Rapid methods : choice and criteria of acceptability in food microbiology

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For a long time, industrials as well as research laboratories have been interested in the possibility of replacing reference methods which are often long and expensive by alternative methods ; they were supposed to present a lot of advantages: they had to be more rapid, less costly, allow to examine a larger number of samples, spare place ...that looked like a dream. In fact, it was a long time ago as the dream became a reality (more than 30 years) ;I think you are, we are, very lucky to be allowed, during those days, to hear a lot on the subject by the "father" of rapid methods who developed the concept "think small" as he was still a young student ! From that time, a lot of rapid / or automated methods dedicated to the detection / enumeration of microorganisms have been developed all around the world; you probably know that Dr Fung organizes a course on rapid methods and automation in microbiology every year from 1980 ; here, in Barcelona, this year is the 4th workshop and , courses or meetings on the same topic are organized in different parts of the world .

However, the choice of methods remains somewhat difficult: what to use, in which objective ? . Will those methods be accepted by the scientific community, or by people in charge of regulations and , maybe in some cases , used instead of classical methods ? I remember well that , in 1987 during a meeting which was organized in Paris, Dr Fung expressed the fear that a young microbiologist may be very disoriented when looking at the explosion of microbiological methods . What to do ? what to choose ?
Surely , from that time , the situation has been improved; anyway, things are not always very clear and , probably , we are still in a transitory period ...

That is the reason why, during the time which I have been allowed for that introduction, I would like to share with you some considerations concerning the choice and criteria of acceptability methods in food microbiology according to the objectives which may influence the choice as of: - epidemiological surveys - controls by public and industrial laboratories – official controls; finally, I would like to comment a project called " e.Cam" which was build to clarify the situation and help microbiologists from different horizons in their choice; but, at first, we shall have a look to some general considerations .

I – General considerations

During the past decades, questions related to Food Safety have taken an increased importance in the mind of consumers, and that in all industrialized countries in order to guarantee the safety of products, foods are produced in such conditions as they allow the control of hygiene by industrials. In that context , HACCP (Hazard Analysis of Critical Control Points) is a fundamental tool. Raw materials as well as foods at different stages of production and after are submitted to controls realized in order to know that critical points are really under control. The results of those controls must be available and recognized by all concerned people and a special attention has to be given to what may happen during official controls.

In fact , one point has especially to be emphasized:

The recognition of the results of controls supposes that two steps are validated:

1- the sampling step: that means to take a representative sample from a production site ; the conditions of sampling have to be perfectly defined and based on statistics as much as possible.

the type of sample has also to be defined and known

2- the analysis step, which supposes that a validated method does exist but also that a competent laboratory is susceptible to use it in good conditions

If those two conditions are not fulfilled, the final results can't be valid ! and that is very important for the considerations which will be developed further.

The general advantages of rapid methods have often been described; among those:

- they are supposed to be rapid, of course; however, it is important to know whether or not the time of sample preparation is included in the performance indicated by the producer !
- the cost, usually far less (even when including the necessary initial funding, the cost of reagents, eventually other costs) than for conventional ones
- the place necessary to perform them ... and that advantage cannot be ignored !

However, in quite all regulations all around the world, the classical methods remain till now the only methods recommended .

II- Choice of methods and criteria of acceptability according to the objectives of the investigations

Epidemiological surveys

The examination of a sample representative of a population is of highest importance for epidemiological surveys. However, when using conventional methods, those surveys may become very expensive. Personally, I made such an experience when, in the 70 ies , I was working in Ploufragan (Brittany), trying, at the request of the Ministry of Agriculture, to set up microbiological criteria for poultry further processed products ; at that time, we pointed out the possibilities of contaminations "from farm to fork " and we began to investigate the *Salmonella* contamination of the same flocks (breeding flocks, hatcheries, poultry farms, transport, following steps of processing and further - processing). At that time, each sample was examined for *Salmonella* using two enrichment media, 2 times of incubation (24 and 48 hours), two isolation media ... moreover, all biochemical tests for identification were performed using conventional tests (10 ml per tube)

The use of miniaturized methods from the time of the intervention of Dr Fung in 1976 lead to improve really the situation as we began to use miniaturized methods in microtiter plates.
The place, the costs were dramatically reduced ; however, we had no doubt about the results as the methods used were strictly identical to the conventional ones .

