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Validating detection techniques

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4.1 Introduction

Microbiological tests are important in governmental food inspection to enforce legal regulations, in international trade to determine compliance with a microbiological standard, in commercial relationships between trade partners to exercise control on agreed microbiological specifications, in the food industry to maintain quality control and process requirements, in academic laboratories for conducting research, and in reference laboratories to confirm the analyses of another laboratory and to provide surveillance data. The results of these tests should be reliable, making it important to determine the performance characteristics of a particular method. Apart from these technical characteristics it is important that all parties involved agree with and accept the methods employed. Mutual recognition of test methods in international trade facilitates commerce.

Standardized methods have been elaborated by international, national or trade organizations such as ISO (International Standards Organization), AOAC International (formerly Association of Official Analytical Chemists), CEN (Comité Européen de Normalisation), NMKL (Nordisk Metodikkomitté för Livsmeddel), AFNOR (Association Française de Normalisation), NNI (Nederlands Normalisatie Instituut), DIN (Deutsches Institut für Normung), IDF (International Dairy Federation) etc. (Bertram-Drogatz *et al*., 2000).These standardized methods for detection of microbiological contaminants in foods are usually classical methods, primarily because the intention is to provide the users of these methods with a reliable and internationally accepted method which enables them to obtain equivalent results in different laboratory settings without having exclusivity of materials related to one manufacturer. Although, in

essence, these standardized methods serve only as guidelines for reliable microbial analysis of foods, historically in many countries governmental agencies and trading agencies recommend or accept them as official methods for detection or enumeration of microorganisms in foods. They are considered as the reference methods (Hitchins, 1996; Lahellec, 1998).

During the last decade several alternative (rapid) methods have been introduced for the detection and/or enumeration of microorganisms in foods as a result of recent developments in immunology, biotechnology and instrumentation. These alternative methods are often more rapid, user-friendly and open to automation and are thus of great interest to the food industry and control laboratories. Therefore, they are often preferred for daily use than the classical reference methods. Before alternative (rapid) methods can be used and accepted by the interested parties, it should be shown that the method is fit for its purpose by an independent organization. In practice, a method validation study needs to be performed to demonstrate that the intrinsic technical performance of these new procedures are acceptable.

The increasing need for validated methods is also due to the concept of official laboratories which must be accredited according to the requirements of ISO 17025 and thus need to use either official (standardized) methods, validated alternative methods or, if using a modified standardized method or own developed alternative method, perform a proper validation. On the other hand it is evident that standardized or validated methods can only give reliable results if used in a food microbiology laboratory with an overall Quality Assurance programme in place.

Section 4.2 defines a number of technical performance characteristics which are commonly determined in a validation study. Section 4.3 gives an overview of the validation protocols in use or suggested by AOAC International and the European validation procedures developed under the MicroVal project, and discusses a number of issues that need to be addressed in the preparation of a solid validation scheme. Some examples of performed validation studies of new techniques are discussed in Sections 4.4 and 4.5 to illustrate the above-mentioned issues. Section 4.6 focuses on the need for the commitment to quality assurance of the laboratory management in order to provide controlled conditions to execute microbiological analytical procedures using validated methods. Finally Section 4.7 looks at future trends.

4.2 Definition of performance characteristics

Validation is the demonstration that the technical performance of the method is comparable to the existing standard method in order to provide confidence to the end user that a method has proven ability to detect or enumerate the organism or group of organisms specified. Validation should include one or a combination of the following procedures

- determination of the performance of the method using reference materials or if not available well characterized pure cultures of relevant test strains
- a methods comparison study
- an inter-laboratory study
- testing to determine the effect of the variables, e.g. period of incubation, food matrix, etc. on the performance of the method.

Validation studies typically determine some or all of the following parameters.

4.2.1 Trueness (bias) (quantitative methods)

The closeness of agreement between the true value or, if not known, the accepted reference value and the mean result which is obtained when the experimental procedure is applied a large number of times is known as the systematic error (Ellison *et al*., 2000; ISO, 2002). The trueness can be obtained by the analysis of certified reference materials (CRMs), derived from a certifying body (e.g. the Community Bureau of Reference (BCR) of the European Commission (EC)). The production of reference materials is based on spray drying bacteria suspended in milk. The highly contaminated milk powder obtained is mixed with sterile milk powder and treated for homogeneity and stability and filled into gelatine capsules. Based on microbial analysis by multiple laboratories with a high level of experience, a number of reference materials were subjected to BCR certification. Based on the BCR certificate, user tables are produced presenting the 95 per cent confidence limits for the number of capsules likely to be examined in practice (In 't Veld, 1998; In 't Veld *et al*. 1999). However, only a limited number of CRMs are available for microbiological purposes. Non-certified reference materials having known values are available from different organizations for more, if not all, microbial parameters.

In microbiology, where almost no stable reference material is available, the analysis of spiked materials (recovery studies) can be performed as an alternative. 'Known values' can also be obtained after many replicated measurements with naturally contaminated samples using the reference method which is independent from the method to be validated. However, under these conditions the true value is not defined precisely and it is not possible to estimate correctly the trueness. An almost accurate approximation can be arrived at by measuring the same subsamples with the reference method and the alternative method. Participitation in proficiency testing schemes can also help in assessing the laboratory performance for the parameter of trueness.

4.2.2 Precision (quantitative methods)

Precision may be defined as the closeness of agreement between independent test results obtained by applying the experimental procedure several times on the same sample under stipulated conditions (= random error) (Ellison *et al*., 2000; Notermans *et al*., 1997). The measure of precision is usually expressed in terms

of imprecision and calculated as a standard deviation of the test results. Less precision is reflected by a larger standard deviation. Checks of precision in routinely used methods should be made to ensure that the result does not change with time as a result of changes in reagents, equipment, staff, etc. Distinction is made between:

- *repeatability*: indicates the variability observed within a laboratory, over a short time, using a single operator, the same apparatus on identical test material
- *reproducibility*: inter-laboratory reproducibility indicates the variability observed when different laboratories analyse the same sample by use of the same method and may be estimated directly by inter-laboratory study. Intra-laboratory reproducibility relates to the variation in results observed when one or more factors, such as time, equipment and operator, are varied within a laboratory.

