BIOMÉRIEUX

New Standardized Methodology to Evaluate Environmental Monitoring Plates Inspection Using an Automated System.

Your Ally in Advancing Quality

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INTRODUCTION

As the main microbiological control performed in the pharmaceutical industry, Environmental Monitoring (EM) ensures the safety and efficacy of pharmaceutical products.

To efficiently control the quality of such products, pharmaceutical manufacturers must monitor for potential microbial contaminants. This is typically performed using irradiated culture media in Petri plates throughout the production process, especially within clean rooms, isolators and controlled production environments.

The widely accepted practice for Petri plate examination involves enumerating discrete microbial colony forming units (CFU) per plate after the plate has been incubated according to the time and temperature parameters validated by the end user. The two-fold challenge, however, is that pharmaceutical environmental monitoring generates thousands of Petri plates per month, and not all microorganisms, which may be rare in clean rooms, grow into distinct macroscopic colonies, thereby increasing the risk of missing them.

The current method is extremely manual, variable, and error prone, but it remains the standard procedure used for hundreds of millions of samples per year. When so many plates are evaluated manually, pharmaceutical QC becomes vulnerable to data integrity failures, incorrect enumerations and missed samples. The net result is a negative impact on patient safety due to time consuming, and expensive investigations.

Therefore, the importance of accurate and precise enumeration and counting of microorganisms has been stressed by global regulatory agencies.

In this white paper, we assessed the performance of visual Petri plate inspection in a pharmaceutical setting and introduced a novel standardized methodology to automate the incubation and enumeration of Petri plates using the 3P® STATION, shown on the right. Results were compared to the ones generated according to the routine EM Standard Operating Procedure in four global pharmaceutical companies.

The 3P STATION automates the incubation and counting of microbiological colonies on EM Petri plates in real time. This innovative system automates the incubation of plates between 20°C and 35°C where high performance algorithms detect and follow the growth of microorganisms.

3P® STATION

Using a high-resolution camera coupled with an advanced telecentric optical lens, the 3P STATION takes one high-definition image every hour and creates a movie to track the growth of microorganisms. In this study, the inoculated plates were incubated in the 3P STATION to generate images of microorganisms throughout the incubation process from start to finish. In the case of swarming colonies that could hide or make the enumeration difficult, the system allowed users to rewind the growth movie to the exact moment where enumeration was possible.

MATERIAL AND METHODS

Microorganims and temperature conditions

The five recommended Pharmacopoeia strains (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404) and three in-house wild microorganisms (two bacteria and one mold) were challenged during the reading evaluation. Three inoculation levels were evaluated: 5 CFU, 25 CFU and 50 CFU per plate, five replicates were realized per inoculum size.

All microorganisms were grown on bioMérieux TSA 3P with Neutralizers ref. 43819 and Count-Tact 3P ref. 43699 at an appropriate temperature and incubation time to optimize the ease of reading for operators.

3P STATION

Introduction to a reference method (the Reference Traditional Count)

Traditionally, individual operators are prone to counting different numbers of microorganisms on the same plate because of manual interpretations and the unpredictability of the microorganisms' morphology (shapes, swarming, etc.), and the natural detection limits of the human eye.[1]

A standardized CFU count must be defined to evaluate the performance of the current methodology. To obtain this gold standard, we developed a methodology to correct the actual operator count with the data generated by all the images captured from the beginning of the incubation. We defined it as the "Reference Traditional Count".

After incubation in the 3P STATION, each plate is counted by several operators independently, following the traditional counting method. The operators are not influenced by the data of the 3P STATION (pictures or counts). Each operator records their count on independent sheets.

In a second step, after the plate has been read using the traditional method, the physical plate was analyzed a second time by the same operators together with help from the 3P STATION. By replaying the video and zooming in specific plate areas, the operators were able to directly tick the colonies on the final image of the plate, and give a corrected count—the Reference Traditional Count.

It is intended to represent the gold standard enumeration, which is as close as possible to the reality.

RESULTS AND DISCUSSIONS

Three different operators from four pharma companies performed the count of each plate and also generated the Reference Traditional Count.

