Automated Counting of Bacterial Colonies on Spread Agar Plates and Non-Gridded Membrane Filters

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ABSTRACT

The Biotran II automated colony counter was compared with a manual procedure for accuracy in counting bacterial colonies using both spread agar plate and membrane filter techniques. Comparative total bacterial counts of 250 samples (14 food, 124 water and 112 raw milk) were analyzed using the spread agar plate technique. Compared to manual enumeration, the Biotran II was found to be inaccurate for counting bacterial colonies on spread agar plates. Only 60 (24%) and 79 (31.6%) Biotran II counts fell within 10 and 20%, respectively, of the corresponding manual counts. Two samples from each of three river and four effluent sources were analyzed for total aerobic, total coliform, fecal coliform, fecal streptococci and total staphylococci bacterial counts using non-gridded membrane filters. A yellow acetate filter was used to mask background growth and enhance the target colonies on the membrane filter. However, the method had limited success. Only 12 (20.7%) and 20 (34.5%) Biotran II counts fell within 10 and 20%, respectively, of the corresponding manual counts. Until the effect of background growth can be eliminated, the Biotran II cannot be relied upon to accurately count bacterial colonies on membrane filters.

The standard plate count and plate loop count (both pour plate methods) have been used to evaluate the performance of automated colony counters (ACC). Compared with manual or photographic counts of bacterial colonies from milk samples, ACC units have been found to be precise, but reports on accuracy have varied among researchers (1,6,7,9,12,15). In one report (1), none of the ACC units tested approached the acceptable criteria established by Standard Methods for the Examination of Dairy Products (10) that 90% of the ACC counts fall within 10% of the corresponding manual counts. Despite improved optics and circuitry, the ACC units had problems distinguishing between and counting individual bacterial colonies from "pulls" or "spreaders", air bubbles in the agar, and scratches and dust particles on the bottom of the petri plate. Even when using pure cultures for enumerating distinctive bacterial colonies in selective agar media, Guthertz and Fruin (7) found the manual method to be more accurate than any of the ACC units tested.

Spread agar plate methods have been shown to produce higher recoveries than pour plate techniques for enumerating bacterial colonies from a variety of sources (4,8,11,14). More recently, a computerized system has been developed utilizing a camera which scans and enumerates specific colonies on hydrophobic grid-membrane filters placed on selective agar surfaces (2,3). A series of colored filters was used to mask background growth while enhancing target colonies. Both spread agar plating (APC) and membrane filtration (MF) techniques should overcome some of the problems inherent in enumerating bacterial colonies in pour plates using ACC units. That is, APC and MF colonies tend to be larger, easier to detect, and "pulls" and "spreaders" are not a problem (11). Except for the spiral plate counter (13), which uses a different spreading and counting principle, there has been no research conducted for evaluating other commercial ACC units using both spread plate and membrane filter techniques.

This study was undertaken to evaluate the Biotran II automated colony counter (New Brunswick Scientific Co., New Brunswick, NJ) for its ability to count bacterial colonies on an agar surface using spread plate inoculation, and to see if it could also be used to differentiate and enumerate target colonies grown on non-gridded membrane filters placed on selective agar media.

MATERIALS AND METHODS

Spread plate preparation and testing

Inocula (100 µl) from routinely submitted raw milk samples (diluted in phosphate buffered saline), municipal drinking water samples (undiluted) and food homogenates (prepared by stomaching with 1% buffered peptone water and further diluted in 0.1% peptone dilution water), were spread evenly on the surface of standard plate count agar (Oxoid) in 100 x 15-mm plastic petri dishes. Spreading of the inoculum was done using a turntable and a sterile "hockey stick". All petri dishes were incubated at 35°C for 48 h. Each manual and ACC count was done in triplicate by a single technologist using a Quebec colony counter and the Biotran II. For both methods, the petri plates were rotated 90 degrees between counts.

Membrane filtration preparation and testing

Four secondary effluent and three different river samples were analyzed for total aerobic count, total coliforms, fecal coliforms, fecal
streptococci and total staphylococci using 0.45-μm HA gridded membrane filters (Millipore Cat. No. HA0471, Lot No. H22E6890C) plated on standard plate count, m-Endo-LES, m-FC, KF, and tellurite polymyxin egg yolk (TPEY) agars, respectively. The m-Endo-LES and m-FC agars were incubated at 35 and 44.5°C, respectively, for 24 h. The KF and TPEY agar plates were placed in a moisture chamber and, along with the standard plate count agar plates, incubated at 35°C for 48 h. During this initial screening stage, the effluent and river samples were stored at 4°C.