4.2.3 Accuracy (qualitative and quantitative methods)

Accuracy is the closeness of agreement between a test result and the true value, or if not known, the accepted reference value. Accuracy is a qualitative concept and involves a combination of random components and a common systematic error. For microbiological analyses the term 'relative accuracy' is sometimes used, defined as the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples. The term 'relative' implies that the reference method does not automatically provide the accepted reference value (ISO, 2002).

4.2.4 The detection limit (qualitative and quantitative methods)

The detection limit is the smallest number of culturable microorganisms that can be reliably detected in the sample. For qualitative methods it can be defined as the smallest number of culturable microorganisms that can be detected on 50 per cent of occasions by the alternative and reference methods. For quantitative methods the detection limit is higher than the critical level, the latter being the lowest level of culturable microorganisms that can be reliably enumerated, for instance, the average of a blank sample plus three times the standard deviation of the blank and can be determined by analysing a relatively high number of blank samples (ISO, 2002).

4.2.5 Linearity (quantitative methods)

Linearity is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample. That is an increase in analyte produces a linear or proportional increase in results (ISO, 2002).

4.2.6 Sensitivity and specificity (qualitative methods)

Both sensitivity and specificity relate to the degree to which a method responds uniquely to the specified target organism or group of organisms and relate to the number of false positive and false negative results that are found with the validated method. Various definitions for sensitivity and specificity have been proposed (Notermans *et al*., 1997).

The sensitivity of a method is the proportion of target organism that can be detected; it can be calculated using the following equation:

$$\text{sensitivity (per cent)} = \frac{\text{number of true positives } (P)}{P + \text{number of false negatives}} \times 100$$

A failure to detect the target when present is a false negative result and will lower the sensitivity of a test. In food microbiology only a very low frequency of false negative results can be tolerated for safety reasons.

The specificity of a method is the ability to discriminate between the target organism and other organisms; it can be calculated using the formula:

$$\text{specificity (per cent)} = \frac{\text{number of true negatives } (N)}{N + \text{number of false positives}} \times 100$$

A positive result in the absence of the target is a false positive result and will lower the specificity of a method. For rapid screening methods, a higher false positive frequency may be acceptable, as positive screening tests are followed by confirmation tests.

In addition to the specificity and sensitivity as described above, also the inclusivity and exclusivity of a qualitative method may be determined, inclusivity being the ability of the validated method to detect a wide range of strains belonging to the target organism, exclusivity being the lack of detection with the validated method of a relevant range of non-target strains (ISO, 2002).

4.2.7 Robustness/ruggedness (qualitative and quantitative methods)

Robustness may be defined as the sensitivity of the method for (small) changes in environmental conditions or method parameters during execution, e.g. time and temperature of incubation, sources of supplies, purity and shelf-life of reagents, etc. (Andrews, 1996).

4.2.8 Practicality

Other considerations which can be important for adopting a new method and are generally referred to as practicality include (Andrews, 1996):

- the safety hazards associated with the procedure
- whether the procedure is quick and easy to perform and shows possibilities for automation
- the need for the analyst to follow extensive training programmes

- the availability of the method and the reputation of the manufacturer (services, quality system covering the production line).

4.2.9 Performance characteristics and standardized methods

Determination of the performance characteristics of a method by validation will facilitate acceptance of the test results by international, national or regional regulators and trade partners. Standardized methods which are published by international, national or regional standardization organizations or trade organizations are considered as validated. In this case the laboratory has to demonstrate that criteria for validation indicated in the standard can be achieved. However, these standardized methods are not necessarily validated methods. In contrast to IDF and AOAC methods, which have gone through a validation study before acceptance, only recently a number of ISO methods have been submitted to a validation scheme by order of the European Commission (Standards, Measurement and Testing Fourth Framework Programme Project SMT4-CT96-2098). The performance characteristics of six ISO methods were determined, namely *Bacillus cereus* (enumeration), *Listeria monocytogenes* (detection and enumeration), *Staphylococcus aureus* (enumeration), *Clostridium perfringens* (enumeration) and *Salmonella* (detection) and will shortly be published in the corresponding ISO methods (Schulten *et al*., 2000; Scotter *et al*., 2001a,b). Nevertheless, ISO methods have historically been considered as internationally accepted standard methods, because they are the outcome of an open discussion between experts of the different participitating nations (recommended by the national committees) in dedicated working groups (ISO/TC34/SC9 for Microbiology) (Lahellec, 1998). Although these standardized methods are regularly revised to include improvements that have been made in classical methods this process is laborious and time-consuming and subsequently these methods are not always up-to-date with new developments. If any modification is made to a standardized method by the user, e.g. change in period of incubation, restriction in the number or use of other culture media or confirmation tests, a limited validation study should be performed.

Recently numerous new methods have been developed which either generate results more rapidly than the classical culture methods and/or are easier to perform and open to automation. These methods can be developed 'in-house' or are available as a commercial test kit. An extensive validation study should be performed if the method is used on a routine basis in a control (official) laboratory applying for accreditation. Where an alternative method is used on a routine basis for internal laboratory use without the requirement to meet (higher) external criteria of quality assurance, e.g. in autocontrol or applied research laboratories, a less stringent comparative validation of the alternative method may be appropriate.

4.3 Validation protocols

An appropriate procedure for a validation study should be developed for each proposed method. The validation protocol will differ for a qualitative or a quantitative method and the stringency of the criteria set for the technical characteristics of the method will depend upon the application of the method, e.g. rapid screening method in a HACCP programme or analytical procedure to detect the cause of a foodborne outbreak, and the scope of the method, e.g. type of microorganism and type of food involved.

