What is an appropriate test method?

Today's regulation and global food safety systems rely heavily on risk assessment and preventative systems to assure quality and safety. These systems all require some element of monitoring and measurement. The quality and performance management expert H. James Harrington is credited with this admonition;

> **"Measurement is the first step that leads to control and eventually to improvement. If you can not measure something, you can not understand it. If you can not understand it, you cannot control it. If you can not control it, you can not improve it."**

All quality and safety methods have limitations. But these limitations are frequently ignored or dismissed for the sake of simplicity, convenience and/or cost. Nonetheless, quality methods are still expected to deliver reliable, accurate information. Physical and chemical parameters are usually stable and their measurement is accurate and precise. By contrast, biological parameters and methods for food safety have more inherent natural variation and a greater uncertainty in measurement.

So which technology or method is appropriate and fit for purpose in support of food safety? This series of articles for 2019 will address some of the test requirements and available methods of measurement.

A fundamental principle for food safety and all codes of practice is good hygiene that is often defined as a pre-requisite in food safety programs.

The primary purpose of cleaning is the removal of residues from product contact surfaces and its environs. Visual assessment of cleanliness is very subjective and can only detect macroscopic defects and gross lapses in practice.

Their application is limited to pest control and foreign bodies detection that are often supplemented by other measurements such on-line optical inspection and metal detection. Measurement of microbes as a hygiene monitor is well established, including specific groups of indicator, index or pathogenic organisms.

However, microbiological methods are often complex, expensive and time-consuming, giving results in several days, which is too late for the production of many foods with short shelf lives. They also tell us nothing about the residues remaining on surfaces after cleaning.

Over the past 40 years, rapid methods that give a direct objective measurement of product residues (including ATP (such as the UltraSnap Surface ATP Test), protein (such as the Pro-Clean protein residue test) and simple sugars (using the SpotCheck Plus glucose and lactose detection device) have been widely accepted as providing assurance of cleanliness and good hygiene. They are not intended to be an alternative to microbiological tests because they are measuring different aspects of hygiene that are more relevant to the cleaning process (the removal of residues). Cleaning should not be confused with disinfection/ sanitisation, which is the reduction of residual microbial contamination to an acceptable level. Trying to disinfect a dirty surface is a waste of time and money.

There are many benefits of deploying good hygienic practices. Safety and quality are assured, while improving shelf life, and product recall and food poisoning incidents are prevented. Consequently, customer satisfaction and brand loyalty are protected. Cost savings in manufacturing are obtained from a reduction in wastage and maintenance costs, and the optimisation of cleaning reduces chemical, water, energy and effluent costs, while minimising environmental impact.

Conversely, the cost of failure can be devastating. Damage to a brand can cost millions and be very difficult to recover from, and directors of food businesses can be fined, the subject of lawsuits, or even imprisoned.

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Measurement of microbial indicator

Microbiological tests are conducted on raw materials, finished products, product contact surfaces and food processing environments in support of quality control and assurance. These facilitate high standards of hygiene and product quality that prevents spoilage, enables long shelf life and reduces risk to maintain food safety compliance. Tests need to be conducted regularly with several replicates preferably using a statistically relevant sampling plan giving rapid results to enable preventative actions to be deployed. This ideal is often impractical and/or has a high cost in routine food processing such that screening tests to monitor trends are frequently used to measure compliance and risk.

The simplest and most common microbial indicator test is the Total Aerobic Bacterial Count (or Total Viable Count). Coliforms and E. coli are indicators of faecal cross contamination. The wider family Enterobacteriaceae are also used as hygiene indicators but can be limited to some products types. Tests for the genus Listeria are frequently used to monitor the potential risk associates with the pathogenic L. mono-cytogenes. Indicators of food spoilage require the measurement of certain types of organism, for example lactic acid bacteria and yeasts that are adapted to certain environmental conditions. Methods to measure microbial indicators are typically non-specific and may require additional tests to confirm the identity of the organisms. Consequently, they have a lower cost and serve a different purpose to other more specific, definitive and sensitive test methods.

