J.Meat Sci., 2012, 8(1&2) : 72 - 75 Research Note

Comparative evaluation of efficacy of manual *vis-à-vis* automated colony counting techniques used for enumeration of microorganisms in meat

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ABSTRACT

Manual counting of colony forming units (CFU) grown over a semi-solid media is a tedious process while enumerating microorganisms in foods such as meat and meat products thereby necessitating automation. In the present study, automated colony counter was validated against manual colony counting by counting CFUs grown on solid agar plates. Colonies grown on agar medium by standard plate count technique of meat samples were enumerated by both counting methods. Upon counting colonies in the range of 10-1000 per plate, it was found that the values of automated counting were on par with the manual counting as revealed by linear eqnation ($y = 1.0011x + 0.588$, $R^2 = 0.9998$). Keeping in view the time consumption and tedious effort exercised during manual counting of colonies on solid media, automated counting technique using imaging technology was found rapid, accurate and convenient.

Key words: *Colony count, CFU, enumeration, imaging*

Enumeration of microorganisms using standard plate count (SPC) over solid agar media requires counting of colonies. Each microbial colony emerges out of a colony forming unit (CFU) as a result of binary fission in microorganisms. Colony formation makes invisible CFU (individual bacteria) detectable thereby aiding counting of microscopic organisms. This is the basis of enumeration of load of microorganisms in foods such as meat and meat products.

However, higher load of microflora in meats per unit weight is unreadable owing to overcrowding and overlapping of CFUs. Hence, the sample is serially diluted and the count is transformed into a countable number (a process of 1:10 serial dilution of sample or 10-fold dilution). Upon incubation for a specified period (usually 18-24 hours), only those plates having countable number of colonies (customarily, 25-250 or 30 -300) are taken for expressing SPC per gram of sample (meat or meat product). Colonies per plate less than 25 (or 30) per plate might arise due to contamination while carrying out experiment; and >250 (or 300) could be too numerous to count (TNTC). Hence, conventionally, only 30 to 300 CFU are enumerated per dilution over plates.

Petri plates containing semi-solid agar medium yield colonies after incubation under appropriate growth conditions required by microorganisms. These colonies are counted to determine number of CFUs by manually counting individual colonies on plates against illumination of transmitted light fitted with a magnifying glass (colony counter). The microbial load is thus expressed based on the assumption that each colony has emerged from one single bacterium (colony forming unit, CFU). However, this process is time-consuming, tedious and error prone. Laboratories analyzing hundreds of meat samples for microbial enumeration have to spend several man-hours for just counting. Further, the analyst has to be highly alert and attentive while counting; moreover, the procedure is tiresome and error-prone due to over-crowding of colonies (Breed RS and Dotterrer, 1916).

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Therefore, there is a need for an automated counter that counts colonies instantaneously and has even capability of storing image into its database (Brugger *et al.* 2012).

In order to overcome the problems encountered by the manual colony counters, automated colony counters (ACC) have been introduced that reliably detect and count colonies. The ACC are built as a system consisting of hardware and softwares. The hardware consists of plate illuminator, image focusing and image-capture devices; while software analyses the image and reproduce it in the form of colony count. Modern ACC systems are user-friendly and cost-effective with in-built algorithms applicable to a variety of culture media and microbial colonies.

Keeping in view the need for an ACC for the routine work in food analytical laboratories, the present study was undertaken with the aim of validating the ACC for the purpose of enumeration of microbial colonies over agar plates using meat samples.

Preparation of semi-solid agar petri plates for getting visible colonies*:* Samples of meat and meat products submitted for microbial analysis to the Department of Veterinary Public Health &Epidemiology, Bombay Veterinary College, Parel, Mumbai were used for this study. Standard plate count (SPC) as Aerobic count was performed as described in the bacteriological analytical manual (BAM, 1998) with slight modifications. Briefly, 10 grams of minced meat sample was homogenized sterile normal saline (NS) and the volume was made to 100 ml; this first dilution was taken as 1:10 or 10^{-1}. Further 10 fold dilutions were from 1st dilution by taking 9 ml of NS into each tube and transferring successively well-mixed 1 ml of previous dilution. One milliliter of each dilution was poured over a sterile petri plate and about 12 ml of molten autoclaved nutrient agar was mixed into it. The plates were then rotated in clock-wise and anti-clock-wise directions and allowed for solidification in the laminar air flow. Finally, plates were incubated up-side down at 37°C for 18-24 hours and colonies were counted manually using a colony counter manually as well as with the help of automated colony counter (ACC). Only those plates that were populated with colonies between 30 and 300 were considered for counting.