In the past different countries have developed different validation schemes. Several standardization organizations such as AOAC, IDF, AFNOR, NMKL, etc. have expanded their activities and started up a validation protocol for alternative methods. This has frustrated kit manufacturers, as they have to undertake a number of different validations in different countries, in order to get widespread acceptance of their test. There is a need of harmonization in validation schemes.

In 2002, the European standard 'Protocol for the validation of alternative methods' was accepted by the CEN. This standard is the outcome of the MicroVal project which started in 1993 with the aim of setting up a European validation procedure (MicroVal Secretariat, 1998; Rentenaar, 1996). As such the first goal, to establish an international accepted protocol for validation of alternative microbiological methods through standardization, has been achieved. Through the CEN/ISO 'Vienna agreement' this European Standard will also be adopted as an ISO standard (ISO 16140) and agreements have been made with AOAC International for mutual recognition of the different validation schemes. The ISO 16140 has been prepared by Technical Committee CEN/TC 275 'Food analysis – Horizontal methods' in collaboration with Technical Committee ISO/TC 34 'Agricultural food products'. A European organization for independent certification of alternative methods based on the European standard is set up (second goal of the MicroVal project) and pilot validation studies are ongoing (MicroVal Secretariat, 1998; Rentenaar 1996). The proposed ISO 16140 standard for validation of alternative methods describes a technical protocol for the validation of qualitative methods and a technical protocol for the validation of quantitative methods which both include a methods comparison study and an inter-laboratory study. Specific recommendations are given relating to the experimental setup of the measurement protocol and the calculation and interpretation of the data obtained using appropriate statistics. Standardization of the validation scheme as provided by the MicroVal is an important step towards consistent validation requirements. Nevertheless, the acceptance criteria are not clearly defined in the protocol. Results of the alternative method should be 'comparable' to those of the reference method. The actual criteria will depend on the type of method and the circumstances under consideration.

AOAC International is an organization that has a long tradition in the validation of methods. The collaborative study forms the essence of the

AOAC validation process. In this study, competent, experienced analysts, working independently in different laboratories, use a specified method to analyse homogeneous samples for a particular microorganism. Although no standardized protocol is available for the setup of the collaborative study, a number of recommendations are given regarding the minimum number of food types to be tested and the number of samples to be analysed for each food type. An Associate Referee, under the guidance of the General Referee and assisted by a statistical consultant, is responsible for the actual development of the protocol for the collaborative study which should be approved through the Methods Committee on Microbiology and Extraneous Materials. The Associate Referee also conducts ruggedness testing and a precollaborative study to determine the applicability of the method for detection of the target organism(s) in a wide range of food matrices and conditions. The Associate Referee is required to be an expert in a particular target organism or a particular type of method or a food matrix or a combination of these. The General Referee must be a recognized authority in the field of interest (De Smedt, 1998; Andrews, 1996).

A number of issues need to be addressed in the preparation of a solid validation scheme.

4.3.1 Choice of reference method

The reference method should be an internationally accepted method. Usually an internationally standardized method is chosen (ISO method, AOAC International accepted method, IDF method) or, if not available, certain national recognized methods or a method that has been published in a scientific journal and has been used succesfully for several years by several laboratories but without official recognition (ISO, 2002; Andrews, 1996). The choice of the reference method is of importance as it is supposed to give the true result. Indeed, for qualitative methods, the alternative method under validation is considered to produce a false positive result if a positive result is obtained while the reference method shows a negative result. This implies that the reference method should reveal all contaminated samples as true positives. For example for validation studies of rapid diagnostic kits for detection of *Salmonella*, the ISO method can be chosen as a reference method. However, as it has been shown that for certain food matrices a modified ISO method using a semi-solid medium such as Diassalm (LabM, Lancashire, England) or modified semi-solid Rappaport-Vassiliadis medium (MSRV, Oxoid, Basingstoke, England) instead of selective enrichment in a broth leads to a higher number of confirmed *Salmonella* positive samples than the original ISO method (De Zutter *et al*., 1991; Van Der Zee *et al*., 2002), this modified ISO method presents a good alternative to function as reference method in methods comparison studies (Poppe and Duncan, 1996).

4.3.2 Number of food types to be tested and number of samples to be analysed

The results of the method under validation are inherently affected by the type of food product under examination. The numbers, character and biochemical activities of the normal accompanying flora (background flora) in the food product affect the behaviour of the target organism(s). In addition the intrinsic and extrinsic properties (pH, a_w, temperature, atmosphere, naturally occurring or added antimicrobial components) of the food product under consideration and the nature and intensity of processing to which it was submitted inflict sublethal injury on the target organism(s) and may reduce its recovery by the proposed method (Struijk, 1996). Moreover, the composition of the food itself can complicate the assay further because of the presence of a number of constituents that can directly interfere with the assay procedure itself as is often noted with PCR methods (Wilson, 1997).

The number of food types to be included depends on the applicability of the method. If the method is to be validated for all foods, usually five categories of foods are included in the validation study. Food categories are predominantly determined by the origin of the product, e.g. meat products, poultry, fish and seafood, fruits and vegetables, dairy products, chocolate/bakery products and others (dressings and mayonnaise, egg products, cereals, etc.). Animals feeds and veterinary samples or environmental samples should be regarded as a separate category. Additionally the modes of processing to increase shelf-life, e.g. raw, heat processed, cured/salted, fermented, frozen, etc., may further be used to select food types within a category of foods (ISO, 2002). The types of food chosen should be relevant to the type of target organism(s) sought. For example validation studies for *Bacillus cereus* might include samples of rice, spices, raw and heat processed dairy products, heat processed vegetable-based products, whereas validation studies for *Campylobacter* might include raw poultry and meat, raw seafood, raw milk. However, if the applicability of the method is restricted the number of food categories may be reduced, e.g. the detection of *Vibrio parahaemolyticus* in fish and seafood products. The outcome of the validation study will recommend the acceptance of the method in the latter case for a particular type or otherwise for all types of foods. Whenever a method is validated for the detection of a particular target organism in all foods, questions arise as to whether the method is really effective with each individual type of food associated with that particular type of microorganism (Andrews, 1996). In the validation study only a limited number of food types are implied. If, for example, a validated method for *Listeria monocytogenes* indeed produces reliable results for five food types from each of five categories of foods (e.g. sliced prepacked cooked ham (heat processed meat product), raw milk based soft cheese (raw dairy product), smoked salmon (processed fish product), salad (raw vegetable product) and pasta (other products)), does this guarantee that the validated method will efficiently detect the presence of *L. monocytogenes* in raw milk, pasteurized cheeses, raw poultry, frozen fish, paté, fermented meat, bakery yeast, etc.? This is still an open question and therefore the validation report

