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Agenda Item 8c) **CX/MAS 02/12**

JOINT FAO/WHO FOOD STANDARDS PROGRAMME
CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING

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SINGLE-LABORATORY VALIDATION

VALIDATION OF METHODS THROUGH THE USE OF RESULTS FROM PROFICIENCY TESTING SCHEMES[1]

BACKGROUND

At the 23rd Session of the Codex Committee on Methods of Analysis and Sampling (CCMAS) it was agreed that a paper would be prepared for discussion at the 24th Session on the validation of methods of analysis through the use of results from proficiency testing schemes (see ALINORM 01/23, paras 84). This paper addresses a number of these issues.

The purpose of a proficiency testing scheme is to test the competence of the laboratory and not to validate a method of analysis. In most proficiency testing schemes participants have a free choice of method of analysis and so there is no opportunity to formally validate a method in a proficiency testing scheme, i.e. a multiplicity of methods may be used by participants. However, in some situations, there is the possibility of validating a method of analysis if:

– there are sufficient participants in the proficiency testing scheme who choose to use the same defined method of analysis or

– a method of analysis is prescribed by the scheme co-ordinators.

For most proficiency testing schemes it is the former situation which will predominate but in the case of microbiological analysis, in particular, it is very likely that a method of analysis will be prescribed. The former situation will tend to occur when a (very) empirical determination is being assessed in the proficiency testing scheme, or if there is a very restricted choice of available methodology for a particular determination, e.g. the determination of 3-Chloro-1,2-Propanediol (3-MCPD) in foods.

In addition there is the possibility of re-confirmation of a prior validation of a method through its use in a proficiency testing scheme.

ORGANISATION OF PROFICIENCY TESTING SCHEMES

The proficiency testing scheme whose results are to be used must be organised according to the AOAC/ISO/IUPAC International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories. This protocol has been recognised by the Codex Alimentarius Commission by reference.

That protocol stipulates the procedures that must be incorporated in any proficiency testing scheme, and in particular the work that must be carried out by the scheme co-ordinators to ensure that samples received by the participants in the scheme are homogeneous. At present there are no internationally agreed stipulations to

[1] (Prepared by the United Kingdom)

ensure sufficient homogeneity of samples used for collaborative trial exercises whereas there are for proficiency test exercises.

COMPARISON OF THE SIGNIFICANT DIFFERENCES IN THE VALIDATION OF METHODS OF ANALYSIS THROUGH COLLABORATIVE TRIALS AND PROFICIENCY TESTING

Number of Samples

In the case of a collaborative trial conforming in design to the Harmonised Protocol a minimum of 5 test materials are required to be prepared. However, in most proficiency testing schemes there are insufficient test materials sent out in any one round (i.e. the dispatch of test material at a specific time) to meet the minimum requirements for number of materials as specified in the Harmonised Guidelines for Collaborative Studies. Because of that it is necessary to "build up" the number of samples used to validate a method over a period of time. This may mean that the time taken to build up sufficient results to ensure validation of a method may extend over one or two years depending upon the test materials which are being used in the proficiency testing scheme.

Replication of Results

In most cases the validation of a method in a collaborative trial results in both within, and between, laboratory precision characteristics (i.e. repeatability and reproducibility). In most collaborative trials test materials are dispatched as either blind duplicates or as split level test materials. This means that one of the aims of a collaborative trial, that of determining the within-laboratory variability, is readily achievable. However, because the aim of a proficiency test is different from a collaborative trial, the results of replicate analyses of any particular test material are normally not reported to the proficiency test co-ordinator - i.e. it is only the single result, as reported to the customer, that is returned. Because of that, it is frequently the case that it is not possible to obtain the within-laboratory variability of the method. In such cases only the overall precision of the method i.e. the between-laboratory precision will be quoted and not the within-laboratory will be estimated.

Laboratories

In the case of validation through collaborative trials all the test materials are sent to participants at the same time; thus the same laboratory will receive all the test materials, analyse them and return results to the trial co-ordinator. In the case of any validation through proficiency testing schemes there will be no guarantee that the same laboratories will participate in the scheme over different rounds and that any one laboratory, even if it participated in all rounds, would participate in all the rounds of the scheme. Thus there would be a moving but hopefully relatively stable population of laboratories.

