=Special Lecture=

ISO Standardization and Validation of Reference Testing Methods —Validation of Alternative Methods in Food Microbiology—

Bertrand LOMBARD*

(Coordinator of Scientific & Technical Support, Community Reference Laboratories
Listeria monocytogenes, Staphylococci and Milk)

Food microbiological analysis is highly variable, given the living status of the analyte and the biological nature of the reagents used. In addition the analytical results are closely related to the method's principle, in particular with conventional Pasteur microbiology and for enumeration methods. Food-borne pathogens represent a direct health hazard for consumer's health. For these reasons, there is a clear need to define common reference methods, properly validated, as well as to get assurance on the quality of commercial test kits which can be used in alternative to these reference methods.

Standardization can be considered to be the preferred mean, since based on consensus, to harmonize reference methods at international level. Standardization structures at international level in ISO and at European level in CEN in food microbiology are at first presented. Some methodological aspects are introduced, as well as the different types of standards developed: reference methods, general aspects. The current programme of work is over-viewed. A special hint is given to the position of novel technologies, as well as to the degree of validation of these Standard reference methods.

Reference methods being most of the time based on conventional microbiology, they require a skilled staff and are labor-intensive, long to perform due to the time needed by the bacteria to grow in/on culture media. Thus a large range of alternative methods, based on recent technologies such as ELISA or PCR, have been developed to get results within a shorter interval of time or to reduce work-load through automation. Manufacturers have commercialized a large range of such test kits, but their users need guarantees on their validity, thus the development of validation schemes operated by third-party organizations. To have a common reference technical protocol to conduct these validations, CEN has developed a standard for the validation of alternative methods in food microbiology, adopted by ISO, EN ISO 16140. This standard is presented, as well as its current revision by ISO. The validation/certification schemes are then shortly introduced.

In a final part, we consider the position of Standard reference methods and of validated alternative methods in the context of laboratory accreditation, based on ISO 17025 Standard, and in the European regulatory frame of Regulation 2073/2005 on microbiological criteria in foodstuffs.

Introduction

The harmonization of analytical methodol-

ogy is crucial in food microbiology for the following reasons.

It is well known that food microbiological analysis is highly variable, mainly due to two factors: (i) the intrinsic nature of the analyte, a living micro-organism, and (ii) the principles of detection/enumeration of micro-organisms by conventional culture techniques, which include the use of biological substances themselves variable, such as culture media, antibodies or bio-

* Chairman of ISO/TC 34/SC 9 and Convenor of WG 2 of ISO/TC 34/SC 9
AFSSA-LERQAP (French Agency for Food Safety—Laboratory for Study & Research on Food Quality & Food Processes): 23 avenue du Général De Gaulle—94706 Maisons-Alfort, France
Tel: 33 1 49 77 26 96, Fax: 33 1 49 77 26 95
E-mail: b.lombard@afssa.fr

chemical reagents. In addition, the analytical results are closely linked to the method's principle for conventional techniques, in particular for enumeration ones: the choice of the different steps (enrichment, isolation, confirmation), the selection of temperature/time of incubation, the composition of culture media will directly impact the number of colonies growing on a plate^{14, 15}. The enumeration result also depends on the physiological initial state of the bacteria, which may be in a growth phase, or altered, or stressed¹². There is therefore a clear need to define common reference methods in food microbiology, as to minimize as much as possible the variability of microbiological analyses, and to define in the less ambiguous way the analytical target: a given micro-organism (or group of micro-organisms) to be detected or a given number of micro-organisms (or group of micro-organisms) to be enumerated.

In addition, the harmonised method will need to be highly reliable, since some of them enable to detect or to quantify food-borne pathogens which represent direct hazards for consumers health. Their presence at very low numbers in a given food product (a few cells in a test portion of 25 g) may be directly responsible of the illness of the consumer of that food.

Standardization has proven to be the preferred way, since based on consensus, to harmonize reference methods at international level or at regional level, such as Europe, and to facilitate trade between or within countries. It is also a mean to ensure the validity of methods since they are selected by expert microbiologists coming from several countries, and their choice is based on a well-established set of scientific criteria.

Finally, international food trade is hindered if different national methods are used by the exporting and importing countries: the analyses are duplicated, trade is delayed due to the shipment blocked until test results are obtained by the importer. On the contrary, trade is facilitated when common reference methods are used, as well as commercial kits validated against them: one test is conducted by the exporter and recognized by the importer.

Standardization and Validation of Reference Testing Methods in Food Microbiology

What is a standard?

According to ISO (International Organization for Standardization)¹⁰, it is "a document established by consensus, and approved by a recognized body, that provides, for common and repeated use, guidelines or characteristics for activities or their results, aimed at the achievement of the optimum degree of order in a given context".

