

# **National Reference Laboratory *Salmonella***

## **(Food, Feed and Animal Health)**

### **Report on 2009 Ring Trial for Laboratories Testing for *Salmonella* spp. in Food, Feed and Poultry monitoring programmes in Ireland.**

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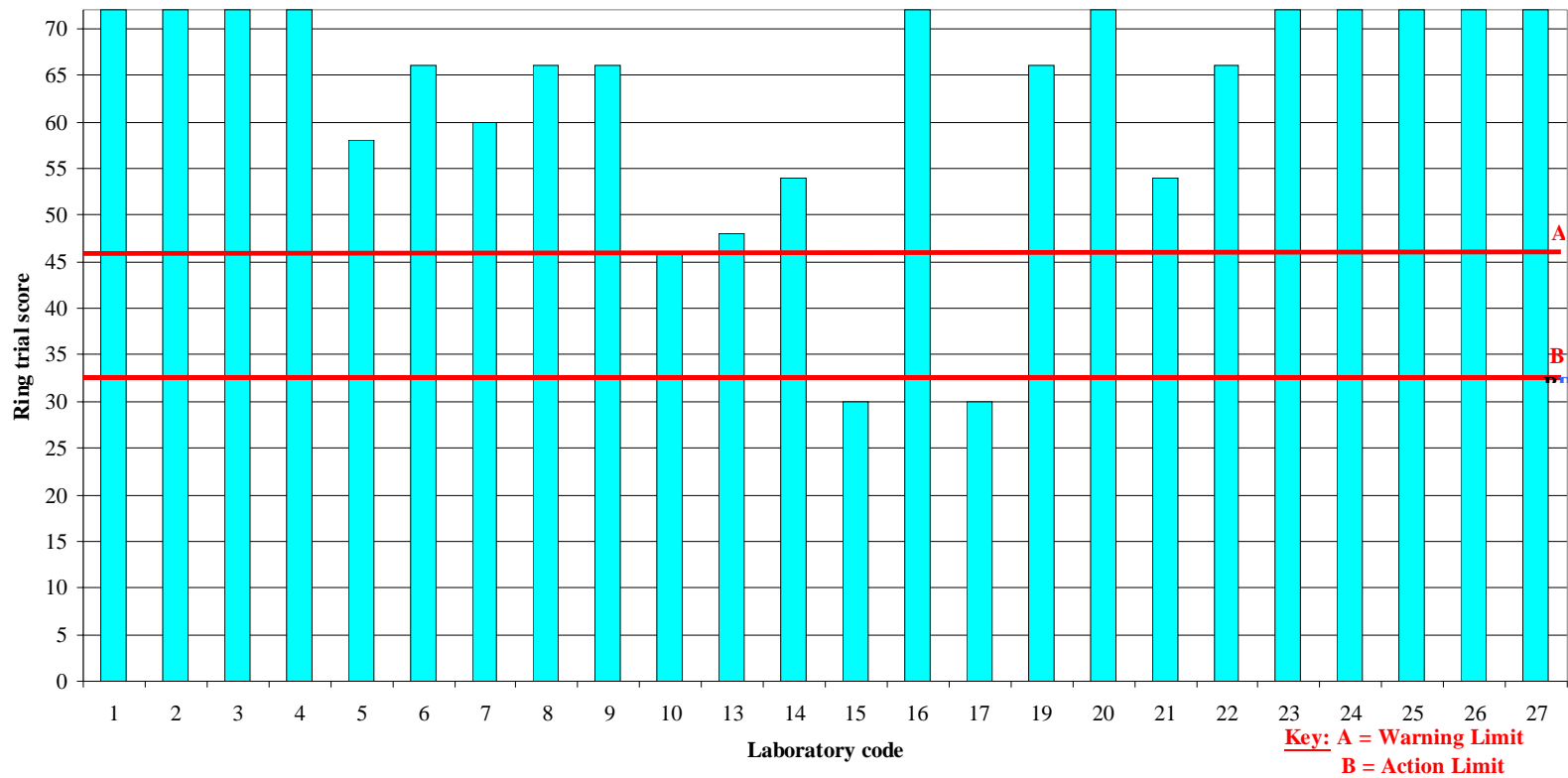
## **Executive Summary**

The Backweston Laboratory Complex hosts the National Reference Laboratory (NRL) functions for *Salmonella* in food, feed and animal health. In Ireland the National control programmes for *Salmonella* spp. in poultry breeding flocks, laying flocks and broiler flocks of *Gallus gallus* required under Regulation 2160/2003 have been implemented through S.I 706 of 2006, S.I. 247 of 2008 and SI 64 of 2009. Although EU legislation specifies a maximum prevalence of 1 % for both *S. Enteritidis* (SE) and *S. Typhimurium* (ST) in such flocks in MS the RoI has maintained virtual freedom from both serotypes in these flocks. Monitoring of the national targets is based on results of both official and food business operator (FBO) testing. FBO testing is conducted in private laboratories which must be approved by the Department of Agriculture, Fisheries and Food (DAFF). These laboratories must demonstrate competency in isolating *Salmonella* spp. and for this purpose participation in any national ring trial is a requirement.

A ring trial was organised by the NRL in June 2009. Lyophilised samples (n = 15) containing various concentrations of *Salmonella* spp., with and without competitive flora, were utilised to determine the ability of participants (n = 24) to isolate and confirm *Salmonella* spp. Samples were assigned a numerical rating in accordance with difficulty associated with the recovery of *Salmonella* spp. Warning and action limits were determined on the basis of the standard deviation calculated from participant scores.

