Evaluating Microbiological Method Equivalence – A Decision Guide

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SUMMARY

Using an appropriate method is a key step in generating reliable results; and, when those results are to be used to make safety-critical decisions, method selection becomes even more important. For microbiological testing, there are national and international standard methods and various other widely accepted methods. Performance of such methods has usually been validated through some kind of collaborative process or independent review. An independent review may have resulted in some kind of certification. Method validation, with or without independent certification, demonstrates that a method has performance equivalent to an established reference method. Many circumstances can arise that cause a laboratory to change methods. In such an event, how is a laboratory to determine that two methods are equivalent to one another if neither of them is a reference method? In this paper we outline a thought process to guide this decision. The process involves comparing existing validation and/or certification data to determine whether two or more methods have been compared against the same reference method for the matrices of interest using a rigorous experimental and statistical approach. If they have, the methods may be considered equivalent, and a laboratory simply needs to verify its ability to perform them. If they have not, then a formal validation may be needed.

OVERVIEW

In previous articles by the International Association for Food Protection Interest Group on Verification and Validation, the increasing need for the most prudent, scientifically and economically sound approaches to method validation and verification was discussed *(5)*. Suggestions for practical, risk-based approaches to address this need focused on matrix grouping and levels of test method evaluation in a second publication *(6)*. Following on this theme, this current article discusses another approach that would alleviate verification and validation testing pressure and reduce the burden of evaluation, particularly when one is faced with choosing between two or more validated methods. The most direct comparison is when the methods are validated for the same target analyte in the same validated matrix. When can we consider these methods equivalent to one another without a direct comparison between them? How does the reference method affect this consideration? What if the validations do not include exactly the same matrices? What other factors would play into method selection? These questions and more are the subject of frequent decisions in microbiology laboratories around the world.

Before addressing these questions, it is helpful to have a basic understanding of the process of method validation. The starting point for validation of a new method is the existing reference method against which the new method is compared. There are minor differences in the definition of reference among sources *(1, 8, 15)*, but all are from recognized sources such as the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM), the U.S. Department of Agriculture (USDA) *Microbiology Laboratory Guidebook* (MLG), the Health Canada *Compendium of Analytical Methods*, standards from the International Organization for Standardization (ISO), and national standards from countries throughout the world. Traditionally all are cultural methods.

The developer of a new qualitative method evaluates several performance parameters including inclusivity, exclusivity, robustness, and stability and the ability to detect the target in a range of matrices following guides to validation such as AOAC Appendix J *(1)* or ISO 16140-2 *(9)*. There are

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TABLE 1. Comparison between AOAC and ISO 16140-2 certification requirements for qualitative methods

Study	AOAC appendix J *(1)*		ISO 16140-2:2016 *(9)*
	PTM submission	OMA submission	
Inclusivity/exclusivity	X	X	X
Matrix suitability:[a]			
POD/dPOD	X	X	
LOD_{50}/RLOD			X
Multicomparison			X
Robustness	X		
Lot to lot consistency	X	X	
Interlaboratory/collaborative		X	X

[a]POD, probability of detection, defined as number of positive samples divided by total number of samples in a fractional recovery study; dPOD, difference between probabilities of detection of candidate and reference methods; LOD_{50}, limit of detection, the level of contamination with an expectation of 50% positive test results; RLOD, relative level of detection, the ratio of the LOD of the alternative method and the LOD of the reference method.

TABLE 2. Comparison between AOAC and ISO 16140-2 certification requirements for quantitative methods