should clearly state exactly which types of food were implicated in the validation study. Although the fact that a method is officially validated is an indication of good functioning of the detection or enumeration characteristic, the user of the method should always demonstrate that the method is producing reliable results if applied to its particular type of food matrix by a in-house limited validation study.

With regard to the number of samples to be included in the validation study, a sufficient number of identical samples should be analysed by both the reference method and the method under validation in order to generate sufficient data to allow the use of appropriate statistics for interpretation. Examples of numbers of samples included in the validation study protocols proposed by MicroVal and used by AOAC International are shown in Table 4.1. It should be mentioned, however, that these numbers of samples are valid for an extended validation study of a commercial kit. The validation of an internal method in food control laboratories, industrial laboratories or applied research laboratories may include variable numbers of samples depending on the type and the scope of the method. This is further illustrated in Sections 4.4 and 4.5.

It is recommended that the food samples in a methods comparison study come from as wide a distribution as possible in order to reduce any bias from local or seasonal factors and broaden the range of validation. It is desirable for qualitative methods to obtain approximately 50 per cent of positive results and 50 per cent of negative results for the same food type although this may not always be feasible when analysing naturally contaminated samples for foodborne pathogens. The reference method and the method under validation should be performed with, as far as possible, exactly the same sample. For example if the first stage of the two methods is the same (the same pre-enrichment broth or the same primary dilution) than take subsamples after this first step (ISO, 2002). Effective comparison of pathogen detection methods often requires testing at around the detection limit, where differences between methods are likely to be apparent. In this case it may be impossible to generate paired samples, where it is known that the number in one sample of the pair is accurately reflected in the other (Baylis *et al.*, 2001).

4.3.3 Naturally contaminated food samples versus artificially contaminated food samples

Whenever possible naturally contaminated samples should be used in methods comparison studies as these represent best real-life encountered samples with the target organism(s) present as a minority (if pathogenic bacteria) in a vast majority of other bacteria and in a non-optimal (stressed) condition due to the intrinsic properties of the food and the storage and processing conditions. Naturally contaminated samples can be collected from products analysed on a routine basis by the organizing laboratory or other laboratories. Storage should be minimized in order to prevent changes in both numbers of target organism present or the stress it is subjected to. Contamination should again be confirmed by the reference method before or during the validation study (ISO, 2002).

Table 4.1 Comparison of validation schemes suggested by MicroVal and AOAC International

MicroVal[a]		AOAC International[b]	
Qualitative method	Quantitative method	Qualitative method	Quantitative method
Inclusivity 50 strains *Exclusivity* 30 strains *Methods comparison study* Five categories of foods 60 samples per food category (minimum 3 food types) *Detection limit* 5 food types (5 food categories) 3 inocula levels: – control (uninoculated) – low (near the detection limit) – high (10× detection limit) 6 replicates each	*Inclusivity* 30 strains *Exclusivity* 20 strains *Methods comparison study* Five categories of foods 5 levels of target organism(s) 2–10 replicates *Detection and quantification limit* Five categories of foods 6–10 blank samples	*Inclusivity* 100–200 strains *Exclusivity* 20–30 strains *Precollaborative study* 20 food types 3 inocula levels (single strain): – control (uninoculated) 5 replicates – low (near the detection limit) – high (10× detection limit) 20 replicates each	*Inclusivity* 30–40 strains *Exclusivity* 10–20 strains *Precollaborative study* 20 food types 4 inocula levels – control (uninoculated) – low – medium – high 5 replicates each
Inter-laboratory study at least 10 laboratories 1 to 3 food types 3 inocula levels 8 to 10 replicates each	*Inter-laboratory study* at least 8 laboratories 1 to 3 food types 4 inocula levels at least 2 replicates each	*Collaborative study* at least 15 laboratories 5 food types 3 inocula levels 5 replicates each	*Collaborative study* at least 8 laboratories 5 food types 4 inocula levels 2 replicates each

[a] ISO (2002), Scotter *et al.*, 2001a, b.
[b] Andrews *et al.*, (1998).

If it is not possible to acquire a sufficient number of naturally contaminated foods for each of the food categories implicated in the validation study, artificially contaminated samples (spiked samples) can be allowed. However, it is recommended that no more than 80 per cent of the samples should be artificially contaminated (Andrews, 1998). When artificially contaminated samples are used, the levels of inoculation and the preparation of the inocula to achieve these levels of inoculation should be similar to those expected to be found in naturally contaminated samples. For example, a protocol for preparation of stressed (sublethally injured) organisms should be defined (e.g. cold storage, freeze-stress, acid stress) (Baylis *et al*., 2001) and the degree of stress demonstrated at the time of inoculation, e.g. by comparison of the lag-phase and/or the numbers of the stressed culture to the lag-phase and/or the numbers obtained for an optimal culture on a non-selective and/or selective culture medium (Baylis *et al*., 2001; Restaino *et al*., 2001; Uyttendaele *et al*., 2001). Also the background flora (numbers, distribution and ecology) of the samples used for artificial inoculation should be representative for the accompanying flora normally found in naturally contaminated samples (Andrews, 1996).