Equipment used for enumeration of colonies*:* Two types of counting methods were used for the enumeration of colonies *viz*. 1. Manual colony counting method and 2. Automated colony counter.

Manual colony counting method: Manual colony counter (M/s. Lapiz, Bacteriological, Digital) consisted of a window for holding petri plate with circular illumination field below the plate.It was provided with a magnifying glass for viewing enlarged colonies for better resolution. A digital counter was provided for counting. In some advanced models, the counter was also provided with a pen like tip to mark and read the colony, pressing of the tip counted a number that added to the sum of colony count.

Table 1: Colony counts obtained by manual and automated counting techniques

No	Media Colour	Mannual counting (Mean ± S.D.)	Automated counting (Mean ± S.D.)
1)	Orange	15.0±0.0	15.0±0.0
2)	White	20.3±1.5	22.0±0.0
3)	Yellow	25.3±0.6	25.0±0.0
4)	Yellow	27.0±0.0	27.0±0.0
5)	White	45.7±0.6	47.0±0.0
6)	White	56.7±0.6	57.0±0.0
7)	Orange	65.7±1.5	66.0±0.0
8)	Orange	94.7±0.6	95.0±0.0
9)	Pink	182.0±2.0	180.0±0.0
10)	White	236.7±2.5	240.0±0.0
11)	White	270.0±3.0	275.0±0.0
12)	Yellow	278.3±1.5	280.0±0.0
13)	White	292.7±2.5	297.0±0.0
14)	White	308.7±1.2	311.0±0.0
15)	White	323.0±3.6	325.0±0.0
16)	Yellow	330.3±1.5	330.0±0.0
17)	Orange	505.0±5.0	502.0±0.0
18)	Pink	651.0±2.0	638.0±0.0
19)	Yellow	772.7±2.5	776.0±0.0
20)	Yellow	811.7±4.2	820.0±0.0
21)	Yellow	906.7±2.9	910.0±0.0

