Practical evaluation of validated microbiological methods

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The validation of alternative microbiological methods requires extensive laboratory investigations based on the principles of ISO 16140. These technical protocols and procedures are used by organisations such as AOAC, MicroVAL, AFNOR, NordVAL etc, who offer third party certification and approval services.

These validation studies require several sample types and many replicates in a process that can take over 12 months with associated high costs.

Method evaluations are typically conducted by companies wishing to verify that a particular validated method works in their hands for their applications and compares favourably to their current methodology.

By contrast, method evaluations are usually limited by time and resource and often conducted inadequately with poor attention to detail and unrealistic understanding and expectations of the underlying variation from the methods and the samples.

Common mistakes include insufficient number and replicates of samples particularly at low levels of contamination, and an unrealistic expectation and assumption that the current method is conducted correctly and gives a result that is 100% correct.

Consequently, this can lead to an inadequate and incorrect assessment of method capabilities.

Variable colony counts

Methods for the enumeration of microorganisms are inherently highly variable due to the incorrect assumption that one colony forming unit (CFU) is derived from a single organism. The reality is that microbes grow by binary division and exist as chains or clumps of many cells. These clumps are not entirely broken up into single cells by homogenisation during sample preparation which creates a mixture of smaller clumps such that a colony is formed from one, two or more cells.

Precision and accuracy takes on a totally different meaning in microbiology such that the normal expected variation in results is typically Log 0.3-0.5 (2-5 fold) and a result that is considered significantly different is Log 0.5-1.0 (anything from 5-10 times different). For an average result at 1,000 bacteria, the actual value can lie anywhere between 32 and 3,200 and still be correct.

	Average	Lower range	Upper range
Expected ACC* 30C	5.45	5.20	6.20
Measured ACC 30C	5.70	5.40	5.90
Expected ACC 22C	5.45	5.15	6.15
Measured ACC 22C	5.65	4.45	5.85
Expected coliform	3.25	3.05	4.05
Measured coliform	3.55 (3500)	1.95 (90)	5.65 (440,000)
Expected Enterobacteriaceae	4.80	4.45	5.45
Measured Enterobacteriaceae	4.95 (90,000)	2.55 (350)	5.75 (560,000)

*ACC = Aerobic Colony Count

Table 1. Typical data from a European proficiency test scheme.

This fact is often conveniently forgotten or ignored by many microbiologists, perhaps because the inadequacy of the technology is hard to explain and admit to non-microbiologists.

This problem is compounded when the levels of contamination are very low, typically 1-100, and the distribution of contamination in the sample is uneven. This reduces the probability of detection and increases the variation and inaccuracy of the test result still further.

The challenges are even greater when trying to detect pathogens where the specification is absent in 25g but the batch size could be 10's of tonnes.

The CFU can only give an estimated value. A unit of measurement is defined as a 'standard quantity of a physical property' and this should be stable and constant. Clearly the CFU is a very poor unit of measurement.

This has significant implications when comparing colony count methods with other enumeration methods that measure every single viable organism in a clump of two or more organisms that would form a single colony. Methods not generating results in CFU can be expected to be more inclusive and precise measurements will be disadvantaged when having to convert back to give the less precise equivalent CFU value.

Lack of correlation between colony count methods and alternative methods should not necessarily be attributed to the alternative method but rather to inherent variation of colony count.

Significant variation is still observed even when the best laboratories certified to formal laboratory accreditation schemes are taking part in laboratory proficiency test schemes, where a uniform sample is supplied and tested under controlled conditions.

Table 1 shows some typical results from a proficiency test scheme that included 60 accredited laboratories across Europe where there was a large range of results and the more selective the method the greater the variation observed.

Given the variation in the results from Enterobacteriaceae method and the fact that it is little more than a semi-selective Aerobic Plate Count, one has to question

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the value of the information generated by the Enterobacteriaceae method.

Accordingly, it is unreasonable to expect good data to be generated from a quick evaluation involving a few random routine samples tested with no replicates as might be conducted within a routine quality control test schedule. A formal evaluation program should be conducted.

Requirements for evaluation

A successful evaluation of an alternative method needs careful consideration and planning and should include:

- Training on both the reference and new method such that the principles, operating conditions, tolerances and critical limits of both are fully understood such that reliable results are generated and correctly interpreted.
- Both positive and negative control samples are tested to ensure that typical results are correctly identified, and atypical results are not mis-interpreted.
- Samples are prepared and tested with a known inoculum and in the required contamination range.
- Representative real samples.
- Statistical data analysis of the results generated.

