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EU Interlaboratory comparison study animal feed I (2008)

Bacteriological detection of *Salmonella* in chicken feed

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A.F.A. Kuijpers
C. Veenman
J. van de Kassteele
K.A. Mooijman

Contact:
A.F.A. Kuijpers
Laboratory of Zoonoses and Environmental Microbiology (LZO)
angelina.kuijpers@rivm.nl

This investigation has been performed by order and for the account of European Commission, Health and Consumer Protection Directorate-General and the Laboratory for Zoonoses and the Environmental Microbiology (LZO) of the RIVM, within the framework of V/330604/08/CS by the Community Reference Laboratory for *Salmonella*

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Abstract

EU Interlaboratory comparison study animal feed I (2008)
Bacteriological detection of *Salmonella* in chicken feed

In 2008, from the 30 National Reference Laboratories (NRLs) in the European Union, 29 were able to detect high and low levels of *Salmonella* in chicken feed. They achieved the desired outcome at once. One laboratory was unable to produce satisfactory results neither in the follow-up test. The reasons for their failures are currently being investigated. These results were shown in the first interlaboratory comparison study on animal feed, organized by the Community Reference Laboratory (CRL) for *Salmonella*. The comparison study was conducted in October 2008, with the follow up in March 2009. The NRLs responsible for *Salmonella* detection from all European Member States are obliged to participate in this study. The CRL for *Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

During the study, three methods for detecting *Salmonella* in chicken feed were used. Two methods are standardised at international level for the detection of *Salmonella* in animal feed. One of those two methods was least effective as only 92 percent of the samples were found to be positive. The third, internationally prescribed method for the detection of *Salmonella* in veterinary samples was not obligatory but requested by the CRL. This method gave the best results with 99 percent being positive.

To perform the test, the laboratories had to follow the given instructions. Each laboratory received a package containing chicken feed and 35 gelatin capsules containing *Salmonella* spp. at different levels. The laboratories were instructed to spike the chicken feed with the capsules and test the samples for the presence of *Salmonella*.

Key words: *Salmonella*; CRL-*Salmonella*; NRL-*Salmonella*; interlaboratory comparison study; chicken feed; *Salmonella* detection methods

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Rapport in het kort

EU Ringonderzoek dierenvoeder-I (2008)
Bacteriologische detectie van *Salmonella* in kippenvoer

In 2008 waren van de 30 Nationale Referentie Laboratoria (NRLs) in de Europese Unie er 29 in staat hoge en lage concentraties van de *Salmonella*bacterie in kippenvoer aan te tonen. Zij behaalden direct het gewenste niveau. Een laboratorium kon ook tijdens de herkansing niet voldoende presteren. De oorzaken van hun fouten worden nog nader uitgezocht. Dit blijkt uit het eerste ringonderzoek dat het Communautair Referentie Laboratorium (CRL) voor dierenvoeders heeft georganiseerd. Het onderzoek is in oktober 2008 gehouden, de herkansing was in maart 2009. Alle NRL's verantwoordelijk voor *Salmonella*detectie van de Europese lidstaten zijn verplicht om aan dit onderzoek deel te nemen. Het CRL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Tijdens de studie zijn drie analysemethodes gebruikt om de *Salmonella*bacterie in kippenvoer aan te tonen. Twee daarvan zijn internationaal gestandardiseerde methoden voor *Salmonella*detectie in dierenvoeders. Hiervan bleek er één niet de meest effectieve methode te zijn omdat slechts in 92 procent van de monsters *Salmonella* werd aangetroffen. De derde, de internationaal voorgeschreven methode om *Salmonella* in dierlijke mest aan te tonen, is niet verplicht maar is op verzoek van het CRL uitgevoerd. Deze methode behaalde het beste resultaat met 99 procent positieven.

De laboratoria moeten de test volgens voorschrift uitvoeren. Elk laboratorium kreeg een pakket toegestuurd met kippenvoer en 35 gelatinecapsules met melkpoeder dat verschillende besmettingsniveaus *Salmonella* bevatte. De laboratoria moesten vervolgens het kippenvoer en de capsules samenvoegen en onderzoeken of er *Salmonella* in aanwezig was.