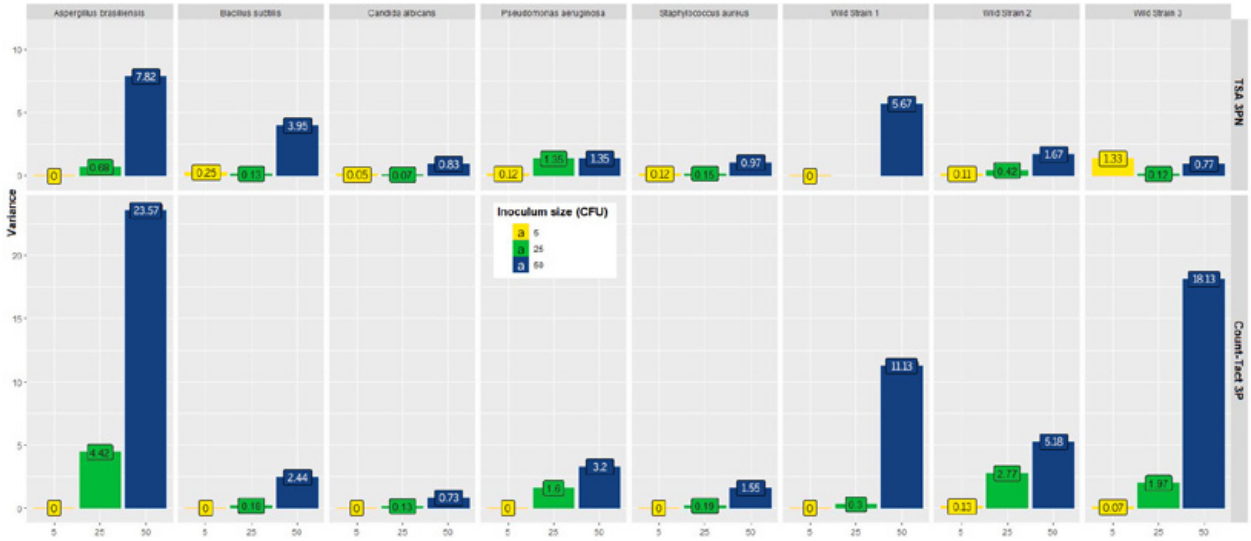

Figure 1: *Variance calculated for all strains per type of plate and inoculum size.*

Figure 1 shows a statistical analysis of the variance calculated from the different enumerations per strain type. This data allowed us to measure how far the set of measurements is spread from the Reference Traditional Count. The higher the variance, the lower the accuracy of the reading. These results have highlighted how the three main parameters that can interfere with the performance of enumeration are:

- The plate format: Count-Tact plates that have a smaller surface (the diameter of 55 mm contact agar plates is lower compared to standard 90 mm agar plates) concentrate the microorganisms that are closer together and more difficult to detect. This seems to be the main factor impacting the quality of the enumeration: $variance_{Count\text{-}Tact} \in [0 ; 23.57] > variance_{monoplate} \in [0 ; 7.82]$.

- The size of the inoculum: the accuracy of the enumeration is decreasing with the increase of the inoculum size. The best enumerations were obtained with 5 CFU inocula (variance $\in [0 ; 1.33]$), and the worst with 50 CFU inocula (variance $\in [0.77 ; 23.57]$), 25 CFU confirmed intermediate results.

- The morphology of the microorganisms: strains that displayed a shape that was not well defined (fuzzy or uneven borders, translucent, etc.) were more difficult to enumerate correctly.

Looking at the wild microorganisms tested, molds (wild strain 3) appear to be the most difficult microorganisms to enumerate accurately and this is emphasized by the type of the plate and the inocula sizes. These different parameters described above exercise a cumulative effect on the ability to discern colonies.

To evaluate the impact of colony morphology on the accuracy of the enumeration, an analysis on the 25 CFU inocula was performed. Results are presented in Figure 2 with the difference of count observed between each operator and Reference Traditional Count.