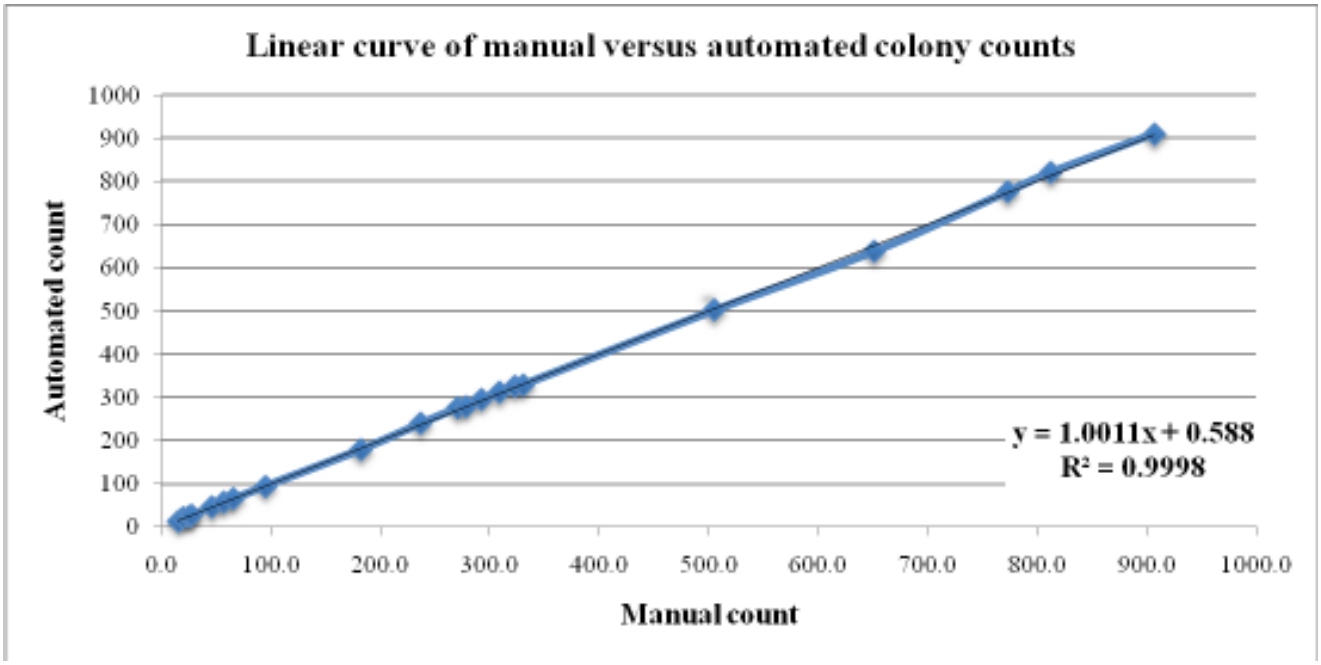

Figure 1. Linear equation plotted using counts taken using a colony counter and ACC.

Automated colony counter: Automated colony counter (BiovisCC200e, M/s. Biovis, Expert Vision Labs Pvt. Ltd.) was used for enumeration of colonies automatically. The hardware configuration of ACC comprised of fully enclosed acrylic system known as 'Colony Imager'. It had a well-spaced slit or compartment in the middle of the enclosure for the easy insertion and removal of petri dish. The Colony Imager consisted of a high resolution camera with zoom lens with provisions for special illumination integrated into the system for the capturing of colony images from a plate. The system used compact imaging technique.

ACC illumination and imaging: Petri dish were illuminated with a circular dark field illuminator and a CCD camera with a resolution of 1.4 megapixels and a zoom lens with 75mm focal length.The illumination set-up consists of illumination from the top and the bottom for generating even light and this compartment was constructed so as to minimize light scattering. Additionally, dark-field mode was used to detect transparent colonies. The camera was connected to a computer *via* an USB interface.

Colony counter software*:* The Software had an interface to the image capturing system and it captured colony images directly using the camera. Once the colony images were captured by running the feature of counting in the software, total number of colonies was displayed instantly. Additionally, the software had imaging functions to measure the morphology of colonies.

Colonies grown on agar medium by SPC technique of meat samples were enumerated by both counting methods *i.e.* manual and automatic. The results of manual and automated colony counting are depicted in Table 1. Based on the observations made in the study, it was evident that the machine counts matched with manual count (Figure 1). When colonies were counted in the range of 10-1000 per plate, it was found that the values of automated counting were in accordance with the manual counting. The linear coefficient ($y = 1.0011x + 0.588$, $R^2 = 0.9998$) revealed good concordance between the two techniques compared in the present study.

In a similar study, Brugger (2012) also obtained similar findings with a novel counting technology. Several investigators have developed novel algorithms for colony counting (Corkidi *et al*, 1998; Putman *et al.* 2005; Bewes *et al.* 2008); however, in the present study effort was made to validate the efficacy of automated counting against

manual counting. On an average 3.4±0.5 minutes are required to count about 250-300 colonies over a plate manually; while ACC takes less than 0.5 minute for the same. Since automated counting of CFUs is on par with the manual counting and takes less time, it is envisaged that laboratories processing huge number of meat samples for enumeration of microorganism may use ACC thereby saving precious time in terms of man-hours.

Further, the advantage of automated colony counters was that the software detected the colonies and counted based on different measurements. It automatically categorized colonies according to shape, size and colour, separation of closely lying colonies, image generation and customized report generation.

However, certain aspects of ACC machine required critical attention. Since illumination was an important aspect of the procedure, good lighting achieved to better clarity leading to accuracy in results. When different illumination techniques were used, circular dark field illuminator was found superior compared to lighting provided from the top and bottom of the compartment.

In conclusion, based on the results of the manual and automated colony counting techniques used in this study, automated colony counting was found superior over the manual enumeration of colony forming units over semi-solid agar medium owing to advantages such as simplicity, ease in performance and savings in valuable time of an analyzer.

Authors thank the Associate Dean, Bombay Veterinary College, Parel Mumbai for providing laboratory facilities for conducting this research.

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