This general definition, designed to cover the very broad scope of standardization activities, underlines that a standard is primarily a text of reference, established by a recognized body, that is for a national standard the national standardization body, in Japan JISC (Japanese Industrial Standards Committee), CEN (European Committee for Standardization) for a European Standard and ISO for an International Standard. It represents a **consensus** of all concerned parties, either at national, European or international level, depending on the type of standard, and this is by far the main characteristic of a standard. It is of **voluntary use**.

ISO

ISO, created in 1947, is an international non governmental federation of national standardization bodies worldwide (158 to date), JISC for Japan. Its coordinating structure, the ISO Central Secretariat, is located in Geneva.

Standardization works are conducted within Technical Committees, Sub-Committees and Working Groups, settled per area of work, each of them under the responsibility (chair and secretariat) of a country which volunteers to take the lead. The secretariat is usually ensured by the standardization body of the country taking the lead, at least for the technical committees and sub-committees. It manages the whole standardization process (circulation of documents, preparation and follow-up of meetings, editorial drafting of standards, ...). The national ISO members and international organizations in liaison decide to become members of a given committee/sub-committee, depending on their interest and their will to become active in the standardization field considered.

The consensus on each standard is vali-

Table 1. Working groups of ISO/TC 34/SC 9 “Food products—Microbiology”

Working Group number	Title	Convenor
WG 1	Meat and meat products	Enne De Boer (Netherlands)
WG 2	Statistics	Bertrand Lombard (France)
WG 3	Methods validation	Paul in't Veld (Netherlands)
WG 4	Proficiency testing schemes	Sue Passmore (UK)
WG 5	Culture media	Melody Greenwood (UK)
WG 6	Parasites	Hugh Smith (UK)
WG 7	Amendment to ISO 7218	Alexandre Leclercq (France)
WG 8	Revision of ISO 6887 series	Sue Passmore (UK)
WG 9	Revision of ISO 6579	Kirsten Moijman (Netherlands)

dated by a series of votes by ISO members, three for the main procedure (full ISO standards):

- Committee Draft vote (CD, 3 months);
- Draft International Standard vote (DIS, 5 months);
- Final Draft International Standard vote (FDIS, 2 months).

Technical comments can be made during the 2 first ones and the draft can be modified according to the comments accepted by the ISO structure in charge of the standard. The last vote is an approval vote before publication, only editorial changes can be made after this vote. The ISO Standards are optionally taken over as national standards by ISO members.

CEN

The central structure of CEN, called CEN Management Centre, is located in Brussels, and has a role similar to ISO Central Secretariat. CEN members are the national standardization bodies of Europe at large: 28 countries are full members and 6 are associated members.

CEN standardization functions on a similar way to ISO. The main difference is that CEN Standards have to be taken over in the collection of national standards of CEN Members, and that conflicting national standards on the same scope have to be withdrawn. This ensures a higher degree of harmonization at European level, made possible by the regional scale involved. Meanwhile, the use of these standards remains voluntary, except if they are cited in European regulatory texts.

Standardization Structures in Food Microbiology

Most of the standardization works in food microbiology are conducted by two structures, one at ISO level and the other one at CEN level:

- ISO/TC 34/SC 9: Sub-Committee 9 “Microbiology” of Technical Committee 34 “Food products” of ISO (in short SC 9);
- CEN/TC 275/WG 6: Working Group 6 “Microbial contaminants” of Technical Committee 275 “Food analysis—Horizontal methods” of CEN (in short WG 6).

ISO/TC 34/SC 9 is under the responsibility of France, chaired by Bertrand Lombard and with a secretariat provided by AFNOR (French Standardization Association), namely Claire De Lamotte. 45 ISO member countries participate to SC 9 and 11 international organizations are in liaison. Japan is currently an observing country and it is hoped it will become a participating country. Several working groups have been settled by SC 9 to deal with specific topics before endorsement by SC 9 (see Table 1). The main SC 9 objective is to develop and to update ISO Standards for the microbiological analysis of a wide spectrum of targets and sample types:

- Mainly bacteria, also yeasts and moulds and recently viruses and parasites;
- In all food and animal feeding stuffs, as well as samples from the environment of food production, food handling and primary production (animal's breeding).