Performance was satisfactory for most laboratories, with two under-performing. A follow-up ring trial was organised for under-performing laboratories, with one participant achieving the desired performance level. Performances of all laboratories approved by DAFF under the poultry regulations were satisfactory.

Scores obtained in the 2009 ring trial by each participating laboratory. Each participant is identified by the laboratory code. The warning and action limits are also displayed.





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## List of Abbreviations

|       |   |
|-------|---|
| ASAP  | Aes Laboratoire <i>Salmonella</i> Agar Plate        |
| ATCC  | American Typed Culture Collection                   |
| BGA   | Brilliant Green Agar                                |
| BPLS  | Brilliant-green Phenol-red Lactose Sucrose Agar     |
| BPW   | Buffered Peptone Water                              |
| CFU   | Colony Forming Unit(s)                              |
| CRL   | Community Reference Laboratory                      |
| DAFF  | Department of Agriculture, Fisheries and Food       |
| EU    | European Union                                      |
| FBO   | Food Business Operator                              |
| HE    | Hektoen Enteric Agar                                |
| ISO   | International Standard Organisation                 |
| MKTTn | Mueller Kauffmann Tetrathionate Novobiocin Broth    |
| MLCB  | Mannitol Lysine Crystal Violet Brilliant Green Agar |
| MS    | Member State  |
| MSRV  | Modified Semi-solid Rappaport Vassiliadis           |
| NA    | Nutrient Agar                                       |
| NRL   | National Reference Laboratory                       |
| RV    | Rappaport Vassiliadis Broth                         |
| RVS   | Rappaport Vassiliadis Soya Broth                    |
| SB    | <i>Salmonella</i> Barranquilla                      |
| SE    | <i>Salmonella</i> Enteritidis                       |
| S. I. | Statutory Instrument                                |
| ST    | <i>Salmonella</i> Typhimurium                       |
| XLD   | Xylose Lysine Deoxycholate Agar                     |

## 1. Introduction

*Salmonella* is the second most important cause of zoonotic infections with *Salmonella* Enteritidis (SE) and *Salmonella* Typhimurium (ST) most frequently associated with human illness in the European Union (EU; Anonymous, 2009, Voogt et al, 2002). Data analysis indicates that eggs and poultry meat are major sources of human *Salmonella* infection (Anonymous, 2007).

The Backweston Laboratory Complex hosts the National Reference Laboratory (NRL) functions for *Salmonella* in food, feed and animal health. In Ireland the National control programmes for *Salmonella* spp., in poultry breeding flocks, laying flocks and broiler meat flocks required under Regulation 2160/2003 have been implemented through S.I 706 of 2006, S.I. 247 of 2008 and SI 64 of 2009. Although EU legislation specifies a maximum prevalence of 1 % for both *S. Enteritidis* (SE) and *S. Typhimurium* (ST) in such flocks in MS the RoI has maintained virtual freedom from both serotypes in breeding and other flocks. Monitoring of the National targets is based on results of both official and food business operator (FBO) testing. FBO testing is conducted in private laboratories which must be approved by the Department of Agriculture, Fisheries and Food (DAFF). These laboratories must demonstrate competency in isolating *Salmonella* spp. and for this purpose participation in any national ring trial is a requirement.

The private testing laboratories that participated in this trial included laboratories approved under the aforementioned poultry regulations in addition to laboratories conducting *Salmonella* testing required under other programmes. Annex D of ISO 6579:2002, outlines the methodology that must be used by laboratories when testing samples for *Salmonella* spp. under the poultry regulations. Laboratories providing testing under other regulations utilise either ISO 6579:2002, the horizontal method for the detection of *Salmonella* spp. in food and animal feeding stuffs, or an approved in house methodology.

The aim of this ring trial was to assess the ability of laboratories to isolate *Salmonella* spp. in the presence of competitive flora.

## **2. Materials and Methods**

### **2.1 Culture Preparation**

#### **2.1.1 Preparation of *Salmonella* spp. and *Citrobacter freundii* Cultures**

Three *Salmonella* serotypes; namely *Salmonella* Typhimurium (ST), *Salmonella* Enteritidis (SE) and *Salmonella* Barranquilla (SB) at, low ( $1.6 \times 10^1$  CFU vial<sup>-1</sup>), medium ( $4.2 \times 10^1$  CFU vial<sup>-1</sup>) and high ( $1.16 \times 10^2$  CFU vial<sup>-1</sup>) concentrations, were utilised in this study. All were wild type strains isolated in the NRL. In addition, *Citrobacter freundii* (*C. freundii*; ATCC 6879), at a concentration of  $7.16 \times 10^2$  CFU vial<sup>-1</sup> was also utilised in this study.

A bacterial suspension adjusted to 0.5 McFarland was prepared and used to perform a 10 fold serial dilution in 18 ml of 0.85% sterile saline (Oxoid). The dilutions were enumerated on Nutrient agar (NA; Lab M) after incubation at 37°C for 24 h. Dilutions were stored at 4°C until required (maximum 24 h). An aliquot of the dilution, calculated to yield the required concentration of CFU ml<sup>-1</sup>, was transferred to lyophilisation buffer to yield a total volume of 180 ml. A 1 ml aliquot of the inoculated buffer was aseptically transferred to each sterile vial.

#### **2.1.2 Preparation of Competitive Flora**

Competitive flora was obtained from a 24h culture of a faecal sample in Buffered Peptone Water. The sample originated in a poultry house of *Salmonella* negative status. A 10 ml aliquot of the overnight culture was transferred to 230 ml of sterile lyophilisation buffer to yield a final concentration of  $2.9 \times 10^6$  CFU vial<sup>-1</sup>. A 1 ml aliquot of the inoculated buffer was aseptically transferred to each sterile vial.