It is important that sufficient replicate samples are tested. This should be at least 5-10 replicates at each level, and the lower the expected contamination level then the greater the number of replicates is required.

Dairy case study

A large cheese making facility with a continuous 24-hour operation has a quality assurance program to test every starter culture vat and ensure that coliforms are absent at <10/g or ml. The traditional method takes 24 hours and a faster result (7-10 hours) was required to get better control and earlier warning of a potential problem.

The MicroSnap Coliform test (Hygiena) provides a result in 6-8 hours and has been certified for milk and several other foods under the AOAC Research Institute Performance Tested Methods Program.

The method involves a short enrichment phase followed by a 10 minute specific detection step using a bioluminogenic, substrate-based test for either coliform or E. coli. Starter cultures for cheese contains >10 million lactic acid bacteria per ml and detecting low numbers of coliform in their presence was a challenge but was nevertheless achieved.

Negative and positive samples were tested to verify test performance and any effects from the sample matrix. The sample gave a slight interference as an elevated background signal that was not sufficient to limit the performance of the rapid test. Three different coliforms in the form of commercial lenticules (Public Health England (PHE) i.e. strains of E. coli NCTC 9001, 13216 and 25922) at three different contamination levels were used to inoculate active starter culture.

Lactococci (CFU/ml)	E. coli (CFU/ml)	MS Coliform (RLU)
1.00E+08	500	7741
1.00E+08	50	2251
1.00E+08	5	112
Negative control	<1	32

Table 2. Quantitation of E. coli using the MicroSnap coliform detection system in lactic starter cultures.

Table 2 shows that <10 E. coli/ml in the presence of 10,000,000 lactococci can be detected in 7.5 hours.

The data shows linearity and quantitation that is used to enumerate the sample; for larger numbers of E. coli the detection time was reduced to 5.5 and 3.5 hours for an inoculum of 500 and 50,000/ml respectively. The company has adopted the MicroSnap Coliform test into its routine testing program.

Agricultural case study

Enumeration of the total bacteria flora (ACC) and Enterobacteriaceae in fresh field tomatoes was considered a poor measure of quality and safety and a rapid test for coliform and E. coli was evaluated using the MicroSnap systems.

Tests for Enterobacteriaceae are not appropriate for fresh fruit and vegetables or for products containing salad vegetables because fresh fruit and vegetables often carry high levels of these organisms as part of their normal flora.

The 10% tomato homogenate did exhibit some enzyme activity relevant for the coliform test but not the E. coli test. This natural background activity decreased during the incubation period and compensation for small residual levels did not affect the result.

Most of the contamination is on the surface of the tomato, whereas the centre of undamaged tomatoes is sterile.

Accordingly, for a more representative cleaner sample with a high concentration of contaminants, it would probably be better to test only the surface layer itself.

The conventional 10% homogenate was used and inoculated with E. coli at low levels (<10 CFU/ml). The MicroSnap Coliform test detected down to 10-100 CFU/ml after six hours and 1-10 CFU/ml after eight hours (see Table 3). Similar results were obtained for the MicroSnap E. coli specific test although the detection time was slightly longer and would require 8-9 hours incubation to determine a negative result of <1/ml.

The results of microbiological analysis are inherently highly variable for several reasons. The CFU gives only an 'estimated value' but it is unreasonably bestowed with some degree of precision and accuracy that it cannot deliver.

Accordingly, great care needs to be taken when comparing and evaluating alternative methods that often have better performance than colony counts that are perceived to be the gold standard. ■

Table 3. Quantitation of E. coli using the MicroSnap coliform and E. coli tests in tomatoes.

Dilution	6 hours	8 hours	10 hours	E. coli per mL
	MS Coliform (RLU)			
-1	3570	6270	5226	80000000
-2	4931	3308	6411	8000000
-3	5840	6156	5088	800000
-4	7644	5411	5269	80000
-5	2038	6692	4504	8000
-6	122	6326	5780	800
-7	32	3431	6382	80
-8	17	28	7577	8
-9	11	20	10	0.8
-10	10	10	10	0
Lenticule 1	16	26	4421	86
Lenticule 2	40	4084	470	5460
Lenticule 3	284	4455	731	60700
Lenticule 4	1949	696	1130	402000
Blank (Tomato)	15	20	10	