ASSUMPTIONS ABOUT COMPETENCY

In a collaborative trial it is assumed that all laboratories are equally "competent". However, in proficiency testing the objective of the scheme is to ascertain the competencies of laboratories. If results from proficiency testing schemes are to be used for method validation then the competency of laboratories in the proficiency testing scheme using the defined method must be assumed to be equivalent to each other. Outlying results would be identified in the normal manner through statistical tests.

EXAMPLE

An example of a method of analysis which has been validated through proficiency testing schemes is given in **Annex I**. Here a method for the enumeration of *Listeria monocytogenes* in meat and meat products has been validated through the use of results from a proficiency testing scheme.

RECOMMENDATION

The use of results from proficiency testing schemes is recognised as being valuable and appropriate. Their use has been much discussed but as yet no defined procedures have been identified.

The International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories will shortly be revised. It would be appropriate for that harmonised protocol to address the issue of method validation through the use of proficiency test results in that revision, thus allowing CCMAS to comment on such procedures which should then be adopted on an international basis.

ANNEX I: METHOD FOR THE ENUMERATION OF *LISTERIA MONOCYTOGENES* IN MEAT AND MEAT PRODUCTS

COSHH AND SAFETY CONSIDERATIONS

Analysts are reminded that appropriate hazard and risk assessments required by the Control of Substances Hazardous to Health Regulations, 1988 (See "Control of Substances Hazardous to Health - Approved Codes of Practice, Control of Substances Hazardous to Health Regulations, 1988") must be made before using this method.

Each laboratory should follow its own safety rules and national regulations, particularly COSHH, with respect to the sample preparation.

The procedures specified in this method shall only be carried out in laboratories with suitable facilities and under control of a qualified microbiologist.

These procedures shall not be performed in quality control laboratories, or in food manufacturing or processing premises, where there is a risk of contamination of the environment.

Full bacteriological precautions shall be taken at all times whilst carrying out the procedure specified in this method. Particular attention shall be given to the sterilisation of used equipment and media after testing suspect samples prior to disposal or reuse.

Note - For further and more detailed safety precautions reference is made to ISO 7218, Microbiology - General Guidance for Microbiological Examinations, in general and the clauses 3,4 and 7 in particular (10.3).

1. SCOPE AND FIELD OF APPLICATION

This method specifies procedures recommended for the enumeration of *Listeria monocytogenes* in meat and meat products.

2. DEFINITIONS

For the purpose of this recommended method the following definitions apply:

2.1 *Listeria monocytogenes*:

Pathogenic bacteria that form typical colonies on the specified solid selective medium and which display the morphological, physiological and biochemical characteristics described, when tests are carried out in accordance with this method.

2.2 Enumeration of *Listeria monocytogenes*:

Determination of the number of viable and confirmed *L. monocytogenes* bacteria per gram of product when the examination is carried out in accordance with this method.

3. PRINCIPLE

In general, the enumeration of *L. monocytogenes* necessitates three successive stages as in 3.1 to 3.3. See also the diagram of procedure in Appendix I.

3.1 Preparation of the test sample

The test sample is homogenised in suspension medium and decimal dilutions are prepared as necessary.

3.2 Enumeration and presumptive identification

The selective agar is inoculated from the initial suspension (3.1) and dilutions thereof, incubated at 30°C and examined after 48 h to check for the presence of colonies which, from their appearance, are considered to be presumptive *Listeria* spp..

3.3 Confirmation of identity

Colonies of presumptive *Listeria* spp. (3.2) are sub-cultured onto a non-selective solid medium for confirmation of identity by means of appropriate morphological, physiological and biochemical tests.

4. CULTURE MEDIA AND REAGENTS

4.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the culture media, dehydrated basic components or complete dehydrated media are used. The manufacturer's instructions shall be rigorously followed.

The chemical products used for the preparation of the culture media and reagents shall be of recognised analytical quality.

The water used shall be distilled or deionised water, free from substances that might inhibit the growth of microorganisms under the test conditions.

When agar is specified, the amount used should be varied according to the manufacturer's instructions to give media of suitable firmness.