### **2.2 Preparation of Sterile Samples**

A 1 ml aliquot of un-inoculated lyophilisation buffer was aseptically transferred to sterile vials.

### **2.3 Sample Storage, Labelling and Distribution**

Lyophilised vials were sealed under vacuum, foil capped with aluminium seal (Bio-sciences Ltd.) and placed in a labelled zip-lock bag. Each sample consisted of a pair of vials, divided into two lots (A and B) and labelled A1 to A15 and BI to B15. The labelled samples (n = 15; consisting of 30 labelled vials) were randomly assigned to

individual zip-lock bags, each constituting an individual batch of samples and stored at  $-20^{\circ}\text{C}$  prior to dispatch. Detail of sample content is listed in Table 1.

## **2.4 Quality Control**

### **2.4.1 Lyophilised Vials**

Vials ( $n = 10$ ) were randomly selected from each prepared batch and re-suspended in 1 ml of sterile distilled water at room temperature ( $20 - 25^{\circ}\text{C}$ ) for 10 min. Of these, replicate vials ( $n = 5$ ) were tested for the presence/absence of *Salmonella* spp. in accordance with ISO 6579:2002, Annex D, to determine the ability of lyophilised bacterial cells to recover and replicate to detectable numbers. An aliquot (500 or 100  $\mu\text{l}$ ) was removed from the remaining vials ( $n = 5$ ) and enumerated on NA after incubation at  $37^{\circ}\text{C}$  for 24 h to determine the number of organisms and the homogeneity of viable bacterial numbers per vial. Sterile vials were also tested to ensure sterility.

### **2.4.2 Lyophilised Ring Trial Samples**

Batches of ring trial samples ( $n = 3$ ) were randomly selected and tested for the presence/absence of *Salmonella* spp. in accordance with ISO 6579:2002, annex D, one week prior to dispatch.

## **2.5 Sample Dispatch**

Samples were dispatched to the participants ( $n = 24$ ) in week number 26 with instruction for testing to commence the following Monday.

## **2.6 Criteria for Acceptable Performances**

Each sample was scored in accordance with the difficulty in recovery of *Salmonella* spp., as outlined in Table 1. The standard deviation for participant scores was calculated. The warning and action limit was set at two and three times the standard deviation, respectively.

## **2.7 Stability of Stored Lyophilised Cultures**

Vials ( $n = 5$ ) were enumerated four months post storage, as described in section 2.4.1 above, to determine the stability and viability of recovered bacterial cells. The

stability of stored lyophilised samples was deemed satisfactory as counts were within  $0.5 \log_{10}$  CFU vial<sup>-1</sup> of pre storage counts.

## **2.8 Follow-up trial**

Samples (n = 15; stock from the previous trial stored at  $-20^{\circ}\text{C}$ ) as outlined in Table 2, were dispatched in week number 42 with instruction for testing to commence the following Monday. A limited number of laboratories (n = 2; laboratory codes 15 and 17) that under-performed in the original trial participated in this repeat trial.

The criteria required to pass was therefore deemed to be:

- No false positive samples, and
- A maximum of 3 false negative samples.

### 3. Results

Table 1 lists the content, *Salmonella* status and sample number of each sample included in this trial. All laboratories completed the trial and returned results before the submission deadline. All results were therefore included for analysis. Results submitted by each laboratory are listed in Table 3. All ST samples were correctly identified, however 23 of 72 SE and 11 of 72 SB were incorrectly identified. In brief, eleven participants correctly identified all samples, the remaining participants reported samples incorrectly as follows: one sample (n = 5), two samples (n = 2), three samples (n = 3), four samples (n = 1) and six samples (n = 2). Each participant was awarded a score based on the results submitted, as outlined in Table 1. A score of 72 was the maximum obtainable. The standard deviation was calculated to be 12.9 with warning and action limits set at of 46.2 and 33.3, respectively.

The score obtained by each participant is outlined in Figure 1. Two laboratories were deemed to have under-performed, both obtaining a score of 30, which was below the action limit. The two under-performing laboratories participated in a follow-up trial, details of which are listed in Table 2. Of these two participants, one was subsequently deemed satisfactory. The results are listed in Table 4.

Due to the frequency of false negative results associated with SE and SB in this trial a more detailed examination of the effectiveness of the media used by laboratories was undertaken. Table 5 shows the recovery of SB and SE in the various selective media utilised by laboratories in the samples supplied. Results show MSR/V to be the most effective selective media for recovery of both SE and SB in the presence of the competitive flora used and of *C. freundii*. Recovery of SE in the presence of competitive flora using RVS was poor. Figures 2 and 3 display the difference in recovery of SE and SB, respectively, on Xylose Lysine Deoxycholate (XLD) and Brilliant Green Agar (BGA) for each of the three selective enrichments investigated. The variation on ability to recover SE using different enrichment media can clearly be observed. Table 6 lists the media utilised by each ring trial participant.

#### 4. Discussion

In total, twenty-four laboratories completed the ring trial. Of these, two failed to achieve the minimum score and were therefore considered to have under-performed in the trial. All negative samples were correctly identified, indicating that confirmatory testing and therefore specificity of testing methodologies employed by laboratories are satisfactory.

The two under-performing laboratories were unable to recover *Salmonella* (all SE and SB samples) in the presence of competitive flora. Recovery of ST, a pure *Salmonella* culture containing low numbers in comparison to both SE and SB, was correctly reported for all three sample replicates. Isolation of *Salmonella* in the presence of competitive flora was therefore deemed a problem. These two laboratories participated in a follow-up trial, one of which under-performed again by identifying as negative four samples containing *Salmonella* spp. in the presence of competitive flora.