A real question occurred when a lot of rapid / alternative methods were proposed on the market for the detection / enumeration of microorganisms ; in the beginnings, the situation was somewhat confused till the proposal in different countries of a validation system more or less similar to that proposed by AOAC.

I would like to insist on one point, i.e that , **whichever the method used , and even for epidemiological surveys, it is important to be able to compare the results obtained by different scientific teams all around the world.**

Nowadays, the possibility of comparison of results of epidemiological surveys is still a topical question: a good example is that of EHEC; if you try to compare the, I am speaking of my own experience) but I am sure the situation is improving ..

Methods used in public and private laboratories

While a few decades ago, the objective in public and private laboratories was especially to demonstrate, a posteriori, the control of hygiene and products quality by the analysis of finished products, they are now also used to be sure of the reliability of processes (HACCP, Risk Assessment) and of the conformity to safety objectives.
The introduction of the Risk Analysis has induced a trend in the methods diversity and has lead to a search in the use of alternative methods .

We must keep in mind the fact that, according to regulation and standardisation status, the methods can be considered as:

- official methods
- reference methods
- alternative methods
- "new" methods

Let me take the example of milk and dairy products:

The list of official methods is updated especially during the meetings of National Reference Laboratories (NRL)organised by the Community Reference Laboratory (CRL). As a priority, proposed methods are those standardised by CEN, ISO, or IDF (most of them can be considered as reference methods) it may be, and at the moment it is the case for staphylococcal enterotoxins, proposed by the CRL.

Till now, the methods proposed are conventional ones.

° The use of alternative methods

At a European level, the CRL has provided the possibility of using alternative methods instead of reference ones, submitted to the condition they have been validated by the standard EN/ISO 16140. In fact, the objective of the standard is, in fact, to be able to replace the actual reference methods by that of alternative ones, faster and easier to use (automation, reagents ready to be used) as ELISA kits, molecular hybridation, PCR .
The technical validation consists in two steps: one comparative study carried out by one laboratory and an inter-laboratories trial concerning the two methods conducted in parallel. For each step and each method, performance criteria are defined as well as the experimental protocol, the mode of calculation and, eventually their interpretation.

The standard sets up also the principles of a certification by a third party, based on that technical validation but adding some requirements on the quality assurance of the alternative method of the producer of the alternative method.

For auto-controls, the possibility of using alternative methods is open ; it must be said that, from a long time, the quality control of milk has been based on instrumental rapid methods.

Official controls

Usually, official methods are those included in regulations and are conventional ones.
However "reference" does not mean "static" and it seems interesting to keep in mind the content of the resolution n° 74 taken during the meeting of CEN TC275/WG6 held in Parma (It), 23rd April 2004 . WG6 agreed on the following points:

- *Each time a standard reference method is established or revised, the possible use of new technologies including PCR will be reviewed alongside the existing culture method to determine the most suitable method for the reference method .*
- *In addition, to the existing reference standard for a given microorganism, the need to develop a standard method with a different objective (such as pathogenicity ..) would be considered in the same way as point 1.*
- *When new technologies including PCR are used as an alternative method to the standard reference method, they shall be validated against the reference method.*

The subject of "alternative" methods is really in complete evolution !

III – E.Cam : Electronic Compilation of Analytical Methods

In order to help microbiologists from different horizons in their choices, a project called "e-CAM" (Electronic Compilation of Analytical Methods) was developed by AOAC INTERNATIONAL with the help of federal funding (Food safety and Inspection Services FSIS) and FDA.
I thought it would be interesting, in an introduction to a course on "rapid methods and automation in microbiology", to be aware of that project in order to classify not only the methods but also our ideas on the possible and adequate use of such or such method in a given context.

The background of that project will be shortly described in a first part in a second part, the basis of classification will be indicated, then discussed finally, we shall try to point out the usefulness of the classification in the context of the present course.

1- Background

As everybody knows, foodborne illness is a significant public health problem all around the world: as an example, in the USA , the Center for Disease Control and Prevention estimates that 325000 hospitalizations and 5000 deaths per year are caused by foodborne pathogens (it must be mentioned that the comparison with data obtained in different countries is quite difficult, as the collect of data is realized according to different ways). In addition to the usual problem of foodborne illness, it must also be added that bioterrorism seems to have been one important factor leading to take rapid methods in microbiology into account.