4.3.4 The source and number of inoculum strains

Reference materials, containing appropriate but well defined levels of target organism in a stable but stressed state, may be used for spiking samples. However, for qualitative studies their use is limited when only a few strains or serotypes of food origin of the target organism are available as reference materials. Strains that have been isolated from the same type of food product are preferred over clinical isolates for spiking. If not possible, then inocula should be fully characterized (reference) cultures (Andrews, 1996; ISO, 2002).

In the selection of strains to test inclusivity and exclusivity the majority of the strains should originate from the type of foods used in the validation study and cover the recognized range of the target organism with respect to the geographical distribution, incidence and diversity of the identification characteristics, its biochemical activity, serotype, phage type, etc. If the proposed method detects a defined target organism at the genus level, a range of species from that genus and if possible all species of the genus should be included, as well as a number of representative species from a range of genera within the same family. If the proposed method detects at the species level, a range of strains from that species should be included originating from different sources as well as representative strains from a range of species within the same genus. A number of representative strains of non-target organisms known to be part of the background flora of the type of foods included in the validation study could be included in the testing of selectivity of the method (ISO, 2002).

4.4 The application of validation schemes: immunological methods

Examples of the experimental design used for the evaluation of rapid methods in a number of studies are given here to illustrate the points made in the previous section. Because food manufacturers and regulators need quick and reliable information on the presence of foodborne pathogens in the food supply, numberous rapid or user-friendly methods for detection of foodborne pathogens (particular for *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157:H7) are continuously introduced to the market. Consequently, the majority of the methods comparison studies performed and published concern qualitative methods for these pathogenic bacteria. The examples mentioned here include both immunological (Section 4.4) and molecular (Section 4.5) methods, and were chosen on the basis of their wide application and/or potential in food microbiological laboratories throughout the world. These examples are intended simply to illustrate the methodology applied for evaluation or validation of the alternative methods, and therefore no judgement on the actual performance should be made on the basis of these data, as more recent information on the actual methods' performance may be available.

4.4.1 The VIDAS *Listeria* enzyme-linked fluorescent immunoassay

The VIDAS *Listeria* (LIS) (BioMérieux, Marcy l'Etoile, France) is a qualitative enzyme-linked fluorescent immunoassay (ELFA) performed in the automated VIDAS instrument for the detection of *Listeria* spp. This method allows rapid screening for the presence of *Listeria* spp. in food and environmental samples after a 44–48 h prior enrichment step. Positive results must be confirmed by standard culture methods.

The test kit's performance was reviewed by the AOAC Research Institute's Performance Tested Methods Programme and found to perform to the manufacturer's specifications. Inclusivity and exclusivity were demonstrated with 206 strains of *Listeria* and 50 strains of non-*Listeria* microorganisms. In a precollaborative study including 980 samples consisting of uninoculated, inoculated and naturally contaminated samples representing 17 different foods, the test was shown to be as good or better than culture method tests (FDA-BAM 8th Edition) of the same samples. The collection of food matrices included dairy products, seafoods, vegetables, raw meats and poultry and cooked and processed meats and poultry. A number of environmental surfaces were also subject to testing.

Subsequently, the VIDAS LIS method and the traditional culture method (FDA-BAM 8th Edition) were evaluated in a collaborative study. Nineteen laboratories participated in the study. Six food types (ice cream, cheese, green beans, fish, roast beef and ground turkey) were selected for the study. Ice cream, green beans and cheese were each inoculated with a different serovar of *L. monocytogenes*, roast beef was inoculated with a strain of *L. innocua*, and fish

were inoculated with *L. welshimeri*. Ground turkey samples were naturally contaminated with *Listeria*. Each food type was divided into three portions; the first two were inoculated (1–5 cells/25 g for the low inoculation level and 10–50 cells/25 g for the high inoculum) and the third served as uninoculated (negative) control. Cheese samples were stabilized by storage for five days at 4ºC. All other samples were stabilized by storage for five days at −20ºC. Each collaborator received a set of 15 samples for each food product (five replicates of each inoculum level and negative control). Of 1558 samples tested, 935 were positive: 839 by the VIDAS method and 809 by standard culture method tests. Overall false negative rates were 10.3 and 13.5 per cent for the VIDAS LIS and culture methods, respectively. The false positive rate for the VIDAS LIS assay was 1.4 per cent based on nine VIDAS LIS positive assays that did not confirm positive by isolation of *Listeria*. The agreement between the VIDAS LIS and culture methods for all samples tested was 86 per cent. Results for each food type and each contamination level were as good or better than those obtained with the traditional culture method (Gangar *et al*., 2000). On the basis of these studies the VIDAS LIS method for detection of *Listeria* spp. was recommended for Official First Action. The recommendation was approved by the Methods Committee on Microbiology and Extraneous Materials, and was adopted by the Official Methods Board of AOAC International.

The VIDAS LIS kit also received an AFNOR validation certificate (No. Bio 12/2–06/94) for the rapid detection of *Listeria* spp. in all food products. Inclusivity and exclusivity (indicated as specificity by the AFNOR certificate) were demonstrated with 217 strains of *Listeria* (207 were isolated from food and 10 were *L. monocytogenes* collection samples) and 35 strains of non-*Listeria* microorganisms. The intrinsic detection limit defined as the number of *Listeria* required to obtain a positive response with the VIDAS LIS kit was between 10^4 and 10^5 cfu/ml obtained on four pure strains of *Listeria*. The detection limit was performed using four different types of food (meat, vegetables, milk products and seafood), each artificially contaminated with four strains of *Listeria* at five different contamination levels: 0, 1–10, 2–20, 5–50 and 10–100 bacteria per 25 g. Agreement between the two methods was found to be 96.4 per cent (80/83). The three false readings involved levels of two or three bacteria per 25 g. Accuracy was determined by a methods comparison study of the VIDAS LIS method with the reference method (ISO 11290-1) using 204 samples (various products) of which 88 were naturally contaminated and 116 uncontaminated. All samples were tested in duplicate by both the VIDAS LIS method and the reference method. Overall the concordance between the VIDAS LIS kit and the reference technique was catalogued as good by AFNOR (eight false negatives were obtained with the VIDAS LIS kit and five false negatives with the reference method). Precision data were determined during an inter-laboratory assay involving 13 laboratories. Analyses were performed using pasteurized milk samples artificially contaminated with a *L. monocytogenes* strain at four different levels: 0, 1–10, 5–50 and 10–100 cells per 25 g. All the results were concordant with expected results. The method was shown to be reliable.