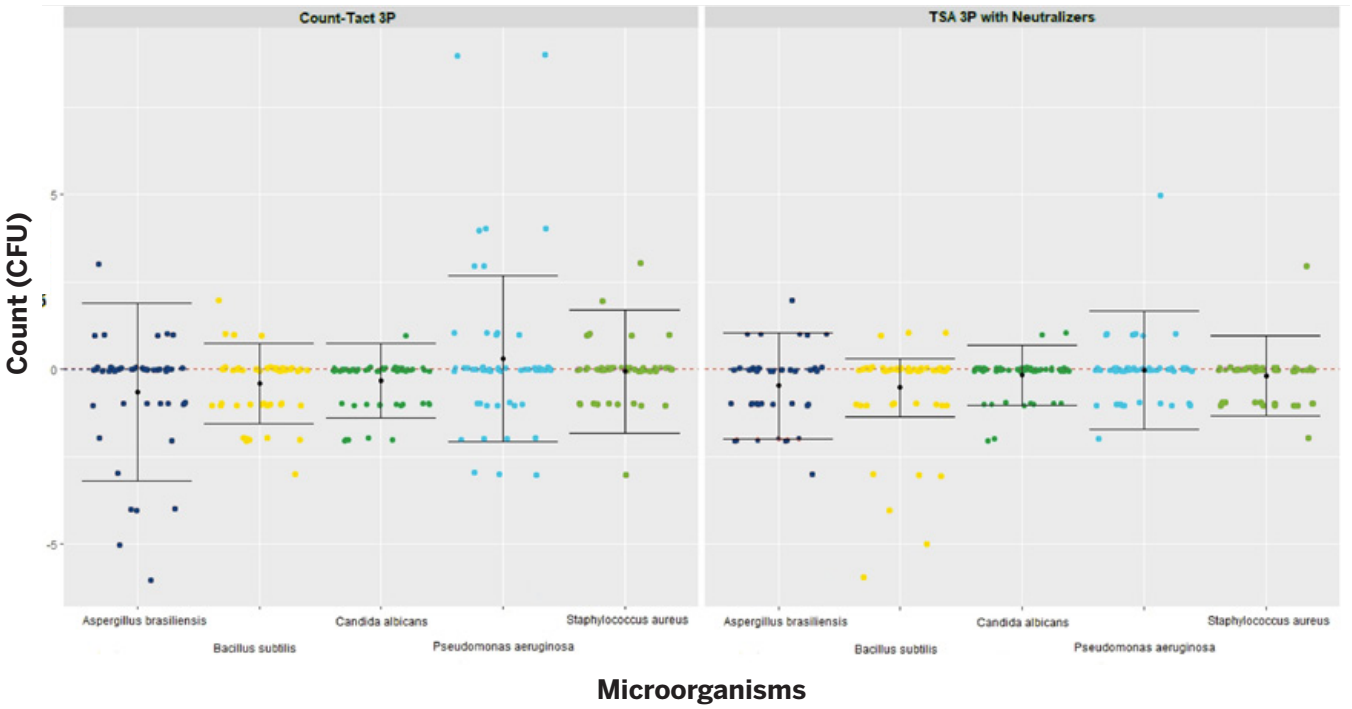

Figure 2: *Difference of enumeration observed between Operators vs. Reference Traditional Count for the 5 Pharmacopoeia strains at 25 CFU.*

Figure 2 confirmed that the type of Petri dish is impacts the ease of detection of the microorganisms on the agar. The standard deviation is higher on the Count-Tact plates, however, outliers can be observed on the 90 mm plate format, especially on the *B. subtilis* strain where underestimated enumerations were observed. This could be explained by human interpretation when swarming events occur and increase the subjectivity of this exercise.

The impact of the morphology in accurate counting is more visible on the Count-Tact format where colonies that did not display a well-defined shape (*A. brasiliensis*) or that were translucent (*P. aeruginosa*) have provided less accurate results.

Another analysis can be done by looking at the distribution of the enumerations observed by the operators and the difference in terms of false detection compared to the Traditional Reference Count, as highlighted in Figure 3.

