Validation of ISO method 11290 Part 1 — Detection of *Listeria monocytogenes* in foods

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Received 5 July 2000; received in revised form 19 September 2000; accepted 26 October 2000

Abstract

The European and International Standard method for the detection of *Listeria monocytogenes*, described in EN ISO 11290 Part 1: 1997 (International Organisation for Standardisation, Geneva) was validated by order of the European Commission (Standards, Measurement and Testing Fourth Framework Programme Project SMT4-CT96-2098). Nineteen laboratories in 14 countries in Europe participated in a collaborative trial to determine the performance characteristics of the method, which are intended for publication in the corresponding standard. An additional objective of this project was to devise a new series of parameters to indicate the ‘precision’ of microbiological qualitative methods. The method was challenged with three food types, namely fresh cheese, minced beef and dried egg powder and a reference material. Inoculation levels ranged from 5 to 100 cfu/25 g. Each participant examined five replicates of each food type at three inoculum levels and five reference materials. Both PALCAM and Oxford media were assessed. All test materials were subjected to stringent homogeneity and stability testing before being used in the collaborative trial. The results demonstrated that the method prescribed in EN ISO 11290-1 had an overall sensitivity of 85.6% and a specificity of 97.4%. *L. monocytogenes* was detected in most cases after primary enrichment, although secondary enrichment often yielded further positives. However, a significant number of false-negative results were obtained with all food types when large numbers of *L. innocua* were present in the test materials. *L. innocua* tended to dominate *L. monocytogenes* during the selective enrichment stages and thus masked small numbers of colonies of *L. monocytogenes* on the isolation media. There was no evidence from this collaborative study to demonstrate a significant difference in performance between Oxford and PALCAM media. Due to the problem of false-negative results with this method as highlighted in this trial, recommendations have been made to ISO to launch a revision of the standard to improve the detection of low numbers of *L. monocytogenes* in foods. New statistical methods devised to advance the

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PII: S0168-1605(00)00462-1
measurement of the performance of qualitative microbiological methods are also described. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Listeria monocytogenes; EN ISO 11290-1; Validation; Performance characteristics; Food

1. Introduction

It is widely accepted that the use of validated methods is an essential part of any sound laboratory quality assurance programme. It is particularly important to have documented performance characteristics for methods that are utilised for statutory purposes or in cases of trade disputes. It is also the policy of the European Committee for Standardisation (CEN) to require methods that are to become European standards to be validated by collaborative study. Until recently, many internationally recognised reference methods were only ‘historically proven’ and had no defined precision parameters. In order to rectify this deficiency, project SMT4-CT96-2098, financed by the European Commission under the Fourth Framework Standards, Measurements and Testing Program (SM&T), was elaborated to determine the precision data in terms of repeatability (r) and reproducibility (R) or performance characteristics (qualitative methods) of six ISO (International Organisation for Standardisation) methods. These were prioritised for acceptance by CEN as follows: Bacillus cereus (enumeration), Listeria monocytogenes (detection and enumeration), Staphylococcus aureus (enumeration), Clostridium perfringens (enumeration) and Salmonella (detection). The determined performance characteristics are intended for publication by CEN in the corresponding CEN methods and subsequently by ISO via the Vienna Agreement.

The objectives of this EU-funded project and the results of the first validation carried out on ISO 7932 1993 ‘Enumeration of Bacillus cereus’ have been described previously by Schulten et al. (2000)

In this paper, we report the results of the second collaborative study in the project to validate EN ISO 11290 Part 1 (Anon., 1997) for the detection of L. monocytogenes in foods. The trial was carried out in March/April 1998. We also describe new statistical methods to enable the precision of qualitative methods to be assessed in the same way that repeatability and reproducibility are used with quantitative methods.

2. Materials and methods

2.1. Design of the trial

Nineteen laboratories from 14 countries in Europe participated in the collaborative trial. All laboratories were accredited or in the process of being accredited according to EN 45001 standard (Anon., 1989a). The design of the trial was described to participants in a detailed trial chronology, standard operating procedure (SOP) and a test report on which to record the results and return to the trial leader for analysis. A SOP was written, based on EN ISO 11290-1, because additional information was required to ensure that all test materials were treated the same way by each participant in the trial. In the test report, participants were required to detail all supplementary information that could have influenced their results. Prior to each collaborative trial, a ‘pre-trial’ was organised among the three contractors to verify that the SOP, as written, contained no potential ambiguities and to establish that the test materials were fit for purpose.