A recovery rate of 68% was recorded for SE in the presence of competitive flora with 47 of 72 positive samples correctly identified by participants. Investigation into the ability of three selective enrichment media identified RVS recovery of SE in the presence of competitive flora to be poor in comparison to recovery from MKTTn and MSR.V. RVS has been reported with poorer recovery of *Salmonella* from artificially contaminated samples than MSR.V (Raes et al, 2001). Voogt et al, 2001, has reported similar findings. Three participants (laboratories 7, 10 and 21) who utilised RV or RVS as the sole selective enrichment media correctly identified 1 of 9 replicate SE samples only. This accounted for 35% of incorrect SE results. It can be concluded, on the basis of these results, that two selective enrichment media be employed for the recovery of *Salmonella* as recommended by the ISO method or alternatively, MSR.V as recommended by Annex D.

The use of semisolid media such as MSR.V is reportedly more efficient in recovery of group D *Salmonella*, which include SE (Voogt et al, 2001). Interestingly, in this study 6 participants employing MSR.V, either alone or in combination with an additional enrichment media, reported 8 of 18 SE samples incorrectly. In addition, the two under-performing laboratories (laboratory codes 15 and 17) employed RV/RVS and MKTTn as recommended by the ISO protocol. Raes et al (2001)

concluded that while the type of enrichment media will impact on positive isolation, the laboratory using the media and the degree of familiarity with that media will also impact on results.

SB was not identified in 15% (11 of 72) of positive samples. The two under-performing laboratories accounted for 6 of 11 incorrect results with one other participant (laboratory code 13) accounting for an additional 3 incorrect results. The unusual *Salmonella* serotype may have been a factor for one participant (laboratory code 13) as only samples containing SB were incorrectly identified on that occasion. These findings are supported by Voogt et al, (2002) who reported the degree of difficulty relative to the level of contamination, presence of competitive flora in addition to the use of serotypes can adversely impact on ring-trial participant results.

There was no observed trend when the manufacturer of each media type utilised by participants was reviewed. Augustin et al, (2006) observed small differences in the counts recovered from media manufacturers for several selective culture media utilised by participants in a food microbiology proficiency-testing scheme, overall this variation was deemed insignificant. The source of the media did not appear to impact on result reported in this study.

A new sample matrix, lyophilised vials, was utilised for the preparation of cultures in this ring trial. Lyophilisation of bacterial cultures can produce stressed and damaged cells. Consequently, samples may be more challenging. This will depict a more accurate representation of natural sample conditions. Previous ring trials have utilised a charcoal swab format for the dispatch of samples, which was less challenging and introduced a time constraint for the preparation of test materials, sample quality control and the timely dispatch of samples.

Laboratories require DAFF approval to conduct testing under Regulation 2160/2003 on the control of *Salmonella* spp. in animal populations, and Regulation 1774/2002, on animal by-products not utilised for human consumption. Of the 24 laboratories that participated in this study, 19 are accredited by an independent quality system. Although all the participating laboratories in this ring trial also participate in commercial PT schemes, current and previous ring trials organised by the NRL

provide a range of challenging samples that mimic those submitted from farms. These ring trials were developed to mimic the challenging trials organised by the CRL for various NRL's in MS of the EU. The performance of all laboratories participating and those approved by DAFF for the national monitoring programmes in poultry show a high standard and quality of testing conducted by participating laboratories. However, the provision of these ring trials by the NRL on an annual basis is costly and time consuming. As the range of PT schemes is now increasing it may be more possible to utilise some of them as an ongoing means of monitoring the quality of testing undertaken in approved private laboratories.

## **5. Conclusions**

Ring trial performance of all laboratories approved by DAFF under the poultry regulations was satisfactory.

## **6. Acknowledgement**

The cooperation of Dr Nola Leonard, UCD is gratefully acknowledged.

## **References:**

Anonymous, 2007. Trends and sources of zoonoses and zoonotic agents in the European Union in 2007. The EFSA Journal, 130, 23 – 352.

Anonymous, 2009. Trends and sources of zoonoses and zoonotic agents in the European Union in 2009. The EFSA Journal, 223, 3 – 320.

Augustin, J. C. and Carlier, V., 2006. Lessons from the organisation of a proficiency testing program in food microbiology by interlaboratory comparison: analytical methods in use, impact of methods on bacterial counts and measurement of uncertainty of bacterial counts. Food Microbiology, 23, 1 – 38.

Raes, M., Nagelkerke, N. and Henken, A. M., 2001. Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms. RIVM report 284500018.

Voogt, N., Nagelkerke, N. J. D., van de Giessen, A. W. and Henken, A. M., 2002. Differences between reference laboratories of the European Community in their

ability to detect *Salmonella* species. *European Journal of Clinical Microbiological Infectious Disease*, 21, 449 – 454.

Voogt, N., Raes, M., Wannet, W. J. B., Henken, A. M. and van de Giessen, A. W., 2001. Comparison of selective enrichment media for the detection of *Salmonella* in poultry faeces. *Letters in Applied Microbiology*, 32, 89 – 92.