Measurements of pH shall be made using a pH meter, measurements being referred to a temperature of 25°C. Adjustments, if necessary, are made by adding either 1 M hydrochloric acid or 1 M sodium hydroxide solution.

If the prepared culture media and reagents are not used immediately, they shall, unless otherwise stated, be stored in the dark at a temperature between 2 and 5°C for no longer than 1 month, conditions which do not produce any change in their composition.

4.2 Diluent

	g/L
Peptone	1.0
Sodium chloride	8.5

Preparation: Dissolve the components in 1000 mL of water by gently heating. Dispense into final containers such that after autoclaving each tube or bottle contains 9.0 ± 0.2 mL. Adjust the pH so that after autoclaving it is 7.0 ± 0.2 at 25°C. Autoclave at 121°C for 15 min.

4.3 Culture media

4.3.1 Sample suspension medium (UVM 1 formulation)

4.3.1.1 Base

	g/L
Protease peptone	5.0
Tryptone	5.0
Meat extract	5.0
Yeast extract	5.0
Sodium chloride	20.0
Di-sodium hydrogen phosphate	12.0
Potassium dihydrogen phosphate	1.35
Aesculin	1.0

Preparation: Dissolve the dehydrated components in 1000 mL of water by boiling. Autoclave at 121°C for 15 min. Cool to 50°C.

4.3.1.2 Supplement per Litre of medium

	mg
Nalidixic acid	20.0
Acriflavine HCL	12.0

Preparation: Dissolve the components in 4 mL of water. Sterilise by filtration through a filter of pore size 0.22 µm (5.1.13).

4.3.1.3 Preparation of the complete medium

Aseptically add the supplement to the basal medium. Invert gently to dissolve. Adjust the pH so that, after sterilisation and the addition of supplement, it is 7.4 ± 0.2 at 25°C. Aseptically distribute the complete medium into 225 mL volumes. The complete medium may be stored for up to one week at 2 - 5°C before use.

4.3.2 Listeria selective agar Oxford formulation

4.3.2.1 Base

	g/L
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Aesculin	1.0
Ferric ammonium citrate	0.5
Lithium chloride	15.0
Agar	10.0

Preparation: Dissolve the dehydrated components in 1000 mL of water by boiling. Autoclave at 121°C for 15 min. Cool to 50°C.

4.3.2.2 Supplement per Litre of medium

	mg
Cycloheximide	400.0
Colistin sulphate	20.0
Acriflavin	5.0
Cefotetan	2.0
Fosfomycin	10.0

Preparation: Dissolve the components in 10 mL of a 1:1 solution of ethanol:water. Sterilise by filtration through a 0.22 µm pore size filter (5.1.13).

4.3.2.3 Preparation of the complete medium

Aseptically add the supplement to the basal medium and mix thoroughly. Adjust the pH so that, after sterilisation and addition of supplement, it is 7.0 ± 0.2 at 25°C. Transfer the complete medium in quantities of about 15 mL to sterile Petri dishes and allow to solidify on a level surface (5.1.14). The complete medium may be stored for up to one week at 2 - 5°C before use.

4.3.3 Tryptone Soya Yeast Extract Agar (TSYEA)

	g/L
Tryptone soy broth	30.0
Yeast extract	16.0
Agar	12.0 to 18.0

Preparation: Dissolve the dehydrated components in 1000 mL of water by boiling. Adjust the pH so that after sterilisation it is 7.3 ± 0.1 at 25°C. Autoclave at 121°C for 15 min and allow to cool to 50°C. Transfer quantities of about 15 mL to sterile Petri dishes and allow to solidify on a level surface (5.1.14).

4.3.4 Tryptone Soya Yeast Extract Broth (TSYEB)

	g/L
Tryptone soy broth	30.0
Yeast extract	16.0

Preparation: Dissolve the dehydrated components in 1000 mL of water by heating gently. Adjust the pH so that after sterilisation it is 7.3 ± 0.1 at 25°C. Transfer the TSYEB in quantities of about 10 mL to tubes or bottles. Sterilise for 15 min at 121°C.

