EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

OPINION OF THE

SCIENTIFIC COMMITTEE ON VETERINARY MEASURES RELATING TO PUBLIC HEALTH

ON

CRITERIA FOR EVALUATION OF METHODS OF *SALMONELLA* DETECTION

(adopted on 19-20 June 2002)

TABLE OF CONTENTS

Acknowledgements

This report of the Scientific Committee on Veterinary Measures relating to Public Health is substantially based on the work of an *ad hoc* working group established by the Committee. The working group included members of the Committee and an external expert.

The working group was chaired by

- Dr Pierre Colin

And included the following members:

- Dr Kirsten Mooijman
- Dr Terence A. Roberts

1. BACKGROUND

Finland and Sweden were granted additional guarantees with respect to *Salmonella* spp. in the context of their Act of Accession as from 1.1.1995. These guarantees entitle these Member States to require that certain animals, and products thereof, exported to these countries have a comparable low prevalence of *Salmonella* contamination, as low as animals and their products produced nationally. These guarantees were granted because of the low incidence of *Salmonella* spp. in animal populations and foodstuffs in these Member States and the control programmes that had been initiated and approved by the Commission (Commission Decisions 94/968/EC and 95/50/EC).

The *Salmonella* control programme covers fresh veal, beef and pork, poultry meat, live poultry for slaughter, breeding poultry and day-old chicks, laying hens, table eggs and live bovine animals and swine. The principle of the guarantees is that either animals or their products are tested for *Salmonella* spp. before export to Finland or Sweden or, alternatively, animals and/or products are derived from holdings or establishments subject to a programme recognised as equivalent to the *Salmonella* control programmes of Finland and Sweden.

The specific rules for the microbiological testing of meat and live animals intended for export to Finland and Sweden are laid down in several Council Decisions and Commission Decisions. These Decisions include testing of meat samples, swab samples and/or samples from faeces, meconium or chick organs. The testing shall cover all *Salmonella* serovars. Originally the method to be applied for the examination was ISO 6579:1993. However, at a later stage, the use of the method NMKL No. 71 was authorised by Council Decisions 97/278/EC and 98/227/EC. According to these Decisions, other methods which are considered to be equivalent to ISO 6579:1993 and NMKL No. 71 or their latest versions may be authorised using the comitology procedure.

The first sets of alternative methods and rapid methods with revised methodologies have been submitted to the Commission for possible authorisation. Consequently a general procedure for the evaluation of such new methods should be developed.

2. TERMS OF REFERENCE

The Scientific Committee on Veterinary Measures relating to Public Health is requested to advise the Commission on criteria to be applied in the evaluation of new methods for *Salmonella* detection, with the aim to determine whether they are at least equivalent to the authorised standards methods (ISO 6579:1993 and the NMKL method No. 71, or their revised versions, currently prescribed by legislation).

The Committee should take into account the previous document issued in 1996 by the Scientific Veterinary Committee (Public Health Section) on "Criteria for equivalence of alternative methods to ISO 6579:1993 for detection of *Salmonella*".

3. INTRODUCTION

The mandate refers to "new methods". The Committee interprets "new methods" to include both new methods and other, already available, alternative methods. A previous Opinion of the Scientific Veterinary Committee (Public Health Section) considered criteria for equivalence of alternative methods to ISO 6579:1993 for detection of *Salmonellae*. Comparison of ISO 6579:1993 and the proposed NMKL alternative method identified differences in the enrichment media, but nevertheless concluded: "… the NMKL method was considered by the group to be equivalent to the ISO method".

That opinion recognised the need for a framework for collaborative studies as a basis for judging whether different alternative methods becoming available, were equivalent to standard methods. One possibility considered was using National Reference Laboratories (NRLs) of the Member States organised and evaluated by the Community Reference Laboratory (CRL), but this was judged not to be feasible because of the number of methods to be validated, the workloads of NRLs and the CRL, and financial reasons. Another option might be to follow procedures laid down by other organisations such as the Association of Official Analytical Chemists (AOAC) and other European organisations (e.g. the French National Association for Standardisation - AFNOR) involved in the validation of alternative methods, including commercial kits.

That opinion noted a EUREKA project entitled "Microval" (Validation of microbiological methods for food and drink - a European perspective), set up in 1993, to establish a European certification mechanism for the validation and approval of alternative methods, especially commercial kits, for the microbiological analysis of foods and drinks. The outputs would be submitted to the European Standardisation Committee (CEN) as a basis for a European Standard, and, through collaboration between CEN and ISO, would also become an ISO standard.