Trefwoorden: *Salmonella*; CRL-*Salmonella*; NRL-*Salmonella*; ringonderzoek; kippenvoer; *Salmonella*detectiemethode

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List of abbreviations

BGA (mod)	Brilliant Green Agar (modified)
BPLSA	Brilliant Green Phenol-Red Lactose Sucrose Agar
BPW	Buffered Peptone Water
cfp	colony forming particles
CRL	Community Reference Laboratory
dPCA	double concentrated Plate Count Agar
dVRBG	double concentrated Violet Red Bile Glucose agar
ELISA	Enzyme-Linked Immuno Sorbent Assay
hcmp	highly contaminated milk powder
ISO	International Standardisation Organisation
LDC	Lysine DeCarboxylase
MKTTn	Mueller Kauffmann TetraThionate novobiocin broth
MLCB	Mannitol Lysine Crystal violet Brilliant green agar
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
OR	Odds Ratio
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RM	Reference Material
RVS	Rappaport Vassiliadis Soya broth
SE	*Salmonella* Enteritidis
SM2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar
XLT4	Xylose Lysine Tergitol 4 agar

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Summary

In October 2008 the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organised the first interlaboratory comparison study on bacteriological detection of *Salmonella* in an animal feed matrix (chicken feed). Participants were thirty National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*) of the EU Member States and of Norway and candidate country Former Yugoslav Republic of Macedonia (FYROM).

The first and most important objective of the study, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in an animal feed matrix. To do so, chicken feed samples of 25 g each, were analysed in the presence of reference materials (capsules) containing either *Salmonella* (at various contamination levels) or sterile milk powder. A proposal for good performance was made and the performance of the laboratories was compared to this proposal. In addition to the performance testing of the laboratories, a comparison was made between the prescribed methods (ISO 6579, 2002) and the requested method (Annex D of ISO 6579, 2007). For the prescribed method, the selective enrichment media were Rappaport Vassiliadis Soya broth (RVS) and Mueller Kauffmann Tetrathionate novobiocin broth (MKTTn). For the requested method the selective enrichment was Modified Semi-solid Rappaport Vassiliadis (MSRV) agar. Optionally a laboratory could also use other, own media or procedures for the detection of *Salmonella*.

Thirty-five individually numbered capsules had to be tested by the participants for the presence or absence of *Salmonella.* Twenty-five of the capsules had to be examined in combination with each 25 gram of *Salmonella* negative chicken feed. These 25 capsules were divided over the following groups: 5 capsules contained approximately 5 colony forming particles (cfp) of *Salmonella* Typhimurium (STM5), 5 capsules contained approximately 50 cfp of *S.* Typhimurium (STM50), 5 capsules contained approximately 20 cfp of *S.* Enteritidis (SE20), 5 capsules contained approximately 100 cfp of *S.* Enteritidis (SE100) and 5 blank capsules. The other 10 capsules, to which no feed had to be added, were control samples, existing of 3 capsules STM5, 2 capsules SE20, 1 capsule SE100, 2 capsules containing approximately 5 cfp of *S.* Panama (SPan5) and 2 blank capsules.

On average the laboratories found *Salmonella* in only 92 % of the (contaminated) samples when using selective enrichment in MKTTn (prescribed food method). The method for testing veterinary samples (MSRV) gave the best results with 99 % of the positive samples, very closely followed by the other food method (RVS).

Twenty-eight out of 30 laboratories achieved the level of good performance for at least one of the prescribed methods (MKTTn or RVS). One NRL achieved the level of good performance when also the requested food method (MSRV) was taken into account. One NRL was unable to reach the level of good performance, neither in the follow up test. The reasons for their failures are currently being investigated.