4.3.5 Blood agar (not required if microwell haemolysis test used)

4.3.5.1 Base

	g/L
Protease peptone	15.0
Liver digest	2.5
Yeast extract	5.0

Sodium chloride	5.0
Agar	12.0

Preparation: Dissolve the dehydrated components in 1000 mL of water by boiling. Sterilise the blood agar base for 15 min at 121°C. Cool the medium to 47 ± 1°C.

4.3.5.2 Supplement per Litre of medium

	mL
Washed sheep red blood cells	70.0

Preparation: Centrifuge defibrinated sheep blood at 900 x g for 30 min, aseptically removing the supernatant liquid and re suspend the pellet in sterile 0.85% saline solution to the original volume. If the centrifuged suspension has haemolysed, a fresh suspension must be prepared.

4.3.5.3 Preparation of the complete medium

Add the washed sheep red blood cells to the sterilised agar base and mix well. Adjust the pH so that, after sterilisation and addition of supplement, it is 7.0 ± 0.1 at 25°C. Transfer the medium in quantities of about 15 mL to sterile Petri dishes and allow to solidify on a level surface (5.1.14).

4.3.6 Brain heart infusion broth

	g/L
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Protease peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.0

Preparation: Dissolve the dehydrated components in 1000 mL water by heating gently. Adjust the pH so that after autoclaving it is 7.4 ± 0.2 at 25°C. Distribute in 10 mL volumes in screw-capped containers and autoclave at 121°C for 15 min.

4.3.7 Phosphate Buffered Saline (PBS)

	g/L
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2

Preparation: Dissolve the dehydrated components in 1000 mL water by heating gently. Adjust the pH so that after autoclaving it is 7.3 ± 0.1 at 25°C. Dispense in 10 mL volumes in screw-capped containers and autoclave at 115°C for 10 min.

4.3.8 Carbohydrate utilisation broth

4.3.8.1 Base

	g/L
Protease peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Bromocresol purple	0.02

Preparation: Dissolve the dehydrated components in 1000 mL water by gentle heating. Distribute into tubes or bottles in quantities of 10 mL. Sterilise at 121°C for 15 min. Adjust the pH so that after sterilising it is 6.8 ± 0.2 at 25°C.

4.3.8.2 Carbohydrates

	g/L
Rhamnose	50.0
Xylose	50.0

Preparation: Dissolve each carbohydrate separately in 1000 mL water, do not heat to dissolve. Sterilise by filtration through a 0.22 µm pore size filter (5.1.13).

4.3.8.3 Preparation of the complete medium

For each carbohydrate, aseptically add 1 mL carbohydrate solution (4.3.8.2) to each tube or bottle of basal medium (4.3.8.1).

4.3.9 Motility medium

	g/L
Casein peptone	20.0
Meat peptone	6.1
Agar	3.5

Preparation: Dissolve the dehydrated components in 1000 mL water by boiling. Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25°C. Dispense in tubes or bottles in quantities of about 10 mL. Sterilise for 15 min at 121°C.

4.3.10 CAMP (Christie/Atkins/Munch-Peterson) test agar

Very thin-layered sheep blood agar plates are required for this test.

4.3.10.1 Base

	g/L
Protease peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0

Preparation: Dissolve the dehydrated components in 1000 mL of water by boiling. Adjust the pH so that after sterilisation it is 7.0 ± 0.1 at 25°C. Sterilise the blood agar base for 15 min at 121°C. Cool the medium to 50°C and transfer quantities of about 15 mL to sterile Petri dishes and allow to solidify on a level surface (5.1.14).

4.3.10.2 Sheep blood medium

	mL
Basal medium (4.3.10.1)	100.0
Washed sheep red blood cells (see 4.3.5.2)	7.0

Preparation: Add the washed cell suspension to the sterilised, molten base cooled to 47 ± 1°C.

4.3.10.3 Preparation of the complete medium

Pour a very thin layer of sheep blood medium (4.3.10.2) over the basal medium (4.3.10.1) using no greater than 3 mL per plate. Allow to solidify in an even layer. If the blood is added to dishes containing the basal medium which have been prepared in advance, it may be necessary to warm the dishes by placing them in an incubator at 37°C for 20 min before pouring the blood layer. Dry plates before use.