CEN/TC 275/WG 6 is also under a French responsibility, chaired by Alexandre Leclercq and with the same AFNOR secretariat. 30 CEN Members and 14 international organizations in liaison are represented in WG 6. Similarly to SC 9, several task forces (TAG) have been settled by WG 6 on given topics (see Table 2). The objectives are shared with SC 9, and in the frame of the CEN/ISO cooperation agreement called “Vienna Agreement”, most of the standards developed by SC 9 are taken over as CEN Standards, and inversely, the standards devel-

Table 2. Groups of CEN/TC 275/WG 6 “Food analysis, horizontal methods—Microbial contaminants”

Task group number/ Ad'hoc group	Title	Convenor
TAG 1	Culture media	None (dormant group)
TAG 2	Validation of alternative methods	None (dormant group)
TAG 3	PCR	Kornelia Berghof-Jäger (Germany)
TAG 4	Viruses	David Lees (UK)
TAG 5	Primary production stage	Rosine Tanguy des Deserts (France)
TAG 6	Sampling technique	Martine Poumeyrol (France)
TAG 7	Parasites	Patrizia Rossi (Italy)
Ad'hoc group	Shiga toxin producing <i>Escherichia coli</i>	Stephano Morabito (Italy)
Ad'hoc group	Listeria	Rijkilt Beumer (Netherlands)
Ad'hoc group	<i>Enterobacter sakazakii</i>	Han Joosten (Switzerland)
Ad'hoc group	Staphylococcal enterotoxins	Jacques-Antoine Hennekinne (France)

oped by WG 6, essentially in the areas covered by the task groups, are taken over as ISO Standards.

Different Types of Standards Developed in Food Microbiology and Current Works

To date, 58 standards/parts of standards have been published in food microbiology (CEN websites: <http://www.cenorm.be> and ISO websites: <http://www.iso.org>). The official work programme includes 16 work items, but more standards are in preparation. The standards mainly consist in reference methods, they also deal with general aspects related to microbiological analysis of food.

Three general aspects could be at first mentioned:

1. A working group has been settled by SC 9, WG 2 “Statistics”, to provide any statistical expertise needed for the standardization works, and with the objective to give a sound scientific basis to the standards developed.
2. The willingness to harmonize standards (a) in different fields of microbiology (dairy water, medical microbiology) and (b) worldwide, as to build a really unique reference system in food microbiology, through a complete harmonization of reference methods, especially with North America (AOAC).

Reference Methods

Existing Standards, some of them under revision, cover the main micro-organisms of interest in food microbiology: food hygiene indicators and food-borne pathogens, micro-organisms responsible for food alteration or for

their nutritive or commercial value, as well as in micro-organisms of technological interest (see Table 3).

For each of the target micro-organism, detection (presence/absence tests) methods and/or enumeration methods, as well as semi-quantitative methods in some cases are standardized. These methods are mostly based on conventional microbiology that is the ability of bacteria to grow in enrichment broths or on/in solid media. Indeed, this choice enables to meet the major characteristic of a standard: the largest consensus and the broadest applicability in food microbiological laboratories at world-wide level, even in developed countries themselves.

The standard methods with such a large scope, applicable to all food families, are called “horizontal”. The rationale to develop such “horizontal” standards is that the principle of the method (culture media, incubation times and temperatures, confirmation reactions) is mainly designed to recover and to characterize the target micro-organism, whatever the food matrix is, given the fact that the initial steps of the analyse (preparation of test sample and of the initial suspension) is dealt specifically, per type of matrix¹⁶.

New horizontal standards are currently being developed for the following micro-organisms:

- Contaminants in starter cultures/probiotics, under the lead of SC 9;
- Enumeration (by miniaturized Most Probable Number technique) and serotyping of *Salmonella*, under the lead of SC 9;
- Detection of *Clostridium botulinum* by

Table 3. List of reference methods in food microbiology standardized by CEN and ISO (CEN and ISO websites)