1 Introduction

An important task of the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*), as laid down in the Commission Regulation EC No 882/2004, is the organisation of interlaboratory comparison studies. The history of the interlaboratory comparison studies on the detection of *Salmonella*, as organised by CRL-*Salmonella* since 1995 is summarised in Annex 1. In earlier ringtrials the detection of *Salmonella* spp. in veterinary and food samples were studied, while this was the first study for the detection of *Salmonella* spp. in animal feed. The organisation of an interlaboratory comparison study on animal feed was discussed with the NRLs for *Salmonella* at the annual CRL-*Salmonella* workshop in May 2008 (Mooijman, 2008). The first and most important objective of the study, organized by the Community Reference Laboratory (CRL) for *Salmonella* in October 2008, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in an animal feed matrix. This information is important to know whether the examination of samples in the EU Member States is carried out uniformly and comparable results can be obtained by all National Reference Laboratories for *Salmonella* (NRL-*Salmonella*). The second objective was to compare the different methods for the detection of *Salmonella* in chicken feed.

The prescribed method for detection of *Salmonella* in a feed matrix is ISO 6579 (Anonymous, 2002). However, it was expected that selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) could also be used as on alternative medium (Annex D of ISO 6579, Anonymous, 2007). Therefore, the participating laboratories were requested also to use MSRV for testing the chicken feed.

The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary and food samples. The contamination level of the low level capsules was just above the detection limit of the method; the level of the high level samples approximately 5-10 times above the detection limit. Ten control samples consisting of different reference materials, had to be tested without the addition of chicken feed. These reference materials consisted of 3 capsules containing approximately 5 cfp of *Salmonella* Typhimurium (STM5), 2 capsules containing approximately 20 cfp of *Salmonella* Enteritidis (SE20), 1 capsule with approximately 100 cfp of *Salmonella* Enteritidis (SE100), 2 capsules containing approximately 5 cfp of *Salmonella* Panama (SPan5) and 2 blank capsules. Twenty-five samples of *Salmonella* negative chicken feed (25 g each) spiked with 5 different reference materials (including blank capsules) had to be examined. The different reference materials consisted of two levels of *Salmonella* Typhimurium (STM5 and STM50) and two levels of *Salmonella* Enteritidis (SE20 and SE100).

2 Participation

Country	City	Institute
Austria	Linz	Austrian Agency for Health and Food Safety (AGES) Zentrum für Analytik und Mikrobiologie
Belgium	Brussels	Veterinary and Agrochemical Research Center (VAR/CODA)
Bulgaria	Sophia	National Diagnostic and Research Veterinary Institute
Cyprus	Nicosia	Ministry of Agriculture, Natural Resources and Environment Veterinary Services Laboratory for the Control of Foods of Animal Origin (LCFAO)
Czech Republic	Prague	State Veterinary Institute
Denmark	Lyngby	The Danish Plant Directorate Laboratory for diagnostics in Plants, Seed and Fodder
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Helsinki	Finnish Food Safety Authority Evira Research Department, Microbiology Unit
France	Ploufragan	L'Agence Française de Sécurité Sanitaire des Aliments (AFSSA)
Germany	Berlin	Federal Institute for Risk Assessment (BFR) National Reference Laboratory for *Salmonella*
Greece	Halkis	Veterinary Laboratory of Halkis Hellenic Republic Ministry of rural development and food
Hungary	Budapest	Central Agricultural Office, Food and Feed Safety Directorate Food Microbiological Diagnostic Laboratory
Ireland	Kildare	Central Veterinary Research Laboratory CVRL / DAF Department of Agriculture and Food
Italy	Legnaro (PD)	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	National Diagnostic Centre (NDC)
Lithuania	Vilnius	Lithuania National Food and Veterinary Risk Assessment Institute
Luxembourg	Ettelbruck	Administration des services techniques de l'agriculture Division des laboratoires de contrôle et d'essais
Macedonia FYROM Former Yugoslav Republic of Macedonia	Skopje	Faculty of veterinary medicine
Malta	Valletta	Public Health Laboratory (PHL) Evans Buildings Dept.
Netherlands the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib) Centre for Infectious Diseases Control Laboratory for Zoonoses and Environmental Microbiology (LZO)
Norway	Oslo	National Veterinary Institute, Section of Bacteriology

Country	City	Institute
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Hygiene of Animal Feeding Stuffs
Portugal	Lisbon	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
United Kingdom	Addlestone	Veterinary Laboratories Agency (VLA) Weybridge Dept. of Food and Environmental Safety
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Reference materials