Voogt, N., in't Veld, P. H., Nagelkerke, N. and Henken, A. M., 1997. Bacteriological detection of *Salmonella* in the presence of competitive micro-organisms. RIVM report 284500007

**Table 1:** The content and *Salmonella* status of each sample selected for inclusion in the 2009 ring trial. The score for a correct and incorrect result are also listed.

| Sample number | Content Vial A   | Content Vial B                          | <i>Salmonella</i> status | Score if correct | Score if incorrect |
|---------------|--|---|--------------------------|------------------|--------------------|
| 1             | <i>Salmonella</i> Enteritidis @ $1.16 \times 10^2$ CFU | Competitive flora $2.9 \times 10^6$ CFU | Present                  | 6                | 0                  |
| 2             | <i>Citrobacter freundii</i> @ $7.16 \times 10^2$ CFU   | Competitive flora $2.9 \times 10^6$ CFU | Absent                   | 4                | -4                 |
| 3             | <i>Salmonella</i> Typhimurium @ $1.6 \times 10^1$ CFU  | Sterile                                 | Present                  | 4                | 0                  |
| 4             | <i>Salmonella</i> Barranquilla @ $4.2 \times 10^1$ CFU | Competitive flora $2.9 \times 10^6$ CFU | Present                  | 8                | 0                  |
| 5             | Sterile  | Sterile                                 | Absent                   | 2                | -8                 |
| 6             | <i>Salmonella</i> Typhimurium @ $1.6 \times 10^1$ CFU  | Sterile                                 | Present                  | 4                | 0                  |
| 7             | <i>Citrobacter freundii</i> @ $7.16 \times 10^2$ CFU   | Competitive flora $2.9 \times 10^6$ CFU | Absent                   | 4                | -4                 |
| 8             | <i>Citrobacter freundii</i> @ $7.16 \times 10^2$ CFU   | Competitive flora $2.9 \times 10^6$ CFU | Absent                   | 4                | -4                 |
| 9             | <i>Salmonella</i> Enteritidis @ $1.16 \times 10^2$ CFU | Competitive flora $2.9 \times 10^6$ CFU | Present                  | 6                | 0                  |
| 10            | <i>Salmonella</i> Barranquilla @ $4.2 \times 10^1$ CFU | Competitive flora $2.9 \times 10^6$ CFU | Present                  | 8                | 0                  |
| 11            | Sterile  | Sterile                                 | Absent                   | 2                | -8                 |
| 12            | <i>Salmonella</i> Barranquilla @ $4.2 \times 10^1$ CFU | Competitive flora $2.9 \times 10^6$ CFU | Present                  | 8                | 0                  |
| 13            | <i>Salmonella</i> Enteritidis @ $1.16 \times 10^2$ CFU | Competitive flora $2.9 \times 10^6$ CFU | Present                  | 6                | 0                  |
| 14            | <i>Salmonella</i> Typhimurium @ $1.6 \times 10^1$ CFU  | Sterile                                 | Present                  | 4                | 0                  |
| 15            | Sterile  | Sterile                                 | Absent                   | 2                | -8                 |

**Table 2:** The follow-up ring trial sample content and *Salmonella* status.

| Sample number | Content Vial A   | Content Vial B                                       | <i>Salmonella</i> status |
|---------------|--|--|--------------------------|
| 1             | <i>Salmonella</i> Enteritidis @ $6.9 \times 10^1$ CFU  | Sterile  | Positive                 |
| 2             | Sterile  | Sterile  | Negative                 |
| 3             | Competitive flora @ $4.46 \times 10^6$ CFU             | Sterile  | Negative                 |
| 4             | <i>Salmonella</i> Enteritidis @ $6.9 \times 10^1$ CFU  | Competitive flora @ $4.46 \times 10^6$ CFU           | Positive                 |
| 5             | <i>Salmonella</i> Typhimurium @ $3.2 \times 10^1$ CFU  | <i>Citrobacter freundii</i> @ $2.35 \times 10^2$ CFU | Positive                 |
| 6             | <i>Salmonella</i> Barranquilla @ $4.3 \times 10^1$ CFU | Sterile  | Positive                 |
| 7             | <i>Salmonella</i> Enteritidis @ $6.9 \times 10^1$ CFU  | <i>Citrobacter freundii</i> @ $2.35 \times 10^2$ CFU | Positive                 |
| 8             | <i>Salmonella</i> Typhimurium @ $3.2 \times 10^1$ CFU  | Competitive flora @ $4.46 \times 10^6$ CFU           | Positive                 |
| 9             | Sterile  | Sterile  | Negative                 |
| 10            | <i>Salmonella</i> Enteritidis @ $6.9 \times 10^1$ CFU  | Competitive flora @ $4.46 \times 10^6$ CFU           | Positive                 |
| 11            | <i>Salmonella</i> Typhimurium @ $3.2 \times 10^1$ CFU  | Competitive flora @ $4.46 \times 10^6$ CFU           | Positive                 |
| 12            | <i>Salmonella</i> Enteritidis @ $6.9 \times 10^1$ CFU  | <i>Citrobacter freundii</i> @ $2.35 \times 10^2$ CFU | Positive                 |
| 13            | Competitive flora @ $4.46 \times 10^6$ CFU             | Sterile  | Negative                 |
| 14            | <i>Salmonella</i> Barranquilla @ $4.3 \times 10^1$ CFU | Competitive flora @ $4.46 \times 10^6$ CFU           | Positive                 |
| 15            | <i>Salmonella</i> Typhimurium @ $3.2 \times 10^1$ CFU  | Sterile  | Positive                 |

**Table 3:** The results reported by each participant in the 2009 ring trial; sample number, content and expected result is also listed. Each participant is identified by the laboratory code.

