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Research paper

Simplified method to automatically count bacterial colony forming unit

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Abstract

Bacterial colony counting is a significant technical hurdle for vaccine studies as well as various microbiological studies. We now show that an automated colony counter can process images obtained with a digital camera or document scanner and that any laboratory can efficiently have bacterial colonies enumerated by sending the images to a laboratory with a colony counter via internet.
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In response to a pneumococcal vaccine, a population such as an elderly population can induce antibodies that are less functional than those induced in other populations (Romero-Steiner et al., 1999). Thus, functional abilities of pneumococcal antibodies should be determined as a part of pneumococcal vaccine evaluation in a population. The most widely accepted functional assay for pneumococcal antibodies is in vitro opsonization assay (Nahm et al., 1997). The assay is classically performed by incubating with an antiserum, complement and phagocytes and determining the number of surviving pneumococci when the mixture is plated on agar plates. Bacterial colony counting is a key step in assessing the functional capacity of antibodies.

Bacterial colony counting is tedious and laborious. Some investigators adapted an imaging system developed for ELISPOT assays (Liu et al., 2004). Our laboratory used a tetrazolium dye to color pneumococcal colonies (Kim et al., 2003) and make them clearly visible for automatic bacterial colony counters. Since the automated colony counters can easily recognize the colored colonies, they can count colonies very rapidly. However, the automatic counters are too expensive for small laboratories which perform the assays only occasionally. On the other hand, big laboratories may have extremely large counting needs to be accommodated with few automatic counters. Thus, colony counting is a significant budgetary

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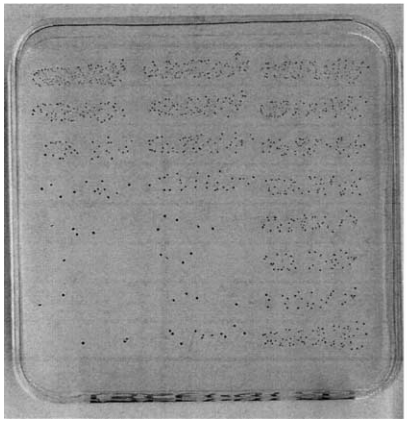
and technical hurdle for laboratories of all sizes. With recent developments in digital cameras and document scanners, electronic images of agar plates with bacterial colonies can be obtained easily and simply transmitted to another laboratory via the internet. Thus, we have directly investigated whether these capabilities can be used to overcome the difficulties currently associated with counting bacterial colonies.

Agar plates with pneumococcal colonies were prepared in a square Petri dish (shown in Fig. 1) as described previously (Kim et al., 2003). The Petri dish contains 24 spots with bacterial colonies, and each spot, which corresponds to one assay condition, has up to about 150 colonies. To obtain images of the Petri dish reproducibly with a digital camera, an imaging station was made with a cardboard box (about $20 \times 25 \times 30$ cm). The distance between the lens and the object was about 18 cm. In one side of the box, we cut a square "Petri dish window" just big enough (9 cm $\times$ 9 cm) to hold a Petri dish snugly. The box was placed on a table with the Petri dish window facing a white wall, which provided a neutral background. A Petri dish was inserted in the Petri dish window in vertical orientation. The side of the box opposite to the Petri dish window was removed to place a digital camera mounted on a mini-tripod (about 10-cm-long legs) in the box, and the Petri dish was photographed through the box. A digital camera (Powershot A75) from Canon (Tokyo, Japan) with a 2-Mega pixel resolution and 32 Mb of internal memory was used.

Images were obtained without electronic flash using manual controls for exposure and distance. To make image files small, the resolution was reduced to 1024×768 pixels and "normal" data compression was selected. Because the resulting jpeg image file was only about 75 KB in size, many (more than 400) image files could be stored in the camera's internal memory. The jpeg files were transferred to a local computer and e-mailed to a remote computer equipped with two programs: Photoshop CS (Adobe, San Jose, CA) and ProtoCOL (Version 4.04 from Synoptics Ltd., Cambridge, UK). The remote computer is part of the dedicated colony counter with its own camera (Synoptics Ltd.) and was distributed by Microbiology International (Frederick, MD). The file was first converted from color jpeg format to a grey scale BMP format using Photoshop CS. The resulting file was about 500 KB in size and had 780×574 pixels with an 8-bit depth. The image file was opened with the ProtoCOL program, which estimated the number of bacterial colonies in each spot.

To determine how well the number of colonies can be estimated with these instruments, we determined the true number of colonies in 70 spots by manually counting the colonies. Also we used a dedicated ProtoCOL colony counter with its own camera to estimate the number of colonies in the spots. Fig. 2A



141	96	109
66	69	89
33	54	59
13	30	53
4	5	46
1	4	43
2	4	29
3	11	74

Fig. 1. A square Petri dish with pneumococcal colonies. The colonies are in 24 "spots," arranged in 3 columns and 8 rows. The image was obtained with a document scanner as described in the text. The number of bacterial colonies in each spot is shown in the table with the figure. When target areas contain 10–100 colonies, the CV of colony counts is about 1–3% for the manual method and about 1–4% for automated methods.