Five batches of *Salmonella* reference materials were prepared. For this purpose milk, artificially contaminated with a *Salmonella* strain was spray-dried (In 't Veld et al., 1996). The obtained highly contaminated milk powder (hcmp) was mixed with sterile (γ-irradiated) milk powder (Carnation, Nestlé, the Netherlands) to obtain the desired contamination level. The mixed powder was filled in to gelatin capsules resulting in the final reference materials (RMs).
The target levels of the five batches of RMs were:

- 5 colony forming particles (cfp) per capsule for *Salmonella* Panama (SPan5);
- 5 and 50 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM5 and STM50);
- 20 and 100 colony forming particles (cfp) per capsule for *Salmonella* Enteritidis (SE20 and SE100).

Before filling all mixed powders into gelatin capsules, test batches of 60 capsules were prepared of each mixture to determine the mean number of cfp per capsule and the homogeneity of the mixture. The remaining mixed powders were stored at –20 ^{o}C. If the test batches fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were completely filled into gelatin capsules and stored at –20 ^{o}C.
The pre-set criteria were:

- mean contamination levels should lie between target level minus 30 % and target level plus 50 % (e.g. between 70 and 150 cfp if the target level is 100 cfp);
- for the homogeneity within one batch of capsules the maximum demand for the variation between capsules should be $T_2/(I-1) \leq 2$, where T_2 is a measure for the variation between capsules of one batch (see formula in Annex 2) and I is the number of capsules.

The contamination levels of the capsules were determined following the procedure as described by Schulten et al. (2000). In short the procedure is as follows:

- reconstitution of each capsule in 5 ml peptone saline solution in a Petri dish at (38.5 ± 1) ^{o}C for (45 ± 5) min;
- repair of *Salmonella* by the addition of 5 ml molten double concentrated plate count agar (dPCA) to the reconstituted capsule solution, and after solidification incubation at (37 ± 1) ^{o}C for (4 ± ½) h;
- after incubation, 10 ml of molten double concentrated Violet Red Bile Glucose agar (dVRBG) was added as an overlayer and after solidification the plates were incubated at (37 ± 1) ^{o}C for (20 ± 2) h.

3.2 Chicken feed samples

3.2.1 General

Before deciding on the type of chicken feed to be used in the ringtrial, the following feed types were tested for the absence of *Salmonella* and the amount of background flora as described in sections 3.2.2 and 3.2.3: scratching chicken feed: natural mixed grains (with crushed corn and sunflower), fine structured natural mixed grain and grain of compressed meal. All feed samples were tested negative for *Salmonella* but showed 10 to 100 times differences in the amount of background flora (cfu/g). Finally it

was decided to use the feed with the highest amount of background flora for the interlaboratory comparison study this was the premium poultry and waterfowl feed consisting of mixed grains (HAVENS).
Twenty five kilogram of chicken feed was bought in a shop for animals (Maarssen, the Netherlands) on 8 September 2008. The feed was tested for the absence of *Salmonella* following the procedure as described in Annex D of ISO 6579 (Anonymous, 2007). For this purpose 10 portions of 25 g were each added to 225 ml Buffered Peptone Water (BPW). After pre-enrichment at (37 ± 1) ^{o}C for 16-18 h, selective enrichment was carried out on Modified Semi-solid Rappaport Vassiliadis (MSRV). Next, the suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA) and confirmed biochemically. The chicken feed was stored at room temperature until further use.

3.2.2 Total bacterial count in chicken feed

The total number of aerobic bacteria was investigated in the chicken feed. The procedure of ISO 4833 (Anonymous, 2003) was followed for this purpose. In summary a portion of 20 gram feed was homogenized in 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into 2 empty Petri-dishes (diameter 9 cm). To each dish 15 ml of molten Plate Count Agar (PCA) was added. After the PCA was solidified an additional 5 ml PCA was added to the agar. The plates were incubated at (30 ± 1) ^{o}C for (72 ± 3) h and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae in chicken feed

In addition to the total count of aerobic bacteria, the Enterobacteriaceae count was determined. The procedure of ISO 21528-2 (Anonymous, 2004) was used for this purpose. In summary a portion of 20 gram feed was homogenized in 180 ml peptone saline solution in a plastic bag. The content was mixed by using a pulsifier (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into 2 empty Petri-dishes (diameter 9 cm). To each dish, 10 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After the VRBG was solidified an additional 15 ml VRBG was added to the agar. The plates were incubated at (37 ± 1) ^{o}C for (24 ± 2) h and the number of typical violet-red colonies was counted after incubation. Five typical colonies were tested for the fermentation of glucose and for a negative oxidase reaction. After this confirmation the number of Enterobacteriaceae was calculated.