The method was challenged with three types of food, i.e. a food product from the dairy group (fresh cheese), meat group (minced beef) and dried food group (egg powder). Test materials used to validate the methods were artificially inoculated to achieve the desired inoculum levels and homogeneity. For each food type, three inoculum levels were prepared with target values of 0 colony forming units (cfu) of L. monocytogenes per 25 g, 5–10 cfu/25 g and 50–100 cfu/25 g. Additionally, each test material contained L. innocua at a level of 50–100 cfu/25 g plus a typical background flora at various levels depending upon the test material type. Each particip-
ant received 15 test materials for each food type, five replicates at each inoculum level. In addition, five reference materials (RM) were included in the trial to identify any serious errors in a participant’s performance. All test materials were tested extensively prior to the pre-trial and collaborative trial to ensure that they could be maintained in a stable and homogeneous state for the duration of the distribution and trial period. The test materials were shipped to the participants by courier in polystyrene insulated boxes containing several ice packs to prevent exposure of the test materials to high temperatures during transport. Participants provided all media and reagents needed for the collaborative trial. Test material codes were randomised for each participant to prevent collusion among laboratories. The examination of the test materials was carried out within a specified time period as prescribed in the trial chronology.

2.2. Method under collaborative trial

The detection of *L. monocytogenes* according to EN ISO 11290-1 required the following successive stages:

(a) Pre-treatment of each food type and RM according to a standardised procedure to obtain an initial sample for primary enrichment.
(b) Primary enrichment in a selective liquid enrichment medium with reduced concentration of selective agents (half-Fraser broth) with incubation at 30°C for 24 h.
(c) Secondary enrichment of a culture obtained from (b) in a selective liquid enrichment medium with full concentration of selective agents (Fraser broth) with incubation at 37°C for 48 h.
(d) Plating out of cultures obtained in (b) and (c) and identification on either Oxford or PALCAM media with incubation at 30°C (Oxford) (Curtis, 1989) or 37°C (PALCAM) and examination after 24 h and, if necessary, after 48 h to check for the presence of characteristic colonies which are presumed to be *Listeria* spp.
(e) Confirmation of the presence of *L. monocytogenes* by means of appropriate morphological, physiological and biochemical tests carried out on five presumptive colonies.

2.3. Preparation of test materials

Cheese test materials were prepared by CECA LAIT (Poligny, France). Liquid cheese was inoculated with the test strains comprising *L. monocytogenes* (serovar 1/2a) and *L. innocua*, both of which had been isolated from milk by CECA LAIT. In addition, a background flora isolated from cheese comprising *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecalis*, *Lactobacillus paracasei* and *Lactobacillus plantarum* was also added at a level of approximately 10⁶ cfu/g. *Listeria* strains were isolated on Oxford (Oxoid) and PALCAM agars (Merck), and then on Trypticase-Soya-Yeast Extract agar (Merck), *Lactococcus* and *Enterococcus* on M17 (Biokar Diagnostics) and *Lactobacillus* on de Man, Rogosa and Sharpe (MRS) (Biokar Diagnostics). Before inoculation, the *Listeria* strains were cultivated in Brain-Heart-Infusion broth (BHI) (Oxoid) for 18 h at 37°C and lactic bacteria in MRS or M17 broth for 18 h at 30°C. After mixing, cultures were diluted in 1/4 Ringer’s solution (Merck) and inoculated into the liquid cheese. The inoculated cheese was clotted by the addition of rennet after dispensing aliquots of 100 g into individual vials. The cheese microflora was stabilised by the addition of an undisclosed bacteriostatic mixture. The bacteriostatic effect diminished when the test material was diluted during examination. Batches of cheese test materials were prepared for the collaborative trial immediately prior to dispatch to participants due to their relatively short stability.

Meat test materials were prepared by MAFF-CSL, UK. The test strains used to inoculate the test materials were *L. monocytogenes* NCTC 5214 and *L. innocua* NCTC 11288. All test materials were additionally inoculated with a simulated autochthonous meat flora comprising approximately 1 × 10⁴ cfu/g *B. subtilis* and *Pseudomonas* sp. and also 1 × 10⁶ cfu/g *Lactobacillus* sp. and 1 × 10⁶ cfu/g *Micrococcus* sp. and 1 × 10⁴ cfu/g *Pseudomonas* sp. Batches were prepared using retail fresh minced beef, which was freeze-dried in bulk, subsampled into vials (portions of 25 g equivalents) and irradiated at 25 kGy (Isotron, UK). The test strains and background flora were cultivated into Nutrient broth (Oxoid), diluted to the appropriate level and added as a ‘cocktail’ to each vial. Finally, the vials were freeze-dried a second time to stabilise the microflora and...
stored at 4°C until use. Different batches were prepared for the pre-trial and collaborative trial.

