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Microbial measurement – the inconvenient truth

This series of articles is intended to take a fresh look at microbiological testing in an attempt to broaden the understanding and accept the limitations and impact on quality and safety assessments.

Microbiological tests are performed on raw materials, finished products and environmental samples to assess risks and monitor manufacturing procedures and control. Raw material and finished product testing are accepted to be of limited value because it is impractical and too expensive to do enough testing to give statistical confidence for the batch. The test results apply only to the samples examined which are random snapshots but it is assumed to be indicative of the whole consignment. More effective control is obtained by the implementation of the principles of quality assurance and preventative systems (e.g. GMP, HACCP) that are now widely adopted and included in food safety regulations. Under these systems there is a greater emphasis and reliance on environmental and in-process samples to manage and minimise cross contamination. Environmental samples give more relevant information about risks to the product. Faster results would also permit timely intervention and corrective action.

The concept of zoning is used to identify, differentiate and segregate processing areas within the facility where potential sources of pathogen and non-pathogen contamination exist (e.g. air, traffic, people, equipment and materials). Controls are identified and implemented appropriate to the business, risk of cross-contamination and proximity to the product.

The gold standard for microbiological testing is the cultural method, the principles of which have remained largely unchanged since the pioneering day of Pasteur and Koch (mid 1800s). However the results are highly variable due to many different factors some of which will be addressed in future articles.

The unit of measurement is the colony forming unit (CFU) which is fundamentally flawed by the incorrect assumption that a single colony is derived from a single bacterium. Each replicate sub-sample will yield a result with a different colony count. It is claimed that Einstein said the definition of insanity is doing something over and over again and expecting a different result. Several replicates for different dilutions of each sample are required to obtain a reasonable approximation but this is seldom done in routine testing.

Even in the best run laboratories the uncertainty of measurement is +/- 40%. This means that the actual value is not known for certain, and for a sample expected to contain 10,000 cfu the value lies somewhere within the range 6000 to 14000 cfu on 95% of occasions but can also be outside this range 5% of the time.

Professional opinion acknowledges that the colony forming unit (CFU) is defined as 'at best, an estimate and should not be reported as absolute' (Compendium of Methods for the Microbiological Examination of Foods (APHA 1992). The working group of the International Laboratory Accreditation Cooperation states: "it is virtually impossible to know the exact microbial concentration in any sample, natural or artificial."

Despite the above, there is often an ignorance and blind belief in the CFU that leads to unreasonable expectations and demands for accuracy and precision in plate count results that cannot be delivered. Several alternative and rapid methods exist yet their adoption has been limited by the requirement to validate their performance against the highly variable culture plate count methods in processes that are not making like-for-like comparisons. Tony Sharpe (1980) stated: "The plate count from its very nature provides data so unique that they can be related to no other analytical data than those from other plate counts" Some microbiological methods can be specific and very sensitive but enumeration is very imprecise.

The challenge is to break away from the reliance and vagaries of the CFU and find a better way to assess and express microbial contamination.

We cannot solve our problems with the same thinking we used when we created them.

Albert Einstein

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Why count bacteria on surfaces?

In the previous article we discussed the limitations of microbial measurement when significant numbers were present in the sample. Even the best laboratories running several replicates in proficiency test schemes are expected to see significant variation between laboratory results on the same sample which can be 1 log (10 fold) but in practice the results for indicator organisms such as coliforms and enterobacteriaceae are worse (3-4 log variation has been observed). So what can we expect from environmental samples where the microbial load is much lower?

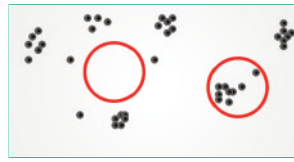
The challenges are many fold, so that enumerating microbial contamination in environmental samples is of limited value and we need to consider a different approach.

Environmental monitoring is required to measure the presence of total bioburden (Total aerobic bacteria), indicator organisms of poor hygiene such as coliform and enterobacteriaceae, spoilage organisms such as lactic acid bacteria or yeast, and pathogen bacteria such as listeria and salmonella. There are no standards for environmental samples due to the varied and unique nature of each manufacturing facility and process.

For many prepared foods it is generally accepted that pathogens should be absent, indicator and spoilage organism should be very low (<10 or <100 cfu) or below a detectable limit and the total bacteria count has broad guidelines of <10cfu as good and >1000 cfu as unacceptable. The closer the environmental sample test location is to the open product, then the tighter the specification since there is a greater risk of cross contamination.