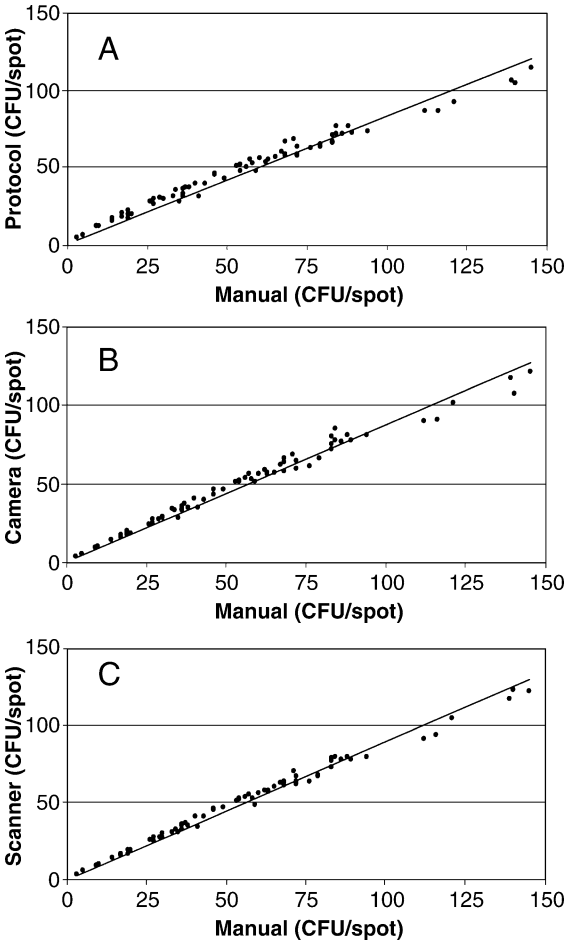


Fig. 2. The numbers of colonies per spot obtained with a dedicated colony counter (Panel A), a digital camera (Panel B), and a document scanner (Panel C) were compared with the true counts per spot. The number of colonies was determined in 70 spots, and the true counts were established by manual counting. The lines indicate the best fit lines and their slopes were 0.84, 0.88, and 0.89 for Panels A, B, and C. Correlation coefficients (r^2) were 0.96, 0.98, and 0.98 for Panels A, B, and C.

shows that the two results were highly correlated ($r^2=0.96$) but that the machine counts were slightly lower (by about 10–15%) than the true counts. The deviation was noticeable when the number of colonies per spot exceeded 50. When the ProtoCOL software was used to process the digital camera image, the count was again highly correlated with the true count ($r^2=0.98$) but slightly less than the true count (slope of the best fit line=0.88) (Fig. 2B). The degree of undercounting is similar to that obtained with a dedicated counter (Fig. 2A). The counts by the ProtoCOL counter method and by the digital camera-ProtoCOL software method were equivalent (slope=1.05 (data not shown)).

Document scanners are as widely available as digital cameras. To determine if scanners can be an alternative method of obtaining images of the colonies in Petri dishes, we scanned Petri dishes with a common document scanner (HP psc 2175 all-in-one printer/scanner/copier) that was connected to an IBM-type PC. Four Petri dishes were scanned at one time at a 200-dpi resolution. The images (approximately 350 KB in size in jpeg format) were then evaluated with ProtoCOL software. When the colony counts obtained with the document scanner-ProtoCOL software method were compared with the true counts (Fig. 2C), the results were highly correlated ($r^2=0.98$) with slight undercounting (slope=0.89). Reflecting almost identical colony numbers we can obtain with different methods, automated counting methods produced opsonization titers that were very similar (less than 10% differences) to the manual counting method and this variability is much less than the analytical variability of the assay itself.

Although no data processing steps were customized or automated, the entire operation is fast. Twelve Petri dishes, which represent data from three 96-well microtiter plates, can be counted in about 10 min using the dedicated ProtoCOL counter as summarized in Table 1. When 12 plates are scanned, the imaging and additional data processing requires another 23 min (Table 1). This is a conservative figure because some counting programs do not require file format conversions. A similar amount of time is required for imaging and data processing using the digital camera

Table 1
Estimated time of counting colonies in 12 Petri dishes with various methods

	Scanner (min)	Digital camera (min)	ProtoCOL (min)	Manual (min)
Image acquisition	2	7	0*	–
File format conversion	11	11	0	–
Enumeration	10	10	10	144
Total time	23	28	10	144

* A new image can be acquired during the enumeration of the previous images.

(Table 1). In contrast, manual counting would have taken 144 min (Table 1). Considering that it takes about 3–5 min to obtain optical densities of three 96-well ELISA plates, immunogenicity evaluations of vaccines have primarily depended on antibody concentration measurements. Since any of these colony-counting methods are much faster than the manual counting method and some of the counting methods are almost as fast as reading ELISA plates, we envision that antibody function measurements (e.g., opsonization assays) would be used more in future vaccine evaluations.

The whole procedure described here can be readily implemented anywhere. Only commonly available equipment was used, and other types of scanners or digital cameras should be satisfactory. For instance, we found another digital camera (Cool Pix 5700) from Nikon (Tokyo, Japan) to be satisfactory. It is very simple to set up the cameras or scanners, and many people found the procedure to be easy to learn. We envision satellite laboratories e-mailing images from scanners or cameras to a central laboratory with a dedicated colony counter and receiving results from the central laboratory. However, the need for the central laboratory may disappear since CFUs may be obtained from the images of Petri dishes with other widely available programs.

CFU determination is important in assessing bacterial vaccines in addition to pneumococcal vaccines. For instance, the efficacy of group B streptococcal vaccines can be evaluated with an opsonization assay (Guttormsen et al., 2002), and meningococcal vaccines are assessed by bactericidal assays (Balmer and Borrow, 2004), which also require colony counting. In addition to these vaccine studies, our approach should be useful for many bacterial species since their colonies can be colored with tetrazolium dyes (Cuthbert, 1967; Coudron et al., 1983; Pourcher et al., 1991; Summanen et al., 1992). The studies described here show that the long-standing technical hurdle of efficiently counting colonies has now been eliminated by using common imaging devices and internet access, which have become inexpensive and ubiquitous in the last several years.

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