Egg powder test materials were prepared by the RIVM-MGB, The Netherlands. Each test material was prepared by the participants immediately prior to examination by compositing a vial containing whole egg powder with an autochthonous flora at approximately 10⁵ cfu/g and a vial with two capsules containing the Listeria species suspended in milk powder. For the ‘negative’ test materials, capsules contained only milk powder sterilised after filling by gamma irradiation (10 kGy). A batch of 25 kg of egg powder was obtained from an egg powder factory (Enthoven bv, Bennebroek, NL). Two series of 10 test materials of egg powder were tested for aerobic plate count (Anon., 1989b), Enterobacteriaceae count (Anon., 1993), Listeria enumeration (Anon., 1998) and Listeria detection (Anon., 1997). The test strains used in the egg test materials for the collaborative trial were L. monocytogenes Scott A, ALM 92 and L. innocua ALM 105. Both strains were cultivated in BHI for 24 h (shaken at 100 rpm) at 37°C. Each culture was centrifuged and the pellet suspended in 100 ml of milk, supplemented with 68.42 g saccharose (2 M). Approximately 10 ml of this suspension was dried onto sterile milk powder (Melkproduct 17, Nestlé) by means of a fluid bed spray granulation (STREA-1, Niro-Aeromatic). This resulted in two highly contaminated milk powders. The required contamination levels were prepared by mixing these powders with sterile milk powder in several steps (to optimise homogeneity of the test material) until the required contamination level was reached. For each prepared batch, the mixed powder was filled into gelatin capsules (ca. 0.3 g/capsule) in a laminar flow cabinet using an aluminium filling apparatus.

For the RM, capsules containing ca. 23 cfu L. monocytogenes Scott A, ALM 92/capsule were prepared by RIVM.

### 2.4. Homogeneity and stability of the test materials

The homogeneity and stability of the test materials were tested according to the procedures of the laboratory expert in their preparation. Acceptance of all production batches for use in the trials was made on the basis of achieving satisfactory homogeneity and stability according to the laboratories’ own criteria.

For the cheese test materials, stability at 4°C was measured by analysing, over several days, three test materials in duplicate. For each analysis, 10 g of cheese was diluted in 90 ml K₂HPO₄ solution (Anon., 1992) and blended for 3 min. This suspension was inoculated onto two plates of Oxford agar using a spiral plating system. Only a batch containing 10⁵ cfu L. monocytogenes was tested. For homogeneity, 10 test materials of the same batch were examined in duplicate. The CECALAIT computer program was used for the statistical treatments. 

χ² (Chi square) was calculated on the total count (log₁₀) of colonies per sample to compare the dispersion of the contamination to the dispersion obtained in a Poisson series.

For the meat test materials, stability of L. monocytogenes in minced beef stored at 4°C was verified over a 30 day period. Ten replicate vials were enumerated on Oxford agar at each interval at days 0, 11, 17, 25 and 30. The homogeneity of the batch was verified by enumerating 10 replicate vials immediately after production and again at the same time that participants examined the test materials. An F-test (α = 0.05) was applied using log₁₀ transformed counts and the stability and homogeneity considered satisfactory if the F-test was not significant.

The homogeneity of both the egg powder test materials and the capsules was determined. For the capsules containing the target organisms at the low and high contamination levels, 25 capsules were examined to determine the variation between duplicate counts, i.e. within test material (T₁ test) and between test materials (T₂ test) (Schulten et al., 2000). The stability of the capsules at −20°C was assessed for two batches (L. monocytogenes 50–100 cfu/25 g and L. innocua 50–100 cfu/25 g). Ten test materials per week were examined by plating on PALCAM agar and incubating for 48 h over a period of 10 weeks.

### 2.5. Statistical analysis of the data

As there is no internationally agreed protocol for the statistical treatment of qualitative microbiological data, both traditional and new applications of routine statistical methods were applied to the data generated.
from this trial to try to obtain useful measures of performance such as those used for quantitative methods. The first parameter to be determined was accuracy; for positive test materials this is termed sensitivity, defined as the percentage of known positive test materials that are correctly identified as positives. For negative test materials it is termed specificity, defined as the percentage of known negative test materials that are correctly identified as negatives. In addition, two new parameters were derived termed ‘accordance’ and ‘concordance’ which were designed to be equivalent to the more familiar parameters of repeatability and reproducibility, respectively, as determined for quantitative methods. Accordance is defined as the percentage chance of finding the same result (i.e. both positive or both negative) from two identical test materials analysed in the same laboratory, under standard repeatability conditions. To calculate it from the results of an inter-laboratory test, the probability that two samples give the same result is calculated for each participating laboratory in turn, and this probability is then averaged over all laboratories. Similarly, the concordance is defined as the percentage chance of finding the same result from two identical test materials analysed in different laboratories. To calculate it from the results of an inter-laboratory test, each observation in each participating laboratory is taken in turn, pairing it with all results from similar test materials from all the other laboratories. The concordance is the percentage of all pairings giving the same results over all possible pairings of data.