| Sample number | Sample content | Result          | 1        | 2        | 3        | 4        | 5               | 6               | 7               | 8               | 9               | 10              | 13              | 14              |
|---------------|----------------|-----------------|----------|----------|----------|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1             | SE             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | Positive        | Positive        | Positive        | <b>Negative</b> | Positive        | <b>Negative</b> |
| 2             | C. freundii    | <b>Negative</b> | Negative | Negative | Negative | Negative | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        |
| 3             | ST             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | Presumptive     |
| 4             | SB             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | <b>Negative</b> | Presumptive     |
| 5             | Sterile        | <b>Negative</b> | Negative | Negative | Negative | Negative | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        |
| 6             | ST             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | Presumptive     |
| 7             | C. freundii    | <b>Negative</b> | Negative | Negative | Negative | Negative | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        |
| 8             | C. freundii    | <b>Negative</b> | Negative | Negative | Negative | Negative | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        |
| 9             | SE             | <b>Positive</b> | Positive | Positive | Positive | Positive | <b>Negative</b> | <b>Negative</b> | <b>Negative</b> | Positive        | Positive        | <b>Negative</b> | Positive        | <b>Negative</b> |
| 10            | SB             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | <b>Negative</b> | Presumptive     |
| 11            | Sterile        | <b>Negative</b> | Negative | Negative | Negative | Negative | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        |
| 12            | SB             | <b>Positive</b> | Positive | Positive | Positive | Positive | <b>Negative</b> | Positive        | Positive        | Positive        | Positive        | <b>Negative</b> | <b>Negative</b> | Presumptive     |
| 13            | SE             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | <b>Negative</b> | <b>Negative</b> | <b>Negative</b> | <b>Negative</b> | Positive        | <b>Negative</b> |
| 14            | ST             | <b>Positive</b> | Positive | Positive | Positive | Positive | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | Positive        | Presumptive     |
| 15            | Sterile        | <b>Negative</b> | Negative | Negative | Negative | Negative | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        | Negative        |

**Table 3 continued:** The results reported by each participant in the 2009 ring trial; sample number, content and expected result is also listed. Each participant is identified by the laboratory code. Incorrect results are underlined and highlighted in red.

| Sample number | Sample content | Result   | 15              | 16       | 17              | 19              | 20       | 21              | 22              | 23       | 24       | 25       | 26       | 27       |
|---------------|----------------|----------|-----------------|----------|-----------------|-----------------|----------|-----------------|-----------------|----------|----------|----------|----------|----------|
| 1             | SE             | Positive | <u>Negative</u> | Positive | <u>Negative</u> | <u>Negative</u> | Positive | <u>Negative</u> | <u>Negative</u> | Positive | Positive | Positive | Positive | Positive |
| 2             | C. freundii    | Negative | Negative        | Negative | Negative        | Negative        | Negative | Negative        | Negative        | Negative | Negative | Negative | Negative | Negative |
| 3             | ST             | Positive | Positive        | Positive | Positive        | Positive        | Positive | Positive        | Positive        | Positive | Positive | Positive | Positive | Positive |
| 4             | SB             | Positive | <u>Negative</u> | Positive | <u>Negative</u> | Positive        | Positive | Positive        | Positive        | Positive | Positive | Positive | Positive | Positive |
| 5             | Sterile        | Negative | Negative        | Negative | Negative        | Negative        | Negative | Negative        | Negative        | Negative | Negative | Negative | Negative | Negative |
| 6             | ST             | Positive | Positive        | Positive | Positive        | Positive        | Positive | Positive        | Positive        | Positive | Positive | Positive | Positive | Positive |
| 7             | C. freundii    | Negative | Negative        | Negative | Negative        | Negative        | Negative | Negative        | Negative        | Negative | Negative | Negative | Negative | Negative |
| 8             | C. freundii    | Negative | Negative        | Negative | Negative        | Negative        | Negative | Negative        | Negative        | Negative | Negative | Negative | Negative | Negative |
| 9             | SE             | Positive | <u>Negative</u> | Positive | <u>Negative</u> | Positive        | Positive | <u>Negative</u> | Positive        | Positive | Positive | Positive | Positive | Positive |
| 10            | SB             | Positive | <u>Negative</u> | Positive | <u>Negative</u> | Positive        | Positive | Positive        | Positive        | Positive | Positive | Positive | Positive | Positive |
| 11            | Sterile        | Negative | Negative        | Negative | Negative        | Negative        | Negative | Negative        | Negative        | Negative | Negative | Negative | Negative | Negative |
| 12            | SB             | Positive | <u>Negative</u> | Positive | <u>Negative</u> | Positive        | Positive | Positive        | Positive        | Positive | Positive | Positive | Positive | Positive |
| 13            | SE             | Positive | <u>Negative</u> | Positive | <u>Negative</u> | Positive        | Positive | <u>Negative</u> | Positive        | Positive | Positive | Positive | Positive | Positive |
| 14            | ST             | Positive | Positive        | Positive | Positive        | Positive        | Positive | Positive        | Positive        | Positive | Positive | Positive | Positive | Positive |
| 15            | Sterile        | Negative | Negative        | Negative | Negative        | Negative        | Negative | Negative        | Negative        | Negative | Negative | Negative | Negative | Negative |

**Table 4:** The 2009 follow-up ring trial participant results. The laboratory code, submitted sample result and overall status for each participant is listed. Incorrect results are highlighted in red.

| Sample number | Sample content | Result   | 15              | 17              |
|---------------|----------------|----------|-----------------|-----------------|
| 1             | SE             | Positive | Positive        | Positive        |
| 2             | sterile        | Negative | Negative        | Negative        |
| 3             | CF             | Negative | Negative        | Negative        |
| 4             | SE             | Positive | <u>Negative</u> | Positive        |
| 5             | ST             | Positive | Positive        | Positive        |
| 6             | SB             | Positive | Positive        | Positive        |
| 7             | SE             | Positive | Positive        | Positive        |
| 8             | ST             | Positive | <u>Negative</u> | Positive        |
| 9             | sterile        | Negative | Negative        | Negative        |
| 10            | SE             | Positive | <u>Negative</u> | Positive        |
| 11            | ST             | Positive | <u>Negative</u> | <u>Negative</u> |
| 12            | SE             | Positive | Positive        | <u>Negative</u> |
| 13            | CF             | Negative | Negative        | Negative        |
| 14            | SB             | Positive | Positive        | Positive        |
| 15            | ST             | Positive | Positive        | <u>Negative</u> |
| Ring Trial    |                |          |                 |                 |
| Result        |                |          | <b>Fail</b>     | Pass            |

**Key:** SE – *Salmonella* Enteritidis; ST – *Salmonella* Typhimurium; SB – *Salmonella* Barranquilla; CF – competitive flora.

