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Economical Method for Estimation of Bacterial Viable Count

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Abstract.- Viable count of six pure cultures of bacteria as well as those of water and raw milk samples were worked out by the standard spread plate method and economically designed miniagar discs using lesser amounts of the aliquots. The transfer of the material was also attempted with the help of micropipette in addition to a metal spreader. Micro-colonies appeared within 6 to 10 hours. The results obtained indicated that colony forming units (C.F.U.) both of natural samples as well as experimental cultures can be established economically by employing the modified miniagar discs method, a modified protocol.

Keywords: Colony forming units; microcolonies; miniagar discs for C.F.U.

INTRODUCTION

Estimating bacterial population densities is important in environmental assessments of air, water and soil as well as in the experimental cultures. Various methods are also employed by public health authorities to keep check on microbial quality of milk, water and food. In this regard a number of techniques are used in food industries. Physical methods are used to estimate total population *i.e.* dead as well as live microorganisms. They include direct counting, measurement of turbidity, determining biomass and estimating growth dependant metabolites. The biological methods are used for estimating the number of viable units (Pelczar *et al.*, 1986; Morgan and Carter, 1996; Banwart, 1998).

The number of viable cells in a sample is assessed from the number of colonies, which develop on incubation of known amount of a sample inoculated on a solid medium. Plate count, roll tube count, drop count, surface count, dip slide count, contact plate, membrane filter count and estimations of most probable number are the familiar methods of measuring colony forming units (C.F.U.) in a sample. A viable count method assumes that a visible colony will develop from each microorganism. However, a single colony may develop from one or from hundreds or even thousands of microorganisms physically associated with each other or distributed at one location during the process of inoculation. Thus each colony develops form one viable unit; therefore viable counts are usually given as number of C.F.U. per unit volume rather than number of microbes (Collins *et al.*, 1995).

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The selectivity of medium and the conditions of incubation may significantly affect the number of viable cells, which give rise to colonies. Viable counting is the only procedure that provides direct estimate of alive cells. In other words, the death or decline phase of a bacterial batch culture can only be worked by C.F.U. method. However, the method is laborious and needs much amount of medium and glassware as compared to other methods for estimating bacterial populations. Therefore many modifications have been attempted to simplify it. Malik (1977) devised three different miniaturized methods for the rapid surface viable counting, which were easier, quicker and in some cases more accurate when compared with seven other methods of viable counts. The technique required about 10% of the material and time needed for conventional spread-plates method and at the same time results were in no way inferior. The author has described that in collections and bacteriological laboratories where conventional methods require large number of plates; the presented techniques could prove most convenient, rapid and economical. Similarly, Senyk

et al. (1987) made an attempt for convenient C.F.U. measurements. They compared dry culture medium method and conventional plating technique for enumeration of bacteria in pasteurized milk and found strong linear relationship between the two methods.

The present protocols were designed and compared with viable counts obtained by a standard method. These include C.F.U. measurement on miniagar discs inoculated with the help of micropipette as well as metal disc spreader. Cells of many bacterial species are arranged into groups of variable sizes. C.F.U. on standard count agar plates are enumerated generally after 24 hrs of incubation, which do not discriminate between the differences of the number of cells from which they developed. Because of this much incubation the colonies attain maximum size, irrespective to the number of cells/group yielding a C.F.U. One of the main objectives besides the economical reason for using mini agar discs in the present study was to correlate the number of cells per group of a bacterial culture to the C.F.U. enumeration emerging on the surface of a solid medium. In this study size of microcolonies, before attaining the maximum size, is also reported and compared with the number of cells per chain or group, determined microscopically. Results of this study allow C.F.U. measurements economically and describe the relevance of observing microcolonies to the number of cells yielding a C.F.U.