3. Results and discussion

3.1. Stability and homogeneity of the test materials

All test materials were considered sufficiently stable and homogeneous for the trial. Cheese test materials stored at 4°C were stable and homogeneous for up to 7 days. Meat test materials were stable and homogeneous when stored at 4°C for up to 1 month. For the egg test materials, separate examinations were carried out on the egg powder and capsules containing L. innocua and L. monocytogenes. Both the egg powder and L. innocua capsules were stable and homogenous over a 10 week period when stored at −20°C. However, for L. monocytogenes 50–100 cfu/capsule, the regression coefficient was significantly decreasing (log$_{10}$ 0.0065 per week), but as the test materials were analysed within a 3 week period this decrease is unlikely to have had a significant effect on the results.

3.2. General results of the trial

Cheese test materials were received by most participants within 48 h of dispatch. One laboratory could not commence their examinations until 4 days after dispatch because their test materials did not arrive in time to start before a weekend. However, the test materials were stored at 4°C and as they were known to be stable for up to 7 days, the laboratory was instructed to continue with the trial. Two laboratories elected not to examine cheese test materials. No problems were encountered with distribution of the meat, egg powder test materials and RM. Temperature control during shipment was satisfactory as no test materials had been subjected to any excessive temperature abuse according to data obtained from the temperature monitoring devices included in the packages.

Due to the level of work required in this trial, participants were instructed to detect L. monocytogenes using only one of the two media prescribed in EN ISO 11290-1, i.e. Oxford or PALCAM (whereas EN ISO 11290-1 requires both media to be used). Nine laboratories used Oxford agar and 11 used PALCAM agar.

3.3. Results excluded from further analysis

For this qualitative trial it was agreed that results from laboratories would not be excluded unless they fell into three specified categories: (a) the test materials had received a significant temperature abuse during shipment, (b) the laboratory had clearly deviated from the specified standard operating procedure or (c) the performance of the laboratory was questionable as indicated by large numbers of false-positive or false-negative results more than would be expected by chance.

Two laboratories fell into the above categories. One returned a significantly high proportion of false-positive results and thus their competence in confirmation of L. monocytogenes, relative to data returned by other participants, was questionable. A
second laboratory failed to rehydrate the meat test materials correctly prior to commencing examination and, as their results for the meat test materials were generally poor, their data (for the meat test materials only) was excluded from further analysis.

3.4. Statistical analysis of data

3.4.1. Summary of participants’ results

A summary of participants’ results depending upon the isolation medium used is given in Tables 1 and 2.

3.4.2. Specificity

A total of 15 negative test materials were examined by each laboratory (five per food type). After the exclusion of data from two laboratories, a total of eight false-positive results were reported. The percentage of false-positive results is shown in relation to food and isolation medium in Table 3. Although there was a higher percentage of false positives with PALCAM, analysis by logistic regression revealed no significant differences between media, food types or laboratories in the proportion of false positives observed. The overall specificity of the method was 97.4%.

Table 1
Summary of participants’ results on PALCAM agar: No. test materials correctly identified from a possible maximum of five

<table>
<thead>
<tr>
<th>Test material</th>
<th>Inoculum level</th>
<th>Laboratory code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cheese</td>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5</td>
</tr>
<tr>
<td>Meat</td>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5</td>
</tr>
<tr>
<td>Egg</td>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5</td>
</tr>
<tr>
<td>RM</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*a B, negative for L. monocytogenes; L, 5–10 cfu/25 g L. monocytogenes plus 50–100 cfu/25 g L. innocua; H, 50–100 cfu/25 g L. monocytogenes plus 50–100 cfu/25 g L. innocua. NT, not tested.

Table 2
Summary of participants’ results on Oxford agar: No. test materials correctly identified from a possible maximum of five

<table>
<thead>
<tr>
<th>Test material</th>
<th>Inoculum level</th>
<th>Laboratory code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cheese</td>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5</td>
</tr>
<tr>
<td>Meat</td>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5</td>
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<tr>
<td>Egg</td>
<td>B</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>4</td>
</tr>
<tr>
<td>RM</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

*a B, negative for L. monocytogenes; L, 5–10 cfu/25 g L. monocytogenes; H, 50–100 cfu/25 g L. monocytogenes plus 50–100 cfu/25 g L. innocua.
### Table 3
Percentage of false-positive results in relation to food and medium

<table>
<thead>
<tr>
<th>Food type</th>
<th>Medium</th>
<th>Oxford</th>
<th>Mean %</th>
<th>PALCAM</th>
<th>Mean %</th>
<th>All</th>
<th>Mean %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese</td>
<td>9</td>
<td>2.2</td>
<td>8</td>
<td>5.0</td>
<td>17</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Meat</td>
<td>9</td>
<td>0.0</td>
<td>9</td>
<td>4.4</td>
<td>18</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>9</td>
<td>2.2</td>
<td>10</td>
<td>2.0</td>
<td>19</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>27</td>
<td>1.5</td>
<td>27</td>
<td>3.7</td>
<td>54</td>
<td>2.6</td>
<td></td>
</tr>
</tbody>
</table>

* ‘n’ refers to the number of sets each of five replicates, e.g. n = 9 implies 45 test materials in total.