4.4.2 Dynabeads anti-Salmonella

Dynabeads anti-Salmonella (Dynal, Oslo) employs magnetizable particles coated with specific antibodies to concentrate all *Salmonella* serovariants selectively from foods and environmental samples. The technique which takes 15–20 minutes, may replace or enhance the performance of the 18–48 h conventional selective enrichments. The originally described protocol included immunomagnetic separation (IMS) from buffered peptone water (BPW) enriched food samples followed by plating (IMS-plating). The direct plating of bead-bacteria complexes onto solid media is suitable for processed foods or samples known to have a history of very low resident flora. For raw food samples, for example raw poultry, in some instances IMS-plating resulted in the overgrowth of target salmonellae by competitive enteric flora on the plating media. A modified IMS protocol consisting of the standard pre-enrichment of samples in BPW followed by IMS and subsequent selective enrichment of the bead-bacteria complexes in Rappaport-Vassiliadis Soya Peptone broth before plating on selective media (IMS-RVS-plating) was evaluated as an alternative to the traditional culture method for detection of *Salmonella*. The performance of both the IMS-plating and the IMS-RVS-plating protocol was compared to the conventional ISO 6579 method using ten food samples (powdered skimmed milk, mayonnaise, cake-mix, raw chicken meat, cooked sausage, cheese, pepper, meat balls, lasagne, casein) inoculated with 20 different *Salmonella* serotypes (two serotypes for each food sample) at low (1–5 cfu/25 g) and medium (10–50 cfu/25 g) level before pre-enrichment. All inoculated samples were frozen for one month before being examined. A 100 per cent concordance was achieved between the direct IMS-plating and IMS-RVS-plating for the ten diverse food samples (processed and raw) but the latter protocol assisted in the formation of well isolated near pure cultures of salmonellae on the plates. The IMS method showed respectively a 90 per cent and 95 per cent concordance with the ISO method and isolated respectively two and one more *Salmonella* positive sample than the conventional ISO method at the low and medium inoculum level. In an evaluation of both IMS protocols using 100 naturally contaminated samples (50 poultry carcasses, 20 cloacal and/or faecal swabs, 15 chicken liver samples, 15 chicken breast meat samples, and 10 poultry feed samples) the advantage of the IMS-RVS-plating method (39 positive samples detected) compared to the conventional ISO 6579 reference method (31 positive samples detected) and the IMS-plating method (only 20 positive samples detected) was clearly demonstrated (Cudjoe and Krona, 1997).

The IMS separation method has been successfully combined with the ELISA technique as the end-detection method for the recovery of *Salmonella* Enteritidis from eggs and skimmed milk powder. However, studies of raw chicken using a combined IMS and ELISA technique gave a significant number of false negative results because of high levels of competing flora. A study was performed to evaluate a modified IMS-ELISA method involving the resuspension of the bead-bacteria complexes and incubation in GN broth at 42ºC for 6 h prior to ELISA analysis. The present study is, however, an example of a study with a (too)

limited experimental setup. In the first part only two food types, animal feed and raw chicken, artificially inoculated, not in the food matrix but in the BPW pre-enrichment broth, using rather high inoculum levels (2000, 200 and 20 cfu per ml) and only three serotypes of *Salmonella* were involved (18 samples in total) to demonstrate that direct application of IMS-ELISA failed most of the time although RV-XLD (conventional culture method) and IMS-XLD succeeded in detecting *Salmonella*. The second part was even more restricted and stated the improved detection of *Salmonella* using IMS-GN-ELISA compared to the conventional RV-XLD methodology on the basis of 15 samples (triplet raw chicken pre-enrichment broths artificially inoculated with *S.* Enteritidis at 5 inoculum levels) (Mansfield and Forsythe, 2001).

4.5 The application of validation schemes: molecular methods

4.5.1 The Probelia™ *Salmonella* PCR system

Recently, the polymerase chain reaction (PCR) has emerged as an increasingly important diagnostic tool in food microbiology. The Probelia™ *Salmonella* PCR amplification and detection kits (Bio-Rad, Marnes la Coquette, France) have received AFNOR approval for detection of *Salmonella* in foodstuffs. The Probelia™ *Salmonella* PCR system is based on the amplification of the *iagA* gene (involved in the bacterial invasion process) of *Salmonella* spp. by PCR followed by probe hybridization in a 96-well format for colorimetric detection. In an independent study (Wan *et al.*, 2000) the Probelia™ PCR system was evaluated for rapid and specific detection of *Salmonella* spp. in dairy products and compared to the Australian Standard Method. Using bacterial DNA preparations derived from ten-fold serial dilutions of a pure culture of *Salmonella* Agona, the intrinsic detection limit of Probelia™ was determined as being between 8 and 79 cfu/ml, equivalent to 0.2–2 cfu per PCR reaction. Next, a methods comparison study was performed using artificially inoculated (with *S.* Agona) skim milk powder at 5–10 cfu/g and subjected to analysis immediately and after storage for two up to four to six weeks at 5, 15 or 25ºC (five replicates at each sampling point). A second food type was included: artificially inoculated ricotta cheese at 1–2, 10–20 and 100–200 cfu per 25 g (three replicates each). For all of the 40 milk powder samples and 12 ricotta cheese samples, the Probelia™ results were consistent with those of the Australian Standard Method. The present study was restricted to dairy products: two food types were included, however, only one *Salmonella* strain was involved, *S.* Agona. Naturally contaminated samples were not included. Recently the Probelia™ *Salmonella* PCR system was replaced by the manufacturer by a real-time PCR based kit iQ-Check *Salmonella*, applying a fluorescent probe hybridizing to the generated amplified products and measuring fluorescence directly in the tubes during the PCR annealing step.