At that time in 1996, differences were noted between the validation procedures of AOAC and those proposed by the "Microval" project to CEN.

After the EUREKA project "Microval" was completed in 1996, a new organisation was set up also called "MicroVal". This organisation proposes to organise certification schemes for the validation of alternative methods for microbiological analysis of foods, animal feeding stuffs, beverages (excluding water analysis) and food environmental samples. These certification schemes will be based on the protocol EN ISO 16140, as soon as that procedure is finally published.

One Subgroup, derived from the "Microval" project and national Standardisation Committees, has collaborated in CEN Technical Committee 275, Working Group 6, to produce an European Standard draft, leading to the document EN ISO 16140 "Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative method" - produced with ISO, Task Committee 34, Subcommittee 9, as recently adopted.

4. CURRENT DEVELOPMENTS

The procedure for the determination of *Salmonella* spp. in foods is described as a standard method in ISO 6579 and applies a traditional microbiological method. This

search for *Salmonella* spp. falls in the category of 'qualitative methods', being translated as the presence or absence of this bacterium in x grams or millilitres of the sample analysed. For some years, faster methods have been developed, drawing on various technologies such as immuno-concentration, immuno-immobilisation, ELISA, DNA probes and more recently molecular biology (D'Aoust, 1995, Van der Zee and Huis in't Veld, 2000).

These methods are subjected to comparative studies aimed not only at verifying their speed, that is the possibility to give a result in a shorter time than the conventional methods, but also their ability to produce a comparable result in terms of sensitivity and specificity. These rapid methods have proven very useful in many circumstances, especially in the implementation of control and surveillance systems in production and processing of foods. Some alternative methods are already being used to reduce the times of storage of perishable foods, allowing more rapid release after satisfactory test results. In such circumstances, it is necessary to establish standard procedures to assure that these alternative methods give results equivalent to those obtained by the official methods.

At present, available procedures to determine equivalence between two methods and/or to validate alternative methods are published by organisations such as AFNOR, AOAC, CEN, ISO, and NordVal. The results are accepted in particular countries or groups of countries, but not universally. The described procedures published by these standardisation bodies are similar in their general outline, but differ in small details. For example the procedures described in EN ISO 16140, AFNOR and NordVal require that the expert laboratory should be accredited for the relevant field; this is not demanded by AOAC. In general the main criteria described below are assessed in all these procedures. However, the detail of ISO/CD 17994: 2001: "Water quality – Criteria for the establishment of equivalence between microbiological methods" differs greatly from the other procedures because

a) this procedure determines only equivalence, whereas the other procedures also concern validation of the alternative method, and

b) the matrix is different from the other procedures (water instead of food).

Excluding ISO/CD 17994:2001, and after evaluation of the other different procedures for validation of alternative methods, which include all criteria requested and follow the same methodology, preference is given to an internationally accepted standard procedure. The EN ISO 16140 fulfilled these criteria, and results would be accepted across Europe. There are active negotiations with AOAC to make results of validations even more widely accepted.

5. DISCUSSION OF PROPOSED VALIDATION PROCEDURE

The document EN ISO 16140 proposes that the validation of an alternative method is defined as follows: "Demonstration that adequate confidence is provided that the results obtained by the alternative method are comparable to those obtained using the reference method". Strict application of the term "equivalent" (as written in the Council and Commission Decisions) would mean that an alternative method that performs "better" (detects more, real *Salmonella* spp. positive samples) than the reference method would not be accepted, as it would not be exactly "equivalent". The terms "comparable" or "at least equivalent" would allow an alternative method

that gives "better" results (detects more, real *Salmonella* spp. positive) than the reference method to be accepted.

In this document (EN ISO 16140) the protocol should be performed in the 'organising laboratory' and some principles for validation and certification of alternatives methods are elaborated. In EN ISO 16140, the 'organising laboratory' is defined as: "Laboratory having the qualified staff and skills to perform the method comparison study and organise the inter-laboratory study. The availability of an experienced statistician is essential for the analysis of the results".

Considering the technical protocol of validation of qualitative methods, corresponding with *Salmonella* spp. methods, two important steps are identified:

- Methods comparison study, described as "study performed by the organising laboratory of the alternative method against the reference method",

- Inter-laboratory study, described as "study of the alternative method's performance using common samples in several laboratories and under the control of the organising laboratory".

5.1. Methods comparison study

The study consists of two components- an initial phase comparing the performance of methods using pure cultures, and a second comparing performance using naturally or artificially contaminated foods.