Study	AOAC appendix J *(1)*		ISO 16140-2:2016 *(9)*
	Performance tested method	Official methods of analysis	
Inclusivity/exclusivity studies	X	X	X
Matrix suitability	X	X	
Accuracy profile			X
Relative trueness profile			X
Limit of quantification			(X)
Robustness	X		
Lot to lot consistency	X	X	
Interlaboratory/collaborative		X	X

minor differences among validation guides *(Tables 1 and 2)*, but all conclude with a statistical comparison between the new (candidate) and reference methods. To be considered equivalent, the limit of detection (LOD) of the candidate method must not be statistically different from that of the reference method in a "fractional recovery study." In this sort of study, matrix is naturally contaminated or artificially inoculated at concentrations so low (nominally ≤1 CFU per test portion) that only a fraction of all samples tested will be positive for the target. Ideally this fraction will be about 50%, and at least in the range 25 to 75%. Any laboratory-developed method that can demonstrate equivalence to an appropriate reference method may be used as an "internally developed method." However, in commercial pathogen testing it is more common for method developers to submit validation results for certification by a body such as AOAC INTERNATIONAL, Association Française de Normalisation (AFNOR), microVAL, NordVal, and others. All methods certified by the same certifying body, for the same analyte in the same matrix and test portion size, are considered equivalent to the reference method. The open question, then, is "Are those methods equivalent to each other?" Equally, what if methods are validated for the same matrix and test portion against the same reference method but are certified by different certifying bodies?

The developer of a new quantitative method applies a similar concept of an experimental comparison between candidate and reference methods, but the statistical evaluation is based not on differences in detection but on differences in enumeration. Thus, the experimental design is necessarily different and can be reviewed in the AOAC *(1)* or ISO *(9)* validation protocols.

The discussion in this paper will focus on comparison of certified, validated, qualitative methods because these are readily available and multiple test kit providers offer methods for significant pathogens such as *Salmonella* and *Listeria*. For pathogen testing, laboratories typically resort to internally developed or internally validated methods only when certified methods are not available or not suitable for a particular matrix. Nevertheless, we will give some examples pertaining to quantitative methods.

ARE CERTIFIED METHODS EQUIVALENT TO EACH OTHER?

Method evaluations

The process of validation outlined above, with certification by an independent certifying body such as AOAC or AFNOR, results in a choice of several test kits that accomplish the same testing goals with respect to the target analyte and food matrix. How does one compare among methods that are all equivalent to the same reference method? Providing a rationale to follow in answering this question allows laboratories to create a "toolbox" from which a variety of appropriate methods could be used interchangeably, thereby reducing the burden of validation or verification for many matrices and facilitating rapid switching between methods in the event of supply disruptions.

Certified methods must have successfully demonstrated equivalence to the reference method. However, all reference methods are not the same, and accreditation protocols are not harmonized. For example, ISO methods are consensus methods for which the validation data are publicly available; however, methods in the FDA BAM are internal methods that have been shared publicly but for which validation data are not generally public. Hence, careful consideration is needed to determine whether certified microbiological methods are interchangeable:

- The methods must be shown to be equivalent to the same reference method by a recognized accreditation protocol.
- The validation study must have assessed the same target analyte in the same matrix and test portion size.

Given these criteria are met, and allowing that the testing lab is accredited and proficient in performing the method, selection could then be based on reasons other than proven pathogen or indicator organism detection. The choice could be based upon ease of use, turnaround time (TAT; also known as time to result), throughput, and ruggedness *(16)*, along with cost of labor, consumables, and training, capital expenditures for equipment, and quality of vendor support. There are several examples of a "toolbox" approach being used in regulatory laboratories where a selection of methods are available for use for the same matrix.

The USDA Food Safety and Inspection Service (FSIS) makes available the list "Foodborne Pathogen Test Kits Validated by Independent Organizations" for the detection of several foodborne pathogen targets (i.e., *Salmonella, Campylobacter, Listeria* spp., *L. monocytogenes, E. coli* O157:H7, and non-O157 STEC) *(12)*. The introduction to that document states that

> Establishments and laboratories should choose test kits that are:
> - Validated for testing relevant foods by a process from:
> - ◊ [A] recognized independent body (i.e., AOAC, AFNOR, MicroVal, NordVal);
> - ◊ [A] U.S. regulatory body (i.e., USDA FSIS, or FDA);
> - ◊ [The] International Organization for Standardization (ISO).

Outside of the United States, laboratories should be free to consider test kits validated by their appropriate national bodies in addition to the organizations listed above, as long as they are not generating data for USDA-regulated foods for sale in the United States. The FSIS document further states that

- The validated method should be:
  - ◊ Fit for the intended purpose and application (e.g., validated for the appropriate matrix and sample size to detect the appropriate foodborne pathogen);
  - ◊ Performed per the conditions of the validated protocol by a laboratory that assures the quality of the analytical results.