4.5.2 The Taqman™ *Salmonella* PCR system

The Taqman assay is a fluorogenic PCR-based format enabling real-time detection of PCR products. The assay utilizes the 5′ nuclease activity of *Taq* DNA polymerase to hydrolyse an internal fluorogenic probe for monitoring the amplification of DNA target (the *Salmonella inv*A gene). Studies were performed to elucidate the specificity and sensitivity of the assay for pure cultures of *Salmonella* and for *Salmonella*-contaminated foods (Chen *et al*., 1997). A total of 164 *Salmonella* strains representing all the subspecies of *Salmonella enterica* were detected while 52 non-*Salmonella* strains were not detected. The intrinsic detection limit of the PCR assay was 2 cfu per PCR reaction when 10-fold serial dilutions of a pure culture of *S.* Typhimurium were used (PCR assays conducted in duplicate and replicated on different days). An appropriate sample preparation protocol was selected for the isolation of PCR-amplifiable DNA from foods. A detection limit of 3–7 cfu/PCR reaction was obtained using post-enrichment spiked food samples (ground beef, ground pork) inoculated with 10-fold dilutions of the *S.* Typhimurium strain and ca. 3 cfu per 25 g were detected when foods (raw milk, ground beef, ground pork) were inoculated with 2-fold dilutions of the same *Salmonella* strain and pre-enriched overnight. In both experiments no replicates were involved. Finally naturally contaminated foods (50 chicken carcass rinses and 60 raw milk samples) were examined for *Salmonella* using both the fluorogenic Taqman™ assay and the MSRV culture method which was used as the reference method. The concordance between the two methods was over 98 per cent. Two samples were *Salmonella* positive by the PCR assay but negative by the MSRV method.

4.5.3 A PCR-ELISA test for detection of Shiga toxin-producing *Escherichia coli* (STEC)

In the last few years, STEC and particularly strains of serogroup O157, have emerged as food-poisoning pathogens. A study was performed to evaluate the sensitivity and specificity of an in-house developed PCR-ELISA test for detection of STEC in dairy products using pure cultures, spiked and naturally contaminated samples (Fach *et al*., 2001). Specificity of the PCR-ELISA was determined with 94 STEC strains (including a large range of STEC serotypes isolated from humans and animals) and 84 non-STEC strains. The sensitivity was determined using duplicates of three individual STEC strains suspended and 10-fold diluted from approximately 10^6 to 1 cfu/ml. The detection limit in dairy products was carried out on five different pasteurized cheeses artificially contaminated with individual STEC strains (three strains) at four levels of contamination (0,10, 100 and 1000 cfu per 10 g, ca. 30 replicates each except for the highest inoculum level using ca. 15 replicates) and immediately subjected to testing. Finally a comparative study of the PCR-ELISA with vero cytotoxicity testing as the reference method was conducted involving 527 naturally contaminated samples (raw milk samples, unpasteurized cheeses, pasteurized cheeses and dairy environment samples). Of the 527 samples tested, both the PCR-ELISA and the vero cell assay detected

STEC in 30 samples. Only one sample which cause a cytotoxic effect on vero cells was PCR negative. PCR-ELISA detected STEC in an additional 74 samples. Regarding these results, the PCR-ELISA and the vero cell assay were not in total accordance. The overall percentage of agreement (negative or positive by both methods) was 85.8 per cent.

4.5.4 PCR/restriction enzyme analysis (PCR/REA) for identification of thermophilic *Campylobacter* species

Thermophilic *Campylobacter* species (*Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) are recognized as the most common bacterial agents responsible for gastroenteritis in humans in industrialized countries. The use of traditional phenotypic tests for the differentiation and species identification of campylobacters is often hampered by the fact that these bacteria are fastidious, asaccharolytic and possess few distinguishing biochemical characteristics. An in-house PCR/REA method was compared with standard phenotypic tests for the identification of these thermophilic campylobacters. In total 182 presumptive thermophilic campylobacters from 12 different animal species were tested by both PCR/ELISA and standard phenotypic tests. By PCR/REA, 95 per cent of isolates were identified as either one of the four thermophilic *Campylobacter* species or as not belonging to these organisms at all. By standard phenotyping, 174 of the 182 isolates were initially identified as either *C. jejuni*, *C. coli*, *C. lari* or *C. upsaliensis*. The PCR/REA and standard phenotypic tests resulted in only 67 per cent concordant species identifications. However, for the majority of the isolates who received discrepant species identifications with PCR/REA and standard phenotyping (52 isolates), additional tests could explain the discrepancies and prove the accuracy of the PCR/REA. For example 19 biochemically hippurate negative isolates initially phenotypically identified as *C. coli* but considered *C. jejuni* by PCR/REA were verified as *C. jejuni* by positive hippuricase gene PCR (Engvall *et al*., 2002).

4.6 The use of validated methods in accredited laboratories

The confidence in the results produced by a microbiological analytical procedure relies on the goodness of the analytical method (determined by a validation study) but also on the competence of the laboratory to provide accurate, reliable and repeatable test results under controlled conditions. Accreditation of laboratories carrying out microbiological food analysis provides to those relying on its services the assurance of the reliability of the test results. Accreditation of a laboratory is the formal approval given by a national authorized body (linked by the European cooperation for Accreditation of Laboratories (EAL)) that the laboratory is competent to carry out specific methods of analysis and has a commitment to quality assurance in the laboratory (Bowles, 2000). A detailed account of the requirements that testing laboratories

have to meet are described in ISO 17025:1999 (ISO, 1999). The criteria required are divided into:

- management requirements:
  - organization
  - quality system
  - document control
  - review of requests, tenders and contracts
  - subcontracting of tests and calibrations
  - purchasing services and supplies
  - service to the client, complaints
  - corrective and preventive actions
  - control of records
  - internal audits, management review
- technical requirements:
  - personnel
  - accommodation and environmental conditions
  - test methods and validation, measurement of uncertainty
  - equipment
  - measurement of traceability, control of data, sampling
  - reporting of results.