### Table 4
Percentage of correct results (sensitivity) in relation to medium used

<table>
<thead>
<tr>
<th>Food type</th>
<th>Medium</th>
<th>Oxford</th>
<th>Mean %</th>
<th>PALCAM</th>
<th>Mean %</th>
<th>All</th>
<th>Mean %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese</td>
<td>18</td>
<td>88.9</td>
<td>16</td>
<td>97.5</td>
<td>34</td>
<td>92.9</td>
<td></td>
</tr>
<tr>
<td>Meat</td>
<td>18</td>
<td>91.1</td>
<td>18</td>
<td>92.2</td>
<td>36</td>
<td>91.7</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>18</td>
<td>66.7</td>
<td>20</td>
<td>75.0</td>
<td>38</td>
<td>71.1</td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>9</td>
<td>82.2</td>
<td>10</td>
<td>96.0</td>
<td>19</td>
<td>89.5</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>63</td>
<td>82.2</td>
<td>64</td>
<td>88.7</td>
<td>127</td>
<td>85.6</td>
<td></td>
</tr>
</tbody>
</table>

* ‘n’ refers to the number of sets each of five replicates, e.g. n = 9 implies 45 test materials in total.

### Table 5
Percentage of correct results (sensitivity) in relation to inoculum level

<table>
<thead>
<tr>
<th>Food type</th>
<th>Inoculum level</th>
<th>Low</th>
<th>Mean %</th>
<th>High</th>
<th>Mean %</th>
<th>All</th>
<th>Mean %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese</td>
<td>17</td>
<td>85.9</td>
<td>17</td>
<td>100.0</td>
<td>34</td>
<td>92.9</td>
<td></td>
</tr>
<tr>
<td>Meat</td>
<td>18</td>
<td>83.3</td>
<td>18</td>
<td>100.0</td>
<td>36</td>
<td>91.7</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>19</td>
<td>53.7</td>
<td>19</td>
<td>88.4</td>
<td>38</td>
<td>71.1</td>
<td></td>
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<td>82.2</td>
<td>10</td>
<td>96.0</td>
<td>19</td>
<td>89.5</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>54</td>
<td>73.7</td>
<td>54</td>
<td>95.9</td>
<td>127</td>
<td>85.6</td>
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</table>

* ‘n’ refers to the number of sets each of five replicates, e.g. n = 9 implies 45 test materials in total.

### 3.4.3. Sensitivity

The main objective of this collaborative trial was to establish the overall performance characteristics of the method and to determine the influence of specific factors, such as the effect of the isolation medium or concentration of target organism, on method performance. As for the negative test materials, the effects of the various factors on the probability of correctly identifying a positive sample were investigated using chi-squared statistics from a logistic regression analysis. Tables 4 and 5 show the sensitivity of the method in relation to food type/medium and food type/inoculum level, respectively.

Large differences in performance of the method in relation to inoculum levels and food types were observed with a significantly lower sensitivity for egg powder test materials. It is not clear why the method showed a poor sensitivity with egg powder test materials as the significant level of homogeneity and stability testing undertaken prior to the trial verified that the materials could be produced to the required quality standards. However, it was noted that, at the higher level, *L. monocytogenes* showed a slight decline in viability with time and it is possible that this may have been more manifest during the trial. There was also evidence that laboratories differed in their relative performance with the different foods. Data indicated that the method was marginally more sensitive when PALCAM agar was used for isolation, but this result was not highly significant (0.05 < *P* < 0.1). The overall sensitivity of the method was 85.6%.

### 3.4.4. Effect of primary and secondary enrichment on method sensitivity

One of the other factors which was of interest in relation to the method under test was whether the sensitivity was influenced by the primary and/or secondary enrichment stages. Table 6 shows the numbers of test materials found positive after primary and secondary enrichment. This variable was examined excluding results from five laboratories as they did not always carry out the secondary enrichment if the presence of *L. monocytogenes* had already been confirmed after the primary enrichment.

Most of the food test materials inoculated at the higher level which were found positive were detected after the primary enrichment (95.5%), whereas 30.0% of those inoculated at the low level required the secondary enrichment. This difference was highly significant (*P* < 0.001). There was also a marked difference in the sensitivity for the different foods (*P* < 0.001), with more cheese test materials being detected after the primary enrichment (97.5% of those found positive) compared to 73.8% for meat and 82.2% for egg. However, this observation should
be treated with some caution because of the significant interaction between laboratories and foods ($P < 0.001$). There was no significant difference in the performance of the isolation media from most of the data.