MATERIALS AND METHODS

Pure cultures of six bacterial isolates including a strain of *Staphylococcus aureus* isolated already in this laboratory were revived in nutrient broth. The isolates were processed for the determination of Gram's reaction and cell morphology, motility, catalase and oxidase tests according to Benson (1994). In addition to it, water and raw milk samples were also processed for viable counts of bacteria. Nutrient agar was prepared, sterilized and poured in sterilized Petri plates to solidify. Each bacterial culture or sample was processed for (i) standard plate count, 100 µl of a given dilution was spread on the entire surface with the help of a sterilized bent glass rod (Three replicates) (ii) Five miniagar discs were formed of a solidified layer of nutrient agar, with the help of an especially designed metal disc cutter (29 mm internal and 32 external diameter) and 10 µl of each dilution was spread on a mini disc with the help of sterilized mini spreader (five replicates) and (iii) nutrient agar layer in a Petri plate was divided into four parts and sample was spread over a quadrant with a sterilized metal disc of 24 mm diameter touched with the contents of a given dilution (four replicates).

All three categories of the plates were incubated in inverted position at 37°C. For standard count agar technique the plates were incubated for 24 hrs and bacterial colonies were then counted with the help of a colony counter. Miniagar discs and quadrant agar plates were incubated till the appearance of microcolonies. Colonies were then counted and measured with the help of an ocular micrometer. The microcolonies were grouped in to big, medium and small, based on their diameters. C.F.U. counts of a sample obtained by standard plate count, miniagar disc count agar quadrants count and total bacterial count by direct microscopic examination were compared. Only the dilutions producing 30 to 300 C.F.U. by standard plate count were used for comparison as suggested by Pelczar *et al.* (1986).

For direct microscopic count of bacteria 10 µl of a dilution was spread within a circle of known diameter on a slide. Three circles were similarly smeared for a given sample. The slides were stained by Gram staining procedure. The number of bacteria was counted in 5 different areas within each circle. All cells falling within visual field, while observing through 100 X objective were counted. Mean from 5 x 3 visual fields was used to calibrate number of bacteria per ml of the sample. In microscopic counting, the bacteria were recorded in to three groups i.e. single cell, association of 2 to 10 and those comprising of more than 10 cells per group. Counts of bacteria in terms of single, 2 to # 10 and more than 10 cells per group were compared to the size of corresponding microcolonies.

RESULTS AND DISCUSSION

Different bacterial isolates, water and milk

samples processed for viable counting of bacteria by standard agar plate count and the modified methods for economic purpose, indicated the validity of miniagar discs and non-reliability for the C.F.U. obtained by inoculating on agar quadrants (Fig. 1, Table I). As can be seen bacterial isolate SA-2 showed C.F.U. value of 22 x 10^7/ml on mini agar disc and 39 x 10^7/ml on standard agar plates. However, when the sample was transferred on agar quadrant by metal spreader, the value was 6.67 x 10^7/ml. This could be due to the error in the amount that was considered to be transferred and partially due to non homogenous spreading. In general the same trend was observed for all samples studied (Table I).

As the well separated colonies on standard agar plates attain the similar size after the incubation. This aspect i.e., number of bacterial cells yielding a C.F.U. cannot be determined from the standard plate count method. However, colonies on miniagar discs can be visualized under a low power microscope before they attain their full size. This enables to measure their diameters and categorize them in three groups based on their size. Thus when the microscopic counts of bacteria classified as single, 2-10 cells and more then 10 cells per group were compared to the corresponding number of small, medium and big sized C.F.U. correlation appeared for some pure cultures of the bacteria (Figs. 2, 3). Thus it is possible to assess the grouping pattern of bacterial cells by determining the size of juvenile, 6-10 hours old bacterial microcolonies appearing on miniagar discs under a low power microscope. Of course besides this additional information the procedure is economical both in terms of amount of medium used and the material required for the process.

Methods employed in this study to economize viable count of bacteria revealed two significant observations. First the amount of medium required is much lesser in mini agar discs as compared to the standard plate count method. Further number of Petri plates needed for an experiment is also less as in case of miniagar discs and agar plate quadrants, five and four spreadings can be accomplished from a single plate, respectively. For the water sample when its dilution 10^4 was spread on nutrient agar plates, the C.F.U. for three replicates were found as 113, 276 and more than 300. When the same dilution with 10µl was inoculated on miniagar discs with the help of mini spreader, the C.F.U.