The appropriate volume and dilution of each sample based on counts obtained by the screening procedure was filtered through a non-gridded 0.45-μm GN-6 membrane filter (Gelman Sciences Product No. 63069, Lot No. 3565072) to obtain colony counts within an acceptable range. Incubation times and temperatures were the same as for the screening procedure, except that m-FC agar was used for total coliform counts in place of m-Endo-LES (2). Manual bacterial colony counts on each membrane were done in triplicate with the plates rotated 90 degrees between counts. On selective agar surfaces, only those colonies having the appropriate differential characteristics for that group of organisms were included in the manual count. Triplicate counts were then done, with rotation, using the Biotran II.

Instrument calibration and use

For aerobic spread plate enumeration using the Biotran II, the diaphragm and sensitivity adjustments were set according to the manufacturer’s recommended procedure using the bottom light source. Petri plates without stacking rings were used, thus eliminating the need for an adjustment factor since the entire agar surface was scanned. For all counts done on membrane filters, the top light source was used. The sensitivity adjustment for the total aerobic counts on membrane filters was done as for the spread plates. For differential membrane filter colony counts on selective agars, sensitivity adjustment was made by placing a sunburst yellow-colored transparency (Xerox Corp. 3R873) over a membrane filter from a total aerobic plate count. The sensitivity threshold for the ACC was adjusted until no colonies were observed on the TV monitor. This procedure had the effect of screening out non-colored background colonies so that only the typical colored colonies would be counted on the membrane filter. Once the sensitivity level was adjusted, the yellow transparency was overlaid on each membrane filter from selective agars and triplicate colony counts made, with rotation of 90 degrees. The colony size discriminator dial was not used for any counting procedure.

Performance criteria

The performance of the Biotran II was evaluated by calculating the percentage of ACC counts which deviated by more than 10 and 20% of the corresponding manual count.

RESULTS

Aerobic spread plate analysis

Shown in Table 1 is the distribution of bacterial counts obtained from all spread plates done in this study. Twenty-four percent (60/250) and 31.6% (79/250) of Biotran II ACC counts fell within 10 and 20%, respectively, of the corresponding manual counts.

Membrane filter analysis

The arithmetic mean colony counts determined by the Biotran II versus manual technique on membrane filters is shown in Table 2. Of the 58 total comparisons, 12 (20.7%) and 20 (34.5%) Biotran II counts fell within 10 and 20%, respectively, of the manual counts. Using highly selective isolation conditions (m-FC at 44.5°C for fecal coliforms and KF agar at 35°C for fecal streptococci), 8 (33.3%) and 12 (50%) Biotran II counts, out of a total of 24 comparative counts, fell within the 10 and 20% range, respectively, of the corresponding manual counts. Of the remaining 34 comparative

TABLE 1. Distribution of mean bacterial counts from all spread plates enumerated manually and comparative performance by the Biotran II.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. tested</th>
<th>Distribution of spread plate counts</th>
<th>Deviation of Biotran II counts</th>
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<tr>
<td>Source</td>
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<td>&lt;30</td>
<td>30-300</td>
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<tr>
<td>Food</td>
<td>14</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Treated water</td>
<td>124</td>
<td>93</td>
<td>18</td>
</tr>
<tr>
<td>Raw milk</td>
<td>112</td>
<td>66</td>
<td>40</td>
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<tr>
<td>TOTAL</td>
<td>250</td>
<td>166</td>
<td>65</td>
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</table>

*Arithmetic mean of three manual counts.

*Number of Biotran II counts which fell within the prescribed limits of accuracy relative to corresponding manual counts.
counts in Table 2 using less selective conditions (m-FC at 35°C for total coliforms, TPEY at 35°C for total staphylococci and standard plate count agar for total aerobic counts), only 4 (11.8%) and 8 (23.6%) Biotran II counts fell within 10 and 20%, respectively, of the corresponding manual counts.

**DISCUSSION**

*Standard Methods for the Examination of Dairy Products* (10) states that laboratory analysts should be able to duplicate counts of other analysts within 10%. However, Fowler et al. (5) found that among analysts the variation was 18.2%, and questioned whether the 10% standard was a realistic performance criteria for reproducible counting. Therefore, in this paper, we have included both a 10 and 20% range in comparing counts between the manual and the Biotran II automated colony counter.