In fact, regulatory agencies have a current primary goal to reduce the prevalence of foodborne hazards, throughout the farm to table continuum, and including all stages. In that context, it is of main importance to be aware of the "status" of such method leading to such result: that is the reason why it was early recognised that the development of a database trying to classify methods should be a key component for a better understanding of data

obtained in different geographical areas. In the century we are living, the computerization of data appeared as the best tool to be used.

In 2001, a cooperative agreement was established between thee USDA / FSIS and AOAC, an advisory group was formed, including 4 subcommittees (chemistry, microbiology, database design planning and implementation, and statistics). These groups have further defined the categories of methods validation, criteria format and other requirements for a successful system.

The challenge was to create an international database including all types of methods which may be used for a given purpose ; in that context , the method should be described as well as the system of validation to which it had been submitted.

2- The e.Cam system and the classification of methods

We have been remembering the variety of methods which have been developed during the last decades for detection / enumeration of microorganisms in food and feedingstuffs, but also the number of validation systems being used in different countries all around the world. The question of an internet based system giving to the users the opportunity of knowing on line the methods as well as the degree of validation was raised many years ago (and I have in mind a question I had to answer after I presented a paper in K.state in 2000 , i.e: which may be the usefulness of internet in that field? isn't it for a very next future ?)

The classification which will be described has been proposed by AOAC and an Advisory group including scientists of different countries; from now, it must be mentioned that the initiative originates from USA, i.e USDA and AOAC INTERNATIONAL which is an independent , not for profit , internationally recognised organisation, showing 120 years of experience in validating and approving methods for food and agriculture.

The purpose of e.Cam is to permit federal state, international organisations and industry conducting food safety work to have electronic access on a subscription basis, to the latest methods of analysis. "use of validated methods will provide uniformity and consistency throughout the scientific community which will allow the sharing of analytical data nationally and internationally. Such data sharing will enhance rapid, appropriate, and effective public health and regulatory responses to food safety threats. The methods would be validated according to international criteria and would be easily accessible by and affordable by users globally.

The system consists of five separate categories of methods: regulatory methods and four other categories differentiated by the degree of validation.

Regulatory (REG) Methods

According to e.Cam, Regulatory Methods are methods which are specified by national and international regulatory agencies for enforcement purposes. These include currently recommended methods and methods specified in regulations.

At that point, it seems interesting to think about what can be found under the word "regulatory". In fact, when we speak about "regulatory", we have in mind what we call "reference" methods; or we can also designate "validated" methods which have been tested against a reference method. In the e.Cam classification, "regulatory" are "reference" methods. The definition and approach seem very simple in fact, we still have to take into account:

- the fact that the definition of the "reference" has sometimes to be clarified and also the necessary steps to be fulfilled before proposing a standard.
Concerning the different steps and requirements necessary to set up a standard, a group of ISO /TC 34 / SC9 is working on that topic.

- the fact that reference methods are not identical all around the world, for example, USDA takes into account AOAC methods and not ISO ones ; however, some progress has been set up during the last years for *Salmonella* detection: during a european project , a collaborative study was realized by european and american laboratories as well in order to compare the results obtained for three matrices (minced poultry meat, cheese curd and egg powder) artificially or naturally contaminated by *Salmonella*.

There was finally an agreement for a mutual recognition of ISO and AOAC methods and the results were recently published in the Journal of AOAC.

- the fact that the approaches of validations systems have also been very different during a very long period and the harmonisation is not yet perfect, howwever, it must be mentioned that a lot of progress has been made: the international EN /ISO protocol for validation of alternative methods, which was published in May 2003 is very close to the AOAC protocol previously published for the same purpose. As was published a few years ago "HANDS TOUCH ACROSS THE SEA". We shall see that **e.Cam refers to both systems.**

Harmonised collaborative Validation (HCV) Methods

"Methods validated through a full collaborative study that meet the standards set forth in the ISO 16140 or AOAC Methods Committee Guidelines for Validation of Qualitative and Quantitative Microbiological Methods for full collaborative study. The collaborative study must report valid data from at least eight laboratories for quantitative methods and ten laboratories for qualitative methods. A methods comparison study conducted in a single laboratory on other foods has to be included"

The proposal is that validation and approval will be administered by AOAC INTERNATIONAL and other reciprocally recognised validation bodies in accordance with the category criteria in e.CAM. Methods submitted in this category must follow program requirements of the *Official methods* program.