3.3 Design of the interlaboratory comparison study

3.3.1 Samples: capsules and chicken feed

On 22 September 2008 (two weeks before the study) the reference materials (35 individually numbered capsules) and 700 grams of *Salmonella* negative chicken feed were packed with cooling devices as biological substance category B (UN 3373) and send by courier service to each participant. After arrival at the participant laboratory, the capsules had to be stored at –20 ^{o}C and the chicken feed had to be stored at +5 ^{o}C until the start of the study. Details about mailing and handling of the samples and reporting of test results can be found in the Protocol (Annex 4) and Standard Operation Procedure (Annex 5). The test report which was used during the study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/prof_testing/detection_stud/ or can be obtained through the corresponding author of this report.

Ten control capsules had to be tested without feed (numbered C1-C10). Twenty-five capsules (numbered 1 – 25) were each tested in combination with 25 grams of chicken feed (negative for *Salmonella*). The types and the number of capsules and feed samples to be tested are shown in Table 1.

Table 1 Overview of the types and the number of capsules tested per laboratory in the interlaboratory comparison study.

Capsules	Control capsules (n=10) No feed added	Test samples (n=25) with 25 g *Salmonella* negative chicken feed
S. Panama 5 (SPan5)	2	---
S. Enteritidis 20 (SE20)	2	5
S. Enteritidis 100 (SE100)	1	5
S. Typhimurium 5 (STM5)	3	5
S. Typhimurium 50 (STM50)	---	5
Blank	2	5

3.3.2 Sample packaging and temperature recording during shipment

The capsules and the chicken feed were packed in 2 plastic containers firmly closed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with four frozen (-20 ^{o}C) cooling devices. Each shipping box was sent as biological substances category B (UN3373) by door-to-door courier services. For the control of exposure to abusive temperatures during shipment and storage, so called micro temperature loggers were used to record the temperature during transport. These loggers are tiny sealed units in a 16 mm diameter and 6 mm deep stainless steel case. Each shipping box contained one logger, packed in the biopack with capsules. The loggers were programmed by the CRL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder immediately after receipt of the parcel to the CRL. At the CRL-*Salmonella* the loggers were read by means of the computer and all data from the start of the shipment until the arrival at the National Reference Laboratories were transferred to an Excel graphic which shows all recorded temperatures.

3.4 Methods

The prescribed method of this interlaboratory comparison study was ISO 6579 (Anonymous, 2002) and the requested (additional) method was Annex D of ISO 6579 (Anonymous, 2007). Additional to the prescribed methods the NRLs were also allowed to use their own methods. This could be different medium combinations and/or investigation of the samples with alternative methods, like Polymerase Chain Reaction based methods.

In summary:

Pre-enrichment in:
- Buffered Peptone Water (BPW) (prescribed)

Selective enrichment in/on:
- Rappaport Vassiliadis Soya broth (RVS) (prescribed)
- Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) (prescribed)
- Modified semi-solid Rappaport Vassiliadis agar (MSRV) (requested)
- Own selective enrichment medium (optional)

Plating-out on:
- Xylose lysine desoxycholate agar (XLD) (prescribed)
- Second plating-out medium for choice (obligatory)
- Own plating-out medium (optional)

Confirmation of identity:
- Confirmation by means of appropriate biochemical tests or by reliable, commercial available identification kits and serological tests. Follow the instructions of ISO 6579.