Many of the problems reported by others (1,6,7,9,12,15) in counting colonies by ACC units using pour plate methods also interfered with the accuracy for spread plated colonies in this study using the Biotran II. Bubbles, cracks in the agar, and scratches on the petri plate caused inaccurate enumeration by the ACC unit. Such inaccuracies in counting were more pronounced when there were fewer numbers of colonies. In addition, the Biotran II was unable to detect clear pinpoint colonies on the agar surface which were easily observed by the technologist using a Quebec counter. This was true even where pinpoint colonies exceeded 300 on a plate. Further adjustment to greater sensitivity could not overcome this obstacle.

Other researchers (7,6,10) have suggested that the accuracy of ACC's would improve if the machine calibration was rigidly standardized and if any plates containing factors such as those listed above, which can cause inaccurate ACC counting, were eliminated from enumeration. In addition, counting only plates containing 30 to 300 colonies was recommended. However, in a routine laboratory setting the time required to screen aberrant plates would nullify the positive aspects of automated counting systems. A time study comparison done during the course

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**TABLE 2. Comparison of Biotran II vs. manual mean colony count for each bacterial group enumerated on non-gridded membrane filters.**

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<td>2</td>
<td>47</td>
<td>56**</td>
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<td>41</td>
<td>41*</td>
<td>61</td>
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<td>4*</td>
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<td>9**</td>
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</table>

*a*Sample source: R = river, E = effluent.

*b*Test: Two bacterial counts were obtained as a result of filtering two different sample volumes.

*c*Standard plate count agar at 35°C for 48 h.

*d*m-FC agar at 35°C for 24 h.

*e*M-FC agar at 44.5°C for 24 h.

*f*Kf agar at 35°C for 48 h in a moisture chamber.

*g*TPEY agar at 35°C for 48 h in a moisture chamber.

*h*TNTC, colonies too numerous to count.

*i*Biotran II count within 10% range of corresponding manual count.

**Biotran II count within 20% range of corresponding manual count.

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of this work showed that it took 131 min manually as opposed to 105 min by the Biotran II to count the same 159 spread plates in triplicate. This was not a large time saving. Many plates enumerated in our laboratory have counts of less than 30 or greater than 300. As shown in Table 1, of the 250 total spread plates in this study only 65 (26%) contained counts in the 30 to 300 range. Routinely, it does not take much time to enumerate manually and record colony densities outside this range because plates containing more than 300 colonies are not counted but only recorded as ≥300.

Guthertz and Fruin (7) found the manual method superior to the ACC units tested (which included the Biotran II) for accuracy in enumerating colonies in selective agar. Inaccurate counts by the machines tested were due to lack of contrast between colony and growth medium, the opacity of the medium used, and, for the coliform counts, interference from background growth. In this study, non-gridded membrane filters were used, making the contrast between the colony and the growth medium consistent from one selective agar to the next, thereby nullifying any effect that medium opacity may have. Other research (2,3) has shown that differential colony counting on hydrophobic grid-membrane filters can be done accurately using a computerized counting algorithm. This system uses a variety of colored filters that effectively screen out background colonies while enhancing target colonies. Preliminary work done in our laboratory (data not shown) showed that, by using a yellow filter over the membrane filter, the Biotran II could selectively enumerate target colonies in inoculated culture work. However, as shown by the data in Table 2, this was not the case using natural water samples. In 44 (76%) of the 58 total comparisons, the colony count produced by the Biotran II exceeded the corresponding manual counts. This elevation of counts by the ACC unit was due to the inability of the yellow filter to effectively mask background colonies that were pigmented in any way on differential media. There was better agreement between the automated and manual counting methods using more selective conditions (KF agar and m-FC at 44.5°C) because there was considerably less interference from background growth. Also, when large swarming bacterial and fungal colonies were observed, especially on total counts, these were often enumerated more than once by the Biotran II. Unsuccessful attempts were made to adjust the sensitivity of this ACC unit to avoid these problems.

The technologist’s eye is trained to detect subtle differences in colony color and size. The Biotran II ACC obviously could not emulate this ability using the one filter approach. The use of different colors and types of filters to enhance certain bacterial groups while masking others may overcome these difficulties so that accurate enumeration of bacterial colonies on non-gridded membranes by conventional ACC units could be made.

This is the first study evaluating an automated colony counter for its ability to count colonies accurately on spread agar plates and non-gridded membrane filters. Compared to manual enumeration, the Biotran II was found to be inaccurate for counting bacterial colonies on spread agar plates and, until the effect of background growth can be completely eliminated, the Biotran II cannot be relied upon to accurately count bacterial colonies on membrane filters.

ACKNOWLEDGMENTS

We express appreciation to Mrs. Jean Bryce for excellent technical assistance during the course of this work and to New Brunswick Scientific Company for the use of the Biotran II automated colony counter.

REFERENCES