Cultural methods involve a test sample that is placed on a nutrient agar and incubated for several days. The result is expressed as a colony forming unit (CFU) defined as an 'estimate of viable cells'. This uniquely variable CFU is neither precise nor accurate and often results in an unfair comparison of novel methods to the plate count and unreasonable expectations of alternative enumeration methods. Ease of use of the methods has been improved by pre-packing (for example pre-poured agar plates and dip slides), convenience dry packaging (self-diffusing dry media in film), and all-in-one swab devices with chromogenic enrichment broth (for example InSite Listeria with fluorescent indication of L. monocytogenes).

Rapid automated methods typically give results in 10-18 hours and generally have a large sample capacity of 24 to >100. The instrumentation incubates and measures the sample continually and typically has a high capital cost. There are several different methods that vary in their detection principle including colour induced redox/pH change, electrical conductivity, fluorescence, oxygen consumption and pressure sensors. Relying on actively growing microbes, these methods can measure different types of bacteria by using selective media although some samples types may cause interference.

Rapid methods giving results in 1-8 hours often require expensive equipment and/or a skilled analyst with some sample pre-treatment. Examples include flow cytometry and fluorescence microscopy (often in combination with filtration). One method using ATP bioluminescence (MicroSnap) can detect a variety of indicator bacteria using convenient disposable devices and a low cost, multi-functional portable instrument that can be used to test product and surfaces.

Trending the results from screening methods enables many more samples to be tested thus increasing the scope and confidence of the surveillance activity and management of quality and risk. Monitoring and trending of results in their own unit of measurements is more meaningful and useful than converting them back to a variable CFU equivalent.

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Testing the sterility of foods

Ultra-Heat Treatment (UHT) is a well-established food processing technology that requires both a steriliser and an aseptic unit (for packaging the product). It is used for low acid (above pH 4.6) products such as UHT milk, UHT flavoured milk, UHT creams, soya milk and many other foods and beverages, such as soups, sauces, desserts, tomato and fruit preparations, and baby food. The process delivers thousands of cartons/sachets of product that vary in size from 5ml to >1 litre with a very low failure rate of <1 in 10,000. So how can we measure microbial content for quality assurance purpose?

Microbial quality assurance of long-life sterile foods requires the demonstration of absence of contamination. Measurement requires the detection of the microflora that are most likely to survive the process and then grow in the product. Neither enumeration nor specific organism measurement is relevant to this screening activity.

The challenge is to detect one or more bacteria per carton from many samples that are representative of the batch. This means testing hundreds of samples most of which will be sterile. Products are stored in a warehouse and often require positive release, so there are significant commercial benefits to be derived from rapid results. Accordingly, the ideal method should be sensitive, rapid with high throughput automation from sampling to testing and deliver reliable results.

There is no available method that can instantly detect one viable bacterium in a litre of product, so the product needs to be incubated in-pack until the bacteria reaches a detectable level (typically 1-2 days at elevated temperatures). All microbiological methods have their own limit of detection, so the incubation time needs to be optimised to the slowest growing bacteria in each product type in a validation study before routine implementation.

Methods that give instant direct measurement after incubation in-pack are preferred over other methods that require an incubation to facilitate detection because of the risk of cross contamination giving false positive result.

Established for >30 years, ATP bioluminescence is the most widely used method for the assessment of sterility of UHT aseptically filled products. It has been applied to a wide variety of long-life food types that are viscous and/or particulate. The instrumentation is a luminometer which can be manual low capacity or 96-test microtitre plate with high throughput capacity processing hundreds of samples per day. The reagents have low running cost with simple user interface and data interpretation. An auto-sampler can even prepare and deliver hundreds of pre-incubated samples to the instrument.

Flow cytometry has a lower limit of detection than ATP and can potentially produce faster results, but sample throughput is lower with limited opportunity for automation, and particulate samples are more challenging.

Growth based detection methods include electrical impedance, pH and dye reduction and oxygen depletion where results are generated in 8-18 hours after the sample has been incubated in-pack. These instruments incubate and measure continually over many hours and each unit has a 24-48 sample capacity. Molecular methods such as immuno-assays or DNA based methods are not appropriate since specific organism detection is not required, non-viable organism could be detected, and the cost are often higher.