Similarly, the FDA notified stakeholders that they had confirmed the equivalency of several rapid testing methodologies for *Listeria* species and *Listeria monocytogenes* in environmental samples *(2–4, 14)*. The agency determined that the methods were "scientifically valid" and "at least equivalent to the reference method of analysis in [CFR] § 112.152(a)(b) in accuracy, precision, and sensitivity" for detecting *Listeria* species and *L. monocytogenes (13)*. In confirming equivalency to the reference method, any of the rapid methods evaluated could be selected for environmental sample analyses within their laboratories.

Application of the proposed selection criteria in practical use is shown using several examples. Information important in determining whether two methods would be interchangeable can be found in method validation studies.

Examples of assessing equivalence

Example 1. A company is considering a switch in methods and would like to know whether the alternative methods being considered for use to assay peanut butter are equivalent to the test methods currently in use *(Table 3)*. Their first interest is in changing from a cultural method for aerobic plate count (APC) to a rapid method. On considering the certification of the rapid method, they find that it was validated against their

TABLE 3. Summary comparison of alternative methods

Methods normally used		Alternative methods		Are methods equivalent?
Method	Accreditation	Method	Accreditation	
APC	FDA BAM Chapter 3	Rapid indicator organism assay	AOAC OMA 990.12	Yes, the method in column A is the standard cultural reference method to which the rapid assay was compared and proven equivalent in an AOAC accreditation study.
Rapid *Salmonella* detection assay I	AOAC OMA 2016.01	Rapid *Salmonella* detection assay II	AOAC OMA 2011.03	Yes, both rapid methods are AOAC accredited to the same level (Official Method of Analysis) and the AOAC method accreditation studies include the same reference method and nut matrices validated at the same sample sizes.

TABLE 4. Summary comparison of tests in response to a supply-chain disruption

Method normally used		Potential alternative method		Are the methods equivalent?
Method	Accreditation	Method	Accreditation	
Rapid *Salmonella* detection assay I	AOAC OMA 2016.01	Rapid *Salmonella* detection assay II	AFNOR NEO 35/02-05/13	No, the methods are not validated by the same accreditation protocol and different reference methods were used. For products under FDA regulation, the accreditation protocol must include an FDA BAM reference method.

current cultural method. Hence both methods are equivalent, and there is no barrier to switching. Their second interest is in switching between two rapid *Salmonella* assays. On examination of the method validations, they find that both are validated against (and, hence, equivalent to) the same reference method. Therefore, both rapid methods can be considered equivalent to each other, and there is no barrier to switching. In both cases the ability of the laboratory to run the new methods effectively should be verified before first use and then monitored through an ongoing proficiency test program.

Example 2. The rapid test kits that a laboratory uses for the detection of *L. monocytogenes* in chocolate are affected by a supply chain disruption and will not be available for some time. Another test kit lists chocolate in its validation studies. However, the accreditation followed the ISO protocol against an ISO reference method, and the product is regulated by the FDA *(Table 4)*. In this case, the methods might be equivalent, but we need to examine the validation certifications to ensure that the appropriate reference method *(14)* was used. If not, the laboratory may still choose the alternative test kit but should undertake some internal verification of its performance against the FDA BAM reference method.

When methods deemed equivalent are examined in detail, technical differences may be seen between them, including the mechanism for detection (molecular or immunological), LOD, and the TAT *(Table 5)*. However, as long as the methods are all sensitive to the level claimed in their validation study, they are all equivalent in their ability to detect a pathogen. In practice, method developers tend to trade increased detection sensitivity for shorter enrichment times and, hence, shorter TATs. Less-sensitive detection technologies will have longer enrichment times to increase the target analyte to levels above the limit of detection. Even if the protocol differs