3.5 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the control samples, and the artificially contaminated samples with chicken feed (negative for *Salmonella* spp.). The specificity, sensitivity and accuracy rates were calculated according to the following formulae:

$$\text{Specificity rate: } \frac{\text{Number of negative results}}{\text{Total number of (expected) negative samples}} \times 100\ \%$$

$$\text{Sensitivity rate: } \frac{\text{Number of positive results}}{\text{Total number of (expected) positive samples}} \times 100\ \%$$

$$\text{Accuracy rate: } \frac{\text{Number of correct results (positive and negative)}}{\text{Total number of samples (positive and negative)}} \times 100\ \%$$

Mixed effect logistic regression (Venables and Ripley, 2002) was used for modelling the binary outcomes as a function of a fixed effect part, consisting of the capsules, enrichment media and isolation media, and a random effect part, consisting of the different laboratories. Differences between media and capsules are shown as odds ratios and were calculated by stratification by medium. The overall performance of each laboratory is also given as an odds ratio, but is compared to the mean of all laboratories, i.e. the outcomes as predicted based on the fixed effects only. 95 % confidence limits and p-values are provided as well.
An odds ratio can be interpreted as an effect size and is the ratio of the odds of detecting *Salmonella* in one group to the odds of detecting it in another group. Groups are for instance two different media, or one laboratory compared to the mean. Results were analyzed using the statistical software R (R Development Core Team, 2008). The lme4 package was used for the mixed effect logistic regression (Bates et al., 2008).

3.6 Good performance

Proposal for criteria testing 'good performance'
The criteria used for testing good performance in this study are given in Table 2. For determining good performance per laboratory all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. For example if a laboratory found for the STM5 capsules with matrix 3/5 positive with RVS/XLD, but no positives with MKTTn or any other selective enrichment medium or isolation medium this was still considered as good result. For the blank capsules also all combinations of media used per laboratory were taken into account. If for example a laboratory found 2/5 blank capsules positive with MKTTn/BGA but no positives with the other media, this was still considered as a 'no-good' result.

Table 2 Used criteria for testing for good performance in the Feed-I study (2008).

Control samples (capsules, no matrix)	Minimum result	
	Percentage positive	No. of positive samples / total No. of samples
SE100	100 %	1/1
STM5	60 %	2/3
Span5 and SE20	50 %	1/2
Blank control capsules	0 %	0/2

Samples (capsules with matrix)	Minimum result	
	Percentage positive	No. of positive samples / total No. of samples
Blank[1]	20 % at max[1]	1/5
STM50 and SE100	80 %	4/5
STM5 and SE20	50 %	2-3/5

1: All should be negative. However, as no 100 % guarantees about the *Salmonella* negativity of the matrix can be given, 1 positive out of 5 blank samples (20 % pos.) will still be considered as acceptable.

4 Results

4.1 Reference materials

The level of contamination and the homogeneity of the final batches of capsules are presented in Tables 3A and 3B. All batches met the pre-set criteria as stated in section 3.1. The enumerated minimum and maximum levels within each batch of capsules are also given in the table. The final batches were tested twice: firstly immediately after preparing the batch and secondly at the time of the interlaboratory comparison study. At the first date of testing the mean contamination level of all batches fulfilled the pre-set-criteria as stated in section 3.1. However, the variation between the SE20 capsules was high. At the second date of testing the mean contamination level of both batches SE capsules were decreased, especially for the SE100 capsules. The reason for this decrease was not clear. Although the mean contamination level of the SE100 capsules was decreased under the minimum target level it was still considered useful for the cause of this study.

Table 3A Level of contamination and homogeneity of SE, SPan and STM capsules.

	SE20	SE100	SPan5	STM5	STM50
Final batch; Test 1					
Date testing capsules	12-06-2008	12-03-2008	09-01-2008	15-11-2008	13-03-2008
Number of capsules tested	50	50	50	50	50
Mean cfp per capsule	19	92	5	6	44
Min-max cfp per capsule	8-49	65-113	1-15	2-11	30-67
T_2 / (I-1)	3.5	1.9	1.4	1.2	1.3
Final batch; Test 2					
Date testing capsules	14-10-2008	14-10-2008	14-10-2008	14-10-2008	14-10-2008
Number of capsules tested	25	25	25	25	25
Mean cfp per capsule	16	48	5	5	43
Min-max cfp per capsule	7-26	30-63	1-12	2-10	26-57
T_2 / (I-1)	1.9	2.5	1.9	1.2	1.7

cfp = colony forming particles; min-max = enumerated minimum and maximum cfp;
formula T_2 see Annex 2; I is number of capsules; Demand for homogeneity T_2 /(I-1) $\leq$ 2