**Table 5:** Recovery of SE and SB in the presence of competitive flora and *C. freundii* using RVS, MKTTn and MSRV.

| Content                 | Replicate | RVS |     | MKTTn |     | MSRV |     |
|-------------------------|-----------|-----|-----|-------|-----|------|-----|
|                         |           | XLD | BGA | XLD   | BGA | XLD  | BGA |
| SB + CF1                | a         | 2   | 2   | 4     | 3   | 5    | 5   |
| SB + CF1                | b         | 3   | 2   | 3     | 3   | 5    | 5   |
| SB + CF2                | a         | 3   | 2   | 3     | 3   | 4    | 4   |
| SB + CF2                | b         | 3   | 3   | 3     | 3   | 4    | 4   |
| SB + <i>C. freundii</i> | a         | 4   | 4   | 4     | 4   | 5    | 5   |
| SB + <i>C. freundii</i> | b         | 4   | 3   | 3     | 3   | 5    | 5   |
| SB + <i>C. freundii</i> | c         | 3   | 3   | 3     | 3   | 5    | 5   |
| SE + CF1                | a         | 1   | 1   | 3     | 3   | 5    | 5   |
| SE + CF1                | b         | 1   | 1   | 3     | 3   | 5    | 5   |
| SE + CF2                | a         | 1   | 0   | 3     | 3   | 4    | 4   |
| SE + CF2                | b         | 1   | 0   | 3     | 3   | 5    | 4   |
| SE + <i>C. freundii</i> | a         | 3   | 3   | 3     | 3   | 5    | 5   |
| SE + <i>C. freundii</i> | b         | 3   | 3   | 3     | 3   | 5    | 5   |
| SE + <i>C. freundii</i> | c         | 3   | 3   | 3     | 4   | 5    | 5   |

Key: SE – *Salmonella* Enteritidis ( $6.9 \times 10^1$  CFU); SB – *Salmonella* Baranquilla ( $4.3 \times 10^1$  CFU); CF1 – competitive flora ( $4.46 \times 10^6$  CFU); CF2 – competitive flora ( $2.23 \times 10^6$  CFU); *C. freundii* – *Citrobacter freundii* ( $2.35 \times 10^2$  CFU).

0 = No typical colonies present

1 = few colonies on one plate

2 = abundant colonies on one plate, mostly isolated

3 = confluent growth on one plate, few isolated colonies on second plate

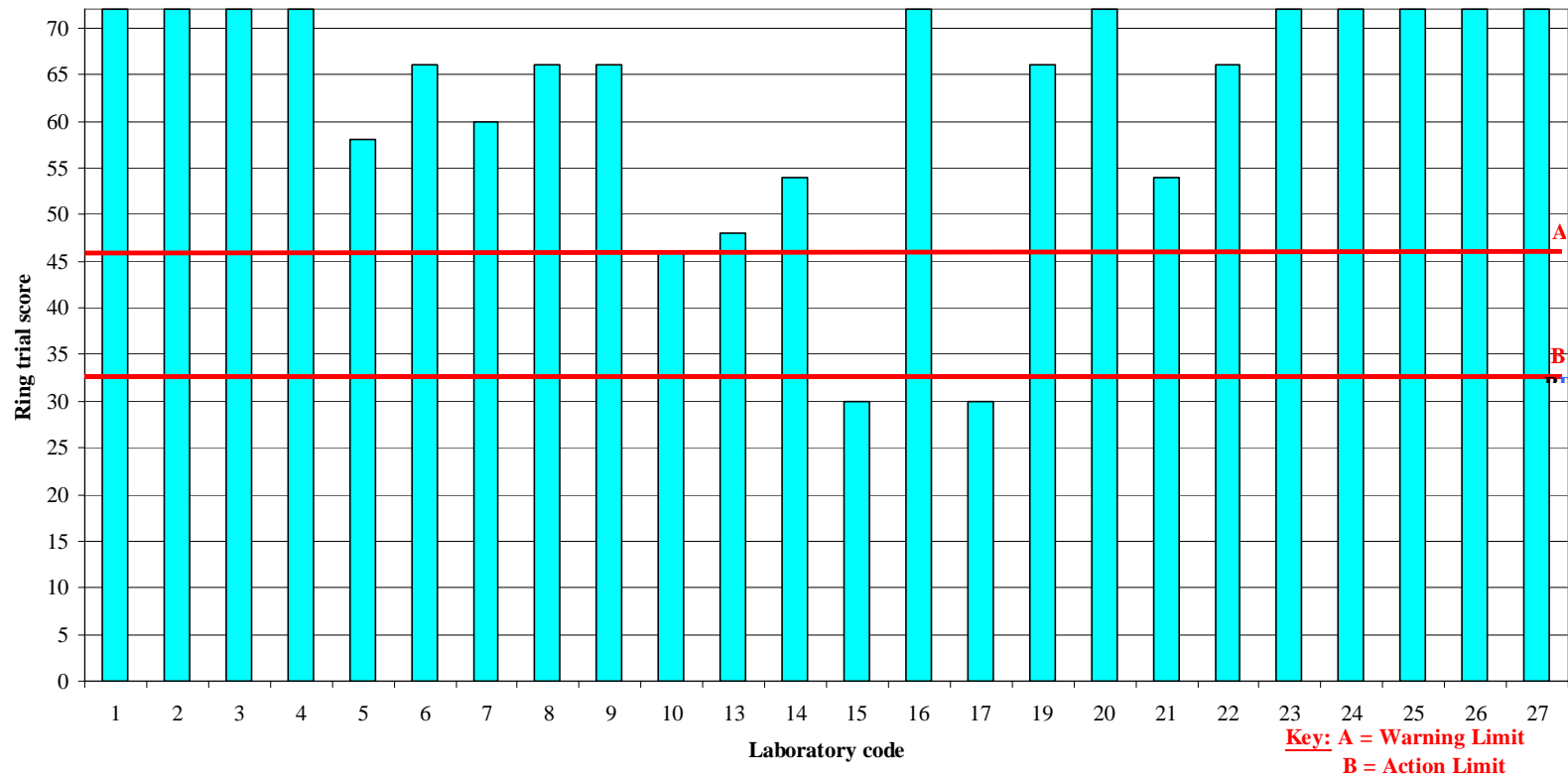
4 = confluent growth on one plate, abundant colonies on second plate