4.3.10.4 CAMP reaction cultures

A weakly β-haemolytic strain of *Staphylococcus aureus* (eg NCTC 1803) and a strain of *Rhodococcus equi* (eg NCTC 1621) are required to undertake the CAMP test. Not all strains of *Staphylococcus aureus* are suitable for the CAMP test.

Maintain stock cultures of *S. aureus*, *R. equi*, *L. monocytogenes*, *L.innocua* and *L. ivanovii* by inoculating TSYEA plates (4.3.3), incubating at 37°C for 24 - 48h, or until growth has occurred and storing at 4°C. Sub-culture at least once per month.

5. APPARATUS & GLASSWARE

Usual microbiological laboratory equipment, and in particular:

5.1 Apparatus

5.1.1 Apparatus for dry sterilisation (oven) or wet sterilisation (autoclave)

Apparatus that will enter into contact with the culture media, the dilution fluid or the sample, except for apparatus that is supplied sterile (particularly plastic apparatus), shall be sterilised either

by being kept at 170 to 175°C for not less than 1 h in an oven or

by being kept in contact with saturated steam at 121°C for not less than 15 min in an autoclave.

An autoclave is also necessary for the sterilisation of culture media and reagents. It shall be capable of being maintained at 121°C.

5.1.2 Incubator: capable of being maintained at 30°C ± 1°C.

5.1.3 Incubator: capable of being maintained at 37°C ± 1°C.

5.1.4 Incubator: capable of being maintained at 25°C ± 1°C.

5.1.5 Waterbath: capable of being maintained at 47°C ± 1°C.

5.1.6 Blending equipment

One of the following shall be used:

a) a rotary blender, operating at a rotational frequency between 8000 and 45000 min^{-1}, with glass or metal bowls fitted with lids, resistant to the conditions of sterilisation.

b) a peristaltic type blender (Stomacher Model 400), with sterile plastic bags.

Note - The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the sample plus diluent.

5.1.7 Loops: of platinum-iridium, nickel-chromium or plastic of diameter approximately 3 mm.

5.1.8 Inoculating needle: of platinum-iridium, nickel-chromium or plastic.

5.1.9 pH-meter: (for measuring the pH of prepared media and reagents), having an accuracy of calibration of 0.1 pH unit at 25°C.

5.1.10 Refrigerator: (for storage of prepared media and reagents), capable of being maintained at 2 to 5°C.

5.1.11 Sterile round-bottom microtitre plates, for microwell haemolysis.

5.1.12 Automatic pipette, capable of dispensing 100 µL volumes.

5.1.13 Filters, of 0.22 µm pore size suitable for the filtration of aqueous solutions and organic solvents.

5.1.14 Level surface, for drying agar plates.

5.2 Glassware: The glassware shall be resistant to repeated sterilisation.

5.2.1 Culture bottles or flasks, for sterilisation and storage of culture media and incubation of liquid media.

5.2.2 Test tubes, of dimensions approximately 16 mm x 125 mm fitted with lids.

5.2.3 Screw-capped bottles of approximately 25 mL capacity.

5.2.4 Flasks or bottles, of capacity 250 mL.

5.2.5 Measuring cylinders, for preparation of the complete media.

5.2.6 Graduated pipettes, of nominal capacity 1 mL graduated in divisions of 0.1 mL.

5.2.7 Sterile Petri dishes, of glass or plastic of diameter 90 to 100 mm.

5.2.8 Spreaders: of glass or plastic.

5.2.9 Microscope slides/coverslips

6. PROCEDURE

See the diagram of procedure in Appendix I.

6.1 Preparation of test sample, initial suspension and dilutions

Add 225 mL sample suspension medium (4.3.1) to 25 g test sample in a Stomacher bag or blender bowl. Blend for 2 min. Prepare dilutions from the initial suspension as necessary in the diluent (4.2).

6.2 Inoculation

Transfer by means of a sterile pipette, 0.1 mL of the initial suspension (10^{-1} dilution) to each of two selective agar plates (4.3.2). Repeat the procedure for 10^{-2} dilution and further dilutions as necessary. Carefully spread the inoculum as quickly as possible over the surface of the agar plate, trying not to touch the sides of the dish, using a glass or plastic spreader (5.2.8). Use a sterile spreader for each plate. Retain the plates at room temperature, on a level surface (5.1.14) for about 15 min with the lids uppermost to allow the inoculum to soak into the agar.