Where test materials were positive after primary enrichment, there were large differences between foods in the proportion that remained positive after secondary enrichment (Table 6). Most meat test materials (92.5%) remained positive, whereas only 44.1% of cheese test materials and 61.4% of egg test materials were also positive after secondary enrichment.

### Table 6
Number of positive samples after primary and/or secondary enrichment

<table>
<thead>
<tr>
<th>Food</th>
<th>Inoculum level</th>
<th>No. test materials</th>
<th>Total No. positives</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Positive</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Positive</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Negative</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Negative</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Positive</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Positive</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; Negative</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese</td>
<td>Low</td>
<td>65</td>
<td>56</td>
<td>16</td>
<td>37</td>
<td>9</td>
<td>3</td>
<td>37</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>65</td>
<td>65</td>
<td>36</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>130</td>
<td>121</td>
<td>52</td>
<td>66</td>
<td>9</td>
<td>3</td>
<td>66</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Meat</td>
<td>Low</td>
<td>70</td>
<td>56</td>
<td>27</td>
<td>2</td>
<td>14</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>70</td>
<td>70</td>
<td>59</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Both</td>
<td>140</td>
<td>126</td>
<td>86</td>
<td>7</td>
<td>14</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>Low</td>
<td>70</td>
<td>38</td>
<td>12</td>
<td>11</td>
<td>32</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>70</td>
<td>63</td>
<td>39</td>
<td>21</td>
<td>7</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>140</td>
<td>101</td>
<td>51</td>
<td>32</td>
<td>39</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM</td>
<td>Rm</td>
<td>70</td>
<td>60</td>
<td>53</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>Low</td>
<td>205</td>
<td>150</td>
<td>55</td>
<td>50</td>
<td>55</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>205</td>
<td>198</td>
<td>134</td>
<td>55</td>
<td>7</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>480</td>
<td>408</td>
<td>242</td>
<td>105</td>
<td>72</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3.4.5. Performance assessment
Tables 7–10 show the overall performance characteristics of ISO 11290-1 in relation to food type and inoculum level. The data obtained from Oxford and PALCAM agars are combined. The specificity of the method did not vary greatly between food types, ranging from 96.5 to 97.9%. With respect to sensitivity, for minced meat and fresh cheese, similar results (83.3 and 85.9%, respectively) were obtained at the lower inoculum level (5–10 cfu $L. monocytogenes$ per 25 g). At the higher inoculum level (50–100 cfu $L. monocytogenes$ per 25 g) the sensitivity increased in both cases to 100%. For the reference
### Table 8
Method performance characteristics with minced meat

<table>
<thead>
<tr>
<th>Sample/level of contamination</th>
<th>Minced meat (negative)</th>
<th>Minced meat (low level)</th>
<th>Minced meat (high level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of laboratories with valid results</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>No. of samples per laboratory</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>No. of accepted samples</td>
<td>85</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Accuracy (specificity) (%)</td>
<td>97.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Accuracy (sensitivity) (%)</td>
<td>–</td>
<td>83.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Accordance (%)</td>
<td>96.7</td>
<td>76.7</td>
<td>100.0</td>
</tr>
<tr>
<td>Concordance (%)</td>
<td>95.6</td>
<td>71.7</td>
<td>100.0</td>
</tr>
</tbody>
</table>

### Table 9
Method performance characteristics with fresh cheese

<table>
<thead>
<tr>
<th>Sample/level of contamination</th>
<th>Fresh cheese (negative)</th>
<th>Fresh cheese (low level)</th>
<th>Fresh cheese (high level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of laboratories with valid results</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>No. of samples per laboratory</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>No. of accepted samples</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Accuracy (specificity) (%)</td>
<td>96.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Accuracy (sensitivity) (%)</td>
<td>–</td>
<td>85.9</td>
<td>100.0</td>
</tr>
<tr>
<td>Accordance (%)</td>
<td>92.9</td>
<td>80.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Concordance (%)</td>
<td>93.1</td>
<td>75.2</td>
<td>100.0</td>
</tr>
</tbody>
</table>

### Table 10
Method performance characteristics with reference materials

<table>
<thead>
<tr>
<th>Sample/level of contamination</th>
<th>Reference material (capsules containing 23 cfu <em>L. monocytogenes</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year of inter-laboratory test</td>
<td>1998</td>
</tr>
<tr>
<td>No. of laboratories with valid results</td>
<td>19</td>
</tr>
<tr>
<td>No. of samples per laboratory</td>
<td>5</td>
</tr>
<tr>
<td>No. of accepted samples</td>
<td>90</td>
</tr>
<tr>
<td>Accuracy (specificity) (%)</td>
<td>–</td>
</tr>
<tr>
<td>Accuracy (sensitivity) (%)</td>
<td>89.5</td>
</tr>
<tr>
<td>Accordance (%)</td>
<td>85.3</td>
</tr>
<tr>
<td>Concordance (%)</td>
<td>80.8</td>
</tr>
</tbody>
</table>
materials containing ca. 23 cfu pure culture of *L. monocytogenes*, a slightly higher sensitivity of 89.5% was obtained. However, the sensitivity of the method, when challenged with egg powder, was poor, with only 53.7% of samples correctly identified as positive at the lower inoculum level and 88.4% at the higher level. The reasons for this disparity in this study are uncertain.