Microorganism detected/enumerated ^a	Standard reference number ^b
<i>Food hygiene indicators/Process micro-organisms</i>	
Enumeration of micro-organisms—CCT at 30°C	EN ISO 4833: 2003
Enumeration of coliforms—MPN technique	ISO 4831: 2006
Enumeration of coliforms—CCT	ISO 4832: 2006
Enumeration of mesophilic lactic acid bacteria—CCT at 30°C	ISO 15214: 1998
Enumeration of psychrotrophic micro-organisms—CCT at 6,5°C	ISO 17410: 2001
Enumeration of yeasts and moulds—CCT at 25°C	ISO 7954: 1987
Enumeration of yeasts and moulds in meat and meat products—CCT at 25°C	ISO 13681: 1995
Enumeration of sulfite-reducing clostridia growing under anaerobic conditions	ISO 15213: 2003
Enumeration of <i>Pseudomonas</i> spp. in meat and meat products	ISO 13720: 1995
Enumeration of <i>Brochothrix thermosphacta</i> in meat and meat products	ISO 13722: 1996
Detection and enumeration of presumptive <i>Escherichia coli</i> —MPN technique	ISO 7251: 2005
Enumeration of β -glucuronidase positive <i>Escherichia coli</i> —Part 1: CCT at 44°C using membranes and BCIG	ISO 16649-1: 2001
Enumeration of β -glucuronidase positive <i>Escherichia coli</i> —Part 2: CCT at 44°C using BCIG	ISO 16649-2: 2001
Enumeration of β -glucuronidase positive <i>Escherichia coli</i> —Part 3: MPN technique using BCIG	ISO/TS 16649-3: 2005
Detection and enumeration of <i>Enterobacteriaceae</i> —Part 1: MPN technique with pre-enrichment	ISO 21528-1: 2004
Detection and enumeration of <i>Enterobacteriaceae</i> —Part 2: CCT	ISO 21528-2: 2004
<i>Food-borne pathogens</i>	
Detection of <i>Salmonella</i> spp.	EN ISO 6579: 2002
Enumeration of coagulase positive staphylococci—Part 1: Technique using Baird Parker agar	EN ISO 6888-1: 1999
Enumeration of coagulase positive staphylococci—Part 2: Technique using Rabbit Plasma Fibrinogen agar	EN ISO 6888-2: 1999
Enumeration of coagulase positive staphylococci—Part 3: Detection and MPN technique for low numbers	EN ISO 6888-3: 2003
Enumeration of presumptive <i>Bacillus cereus</i> —CCT at 30°C	EN ISO 7932: 2004
Detection and enumeration of presumptive <i>Bacillus cereus</i> in low numbers—MPN technique	EN ISO 21871: 2006
Enumeration of <i>Clostridium perfringens</i> —CCT	EN ISO 7937: 2004
Detection of <i>Campylobacter</i> spp.	EN ISO 10272-1: 2006
Enumeration of <i>Campylobacter</i> spp.—CCT	ISO/TS 10272-2: 2006
Detection of presumptive pathogenic <i>Yersinia enterocolitica</i>	EN ISO 10273: 2003
Detection of <i>Listeria monocytogenes</i>	EN ISO 11290-1: 1996/Amd 1: 2004 ^c
Enumeration of <i>Listeria monocytogenes</i>	EN ISO 11290-2: 1998/Amd 1: 2004 ^c
Detection of <i>Escherichia coli</i> O157	EN ISO 16654: 2001
Detection of <i>Shigella</i> spp.	EN ISO 21567: 2004

^a “MPN technique” means Most Probable Number technique, “CCT” means Colony-Count Technique.

^b “EN ISO” means that a common CEN/ISO Standard is concerned, “ISO” that it is only an ISO Standard. “ISO/TS” means ISO Technical Specification. The year of publication of the last version is indicated after the reference number.

^c The standards were amended in 2004.

TAG 3 of WG 6;

- Detection of STEC (Shiga-toxin producing *E. coli*), by an ad'hoc group of WG 6;
- Detection of staphylococcal enterotoxins, under the lead of WG 6;
- Detection of the major food-borne viruses in food and bottled water (noroviruses and hepatitis A viruses), by TAG 4 of WG 6.
- Recent decision to launch works on detection of parasites: *Cryptosporidium* and

Giardia (SC 9) and *Trichinella* (WG 6).

Validation of Reference Methods

Criteria have been set up to select a method for a new/revised Standard: it shall be (i) at least published in scientific literature and/or in a national Standard, (ii) experimentally tested on artificially and naturally contaminated food-stuffs, these tests being often conducted by SC 9 members.

These Standard methods have not been

usually fully validated by a proper collaborative study: due to the living nature of the analyte, the organization of such studies is particularly difficult and costly in food microbiology. Meanwhile, a project, funded by the European Commission, has been conducted in 1996–2000, and 5 main Standard methods, still valid today, have been fully validated: enumeration of *Bacillus cereus*, enumeration of coagulase positive staphylococci (2 methods), enumeration of *Clostridium perfringens*, detection and enumeration of *Listeria monocytogenes* and detection of *Salmonella*⁽¹⁾. In addition, the European Commission is currently considering to give to CEN/TC 275/WG 6 an ambitious mandate to further validate 15 Standard horizontal methods.

WG 3 of SC 9 is currently drafting an ISO Technical Report defining the technical requirements for standardization of reference methods.

To illustrate this topic, let us take two examples.

1. Detection of *Salmonella*

The last version of the standard (EN ISO 6579: 2002) has not been prepared by a real project group, but directly by SC 9. It has been fully validated by an inter-laboratory study with 21 participants from Europe (in the frame of the above mentioned European project) and from USA (AOAC). The results have been published⁽⁶⁾ and the method has been also recognized by AOAC as Official Method 2002.10.

2. *Listeria monocytogenes*: detection (EN ISO 11290–Part 1, 1996) and enumeration (EN ISO 11290–Part 2, 1998)

Both parts of this standard have been amended in 2004, to replace PALCAM and Oxford agars by agar *Listeria* according to Ottaviani and Agosti (LOA), a more specific agar towards the species *L. monocytogenes*. A Project Group has been set up, lead by Rijkelt Beumer (Netherlands) and comprising 11 members from 8 countries, as well as representing the European Community Reference Laboratory for *L. monocytogenes* and IDF. Three sets of trials have been conducted:

- (i) 2 trials (isolation—18 participants and direct count—20 participants) to compare 7 isolation agars (PALCAM, BCM from BioSynth), ALOA from AES,

Rapid' L. mono from BioRad, CHROM-agar, Chromogenic *Listeria* agar from Oxoid and *L. monocytogenes* blood agar from Sifin). These trials, published⁽¹⁾, have enabled to select an agar of ALOA formulation.