6.3 Incubation

Invert the plates prepared according to 6.2 and incubate them at 30 $\pm$ 1°C for 48 h.

6.4 Counting and selection of colonies

Select dishes at two consecutive dilutions containing less than 150 typical colonies, that is colonies surrounded by a dark brown or black halo. Count these suspect colonies.

6.5 Confirmation

6.5.1 Selection of colonies for confirmation

From each plate containing less than 150 typical colonies (6.4) select five typical or suspect colonies or, if there are fewer than five such colonies, select all for confirmation.

6.5.2 Subculturing

Streak the selected colonies onto the surface of TSYEA plates (4.3.3) in a manner which will allow well isolated colonies to develop. Incubate the plates at 30°C for 24 h or until growth is satisfactory.

6.6 Confirmation

6.6.1 Catalase reaction

From each TSYEA plate (6.5.2) pick a typical colony and place it on a coverslip (5.2.9). Add a drop of 3% hydrogen peroxide solution to a microscope slide (5.2.9). Invert the coverslip and place onto the slide. This technique is used to prevent aerosol formation. All *Listeria* spp. are catalase positive demonstrated by the formation of gas bubbles.

6.6.2 Morphology and staining properties

Test for Gram reaction. From each TSYEA plate (6.5.2) pick a typical colony and prepare a heat-fixed mount on a microscope slide (5.2.9). Gram stain and examine under oil immersion on a light microscope. All *Listeria* spp. are Gram-positive short rods.

6.6.3 Motility at 25°C

From each TSYEA plate (6.5.2) select a well isolated typical colony and remove by means of a sterile inoculating needle (5.1.8). For each isolate stab inoculate a tube of motility medium (4.3.9) and incubate at 25°C for 48 h. Examine for growth around the stab, if negative reincubate for a further 5 days. *Listeria* spp. are motile giving a typical umbrelliform growth pattern.

6.6.4 Haemolysis (see also 6.6.5)

If the morphological and physiological characteristics, and catalase reaction indicate the possibility of *Listeria* spp., inoculate blood agar plates (4.3.5) to determine the haemolytic reaction.

Dry the agar surface well before use. Select a typical colony from each TSYEA plate (6.5.2) and streak the colony onto the blood agar by means of a loop (5.1.7). Use one plate per isolate. Simultaneously inoculate blood agar plates (4.3.5) with positive and negative control cultures (*L. monocytogenes*, *L. ivanovii* and *L. innocua*).

After 48h incubation at 37°C, examine the test strains and controls. *L. monocytogenes* shows narrow, slight zones of clearing (β-haemolysis): *L.innocua* should show no clear zone. *L. ivanovii* usually shows wide, clearly delineated zones of β-haemolysis. Remove the colony to examine the haemolysis underneath the colony. Hold plates up to a bright light to compare test cultures with controls.

6.6.5 Haemolysis using microwell technique

As an alternative to the preparation of blood agar plates for the determination of haemolytic activity, a microwell method may be used.

From each TSYEA plate (6.5.2) select a well isolated typical colony and remove by means of a sterile loop (5.1.7). For each isolate inoculate a Brain Heart Infusion Broth (4.3.6) and incubate at 37°C for 48 h.

Prepare a 2% sheep erythrocyte suspension by washing i.e. centrifuging and resuspending, sterile defibrintated sheep blood three times in PBS (4.3.7). From this suspension pipette 100 µL in duplicate into wells of a round bottom microtitre plate (5.1.11).

To the erythrocyte suspension add 100 µL of Brain Heart Infusion broth culture. Incubate the microtitre plate for 45 min at 37°C followed by incubation for 2 h at 4°C. The presence of haemolysins are shown by a homogeneous red liquid. A clear supernatant with a layer of red blood cells on the bottom of the well indicate no haemolytic activity. Reference strains of *L. monocytogenes* and *L. innocua* should be run concurrently with this test.