It is clear that to comply with the requirements of ISO 17025:1999, the laboratory should use validated methods which are documented in standard operating procedures, and needs a systematic approach to quality control (Bolton, 1998; Lightfoot and Maier, 1998). For example:

- The performance of culture media should be tested before use.
  For solid media the modified Miles-Misra method can be used. Detailed information can be found in the recommendations of the IUMS-ICFMH's Working Party on Culture Media (Corry, 1995).
- The performance of the equipment should be checked by daily monitoring and working out schedules for regular cleaning and maintenance
  This can include, for example, the monitoring of the temperature of an incubator using calibrated thermometers. Recently, in the framework of the European FOOD-PCR project a biochemical test was developed to check the efficiency of the thermocycler for performing PCR reactions.
- Defined internal quality control procedures should be used including first, second and third lines of control.
  The first line of control is quality control checks performed by the analyst during each series of tests executed under similar conditions, and can include the analysis of blank samples (no test organism) or positive (target organism) and negative controls (non-target organism) using reference materials or spiked samples. The results of the positive controls can be used to produce control charts to give an indication of the overall performance and consistency of the results in time.

The second line of control is performed less frequently. It includes checks which are initiated by the quality manager of the laboratory and need to be performed by the various analysts of the laboratory, e.g. testing of an identical sample (naturally contaminated or artificially inoculated) or interpretation of incubated culture media by more than one analyst. Results can be used to determine the intra-laboratory reproducibility.

The third line of control comprises the participation of the laboratory in approved proficiency testing schemes and aims to compare the overall performance of the laboratory with others. The results can be used by the laboratory to determine the parameter 'trueness' for the microbiological analytical procedure implied. The data obtained by the internal quality control along with the data from in-house validation studies can be used to estimate the uncertainty of measurement of a microbiological analytical method.

- Of major importance for laboratory accreditation is an appropriately qualified *staff* competent to perform the microbiological tests and supported in its continuing professional development.

The introduction of a quality system as a requirement of laboratory accreditation enables tight control over the laboratory's activities and enhances the confidence in the results produced. Formal accreditation of laboratories performing microbiological testing in the food industry is demanded increasingly by food manufacturers and government representatives.

4.7 Future trends

Dialogue between the international organizations which are involved in the development and validation of microbiological methods is of major importance in order to achieve uniformity and should ensure the acceptance of microbiological results by governmental inspection laboratories and laboratories in the food trade, thus facilitating international commerce. International cooperation is a fact which is illustrated by International Symposia and Workshops jointly organized by ISO, CEN, AOAC International and IDF to create a forum for the exchange of ideas and experiences between microbiologist colleagues. Meetings of ISO TC 34/SC9 and CEN TC 275/WG6 are held annually in the same week and at the same location in order to facilitate good coordination between the two groups. Experts from AOAC International and IDF also attend these meetings. This should result in the adoption of more harmonized standardized protocols although this is not always easy to obtain due to the wide variation in interests and experiences (Andrews, 1996; Hitchins, 1996; Lahellec, 1998). For alternative methods it is in the commercial interest of the suppliers and users of novel test methods to agree on harmonization in protocols for validation of these alternative methods. In 2002 the European standard 'Protocol for the validation of alternative methods' was accepted by the

CEN and will also be adopted as an ISO standard (ISO 16140). Agreements have been made with AOAC International for mutual recognition of the different validation schemes. This should lead to harmonization of validation procedures within Europe and, it is hoped, also acceptance in other countries. It will certainly help the unification of the food trade in Europe.

The use of validated methods is only part of providing a reliable result. Of equal importance is the execution of the method by qualified staff with validated equipment and utensils in a laboratory committed to quality assurance. In addition there should be recognition that the result of an analytical method, particularly near the detection limit, is not absolutely defined as presence or absence of a target organism (in the case of qualitative methods) or as a single number of colony forming units of target organisms (in the case of quantitative methods). The outcome of an analytical method is subject to an uncertainty of measurement which should be known and within the precision characteristics of the method as determined during the validation study. The present ISO standard for accreditation of testing laboratories (ISO 17025:1999) explicitly refers to the necessity of determining the uncertainty of measurement of a microbiological analytical method. The uncertainty of measurement can be estimated by the utilization of validation data (external or in-house validation studies) and quality control data (obtained as part of the accreditation scheme).

Many important decisions are based on the results of microbial analytical procedures. The use of validated methods in accredited laboratories leads to reliable results and provides confidence in the data obtained. Interpretation of these results, however, demands knowledge of the sampling procedure applied, appropriate definition of the goal (the purpose) of the microbial analysis, and a good knowledge of the ecology of the food product involved and the technology used for processing in order to set realistic criteria for interpretation of the test results. Apart from good analytical skills to perform the microbial analyses, also a broad knowledge of food microbiology, food technology and food chemistry is required for interpretation of the test results.

4.8 Sources of further information and advice

Information concerning the definitions of the technical performance characteristics, the determination of these parameters and the measurement of uncertainty can be found in the Eurachem/CITAC guide (2000) on Quantifying Uncertainty in Analytical Measurement as well as in the appropriate ISO 5725 Accuracy (trueness and precision) of measurement methods and results.

For more details on the design of an appropriate validation protocol, see CCFRA (Campden and Chorleywood Food Research Association) Guidelines for Establishing the Suitability of Food Microbiology Methods (2001), the special issue of *Food Control* (1996 Vol. 7, No. 1) on the validation of rapid methods in food microbiology and ISO 16140 Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods (ISO, 2002).

On the accreditation of laboratories and quality assurance, see the actual ISO 17025 *General requirements for the competence of testing and calibration laboratories*, and the book by Lightfoot and Maier (1998) *Microbiological Analysis of Food and Water – Guidelines for Quality Assurance*, published by Elsevier Science.

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