With respect to the accordance value, i.e. the percentage chance of obtaining the same result on two identical test samples under repeatability conditions, broadly similar results were obtained for each of the negative food types, ranging from 92.9 to 96.7%. For samples containing only a low level of *L. monocytogenes*, results in excess of 76% were obtained for meat, cheese and reference materials. However, an accordance of only 48.4% was achieved for the egg powder. This value improved to 83.2% at the higher inoculum, but was still poor compared to meat and cheese at 100%. It is observed from this data that there is a direct relationship between sensitivity and accordance/concordance values in that, when the sensitivity is high, there is also a high value for the accordance/concordance.

With respect to concordance, i.e. the percentage chance of obtaining the same result on two identical test samples under reproducibility conditions, again broadly similar results were obtained for each of the negative food types, ranging from 93.1 to 95.8%. For samples containing only a low level of *L. monocytogenes*, results in excess of 71% were obtained for meat, cheese and reference materials. However, a concordance of only 49.8% was achieved for the egg powder. This value improved to 79.1% at the higher inoculum, but was still poor compared to meat and cheese which again had excellent concordance values of 100% at the higher inoculum level. It is unclear why the results obtained with dried egg powder were poor compared to the other food types as the test materials were known to be stable and homogeneous for the duration of the trial period. Their method of preparation was different from the meat and cheese test materials as participants were provided with capsules containing the required levels of *L. monocytogenes* and *L. innocua* in skimmed milk powder and used these to inoculate the base product immediately before testing, whereas the meat and cheese test materials were directly inoculated, using broth cultures, by the responsible contractor. It is possible that the target organisms in the egg powder were exposed to a higher degree of stress and therefore the method did not perform as well with these test materials.

### 3.5. Confirmation test results

The method for the detection of *L. monocytogenes* requires a number of procedures to be carried out to confirm presumptive colonies isolated on PALCAM or Oxford media. These tests are carried out in a series of stages. Three or four preliminary tests (catalase, Gram staining, motility and Henry illumination) are carried out in order to select *Listeria* spp. colonies. If these tests are indicative of *Listeria* spp., haemolysis, carbohydrate utilisation and the CAMP tests must be performed to confirm the presence of *L. monocytogenes* and also differentiate between some other *Listeria* spp.

As large numbers of presumptive colonies were expected in this trial, participants were allowed some flexibility in the confirmatory tests used and thus the Gram stain, motility and CAMP tests were considered optional. Participants were also permitted to use one of two types of haemolysis tests; a microwell test using a 2% suspension of sheep erythrocytes suspended in PBS or the more traditional sheep blood agar plates. However, participants were instructed that where haemolysis testing was carried out using only blood agar plates, the performance of the CAMP test became obligatory. The haemolysis test is the critical test for the differentiation of *L. monocytogenes* and *L. innocua* and, as both strains were present in the trial test materials, it was this test that caused most problems for some laboratories. Some laboratories obtained poor results with sheep blood agar plates and did not always supplement this test with the CAMP test as instructed. Most laboratories used the microwell method (despite the current discrepancy in the correct concentration of sheep erythrocytes to be used in the test). It is probable that many of the false-negative results were due to difficulties in interpretation of the haemolysis reactions. However, of greater concern is the performance of the method with respect to its selectivity for *L. monocytogenes*. It has become apparent both during the preparation of test materials for use in the trial and also from the trial results that when *L. monocytogenes* is in association with *L. innocua* in a food, the dual enrichment procedure allows *L. in-
nocua to become dominant. Consequently, the picking of only five colonies for confirmation may result in the reporting of false-negative results, as *L. monocytogenes* colonies will always be fewer in number. Several participants reported that the picking of more colonies resulted in a higher detection rate.

4. Conclusion

The method prescribed in EN ISO 11290-1 has an overall sensitivity of 85.6% and a specificity of 97.4%. As a general indication of accordance and concordance, respectively, values of 95.1% (negatives), 81.4% (positives), 85.3% (RM) and 94.8% (negatives), 79.3% (positives) and 80.8% (RM) can be used.