- (ii) 1 trial having involved 22 laboratories to compare ready-to-use ALOA to a medium prepared from individual components. This trial, published⁽²⁾, has shown that both types of media gave equivalent results, a condition to standardize the agar.

Recently, a revision of both parts of the standard has been launched, aiming to enlarge the scope to all *Listeria* spp., to adapt the confirmation stage to the LOA agar and to open the possibility to replace, in the formulation of LOA agar, phosphatidylinositol by soy lecithin with high concentration of phosphatidylinositol. The latter has been substantiated by a comparative trial with 22 laboratories, published⁽²⁾.

Standards on General Aspects

A set of standards deals with general aspects related to the microbiological analysis of food. The following topics are covered, also taking into account the current works:

- a. Good laboratory practices (GLP) in food microbiology (ISO 7218). This standard gathers common aspects of the horizontal standards developed by SC 9 and WG 6, and, defining GLP, represents the basis for laboratory accreditation in food microbiology. It has recently undergone a deep revision/update, published in 2007.
- b. Quality assurance/performance testing of culture media (EN ISO/TS 11133). The rationale to establish this standard was that the description, in the detection / enumeration horizontal standards, of a culture medium with its composition is not enough to ensure to the users that the culture medium will be fit for purpose, and will have the appropriate performance. The existing EN ISO Technical Specification is being converted into a full standard, at ISO level in a joint working group with water microbiology as to prepare

a common standard for food and water microbiology.

- c. Preparation of test samples, initial suspension and decimal dilutions (EN ISO 6887).

Part 1 of this standard deals with the first steps of enumeration methods (preparation of initial suspension and decimal dilutions). The 5 other parts of the standard cover the initial step of microbiological analysis, *i.e.* the preparation of test sample (or taking of test portion from the laboratory sample), on a specific way, that is per type of food. The main families of food and feed are dealt with: meat and meat products, fish and fishery products, milk and milk products, other food products (animal feeding stuffs, egg products, cereal products, ...), as well as in an additional part the sample preparation from primary production stage. A revision of the whole series has just been launched.

- d. Measurement uncertainty (MU) for quantitative determinations (ISO/TS 19036).

This ISO Technical Specification, published at the beginning of 2006, provides a guide on a topic currently of main concern for food microbiological laboratories. In the frame of laboratory accreditation and according to EN ISO 17025⁵⁾, laboratories have to report the uncertainty associated to their results, if requested by their customers. The guide recommends a “global” approach for the MU estimation. It has been amended in 2009 to cover the case of low counts.

- e. Method validation.

In this key topic for the community of microbiological laboratories and for the users of the analytical results, a set of standards, to become different parts of the Standard EN ISO 16140 revised, is under development by WG 3 of SC 9, to deal with:

- Terminology,
- Validation of proprietary kits,
- Intra-laboratory (or in-house) method

validation,

- Method verification: in the particular context of accreditation, to verify that a method already developed and validated is correctly operated in a given laboratory.

- f. Proficiency testing (PT) schemes (draft ISO/TS 22117).

This topic is covered by WG 4 of SC 9. In addition to the general standard under finalization (ISO 17043), the purpose is to develop a guide dealing on the aspects of PT organization and interpretation which are specific to food microbiology¹³⁾.

Status of Novel Technologies

In the context of novel technologies, it is at first necessary to mention one important characteristic of any standard: the requirement/recommendation to use a commercial product should be avoided as far as possible in order not to give a commercial exclusivity to a given manufacturer and not to link the use of a standard to the commercial availability of the product.

Three aspects on the use of novel technologies in a standardized frame can be distinguished:

- 1) Introduction of novel technologies (such as PCR) in specific standardized reference methods.

Two cases allow such an introduction:

- a. If conventional microbiology is recognized to be not satisfactory to detect/enumerate the given target micro-organism at the level of interest. To date, examples are the detection of STEC, of *Clostridium botulinum*, of viruses and partly of *E. coli* O157. In the three first cases, the PCR methods target the pathogenicity genes of the bacteria considered. For viruses, the PCR methods could also target some RNA/DNA sequences specific of the targeted viruses. For *E. coli* O157, the Standard EN ISO 16654 is based on conventional microbiology, but with the introduction of an extraction step by immuno-magnetic separation.