TABLE 5. Characteristics of common test methods

Characteristic	rt-PCR	PCR	ELISA
Specificity based upon *(10)*	DNA or RNA sequences	DNA or RNA sequences	Antibody–antigen binding
Limit of detection (CFU/mL)	10^2–10^4 *(17)*	10^2–10^5 *(17)* 10^4 *(11)*	10^2–10^5 *(17)* 10^4–10^5 *(11)*
Typical TAT for *Salmonella* and *Listeria*	1 day	1–2 days	2 days

TABLE 6. FDA equivalent rapid methods based on validation accreditation and reference method only

Example	Test	Validation protocol	Reference method	Equivalent
1	Rapid PCR test	AOAC	FDA BAM	Yes
	Rapid ELISA test	AOAC	FDA BAM	
2	Rapid PCR test	AOAC	FDA BAM	Yes
	Rapid ELISA test	AFNOR	FDA BAM	
3	Rapid PCR test	AOAC	FDA BAM	No
	Rapid ELISA test	AFNOR	ISO	
4	Rapid PCR test	AOAC	FDA BAM	No
	Rapid ELISA test	AOAC	ISO	
5	Rapid PCR test	AFNOR	FDA BAM	Yes
	Rapid ELISA test	AFNOR	FDA BAM	
6	Rapid PCR test	AFNOR	ISO	Yes–but reference method not recognized by FDA
	Rapid ELISA test	AFNOR	ISO	

among methods, the validated protocol for the matrix results in an equivalent probability for detection. DeMarco et al. *(7)* studied the effects of different lysis procedures and volumes delivered to the final molecular detection reaction when comparing PCR, rt-PCR, and loop-mediated amplification methods. In doing so, they observed differences in sensitivity among the detection technologies but concluded that this finding would not be expected to impact reported results. The methods were performed using their validated protocol and were all sensitive to the level claimed in their validated protocol.

FINDING EQUIVALENT METHODS

In the examples above, the methods to be compared were already known. In circumstances for which an alternative method must be found, the critical criteria are the test-portion size(s) and target analyte(s). An approach such as that outlined in *Table 6* could be used to find an equivalent rapid method that has certifications equivalent for the intended use, though compromises may need to be made on TAT, ease of use, or cost. Looking at example 1 outlined in *Table 6* we see that if comparing a rapid PCR method against a rapid enzyme-linked immunosorbent assay (ELISA) method, where both are validated or certified for the same matrix and test portion size against an FDA BAM reference method according to the AOAC Appendix J protocol, we can consider them equivalent. In example 2, with different validation protocols but the same reference method, we can also consider these methods equivalent. Conversely, looking at example 3, we see that if the same two methods were validated or certified against different reference methods we cannot consider them equivalent without doing some additional comparisons. *Table 6* outlines interpretations for additional permutations of validation conditions to assist with a range of commonly encountered decisions.

A QUICK REFERENCE GUIDE

Selection of an equivalent rapid method could depend on a required TAT. Generally speaking, the TAT to a negative or presumptive result is correlated to cost. More sensitive detection technologies allow shorter enrichments and quicker TAT, but the testing kit cost and equipment to run the test can result in greater operational expense. Thus, real time PCR molecular methods are quicker but can cost more than ELISA test methods. However, both methods are usually more expensive than slower cultural reference methods. If a presumptive result is detected, all rapid methods will follow the confirmation procedure of the reference method against which they were validated, and each method will likely result in a similar TAT.

CONCLUSIONS

The selection of a validated microbiological method, whether a reference method or alternative method, is a critical decision that can potentially affect a company's profits and losses, reputation, and assessment of the appropriateness of their Food Safety system (Prerequisite programs, Preventive Controls, HACCP, or Food Safety Plan). There are various factors that a laboratory can consider when determining which equivalent method to use, including cost, TAT, or an affinity for a method they have been using for years. However, this document provides a rationale to determine equivalency among a variety of accredited methods validated for the same matrix and sample size, thereby allowing flexibility and choice based on whatever criteria are used. This flexibility allows laboratories and end users to meet critical needs efficiently when undesirable situations arise, such as kit shortages, equipment malfunction, and increasing costs. We hope that this paper provides a framework to help decision makers when these situations arise.

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