For sterility screening, ATP bioluminescence has stood the test of time and continues to provide a reliable cost-effective solution.

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Measuring specific food safety markers

Tests for specific analytes are often driven by regulation to protect food safety and have defined limits, e.g., pathogens, toxins, drug residues. Other specific analytes of concern have few or no limits, e.g., allergens and hygiene indicators. There are several methods available to detect each analyte, but the method of choice is dependent on the purpose and application together with the user requirements of hands-on time, work flow, throughput, time to result, data interpretation and cost.

For pathogens in finished products, there are several methods giving definitive results for presence or absence and differing detection times, but it is more important to obtain a representative number of samples to give an acceptable level of assurance. Enumeration of pathogens is required in some cases for raw materials, but levels of pathogens are typically very low. Historically the most probable number (MPN) method has been used which requires a minimal nine-fold increase in workload. Recent improvements using qPCR with short enrichments provide an easier, faster and quantitative method, e.g., BAX System PCR Assay for Salmonella in poultry.

For pathogens in environmental samples, the same principle can be adopted. However, the purpose is slightly different to allow an alternative approach. The potential for listeria cross contamination from the production environment into product is monitored by testing a variety of surface samples from product contact surfaces to floors and drains after cleaning. The greater the number of samples tested and the faster the result, then the greater the level of assurance and control. Under these circumstances, a lower-cost screening method giving presumptive positive results (e.g., InSite Listeria Testing) can provide an additional approach that supports greater surveillance and trending of risk. InSite gives a simple visible chromogenic result in 24-48 hours and is compatible with PCR for confirmation.

Bacteria used as indicators of hygiene for most foods are typically coliform and E. coli, or Enterobacteriaceae, whereas for low pH or high sugar foods, lactic acid bacteria and yeast are more relevant. Several different methods are available for these organisms from the traditional cultural colony count method or more recent versions with different end point detections giving results in seven hours (e.g., MicroSnap indicator organism testing using bioluminescence) and/or other instrumental high-throughput systems relying on electrical conductivity or chromogenic/ fluorescence changes to the medium. PCR test are now available to detect many different non-pathogenic spoilage organisms giving semi-quantitative results in a few hours. However, as discussed in an earlier series, methods for enumerating bacteria on surface are incapable of providing precision and accuracy. Monitoring and trending of results of alternative rapid methods in their own unit of measurements is more meaningful and useful than converting them back to a variable CFU equivalent.

Immunoassays achieve specificity from antibody – antigen reactions in the form of ELISA tests with instrumental measurement of colour or fluorescence after 60 minutes or more. The simpler, more convenient format is a lateral flow dipstick device that gives a faster visual qualitative result in <10 minutes and a reader can give a semi-quantitative result. The methods are well established and applicable to pathogen detection but also to detecting non-microbiological hazards, such as allergens, mycotoxins, and antibiotic and drug residues in foods. ELISA is more sensitive and quantitative, detecting down to parts per trillion. Successful performance of an immunoassay is dependent on the specificity of the antibody, the design of the assay and the sample preparation procedures.

The food safety testing methods we choose need to be fit for purpose and the choices we make determine our future.

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After detecting microbes, what next?

After detecting and enumerating microbes by a number of different methods, the next questions are typically: what are the microbes, what risk do they pose and where did they come from?

To answer these questions, we first need to identify the organisms for which numerous techniques have been developed and deployed. These methods are based on phenotypic or genotypic characteristics of a micro-organism isolated as a single colony often with a characteristic morphology.

The first methods included individual chemical or biochemical tests that measured specific metabolic activities that were subsequently used to differentiate and classify bacteria i.e. their phenotype.

As knowledge and technology improved these tests were compiled into convenience packaging formats, such that genera and species could be easily differentiated by visual colour end point reactions, for example API and EnteroTube.

Automation supported by analytical software and large databases have increased the sophistication and probability of identification, for example Vitek and Biolog to produce results in hours.