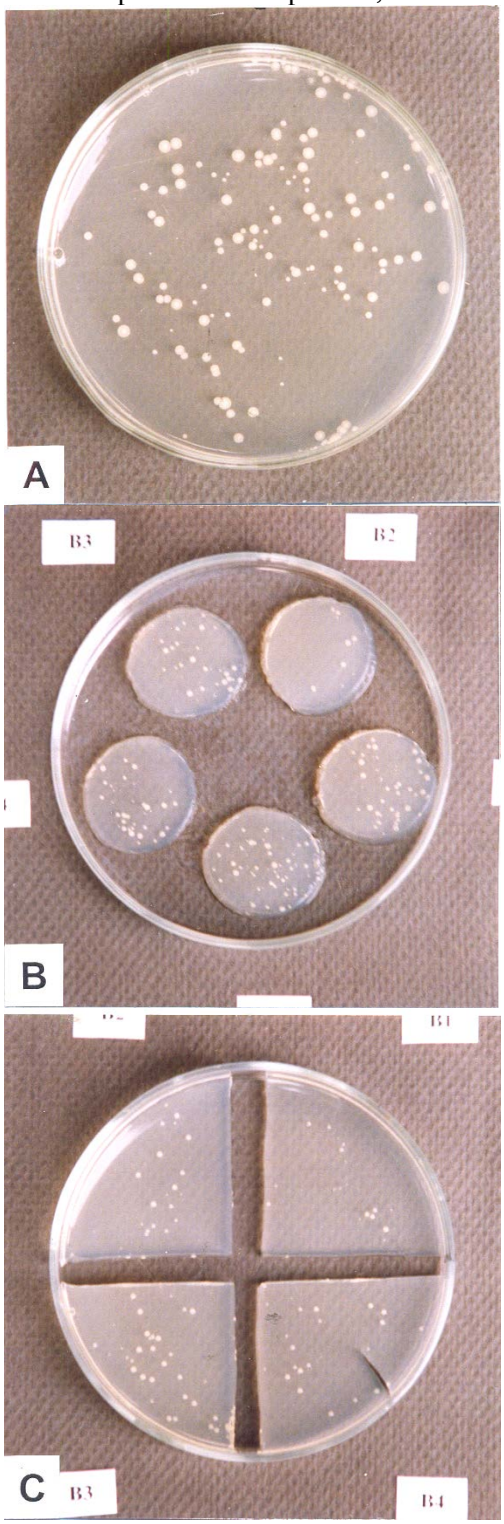

Fig. 1. Appearance of bacterial C.F.U.on stanandard agar plate(A) and microcolonies on miniagar discs(B) and agar quadrants(C).