Multiple – Laboratory Validated (MLV) Methods .

"MLV methods are methods that do not meet the requirements of the HCV category but have undergone validation studies following a third party approved protocol. The study manuscript must include valid data from two or more laboratories analyzing at a minimum the same five different test materials as blind replicates under the same test conditions. Submission must comply with ISO 16140 or "AOAC Microbiology Guidelines for Validation of Qualitative and Quantitative Microbiological Methods except that the collaborative study requirement is removed. The alternative test method results are compared with those from a reference method using common samples of a minimum of 2 foods with three contamination levels. For quantitative methods, 5 test portions are run for each of three levels of inoculation or three lots of contaminated food. A methods comparison study conducted in a single laboratory must be included"

Validation and approval will be administered in the same conditions as for the previous category . AOAC will enter a reciprocal relationship with third party validation bodies that follow specified criteria for methods validation and who agree to administer validation in accordance with e. Cam criteria. Specific arrangements with each organization will be made through a Memorandum of Understanding or an equivalent mechanism.

Single –laboratory Validation (SLV) Methods

Those are "Methods validated through single laboratories studies meeting the standards set forth in the EN / ISO 16140 or AOAC Methods Committee Guidelines for validation of Quantitative and Qualitative Microbiological Methods. Microbiology Methods must include a methods comparison study conducted in a single laboratory . Submission must comply with EN / ISO 16140 or "AOAC Microbiological Methods Committee guidelines for Validation of Qualitative and Quantitative Microbiological Methods" except that the collaborative study requirement is removed. The methods comparison study is conducted in a single laboratory. The conditions of submission are identical to those described in the previous category.

Developmental Non Validated (DNV) Methods.

"DNV methods may be of interest to the analytical community, but have not been validated to the SIV criteria. They may include evolving methods which may not yet be optimized or fully characterized with respect to performance characteristics. A factual disclaimer will be incorporated stating that this method has not been peer-reviewed by technical experts, that it has not been validated through a third party approval scheme and therefore it is provided on an informational basis only".
The conditions of submission and validation are identical to those required for the previous category.

Methods submitted into HCV, MLV, SLV and DNV must include the following , as applicable:

- Submission date;
- Title
- Appropriate identification (AOAC will assign reference number);
- Regulatory reference (if applicable);
- Abstract;
- Safety warnings;
- Scope which includes method applicability; i.e food matrices, analyte, range and interferences;
- Definitions;
- Method principle;
- Reagents / Materials / Media;
- Apparatus and Equipment (with any technical requirements) ;
- Preparation of analytical samples;
- Test Procedure;
- Test Result Acceptance Criteria;
- Calculation;
- Precision (if applicable); recovery;
- Quality control requirements.

3- General considerations and prospective

"When methods are reclassified to another category, they will be removed from the existing category in which they are curently listed, as for example, when a Single Laboratory (SLV) method is later validated as a Multiple –Laboratory Validated (MLV method or a Harmonised Collaboratively Validated (HCV) Method". The possibility of an" on line validation" has been considered.

However, the question is raised to know who will "filter" the acceptability of a method

That is an important topic as it seems absolutely necessary to have a consensus, the possibility of using an "on line" system of validation seems very attractive as a principle but maybe difficult to apply
So, different questions need to be solved.

Presently, e.Cam has been proposed but is not yet used especially for financial reasons (the project is now in the hands of AOAC).
Anyway, should we use the system as such, or keep the idea and try to find an international consensus from the basis which is available actually ?. What has been proposed must surely be kept in mind. At the moment, and far from financial problems, a good exercise may be to classify the methods which will be described and used during this week according to the e.Cam system.

Conclusions

What is it possible to draw as conclusions ?

- it seems to me that the trends in the development of rapid / automated methods in food microbiology look somewhat like the first parts of the life of bacteria: latence, growth, stationary phase ; I don't know if the number of methods proposed is now decreasing but there is surely an evolution towards a more rational use.

- the choice of a method is a critical point which has a direct incidence on the validity of results.

- the working groups in charge of standardisation of methods have to be more and more aware of the impact of their work on the content of regulations.

- the terms "criteria of acceptability" must be present in all minds when choosing a method. That is probably true for all types of investigations and not only in food microbiology.

We are probably living a crucial period in the "history" of rapid methods in microbiology.