In most cases, proceeding with a secondary enrichment will yield a higher sensitivity. However, when *L. monocytogenes* is known to occur in a foodstuff in close association with *L. innocua*, it is possible that *L. monocytogenes* will not be detected using this method due to the overgrowth of *L. innocua* during selective enrichment thus masking the presence of *L. monocytogenes* on both Oxford and PALCAM agars. The importance of picking off sufficient colonies for confirmation and carrying out the haemolysis tests correctly should not be underestimated. The value of the motility test and the tumbling action being highly characteristic for *L. monocytogenes* should also not be forgotten. Additionally, isolation from both the primary and secondary enrichment should be carried out as it is possible, with some food types, that false-negative results may be obtained if isolation is effected only after secondary enrichment.

From the overall results of this collaborative study it has been recommended to CEN and ISO to:

- Incorporate the performance criteria derived from this trial, together with appropriate explanations of how to interpret the various parameters, as an amendment to the Standard EN ISO 11290-1.
- Include in this amendment the correct value (2%) of the percentage of the sheep erythrocytes for the microwell haemolysis test in line with the enumeration method EN ISO 11290-2.
- Launch a revision of EN ISO 11290-1 in order to address the problem of potential false-negative results. For example, by improving the isolation stage by replacing one of the current media with a medium which can differentiate *L. monocytogenes* from other species, especially *L. innocua* (e.g., a medium which reveals haemolytic colonies at the isolation stage). Moreover, the possibility of using molecular typing should be introduced as an alternative to the classical confirmation tests, since several commercial sources are now available.

Acknowledgements

The validation of International Standard EN ISO 11290-1 has been carried out under the framework of a Standards, Measurement and Testing project No. SMT4-CT96-2098. The authors wish to thank the following laboratories for their participation and cooperation in this collaborative trial: Mr H. Stegeman, RIKILT-DLO, PO Box 230, 6700 AE Wageningen, The Netherlands; Mr P.H. in’t Veld, National Institute of Public Health and Environment, Microbiological Laboratory for Health Protection, Antonie Van Leeuwenhoeklaan, 93720 BA Biltoven, The Netherlands; Mrs M. Lambiri, National School of Public Health, Bacteriology Department, 196 Alexandras Avenue, 115 21 Athens, Greece; Dr Boleslaw Wojton, National Veterinary Institute, Al Partyzantow 57, 24-100-Pulawy, Poland; G. Vlaemynck, Dairy Research Station, Microbiology Laboratory, Brusselsesteenweg 370, 9090 Melle, Belgium; I.M. Vicente da Cruz, INETI-IBQTA-DTIA, Estrada do Paco do Lumiau-Edificio S, Lisboa 1699 cedex, Portugal; Prof. M.L. Stecchini, Dipartimento di Scienze Degli Alimenti, Via Marangoni 97, 33100 Udine, Italy; Dr H. Asperger, Veterinarmedizinische Universitat Wien, Institute fur Milchhygiene und Milchtechnologie, Joseph Baumann-Gasse 1, A-1210 Wien, Austria; Mr V. Young, EHB Public Analysts Laboratory, Sir Patrick Dun’s, Lower Grant Canal Street, Dublin 2, Republic of Ireland; Prof. Debevere, Universiteit Gent, Faculteit Landbouwkundige En toegepaste Biologische Wetenschappen wak groep Levensmiddelentechnologie En Voeding, B-9000 Gent; Mrs C. Allaert, Lab of Microbiology of the Food Technology Department, Avda. Alcalde Rovira Roure, 177, 25198 Lleida, Spain; Dr T.
Johansson, National Veterinary and Food Research Institute, Dept of Food Microbiology, PO Box 368, Fin-00231 Helsinki, Finland; DVM Marylene Bonhert, CNEVA Ploufragan, B.P. 53, Zoopole Beaumecamrie, 22440 Ploufragan, France; Dr D. Roberts, Central Public Health Laboratory, Food Hygiene Laboratory, 61 Colindale Avenue, London NW9 5HT; J. Anderson, Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ; Prof. P. Teufel/Dr E. Bartelt, Federal Institute for Health Protection of Consumers, Unit Food Microbiology, Thielallee 88–92, 14195 Berlin, Germany; Dr J.L. Cordier, Nestlé Research Center Lausanne, Quality and Safety Assurance Department, PO Box 44, 1000 Lausanne, Switzerland; Dr K. Friedrich, MUVA Kempten, Qualitats-Und Laborzentrum, Hirnbeinstrasse 10, D-87435 Kempten (Allgau), Postfach 2025, Allgau, Germany; Mr J.C. van Dijk, Stichting COKZ, PO Box 250, 3830 AG Leusden, The Netherlands. The authors also wish to acknowledge the expert statistical advice supplied by Dr Remi Chevennement and also the technical assistance of all their laboratory staff in the preparation and analysis of the test materials used in this study.

References