Environmental monitoring is typically conducted after cleaning to verify sanitation procedures. Similarly, in-process samples are tested to monitor the cross contamination hazard or build-up of contamination during manufacture.

Accordingly, it is expected that microbial contamination will be low. Difficult to clean surfaces are usually used as test locations but microbes are not evenly distributed even on flat surfaces. Microbial distribution on surfaces is often described as contiguous (see image below) so obtaining a representative sample is very difficult and the probability of detection is low.



As discussed previously, micro-biological enumeration methods lack precision and the uncertainty of measurement is compromised further by the low numbers. Plate counts should have a minimum of 25 colonies to give a reasonable probability of enumeration (25% error). As the number of colonies per plate decreases so the standard error of the result increases to 50% for 5 colonies and 100% for 1 colony, so the enumeration in the range of 1-10 colonies per plate is very unreliable.

Validation studies for surface contamination usually use factorial designs where 20 replicate samples are used and the percentage positive samples are recorded and not the number of colonies. Since most environmental samples typically yield results of 1-10 colonies or fewer then enumeration has a high inherent variability and begs the question as to the value of the information generated. It would be better to test a greater number of samples and express the results differently in order to get a better understanding of the levels and distribution of contamination and to trend these over time rather than rely on a single random one-off determination and record the number of colonies present as an absolute number.

Using a statistical 'binning' technique is a better way to analyse data from surface contamination and to compare methods. This smooths the variation due to sample distribution and within the method itself to improve the correlation from <60 to >90% and give greater confidence in the result.

You can get a better understanding of surface contamination and risk if you change the perspective and the yardstick.

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Enumerating surface contamination – an impossible challenge?

Although the concept of environmental monitoring by testing surface swabs is well recognised, the problems and challenges are frequently overlooked or conveniently forgotten for the benefit of simplicity, convenience and convention. Despite advances in swab materials and wetting agents, little has changed since 1917 when the principle of 'swab and rinse' was introduced.

In their review, Moore and Griffiths (2007) state that swabbing efficiencies are often poor with recovery rates ranging from 1% to 25%. The inability to control the reproducibility and repeatability of swabbing techniques can result in extreme variability of results obtained. They concluded that "traditional microbiological methods should neither be presumed to be the gold standard nor the optimum means to assess the efficiency of a company's sanitation program and swabs should not be relied upon to give an accurate indication as to the level of micro-organisms present".

What are the issues? There is no universally accepted swabbing protocol. Several researchers suggest that sampling methods and components should be chosen based on the type and species of bacteria rather than by the most bacteria recovered. There are many factors affecting swab sampling and the subsequent results obtained. Ideally the sample should be tested immediately after collection to prevent deterioration or abuse of the sample but this is usually impractical.

Delays between sample collection and testing are often inevitable. Protracted delays during transport need to be mitigated by controlled storage and transport. Removing bacteria from surfaces is considered a primary objective but their survival on the swab material and/or in the wetting agent, and their subsequent release into a diluent are equally important. Similarly the surface area covered and pressure applied between the swab bud and surface will influence results. Thereafter the resuspended sample is subject to the inherent variability of the detection method itself.

There are no agreed standards for clean surfaces. There are common proposal from several different industries that quantitative aerobic colony counts should be <2.5 per cm^2, or <250 bacteria in a 10 x 10cm square. This is equivalent to a detection limit of 1 part in 100 million and is comparable to trying to collect, detect and quantify a single ant on a tennis court. This contamination level is also the limit of quantitation of the aerobic plate count when using the standard swab and rinse procedure with 10ml diluent.

Counting <25 colonies on a plate gives imprecise and variable results that should be considered semi-quantitative or indicative. Grouping contamination levels as bands (or in bins) is a statistically more relevant and pragmatic approach which yields a better representation and correlation of low numbers of micro-organisms on surfaces. Under these circumstances, the concept of Pass/Caution/Fail is more realistic, meaningful and easy to understand where Pass is <250 and Fail is >1000.

The ideal solution would be to have a swab detection system that collected a representative sample to allow maximum recovery from surface without losses due to resuspension, dilution or time to initiate the test, and deliver results in the shortest possible time for trend analysis. This can be achieved if the sample collection device is also the detection device where 100% of the sample can be tested immediately.