Table 4. List of CEN/ISO standards on the use of PCR for the detection of pathogenic micro-organisms in food (CEN and ISO websites)

Topic	Standard reference number ^a
General requirements and definitions	EN ISO 22174: 2005
Sample preparation for qualitative detection	EN ISO 20837: 2006
Amplification and detection for qualitative detection	EN ISO 20838: 2006
Performance testing for thermocyclers	EN ISO/TS 20836: 2005

^a: “EN ISO” means that a common CEN/ISO Standard is concerned, “ISO” that it is only an ISO Standard. “ISO/TS” means ISO Technical Specification. The year of publication of the last version is indicated after the reference number.

- b. In addition to the standard reference method on a given micro-organism, based on conventional microbiology and with a taxonomical target, another standard based on novel technology can be developed with a different target than taxonomy, such as the pathogenicity of the given micro-organism. Example: detection of pathogenic *Vibrio* and of *Yersinia*.
- 2) Validation of commercial products (such as PCR systems, ELISA kits).
Given the characteristic of any standard recalled in the heading of this section, these products are not to be mentioned in specific standards but need to be validated against the corresponding standard reference method, as to show that they give at least equivalent results, in accordance with the validation protocol of the Standard EN ISO 16140 (see below)
- 3) Guidelines on the use of PCR for food microbiology

General guidance standards on the use of PCR in food microbiology are prepared by TAG 3 of CEN/. TC 275/WG 6. Several of them have been published in 2005/2006 and others are under development (see Table 4).

TAG 3 has been also recently entrusted by WG 6 to develop specific standard methods based on PCR. Currently 3 standards are being prepared: one for the detection of neurotoxin genes of *Clostridium botulinum*, one for the detection of *Yersinia enterocolitica*, and the other for the detection of pathogenic genes of *Vibrio*.

Validation of Alternative Methods in Food Microbiology

As seen before, reference methods in food microbiology are mostly based on conventional

microbiology, thus they require a skilled staff, are labour-intensive, long to perform due to the time needed by micro-organisms to grow in or on culture media.

A large range of alternative methods, based on innovative technologies, have been developed to get results within a shorter interval of time. They are also designed to be more convenient to use, requiring less labour, in particular when they are automated. Automation enables also to analyse a large set of samples at the same time. Test kit manufacturers have commercialised a wide choice of these innovative methods, often based on proprietary components (*e.g.* the chromogenic substrates, the ELISA antibodies, the DNA probes).

Given the need of the users of these methods to have guarantees on their performance, validation schemes, operated by third-party organizations independent from the manufacturers, have been settled. In order to have a common reference technical protocol to conduct these validations, a standard on the validation of alternative methods in food microbiology, EN ISO 16140, has been developed.

Standard EN ISO 16140—Overview

The first version of Standard EN ISO 16140, published in 2003⁴⁾, was developed by TAG 2 of CEN/TC 275/WG 6. It was based on the outcome of the project “MicroVal” of the European R & D programme Eureka, 1993 to 1998, having included an experimental phase¹⁷⁾.

It is under deep revision, conducted by WG 3 of ISO/TC 34/SC 9, with the support of WG 2 for statistical aspects and calculation tools. The main purpose of this revision is to cover the different types of method validation, not only the validation of commercial methods, and to introduce acceptability criteria for the perfor-

mance characteristics.

A clause of definitions can be found, including “validation of an alternative method”: “demonstrating that results obtained with the alternative method are comparable to the results which would have been obtained with the reference method”. In this definition, the word “comparable” can be understood as at least equivalent.

The standard settles the validation process in two phases:

- 1) A methods comparison study of the alternative method (AM) with the corresponding reference method (RM), conducted by one laboratory.
- 2) An inter-laboratory study on the 2 methods in parallel, organized by the laboratory in charge of the first phase.

If a certification of methods is sought in addition to their technical validation, general principles for this certification are given.

Then the standard details two validation technical protocols, for qualitative and quantitative methods. Each of them consists in the selection of a set of analytical performance characteristics. For each criterion, are given: a definition, an experimental design (type/number of samples to be tested . . .), calculations and in some cases interpretation. The current version often lacks of acceptability criteria.

If the alternative method has been already validated prior to the implementation of the standard, previous results may be taken into account if they have been obtained according to the same experimental design. Annex A of the standard gives specific rules for the acceptance of such external results.

Validation Protocol for Qualitative Methods

Methods comparison study

The first phase (clause 5.1 of the standard), aims at comparing AM to RM through 3 different sets of criteria:

- 1) **Trueness** (called “relative accuracy” in the standard) is defined as the degree of correspondence between AM and RM on identical samples.

The experimental design gives priority to naturally contaminated samples. If not enough naturally contaminated samples are available, artificial con-

tamination can be performed, according to a protocol defined in annex C of the standard. If a method is sought to be validated for all foods, 5 food categories are tested (categories defined in annex B of the standard, consisting in the main food families). Samples from environment of primary production and food production are treated as separate categories. For each category and each method, 60 test portions are analysed, belonging to 3 food types defined in annex B.