For *Salmonella* spp. at least 30 pure cultures, representing the serovars of most concern, are selected, as well as 30 pure cultures of non-target microorganisms. These non-target microorganisms are chosen from both the strains known to cause interference with *Salmonella* spp. and from strains naturally present in each food category.

Each test is performed once and inoculation is carried out using dilution of a pure culture of each strain. No food sample is added. The results are expressed in term of **inclusivity** (defined as "the ability of an alternative method to detect the target analyte from a wide range of strains"), and **exclusivity** (defined as "the lack of interference from a relevant range of non-target strains of the alternative method").

In the second step, if the method is to be validated for all foods, five different categories of food must be studied. This number may be reduced to 1, 2, 3 or 4 categories if the validation is restricted to those stated categories. Appropriate environmental and veterinary samples may be included as 2 categories. Artificial contamination of food samples can be used if it is not possible to analyse naturally contaminated foods.

The results are expressed as presence or absence of *Salmonella* spp. in each sample and validation/comparison results are translated into the following parameters (as defined in EN ISO 16140):

"**Relative accuracy** is 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;

Relative sensitivity is the ability of the alternative method to detect the analyte when it is detected by the reference method;

Relative specificity is the ability of the alternative method to not detect the analyte when it is not detected by the reference method".

Additionally, a protocol should be applied, using 5 levels of target microorganisms per food, including the negative control as the first level; the second level should be the theoretical detection level, the third level shall be just above the theoretical detection threshold and any further levels should be higher than the previous level. For each level and each food/strain combination, 6 replicates are performed. Both methods are compared and the relative detection level as described "lies between the two contamination levels giving respectively less and more than 50% detection level ….(and) is therefore expressed as a range". In this case, the **"Relative detection level** is the smallest number of culturable microorganisms that can be detected with 50% of chances in the sample by the alternative and reference methods".

5.2. Inter-laboratory study

The aim is to "determine the variability of the results obtained in different laboratories using identical samples and to compare these results with those obtained in the methods comparison study".

According to EN ISO 16140, "the inter-laboratory study shall produce at least 10 collaborative laboratories having results without outliers". Test samples are prepared in the organising laboratory, artificially inoculating individual samples. At least 3 different levels of contamination shall be used, including a negative control, and 8 blind replicates at each level are analysed by both reference and alternative methods. In this protocol, 480 results (240 by each method) are generated for further analyses. This protocol is in accordance with the AOAC procedure with more replicates (8 instead of 6). The organising laboratory compares the results for each level to calculate the percentage of sensitivity and specificity, the relative accuracy and to examine the discordant results.

5.3. Summary of validation criteria proposed in EN ISO 16140

To summarise, the procedure in EN ISO 16140 emphasises the following criteria:

- The need for the validation to be managed by an independent, accredited laboratory that has proved its competence in the study of the particular microorganism;

- The study of inclusivity/exclusivity by using an appropriate and sufficient number of strains and serovars of *Salmonella* spp. (inclusivity), and of other closely related microorganisms (exclusivity);

- The study of relevant accuracy as well as sensitivity and specificity, by testing naturally, or if not possible artificially, contaminated and uncontaminated samples; the reference and alternative methods shall be performed with, as far as possible, exactly the same sample. Different

categories of food, but also environmental and veterinary samples, including faeces and organ samples, should be used; a list of veterinary samples is given in Table B.3 of Annex B of EN ISO 16140;

- The relative detection level, defined as the smallest number of culturable microorganisms that can be detected by the alternative and reference methods, by testing different levels of inoculations, including the negative control, in different categories of foods;

- The reproducibility of the method from an inter-laboratory study, involving competent laboratories and using identical samples. The aim is to determine the variability of the results. This study should cover not only "detection", or failure of "detection", of the microorganism of concern by testing contaminated and uncontaminated samples, but also the ability of laboratories to detect the presence of the microorganism in various types of samples and at different levels of contamination;

- The results should be evaluated objectively by a Committee of independent experts;

- Additionally, quality assurance requirements for the manufacturer of the alternative method have to be met, in order to ensure consistent product quality over time. This is controlled by an audit of the manufacturer's site and records;

- Following this evaluation, the alternative method obtains its validation for the specified microorganism(s) for one, or several, categories of foods. This validation is given for specified duration and can be re-evaluated periodically;

- If an alternative method has been already validated for one or more foodstuffs, according to the full procedure as described in EN ISO 16140, the validation of this same method for veterinary and/or environmental samples can then be tested in the expert laboratory only.