MicroSnap Surface Express is able to deliver these attributes but like all modern rapid methods relying on metabolic activity, the challenge is how to compare these different approaches to the unique and variable colony count method, particularly at very low contamination levels.

As Einstein said "we cannot solve our problems with the same thinking we used when we created them".

We need to acknowledge the reality of the problem and adopt a different perspective.

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Measuring swab performance

There are many factors affecting the recovery of bacteria from environmental surfaces that have a significant impact on detection and method validation. Most researchers measure what has been collected on the swab; however an alternative approach is to measure what is left behind on a surface.

The 'swab and rinse' method for measuring environmental contamination has remained relatively unchanged since 1917.

As discussed in previous issues, there are a large number of variables that affect swab methods giving low recovery and detection rates of 1-25%. Enumeration is therefore very imprecise so as to be virtually meaningless, particularly at low contamination levels.

Integral to the overall recovery of contamination is the release of bacteria from the swab so as to become available to the final detection method. Bacteria will bind to the material of the swab bud (>50%) and not be released although some modern materials may have a lower retention rate. The greater the perturbation of the swab in the rinse solution, the more contamination is liberated from the swab material but it is rarely 100%.

Methods for environmental pathogens frequently use sponge swabs where the release of contamination from the swab itself is of less importance provided that the whole swab and wetting solution are part of the overall test procedure.

Method validation studies for pathogens of surfaces usually require inocula to be dried on to surfaces, however this causes a large loss of viability (typically 4-6 logs). Consequently, the actual residual inoculum is not known (or no attempt is made to measure it because it is too difficult), and factorial design is used to compare detection methods in a presence/absence test format.
A model system was designed to assess and differentiate swab sample recovery from the overall detection methods by measuring the contamination on the foil surface before and after swabbing. An inoculum dried on to aluminium foil was used as the surface material and two surface test methods were compared.

MicroSnap Surface Express (MSX) is designed to swab large areas (up to 12" x 12") and to retain the sample on the swab for subsequent enrichment and detection of the entire sample. MSX was compared to contact plates.

The table below shows the results of five replicate tests for each method compared with the control (no swabbing) and conducted several times over a nine day period, and demonstrates the efficacy of the MSX device.

Swabbing a large surface area requires a swab bud that is pre-moistened with sufficient swab wetting agent to cover the desired surface area to collect a representative sample, and is convenient to use. This does not necessarily mean it has to be a large foam swab with associated volumes of liquid.

A high swab capture and recovery rate together with 100% detection on the swab with a rapid end point detection method is a major step forward for microbial environmental measurement.

	Sample recovery rate (%)				
	Day 1	Day 2	Day 6	Day 8	Day 9
Control	100	100	100	100	100
Contact plates	51	61	73	88	85
MSX	85	99	84	92	91

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Environmental monitoring: an astronomical challenge!

Environmental monitoring is an essential part of a quality assurance program and driven by global food safety programs and regulation. It provides an early warning of potential cross contamination hazards, verifies good manufacturing practices and supports the limitation of finished product testing. The previous articles in this series highlighted the vagaries of microbiological methods and concerns of measuring microbes on surfaces. Firstly the test methods themselves have high variability and uncertainty which is further compounded by low level contamination after cleaning and the uneven distribution of contamination. It is also recognised that swab methods only recover a small percentage of the total microbial population present on a surface. Given that few samples and replicate tests are usually conducted, it is perhaps not surprising that the probability of detection and reliability of swab test result is very low. This has important considerations for risk management as well as for sample collection and test method procedures.

The scope of the problem is enormous. For Total aerobic bacteria, a generally accepted standard for a clean surface should be <250 cfu per 10 x 10cm area which is equivalent to detecting a single ant on a tennis court, or 1 second in a 5-year period. The task is 2000 fold greater to detect the presence of a single pathogen in a 30 x 30cm area. Thus the probability of detection is very low and is largely dependent on the sample rather than the detection method. Two class sampling plans for pathogen in foodstuff (see table below) are based on relatively high levels of defective units that are probably far greater than those actually achieved in practice. This shows there is a requirement to test large numbers of samples that leads to onerous and expensive testing. Much greater levels of testing are required to detect a lower rate of defective units but this is neither practical nor cost effective. Habraken et al (1986) showed the benefit of different sampling procedures to increase the probability of detecting low numbers of salmonella in milk powder. They showed that 'the only workable solution' relies on increasing the number and amount of sample tested. They used continuous sampling procedures and the testing of larger aliquot samples together with monitoring indicator organisms to dramatically improve the reliability and confidence in the test results and process control.

