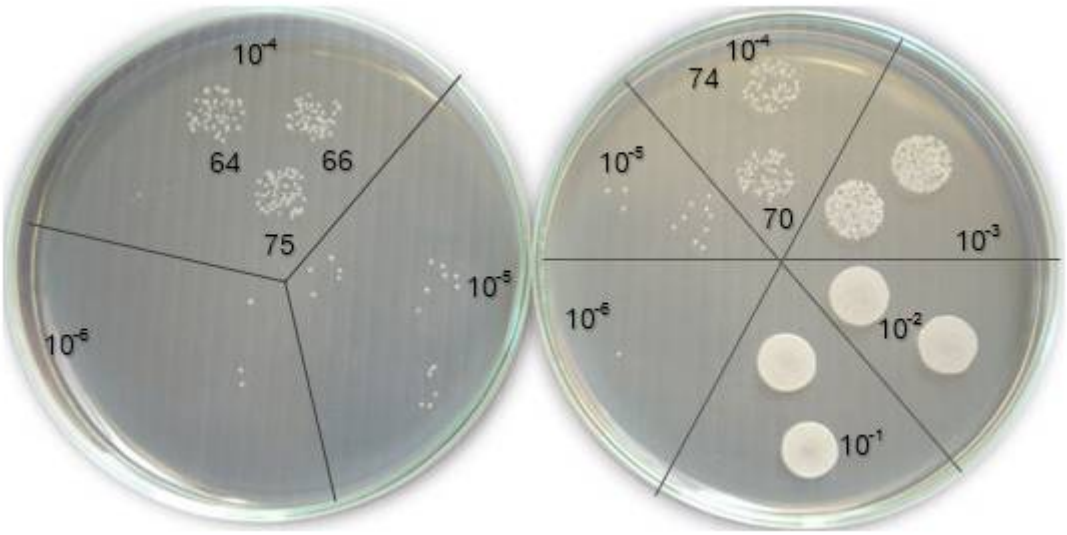

Figure 3. Dropping patterns of the DP method: (A) triplicates of 3 dilutions; (B) duplicates of 6 dilutions (photographed by P. Wongsawad)

CONCLUSIONS

The most obvious cause of error in the DP method is the inability to control the drops to be of equal volume**.** This in turn prevents the method from becoming standardised**.** However, we have demonstrated that this error can be eliminated. Accurate and reproducible CFU counts are possible using the DP method described in this article. The results of the test experiments carried out by three operators show that the DP counts are much more accurate than the SP counts and can be used as a substitute for the conventional SP and PP methods.

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