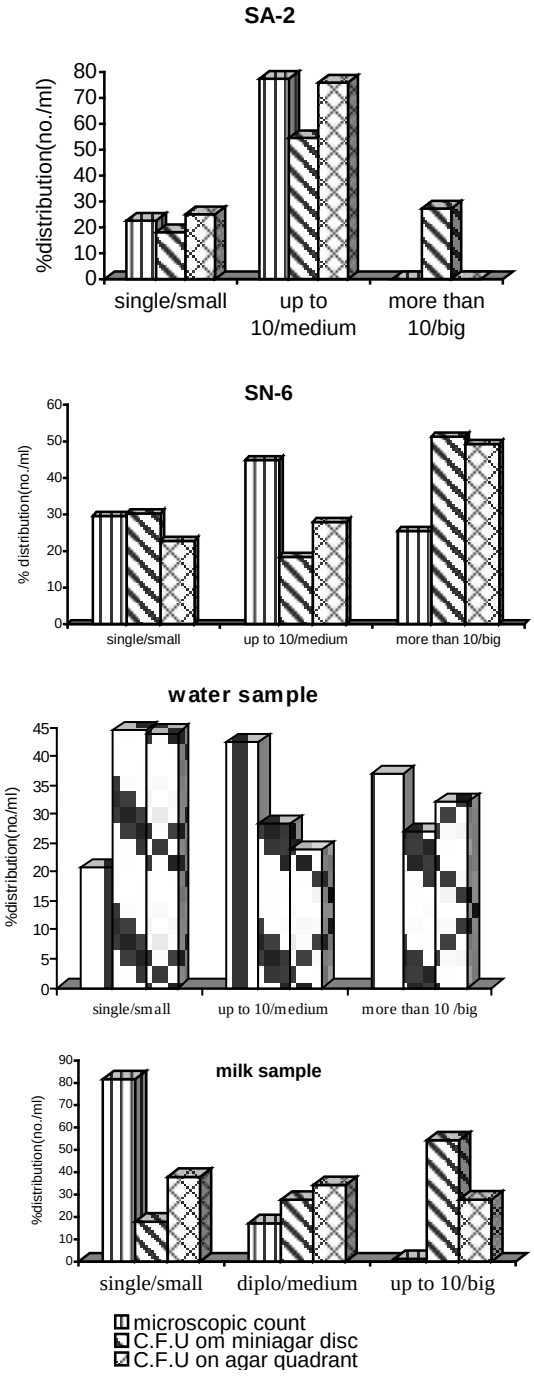

Fig. 2. Comparison of %age of microscopic counts of single, diplo up to 10 and more than 10 cells /group and microcolonies categorized as, big, medium and small which appeared on mini nutrient agar discs and agar plates quadrants of the bacterial isolates SA2, SN 6 milk and water sample.

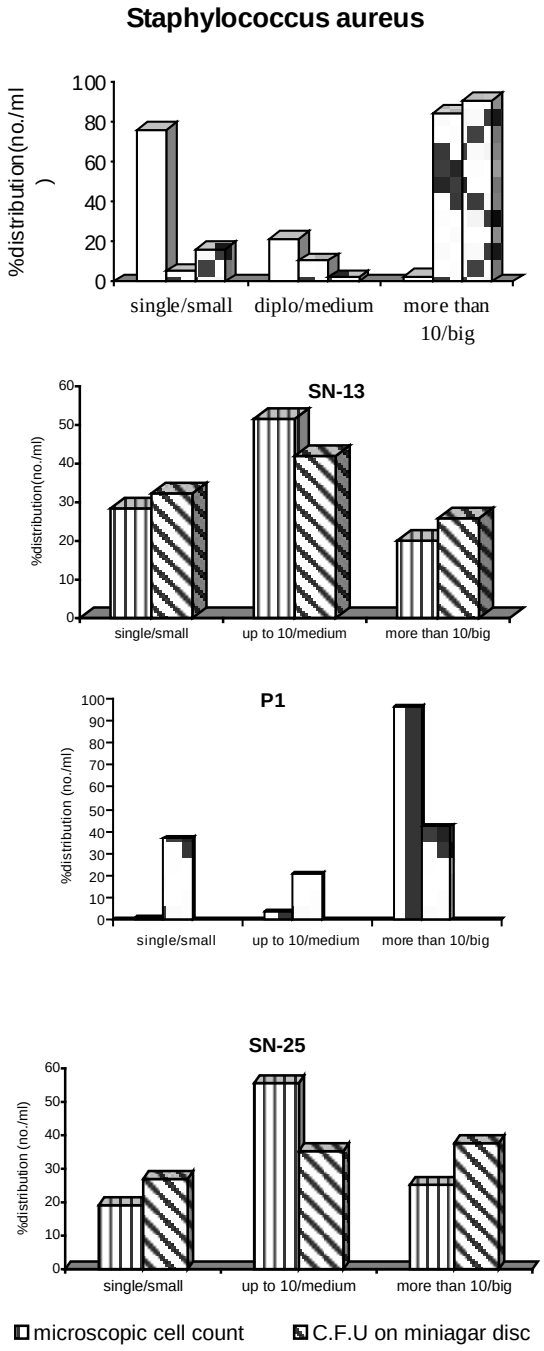

Fig. 3. Comparison of %age of microscopic counts of single, diplo up to 10 and

more than 10 cells/group and microcolonies categorized as, big, medium and Small which appeared on mini nutrient agar discs and agar plates quadrants of the bacterial isolates. *Staphylococuss aureus*,SN13,SN25 and P1.