5 = confluent growth on two plates

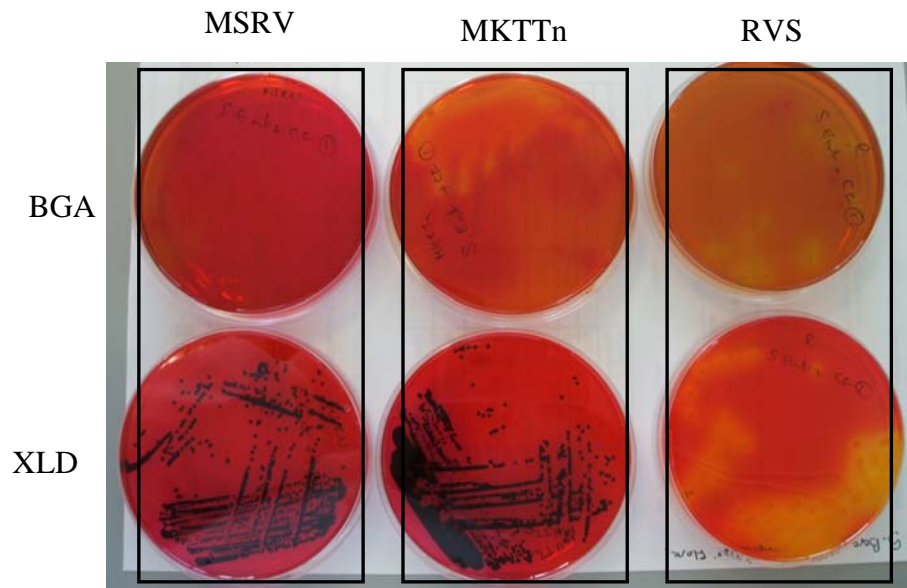
**Table 6:** The media employed by each participant for selective enrichment and plating. Each participant is identified by the unique laboratory code.

| Laboratory code | Selective Enrichment 1 | Selective Enrichment 2 | Plating Media 1 | Plating Media 2 |
|-----------------|------------------------|------------------------|-----------------|-----------------|
| 1               | MKTTn                  | RV                     | XLD             | BGA             |
| 2               | MSRV                   |                        | ASAP            | XLD             |
| 3               | Tetrathionate          | RV                     | XLD             | BGA             |
| 4               | MKTTn                  | RVS                    | XLD             | BGA             |
| 5               | RVS                    | MKTTn                  | BGA             | XLD             |
| 6               | MKTTn                  | RVS                    | XLD             | HE              |
| 7               | RVS                    |                        | XLD             | BGA             |
| 8               | MKTTn                  | RV                     | XLD             | BGA             |
| 9               | MSRV                   | MKTTn                  | XLD             | BPLS            |
| 10              | RV                     |                        | XLD             | BGA             |
| 13              | MKTTn                  | RVS                    | XLD             | BGA             |
| 14              | MSRV                   |                        | XLD             | BGA             |
| 15              | RVS                    | MKTTn                  | XLD             | mBGA            |
| 16              | MKTTn                  | RV                     | XLD             | SABC            |
| 17              | RV                     | MKTTn                  | XLD             | BGA             |
| 19              | RVS                    | MSRV                   | XLD             | BGA             |
| 20              | RV                     | MKTTn                  | XLD             | MLCB            |
| 21              | MSRV                   | RV                     | BGA             | XLD             |
| 22              | RVS                    | MKTTn                  | XLD             | BPLS            |
| 23              | MSRV                   | MKTTn / RVS            | XLD             | BGA             |
| 24              | RVS                    | MKTTn                  | XLD             | mBGA            |
| 25              | RVS                    | MKTTn                  | XLD             | BGA             |
| 26              | RV                     | MKTTn                  | XLD             | MLCB            |
| 27              | RV                     | MKTTn                  | BGA             | XLD             |

**Figure 1:** The score obtained in the 2009 ring trial by each participating laboratory. Each participant is identified by the laboratory code. The warning and action limits are also displayed.



**Figure 2:** Isolation of SE in the presence of competitive flora on BGA and XLD from each selective enrichment media investigated, namely, MSR/V, MKTTn and RVS.



**Figure 3:** Isolation of SB in the presence of competitive flora on BGA and XLD from each selective enrichment media investigated, namely, MSRV, MKTTn and RVS.