6.6.6 Further biochemical confirmation

For these assays a culture in TSYEB (4.3.4) corresponding to the typical colony used for the haemolysis reaction (6.6.4 or 6.6.5) is required. Pick a typical colony from each TSYEA plate (6.5.2) and suspend in a tube containing TSYEB (4.3.4). Incubate for 24 h at 37°C.

6.6.6.1 Carbohydrate utilisation

Inoculate the carbohydrate fermentation broths (4.3.8) each with one loopful of the TSYEB culture (6.6.6). Incubate for up to 7 days at 37°C, although positive reactions (acid formation indicated by a yellow colour) occur mostly within 24 - 48 h. Reference strains of *L. monocytogenes, L. ivanovii* and *L. innocua* should be run concurrently with this test.

6.6.6.2 CAMP test

Streak the *S. aureus* and *R. equi* cultures in single lines across the blood agar plate (4.3.10) so that the two cultures are parallel and diametrically opposite. A thin, even inoculum is required. This can be obtained by using an inoculating needle (5.1.8) or a loop (5.1.7) held at right angles to the agar. Streak the test strain in a similar fashion at right angles to these cultures so that the test culture and reaction cultures do not touch but at their closest are about 1 - 2 mm apart. Several test strains may be streaked on the same plate.

Simultaneously, streak control cultures of *L. monocytogenes*, L. *innocua* and *L. ivanovii*. Incubate the plates at 37°C for 18 - 24 h.

Positive reactions are indicated by an enhanced zone of β-haemolysis at the intersection of the test strain with either the *S. aureus* or *R. equi* culture. However, the appearance of positive results varies with the reaction culture. A positive reaction with *R. equi* is seen as a wide (5 - 10 mm) ‘arrow-head’ of haemolysis. Small

(about 1 mm) zones of weak haemolysis around the intersection of the test and *R. equi* cultures are negative reactions. A positive reaction with *S. aureus* is seen as a small rounded zone of enhanced haemolysis extending only about 2 mm from the test strain and within the weakly haemolytic zone due to growth of the *S. aureus* culture. Large zones of haemolysis around the *S. aureus* culture do not occur.

L. monocytogenes and *L. seeligeri* show a positive CAMP reaction with *S. aureus* but not *R. equi*. *L. ivanovii* reacts with *R. equi* but not with *S. aureus*. The other *Listeria* spp. show negative CAMP reactions with both *S. aureus* and *R. equi*.

6.7 Interpretation of morphological and physiological properties and biochemical reactions

All *Listeria* spp. are small Gram-positive rods (only with 24 h old cultures) that demonstrate an umbrelliform growth pattern in the motility medium. They are catalase positive. *L. monocytogenes* utilises rhamnose but not xylose.

L. monocytogenes, *L. ivanovii* and *L. seeligeri* (weak) produce β-haemolysis on blood agar plates and positive reactions in the microwell haemolysis test. Of the three haemolytic *Listeria* spp. only *L. monocytogenes* fails to utilise xylose and is positive for rhamnose utilisation.

L. monocytogenes and *L. seeligeri* show a positive CAMP reaction with *S. aureus* but not with *R. equi*. *L. ivanovii* reacts with *R. equi* but not with *S. aureus*. The other *Listeria* spp. show negative CAMP reactions with both reaction cultures.

7. CONTROL CULTURES

Control cultures of *L. monocytogenes*, *L. ivanovii* and *L. innocua* should be run concurrently with all confirmatory tests.

8. EXPRESSION OF RESULTS

8.1 General

If all of the selected typical colonies (6.4) confirm as *L. monocytogenes*, the number of organisms present will be the same as that given by the count in 6.4. In all other cases the number shall be calculated from the percentage of isolates confirmed positive in relation to the total number of selected colonies (6.4).

Round the result to a whole number of colonies.

8.2 Calculation of the weighted mean

Calculate the number, N, of *L. monocytogenes* per gram of product using the following equation:

$$N = \frac{\Sigma c}{(n_1 + 0.1 n_2) 0.1 d}$$

where

Σc = the sum of confirmed colonies on all dishes retained

n_1 = the number of dishes retained at the first dilution

n_2 = the number of dishes retained at the second dilution

d = the dilution factor corresponding to the first dilution

Round the result calculated to two significant figures.