Three criteria are calculated: (i) relative accuracy, *i.e.* the number of pairs of identical results for both methods divided by the total number of results, (ii) relative specificity, *i.e.* the number of pairs of negative results for both methods divided by the number of negative results by RM; and (iii) relative sensitivity, *i.e.* the number of pairs of positive results for both methods divided by the number of positive results by RM. The degree of difference between the 2 methods is assessed by a χ^2 test (McNemar) or a comparison to a binomial law, as detailed in annex F of the standard. Only these tests in the standard enable to assess the equivalence of qualitative methods.

- 2) **Limit of detection** (called “relative detection level” in the standard) is defined as the smallest number of culturable micro-organisms that can be detected in 50% of occasions.

It is tested on artificially contaminated samples of one food matrix chosen within each category tested (see before), at preferably 5 (minimum 3) contamination levels (including a negative control), using one target microorganism per category and 6 replicates per method.

The calculation is quite basic: the limit of detection is characterised by a range, between the 2 contamination levels giving for the first less than 50% of positive results (*i.e.* less than 3 positives out of 6 replicates) and for the second more than 50% of positive re-

sults (*i.e.* more than 3 positives out of 6 replicates).

- 3) *Inclusivity* and *exclusivity* are combined criteria, they assess the ability of AM to detect the target micro-organism and its lack of interference from a range of non-target micro-organisms. Pure strains are tested: at least 50 target strains (except for *Salmonella*: 30) and 30 non-target. Criteria for selecting the strains are given in annex G of the standard. Inoculation methods are also described.

Regarding this initial phase, the revision of the standard will mainly concern: (i) the protocol of artificial contamination, adopting the AOAC one to better mimic a natural contamination, (ii) the food classification and (iii) the performance criteria, centred on relative LOD, whose calculation will use a generalized linear model, added with inclusivity and exclusivity.

Interlaboratory study

The second phase, the inter-laboratory study (clause 5.2 of the standard) aims at assessing the variability of AM in an inter-laboratory context ("transferability" of the method).

The experimental design requires at least 10 collaborative laboratories having obtained exploitable results. The samples are prepared with one matrix, individually contaminated at 3 levels (including a negative control). Eight blind replicates are prepared at each level, to be analysed by both AM and RM. Annex H of the standard provides detailed guidance on the organisation of the interlaboratory study.

Laboratory results are excluded only in case of reported technical problems: samples damaged, use of inappropriate culture media, transport conditions (time, temperature) not respected, acceptability rules for colony-counting/enumeration values not respected, and deviations from SOP. They are detailed in annex K of the standard.

The trueness criteria (accuracy, sensitivity and specificity) are calculated for each contamination level and their values are compared to the ones obtained in the first phase. Annex L of the standard gives further optional criteria (accordance, concordance and concordance odds ratio), attempting to better address the random

variability of a qualitative method, by introducing parameters equivalent to repeatability and reproducibility for quantitative methods.

For this study, the revision of the standard is expected to include the possibility to have several participants in the same "laboratory" (geographical site) and the main criterion will be relative LOD.

Technical Protocol for Quantitative Methods

Methods comparison study

This first phase of the study (clause 6.2 of the standard) enables to assess 4 sets of criteria:

- 1) **Linearity**, defined as the ability of AM, for a stated matrix, to give results that are in proportion to the amount of analyte present in the sample. For *true-ness*, the term "relative accuracy" is also used with the same definition than for qualitative methods (see before).

The experimental design requires a minimum of 5 contamination levels with a priority given to natural contamination. At least duplicates (preferably 5–10 replicates) are prepared. The rules defined above for qualitative methods on the categories to be studied apply here also.

A regression analysis of the data is conducted. Two methods for linear regression are suggested in annex R of the standard: orthogonal and least-squares linear regressions. The linearity of the AM against the RM is then deduced (lack-of-fit test), as well as its trueness (bias in the linear equation).

- 2) **Limit of detection** (LOD), defined as the smallest amount of analyte which can be detected but not quantified with a high probability, *i.e.* 95%, whereas the *limit of quantification* (LOQ) is the smallest amount of analyte which can be measured and quantified with defined precision and trueness.

As for analytical chemistry, the experimental design and calculation refer to blank sample analyses and their standard deviation (s_0): $LOD = 3.3 s_0$ and $LOQ = 10 s_0$. This is applicable to indirect instrumental methods but not to alternative methods consisting in col-

ony-count/most probable number techniques, for whose the standard does not propose any clear solution.

- 3) *Sensitivity*, the ability of AM to detect two (slightly) different amounts of analyte measured by RM, refers for the experimental design and calculation to linearity.
- 4) *Inclusivity* and *exclusivity* are defined as for qualitative methods; the experimental design is similar except that the minimum of target strains is settled at 30, and 20 for non-target strains.