Table I.- Comparison of estimate of bacterial population density by direct microscopic count, colony forming units C.F.U. on mini nutrient agar discs, agar plates quadrants and on standard agar plates.

Bacterial isolate /sample	Microscopic counts /ml			Microcolonies (C.F.U) on the mini agar disc after 7 hrs			Colonies (C.F.U/ml) on standard agar plates	Microcolonies (C.F.U)/ml on agar quadrants after 8 hrs		
	Single Cell	Diplo up to 10 cells /group	More Than 10 cells /group	Small	Medium	Big		Small	Medium	Big
SA-2[a]	10.33±2.40 [61.2x10^7± 61x10^7][b]	17±3.51 [209x10^7 ±209x10^7]	15±10.01 [1255628x10^7 ±850599.9 x10^7]	3±0 [4x10^7 ±2.4]	1.5±0.28 [12 x10^7 ±3.7]	±0 [6 x10^7 ±2.4]	29.75±9.39 [39x10^7±2.3]	0.5±0.28 [1.67x10^7 ±0	0.75±0.75 [5 x10^7 ±0	0 0
SN-6[a]	20.66±6.11 [11238773 x10^7±8876318 x10^7]	35±10.58 [17041646 x10^7±1632 3426 x10^7]	19±7.50 [9694589x x10^7± 9533599x10^7]	8.6±1.32 [1.68x10^7 ±0.4]	10.2±3.0 [1.02 x10^7 ±0.30]	16.±4.46 [2.8 x10^7 ±1.09]	176.2 ±47.65 [242x10^7±27]	4.75±2.4 [0.07 x10^7 ±0.041]	7.5±1.25 [0.09 x10^7 ±0.0]	21.75±2.6 [0.16 x10^7 ±0.05]
Water sample	39±12.58 [13969311x10^7 ±12635923 x10^7]	43±5.85 [28749805 x10^7±2748 1148 x10^7]	28±3.46 [24904305x 10^7± 24172017 x10^7]	11.±1.8 [1.8 x10^7 ±0.39]	12±1.14 [1.2 x10^7 ±0.11]	18.8±3.9 [1.14 x10^7 ±0.8]	80.87 ±34.52 [18.6x10^7±16]	34.6±5.5 [0.57 x10^7 ±0.09]	18.66±2.0 [0.31 x10^7 ±0.03]	26±2.2 [.42 x10^7 ±0.03]
Milk sample	16.33±0.88 [13238576 x10^7± 13001755x10^7]	34.33±3.28 [2784752x 10^7±22886 32 x10^7]	18.66±9.95 [194790x10^7± 190370x10^7]	69.2±7.3 [2.28 x10 ±0.4]	35.4±9.4 [3.54 x10^7 ±0.944]	22.8±5.4 [6.9 x10^7 ±0.73]	92 ±42.0 [92x10^7±42]	56.75±14 .16 [.92 x10^7 ±21]	53.7±6.5 [0.83 x10^7 ±0.12]	42.5±7.2 [0.92 x10^7 ±0.21]
Stap. aureus	16.33±5.48 [62449x10^7 ±60339x10^7]	26.66±4.84 [17620x10^7 ±13728 x10^7]	19.33±10.47 [1945x10^7± 1898x10^7]	12.4±1.3 [.003x10^7 ±0]	6.6±2.24 [0.006 x10^7 ±0.002]	3.2±0.86 [0.05 x10^7 ±.02]	14.75 ±7.375 [.0013x10^7±0. 06]	0.5±0.2 [0.00005 x10^7 ±2.8]	1.25±0.47 [0.00017 x10^7 ±0.00]	6±2.6 [0.0022 x10^7 ±0.00]
P1[a]	21.66±9.5 [45545x10^7± 42655x10^7]	103±29.56 [326069x1 0^7± 324293 x10^7]	50.33±19.64 [9030346x10^7±8 547548 x10^7]	72.2±11. [6.4x10^7 ±4.22]	36±5.94 [3.6 x10^7 ±0.59]	8.2±3.86 [7.22 x10^7 ±1.12]	169.3 ±8.96 [169x10^7±8.9]	-[c]	-	-
SN-13[a]	43.66±9.61 [2612197x 10^7±1997606 x10^7]	61.33± 20.01 [4743575x 10^7±43056 7x10^7]	30.33±46.93 [1846211x10^7±1 450131 x10^7]	8±0 [20 x10^7 ±0]	13±0 [26 x10^7 ±0]	10±0 [16 x10^7 ±0]	34.6 ±11.62 [10.91x$10^7$10. 5]	-	-	-
SN-25[a]	14.66±2.40 [889100x10^7± 863558x10^7]	37.66±5.84 [2579962x 10^7±21278 02x10^7]	18±3.7 [1171325x10^7±9 89485x10^7]	9.6±1.4 [5.2x10^7 ±1.8]	6.4±0.50 [6.8 x10^7 ±0.73]	5.2±1.85 [7.26 x10 ±1.8]	49 ±8.08 [49x10^7±8.08]	-	-	-