6. CONCLUSIONS

(1) There are several methods that give results on the presence or absence of *Salmonella* spp. in foods more efficiently than the standard conventional methods. According to the Council Decisions, alternative methods considered to be "equivalent" to the standard methods may also be used. A strict interpretation of the word "equivalent" implies that alternative methods that show better performance than the reference method, in terms of sensitivity and specificity, would not be accepted. However, if the words "comparable" or "at least equivalent" are used, it is possible to accept these alternative methods.

(2) The procedures for validation of these new, alternative, methods are applied at the level of national authorities. Official standards are in the course of elaboration, notably at the European (CEN) and international (ISO) level.

(3) With the exception of ISO/CD 17994 applying only to water quality, the procedures for validation, as described by the different standardisation bodies (AFNOR, AOAC, CEN, ISO, NordVal), are similar in their general outline, but differ in small details.

(4) According to the scope of EN ISO 16140, the procedure can be applied for validation of new, alternative, methods in the field of microbiological analysis of food, animal feedingstuffs and environmental and veterinary samples, and offers a standardisation procedure across Europe.

(5) The demand for validation of new, alternative, methods will become more and more frequent due to the development of new technologies and the increased trade in food.

7. RECOMMENDATIONS

(1) Validation of new, alternative, methods should follow an official procedure. The Committee favours the procedure EN ISO 16140, as recently adopted.

(2) Validation of methods should be supervised by an internationally recognised body. Alternative methods may be validated based on existing data that fulfill the requirements of EN ISO 16140.

(3) The recognition of a new, alternative method for the detection of *Salmonella* spp. in foods can be accepted subject to the following main conditions:

– the criteria of sensitivity and specificity of the method should be addressed by the study of a sufficient number of strains belonging to the genus *Salmonella* and to other closely related bacteria;

– for a *Salmonella* spp. comparison study, at least 30 pure cultures representing the serovars of most concern, as well as 30 pure cultures of relevant non-target microorganisms should be selected.

– the tests of reproducibility should be made within the framework of a study involving competent laboratories, operating blind, and taking account of expected levels of contamination and the various food matrices most likely to be contaminated.

(4) The different standardisation bodies should continue their negotiations to reach further agreement on procedures for validation.

(5) Simpler and more transparent procedures for validation should be developed.

(6) Validation should take account of improvements in analytical methods and make allowance for methods that may be more accurate in terms of sensitivity and specificity than reference methods.

8. GLOSSARY

A.F.N.O.R.: Association Française de Normalisation.

A.O.A.C.: Association of Official Analytical Chemists.

C.E.N.: European Committee for Standardisation.

I.S.O.: International Organization for Standardization.

N.M.K.L.: Nordic Committee on Food Analysis.

NordVal: Nordic system for validation of alternative microbiological method.

9. REFERENCES

Anon., 1993. Microbiology of food and animal feedingstuffs. *Salmonella*. ISO 6579:1993.

Anon., 1994. Commission Decision of 28 December 1994 approving the operational programme for the control of *Salmonella* in certain live animals and animal products presented by Finland (94/968/EC). Off. J. Eur. Communities L371, 36-37.

Anon., 1995. Commission Decision of 23 February 1995 approving the operational programme for the control of *Salmonella* in certain live animals and animal products presented by Sweden (95/50/EC). Off. J. Eur. Communities L053, 31-32.

Anon., 1997. Commission Decision of 11 April 1997 amending Decisions 95/160/EC, 95/161/EC and 95/168/EC concerning the methods to be used for microbiological testing (97/278/EC). Off. J. Eur. Communities L110, 77-78.

Anon., 1998. Council Decision of 16 March 1998 amending Decisions 95/409/EC, 95/410/EC and 95/411/EC concerning the methods to be used for microbiological testing to be undertaken on meat intended for Finland and Sweden (98/227/EC). Off. J. Eur. Communities L087, 14-16.

Anon., 2001. Water quality - Criteria for the establishment of equivalence between microbiological methods. ISO/CD 17994.

Anon., 2002. Microbiology of food and animal feedingstuffs. Protocol for the validation of alternative method. EN ISO 16140.

D'Aoust, J.Y. 1995. Methods for the detection of foodborne *Salmonella* spp.: a review. Southeast Asian J. Trop. Med. Publ. Hlth., 26, suppl. 2, 195-208.

Scientific Veterinary Committee 1996. European Commission. Opinion of the Scientific Veterinary Committee (Public Health Section) on criteria for equivalence of alternative methods to ISO 6579:1993 for detection of *Salmonellae.*

Van der Zee, H., Huis in't Veld, H.J., 2000. Methods for the rapid detection of *Salmonella.* In "*Salmonella* in Domestic Animals", chapter 22, 373-391, (Wray, C., Wray, A., eds).