Take as the result the number of micro organisms per gram of product, expressed as a number between 1.0 and 9.9 multiplied by 10^x, where x is the appropriate power of 10.

8.3 Estimation of small numbers

If the two dishes corresponding to the initial suspension contain less than 15 colonies, calculate the arithmetic mean *m* of the colonies counted on both dishes.

Report the result as follows:

estimated number N_E of *L. monocytogenes* per gram:

$N_E = m \times d^{-1}$ where d is the dilution factor of the initial suspension.

8.4 No characteristic colonies

If the two dishes corresponding to the initial suspension contain no characteristic colonies report the result as follows:

less than $1 \times d^{-1}$ *L. monocytogenes* per gram, where d is the dilution factor of the initial suspension.

9. VALIDATION

The procedure as described in this protocol has been used in an on-going proficiency test exercise organised by the Ministry of Agriculture, Fisheries and Food, CSL Food Science Laboratory, Norwich (10.5). A summary of results obtained is given in Appendix II. Test materials were distributed on four occasions. These comprised freeze-dried minced beef test materials, artificially inoculated with the target organism and a simulated autochthonous flora; in order to simulate, as closely as possible, a natural foodstuff. On each occasion analysts received duplicate blind test materials and were asked to use the method prescribed in this protocol.

All test materials used in the proficiency testing exercise were assessed for homogeneity (Appendix II) using the recommended procedures described in the ISO/IUPAC/AOAC International Harmonised Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (10.6). Homogeneity was assessed immediately following preparation of the test materials (day 0) and again on the date of examination by laboratories (test day). For distribution rounds 1 to 3, test day was 12 days after preparation of the test materials. For round 4, test day was 19 days after preparation.

Statistical analyses of the results for any one test material are as described in the ISO/IUPAC/AOAC International Protocol for the Design, Conduct and Interpretation of Collaborative Studies (10.4).

10. REFERENCES

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APPENDIX I: METHOD FOR THE ENUMERATION OF *L. MONOCYTOGENES*

25 g test portion + 225 mL UVM I

Homogenise

Decimal dilutions as required

Enumerate by direct plating

Incubate for 48 h at 30°C

Count typical colonies on retained dishes of less than 150 colony forming units

Confirm 5 typical colonies from each plate retained

Calculate the weighted mean from numbers of confirmed *L. monocytogenes*

APPENDIX II: STATISTICAL ANALYSIS OF THE RESULTS FROM AN ON-GOING PROFICIENCY TESTING SCHEME

A.1 Matrix

All test materials were prepared on a minced beef matrix

A.2 Laboratories

Data points from participating laboratories were used in statistical analyses after the removal of aberrant results

A.3 Statistical outliers

Entries among tables derived from the original test results that deviate so much from comparable entries that they are considered to be irreconcilable with other data (10.4).

A.4 Assigned value

The robust mean calculated from data returned by all participants who carried out the method as prescribed

A.5 S_r The standard deviation of the repeatability

A.6 r repeatability (within laboratory variation) - the value below which the absolute difference between two single test results obtained with the same method on an identical test material under the same conditions may be expected to lie within a 95% probability

A.7 S_R the standard deviation of the reproducibility

A.8 R reproducibility (between laboratory variation) - the value below which the absolute difference between two single test results obtained with the same method on an identical test material under the same conditions may be expected to lie within a 95% probability

Table 1: Precision characteristics (Log_{10} colony forming units per gram) of the method derived from the results of an on-going proficiency testing scheme

Date of Testing	No. of laboratories	Homogeneity	No. of statistical outliers	Assigned value	S_r	r	S_R	R
May 1994	12	satisfactory	0	4.70	0.08	0.22	0.15	0.42
November 1994	15	satisfactory	0	3.87	0.08	0.22	0.15	0.42
November 1995	18	satisfactory	2	5.19	0.09	0.24	0.16	0.44
November 1996	20	satisfactory	1	4.46	0.09	0.24	0.23	0.64
January 1997	20	satisfactory	1	3.53	0.07	0.19	0.11	0.31