[a]The symbol represents cultures of different bacteria isolated in this lab. but not identified [ ][b], Values calibrated as C.F.U. x 10^7/ml of sample, [c]Test not performed

All the isolates used were motile, Gram positive and of coccus – streptococci except the SN-6 that depicted bacillus – streptobacilli morphology.

appeared for the five replicates as 44, 3, 37, 27 and 64. Similarly, for the bacterial isolates, SN-6 when the second dilution was spread on nutrient agar plates, the number of C.F.U. for three replicates were greater than 300, while for the same dilution whenss 10µl was inoculated on miniagar discs, the C.F.U. emerged for the five replicates were 57, 11, 33, 32 and 45. Thus employing the small discs with fewer amounts of inocula, better results could be expected for C.F.U. in terms of repeatability of the test. This probably indicates the proper ratio of amount of the liquid to be inoculated on the surface area and ease of spreading. In a comparable study Malik (1977) found that viable counts on miniagar discs was the more convenient method. Use of metal spreader needs few equipments and procedure

instead of micropipette for processing a sample for viable count. Such methods can be adopted for selected situations. However, processing sticky viscous material and ability of surface texture of the medium to hold the material after first inoculation have to be worked out.

A very strange observation was that in most of the cases when 100µl of the sample was inoculated on the medium in Petri plate it gave rise to uncountable colonies, but when the same sample was inoculated on miniagar discs both with micropipette delivering 10 µl and the metal spreader transferring 55.56 µl, the C.F.U. appeared usually countable and within permissible limits. Moreover, concerning the results of replicates for the mini agar discs, the values fluctuated within narrow range. These observations clearly indicate that the miniagar discs apart from being more economical also provide accuracy. In fact the amount of inoculum for a given dilution and nature of the spreader bear many relationships with the result of C.F.U. For instance, Pye *et al.* (1995) reported single method for quantifying viable bacterial number in sputum and the spreader termed as "hockey stick" resulted in least variation between C.F.U. values of different plates (less than 16%) and an even distribution of bacterial colonies. Regarding the amount of inoculum it is very important to establish optimum size of expected dilution for a given spreading surface, while minimizing the inoculum and spread surface. For example, Frimodt and Espersen (2000) have shown that for sample having low bacterial contents, 1 microloop did not deposit any bacteria on the agar plate, when colony counts were lower than 10^4 C.F.U. per ml. While 10 microloops constantly deposited 1.5 times higher number of bacteria than predicted and variation coefficients increased with decreasing volumes sampled but 10 microloops were found sufficient to detect counts for 10^3 to 10^4 C.F.U./ml.

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