Advanced chemical methods such as Pyrolysis Mass Spectroscopy was used in the 1970s to measure the fatty acid composition of cells and thereby identify and differentiate bacteria. The latest development in cell compositional analysis is Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectroscopy (MALDITOF).

This volatises the organisms and measures its composition of highly abundant proteins to produce a proteomic fingerprint pattern that is used to identify the organism in minutes. For epidemiological investigation of food poisoning incidents or simply tracing an isolate to its source, further characterisation is required to differentiate between strains of the same species. To this end, the surface characteristic of the bacterial cell have been used to sub-divide bacteria. Antibodies raised to cell wall components, for example, lipopolysaccharides or flagellar proteins, have several applications including serotyping. Similarly, phages are viruses that infect bacteria by first attaching to cell wall recognition sites and have long been used to characterise salmonella to sub-species level due to their high individual specificity.

The genetic code was cracked in 1953. Using enzymes to break up DNA into smaller chain lengths, a characteristic fragment pattern can be generated for each organism after separation by electrophoresis.

Manual systems including Pulse Field Gel Electrophoresis have been widely used and automated systems such as the Riboprinter (shown below) give results in a few hours.

The first DNA sequencing method was invented by Frederick Sanger in 1980 which has changed our understanding of the inter-relatedness and evolution of bacteria. Techniques such as Multi Locus Sequence Typing (MLST) have targeted selected areas of the genome to identify organisms.

DNA sequencing has advanced even further to enable the whole genome to be sequenced not only from a pure culture but also from mixed cultures such that the whole microbiome of a sample can be measured. However environmental conditions have a big impact on what stored genetic information within an organism is used. The phenotypic expression of active bacteria in a mixed population can now be measured and is an alternative assessment of the microbiome and fluctuations within it.

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What's next for rapid methods?

In this series we have discussed different methods for the measurement of quality and safety and their suitability for different applications. Modern methods have provided increasing sensitivity and specificity to detect analytes in the parts per trillion range and able to differentiate minute variations at the molecular level. So where do we go from here?

Sensitivity and specificity appear to have reached their limits although new variations on a theme continue to be developed. Enumeration in microbiology (the colony forming unit, CFU) is at best an imprecise estimate. Its significance is measured in orders of magnitude where precision and accuracy are relative terms offering little value.

The probability of detection of colony counts is low at low level contamination, such as the verification of surface cleanliness at <250cfu/100cm^2, or the equivalent of trying to detect a single ant on a tennis court. Using a rapid method of measuring microbial activity rather than trying to equate the results back to cell numbers would be a more meaningful and useful result.

The detection of pathogens (presence/absence) is limited by the ability to find the proverbial needle in a haystack. Uncertainty of measurement in microbiology is greater than other analytical methods because the analyte (microbe) is a discrete entity that exists in different stages of metabolic activity and has a variety of characteristics that enable it to adapt to different conditions. This is both an advantage and disadvantage for methods that have been used extensively in the past.

Detection methods can be improved using separation and concentration techniques as part of sample preparation procedures to reduce the time to result and improve the probability of detection. Several methods based on centrifugation, filtration, and immuno-magnetic separation are available but add to the workflow and cost of the overall procedure.

Consequently, the requirements to obtain the best and fastest test result are a combination of technologies and optimised procedures customised to fit the acceptable criteria for specific applications. One size does not fit all. The remaining limitations appear to be finding the analyte in the test aliquot (sub-sample) from a representative sample of the lot. Contamination is rarely homogenously distributed but heterogeneously mixed as pockets or nests with the whole.

Obtaining a test sample from a batch with a high statistical probability of detection usually means testing many samples which is often impractical and has high associated costs so there is a need for better sampling methods supported by preventative quality assurance practices.

The revolution in DNA sequencing technologies promises exciting possibilities for identification and microbiome measurement but these are unlikely to be suitable for routine quality analysis. The ideal microbiology test is often considered to detect and enumerate viable organisms giving results in minutes, preferably in-line during manufacturing. However, this Holy Grail is likely to remain unobtainable for the foreseeable future.

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