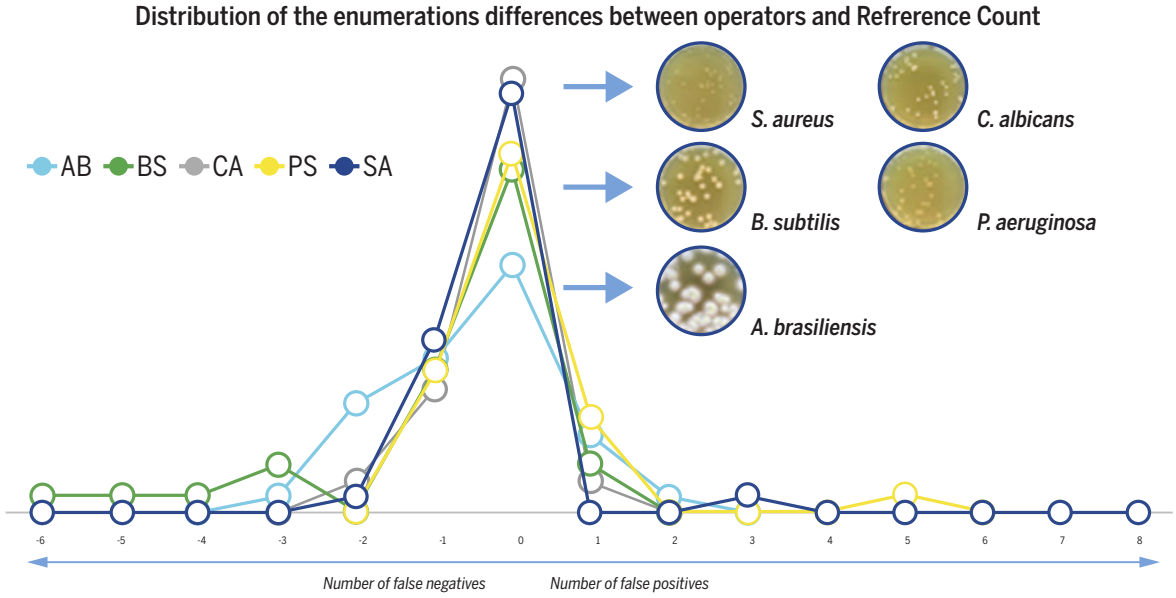

Figure 3: *Distribution of the count differences between operators and Reference Traditional Count (TSA3P N results only) – Morphology of the strains evaluated are presented on the right part of the chart.*

The left section of the chart shows the number of operator enumerations with missed colonies (from 1 to 6) and the right section of the chart shows the number of operator enumerations with additional colonies falsely counted (from 1 to 5). This figure demonstrated that the number of colonies missed is higher than the number of colonies added by mistake. Hopefully, the vast majority of the counts are equal to the Traditional Reference Count and the discrepancies are only due to a difference of 1 CFU. A difference higher than 2 CFUs can occur, but is less frequent.

The impact of the morphology is easily seen when looking at the number of correct enumerations (difference between operator counts and Traditional Reference Count = 0). The microorganisms with the most defined appearance (*S. aureus* and *C. albicans*) were counted more accurately. Bacteria with a more challenging shape (*B. subtilis* and *P. aeruginosa*) were slightly less enumerated. Finally, the mold (*A. brasiliensis*) was far below the others in term of correct enumeration.

Table 1 shows a global view of the enumeration performance per operator on all colonies seen on different dishes. The rate of False Positive is lower than the False Negative rate. The percentage of False Negative can vary from 1.26% to 6.08%. This is a surprising result that questions the reliability of the traditional method for plate inspection.

			False Negative		False Positive	
Op. #	**Pharma Industry**	**Sum of All Reference Count**	**Number**	**Percentage**	**Number**	**Percentage**
1	A	3143	138	4.39%	5	0.16%
2	A	3143	191	6.08%	13	0.41%
3	A	3143	94	2.99%	6	0.19%
4	B	2469	31	1.26%	76	3.08%
5	B	2469	51	2.07%	13	0.53%
6	B	2469	26	1.05%	30	1.22%
7	C	3490	62	1.78%	14	0.40%
8	C	3490	108	3.09%	28	0.80%
9	C	3490	88	2.52%	17	0.49%
10	D	3531	81	2.29%	23	0.65%
11	D	3531	74	2.10%	12	0.34%
12	D	3531	72	2.04%	19	0.54%

Table 1: *False positive and false negative results generated during reading*

CONCLUSIONS

This study presents a new methodology based on a Reference Traditional Count generated thanks to the 3P STATION.

Natural human variability must be taken into consideration as it will always be present. With this approach, that variability is now better qualified. The enumeration of Petri dishes may be difficult due to the morphology, size, and swarming of the microorganisms which introduces increased variability of the results and mainly leads to an underestimation of the real number of microorganisms on a plate. A simple enhancement could be automated reading to provide a robust and standardized counting methodology.

The pharmaceutical industry should consider replacing the traditional method of plate enumeration with automated incubators and plate counters. In addition to consistency in the results, this technology will improve data integrity, lead to more robust trend analysis, and ultimately will alert earlier when a sample exceeds its specification, thereby enabling faster decision making.

REFERENCES

1. Detection of Small Events in Environmental Monitoring Culture Media - How accurate is the visual inspection? Laurent Leblanc, Katia Imhoff

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