The revised standard should replace these criteria by a new approach, the accuracy profile, combining for the interpretation trueness and precision and already implemented in the pharmaceutical field⁷⁻⁹. LOQ could be deduced directly from the graph.

Interlaboratory study

The interlaboratory study (clause 6.3 of the standard) aims at assessing the interlaboratory precision and trueness of the AM against the RM.

The experimental design prescribes at least 8 laboratories having obtained exploitable results. The samples are prepared from one matrix, contaminated at four levels (including a negative control) and analysed in blind duplicates by both the AM and RM.

As for qualitative methods, results are excluded only on the basis of reported technical deviations. The laboratory results are at first $\log_{10}$ transformed, a usual step in quantitative microbiology in order to approach a Normal distribution of the data and to stabilize the variance over contamination levels.

The *precision* characteristics are then calculated with robust statistics, based for the repeatability standard deviation on the median of the duplicate standard deviations and for the reproducibility standard deviation on the Rousseuw's recursive median S_n . Robust statistics have been chosen since they are less sensitive to outlying data than parametric statistics, they accommodate with these extreme data and enable to avoid the use of statistical tests to exclude outliers. Per contamination level, the *bias* (relative accuracy) of AM against RM is estimated by the median of the laboratories'

differences between duplicate means for AM and for RM.

The revision of the standard is intended to introduce the possibility, as for qualitative methods, to have several participants per laboratory and to use the accuracy profile for interpretation.

Validation/Certification Systems Based on EN ISO 16140

Validation or certification schemes all include a third-party assessment, independent from the test kit manufacturer.

Technical validation systems of commercial methods in food microbiology can be found in:

- the USA, operated by AOAC International: the Official Methods scheme (full validation partly following EN ISO 16140), Performance Tested Methods scheme of AOAC Research Institute (without an inter-laboratory study);
- Northern Europe: NordVal operated by NMKL, with a protocol near to EN ISO 16140.

In addition, 2 schemes currently operate a certification system, applying entirely EN ISO 16140: AFNOR Certification in France and the newly set MicroVal, managed by NEN (Dutch standardization body), derived from the MicroVal project (see before). A test kit's certification based on EN ISO 16140 includes obviously that the technical validation follows the entire protocol defined in the standard. The added value of certification is that the method's performance is ensured to remain at the same level in time, with quality assurance requirements on the production site. The organizational aspects of the certification are defined by the certification body itself and described in documents called certification rules.

Standardized Methods and Validated Alternative Methods in the Context of Laboratory Accreditation and European Regulations

The Standard EN ISO 17025, defining general requirements on the competence of laboratories and being the reference standard for laboratory accreditation, contains a clause (5.4.5) on method validation. It states in particular

that a laboratory needs to validate the methods which are not standardized, including the alternative methods. The Standard EN ISO 16140 is the tool of choice to validate the alternative methods in food microbiology and thus to meet the requirement of EN ISO 17025. If a proprietary method has been validated/certified according to EN ISO 16140, it is obvious that the EN ISO 17025 requirement is met and the laboratory does not need to conduct itself any more validation when using this method for accreditation purpose.

The Commission Regulation (EC) 2073/2005³⁾ gathers and updates microbiological criteria for food in the European Union (EU). This regulation is formally applicable to food business operators, along the whole food chain from production to distribution, but it is also indirectly applicable to official controls performed by public authorities of EU's Member States. It defines for each microbiological criterion a reference method, generally an EN ISO Standard, but states in an article of the main text (Article 5) that other methods can be applied, under the following conditions: "when the methods are validated against the reference method in Annex I and if a proprietary method, certified by a third party in accordance with the protocol set out in EN ISO standard 16140 or other internationally accepted similar protocol". From this text, it is clear that if a method has been validated according to EN ISO 16140 or if a proprietary method has been certified according to this standard, it can be used in the frame of this regulation for own checks and for official controls, as to assess that food products meet the microbiological criteria defined in the regulation.

Conclusion

It could be questioned why to spend so many efforts to standardize methods in food microbiology, whereas it is well known that each "good" microbiologist is convinced to have the best method, adapted to its laboratory and to its objectives. Several reasons of scientific nature have been already envisaged in the introduction. In addition to these, we can mention some reasons of different nature.

The use of Standard methods favours the recognition of analytical results in the frame of

the supplier/customer relationship. The use of Standard methods also improves the recognition of analyses conducted in the frame of official controls at national level and in the frame of international trade, as mentioned earlier.

Finally, the set of Standards developed and validated commercial methods is also a sound and common basis for accreditation of laboratories and for setting microbiological criteria in food microbiology. ISO and CEN in this field entirely play their role of preparing reference documents that can be after directly used for accreditation and regulatory purposes.

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