The same principle should apply to environmental samples. In order to increase the probability of detection, more samples covering larger surface areas in high risk locations are required. For surface swab samples, the size of the swab is irrelevant. The swab design should be sufficient to cover the required surface area with sufficient wetting agent to collect the sample and support the survival and detection of the target organism. Detection on the swab is preferable in order to measure 100% of the collected sample that might otherwise be lost or destroyed by subsequent preparation procedures. Combining several samples into a single test is feasible but the identity of the precise sample location is lost, thus reducing the usefulness of the information.

The challenges of surface microbiology can be likened to those of astronomy i.e. the measurement of small objects with faint signals that are sparsely and unevenly distributed against a background of interferences from several sources. Astronomers collect and analyse large amounts of data and look for trends and perturbations. Can microbiologists learn something from this approach?

Number of samples examined	Probability of acceptance Actual percentage of defective samples			
	10%	20%	30%	40%
3	73	51	34	22
5	59	33	17	8
10	35	11	3	1
20	12	1	0.5	<0.5

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The days of the CFU are numbered!

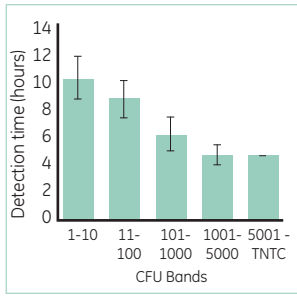
This series of articles has demonstrated that the basic unit of measurement of colony plate count methods (the CFU) gives a highly variable 'estimate' of microbial contamination. The inherent variation within colony count methods itself is due to the incorrect assumption that a single colony is derived from a single organism. This problem is compounded by several other contributory factors including uneven distribution of contamination, low swab efficiency and recovery, and high operator dependency. Similarly, low sample numbers and the use of single replicates generates poor quality data that is mistakenly considered to be an absolute, reliable and definitive measurement. Nothing can be further from the truth. This begs the questions; what information do we require, how can we achieve it and what are reasonable expectations within the limits of existing or alternative methods? Albert Einstein said that "we cannot solve our problems with the same thinking we used when we created them". We need to re-examine the over-reliance on the colony counts and CFU to get a more pragmatic and meaningful measurement of microbial contamination and risk.

Risk assessment has driven the concept of zoning to prioritise sample locations however the frequency and number of samples tested determines the probability of detection and hence the relevance and value of the information generated. Alternative methods offer a faster time to result and often give more meaningful data particularly if they measure the metabolic activity from indicator or spoilage organisms rather than an imprecise number produced after several days. In the absence of any other established metric, alternative methods are compared to colony count methods and this creates a problem for validation due to the vagaries of enumeration from CFUs. Certification bodies such as AOAC typically compare methods over a 3 log cycle within a range of 1000 to 10 million cfu per gram of foodstuff and define equivalence as ≤0.5 log cfu difference (i.e. a 3 fold difference in CFU is acceptable). Validation of enumeration methods for environmental samples is much more difficult because the samples contain fewer organisms (<10 – 500 cfu) and the variation from all sources above is even greater.

Alternative rapid methods are frequently based on metabolic activity that detects every viable organism in the sample whether it is present as an individual cell or part of a clump or chain. All methods have variation but microbiological methods are unique in that the analyte itself is a viable changeable entity that responds to its environment throughout sample preparation and testing. When comparing microbiological methods we have to account for all these sources of variation and set our expectations accordingly. Since the CFU is very variable it is unreasonable to seek correlation to a discrete number, and better still to seek a more meaningful metric such as microbial activity. Expressing results within prescribed bands of time to detection and bands of CFU using statistical binning techniques is a more pragmatic and meaningful way to compare two variable methods. This is particularly useful at low contamination levels and when working within a narrow dynamic range e.g. in environmental samples for cleaning verification (see example in Fig. 1 below where the correlation coefficient was calculated as $R^2 = 0.9406$).

It is time to accept the limitations of the plate counts and CFU, and to seek a more meaningful metric and data analysis of the measurement of microbial contamination. ATP surface testing for product residues has changed the way that cleaning is monitored and verified, and it is now widely accepted. Surely we can adopt a similar approach for measuring microbial activity and contamination with existing and alternative methods.

Fig. 1. Rapid microbial activity test for environmental monitoring.



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