Table 3B Level of contamination and homogeneity of SE and STM capsules used in the follow up study

	SE20	SE100	STM5	STM50
Final batch; Test 1				
Date testing capsules	19-02-2009	29-01-2009	21-01-2009	07-01-2009
Number of capsules tested	50	50	50	50
Mean cfp per capsule	18	67	6	62
Min-max cfp per capsule	11-29	45-107	3-12	39-78
T_2 / (I-1)	0.9	2.7	1.1	1.6
Final batch; Test 2				
Date testing capsules	19-03-2009	19-03-2009	19-03-2009	19-03-2009
Number of capsules tested	25	20	25	25
Mean cfp per capsule	18	84	6	53
Min-max cfp per capsule	8-27	56-115	2-14	33-66
T_2 / (I-1)	1.3	2.0	1.4	1.3

cfp = colony forming particles; min-max = enumerated minimum and maximum cfp;
formula T_2 see Annex 2; I is number of capsules; Demand for homogeneity $T_2 /(I-1) \leq 2$

4.2 Chicken feed samples

The chicken feed was tested negative for *Salmonella* and stored at room temperature. On Monday 22 September 2008 the chicken feed was mailed to the NRLs. After receipt the NRLs had to store the chicken feed at 5 °C.

The number of aerobic bacteria and the number of Enterobacteriaceae were tested twice; firstly at the day the chicken feed arrived at the CRL (9/9/2008) and secondly at the planned date (7/10/2008) of the interlaboratory comparison study. The results are shown in Table 4.

Most of the laboratories (twenty-eigth) performed the study in week 41, starting on 6 October 2008. One laboratory (labcode 22) performed the study one week earlier and one laboratory (labcode 5) performed it one week later.

Table 4 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of chicken feed.

Date	Aerobic bacteria cfp/g	Enterobacteriaceae cfp/g
9 September 2008 stored at 20-24 °C	$2.4*10^6$	$8.4*10^3$
7 October 2008 after 2 weeks at 5 °C	$1.6*10^6$	$1.3*10^4$

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study 30 NRLs participated: 28 NRLs from 27 EU Member States, 1 NRL from a European Economic Area country and 1 NRL from an European candidate country.

4.3.2 Accreditation/certification

Twenty-five laboratories mentioned to be accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005) (labcodes 1, 2, 3, 4, 7, 8, 9, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28, 29 and 30). Four laboratories (labcodes 5, 6, 12, 24) are planning to become accredited or certified in the near future and for one laboratory (labcode 10) the accreditation is already in process.
The NRLs without accreditation needs to take into account that according to EC Regulations No. 882/2004 each NRL should be accredited for their relevant work field before 31 December 2009 (EC Regulation No. 2076/2005).

4.3.3 Transport of samples

An overview of the transport times and the temperatures during transport of the parcels is given in Table 5. The temperature recorders were returned immediately after receipt to CRL-*Salmonella* by all NRLs. The temperature recorder of laboratory 3 was broken when it arrived at the CRL, it was therefore not possible to read the results. The majority of the laboratories received the materials within 1-2 days. However, the parcels of three laboratories (labcodes 1, 7 and 29) were delayed for more than 5 days because of problems with the courier service. The parcels returned at the CRL *Salmonella* in Bilthoven at 29/9/2008 and a new set of materials was sent the same day to those laboratories. The total transport time of those latter three parcels was 5 days. Only the transport time of the second mailing for the laboratories 1, 7 and 29 are indicated in Table 5. When those latter parcels are not taken into account, the average transport time was 25 hours. For twelve parcels the transport temperature did not exceed 5 ^{o}C and for thirteen other parcels the temperature was only for a few hours above 5 ^{o}C but did not exceed 10 ^{o}C. The transport time of the parcel of laboratory 30 was relatively long, but most of the time the temperature did not exceed 10 ^{o}C. The delayed parcels of the laboratories 1, 7 and 29 were kept for the majority of the transport time below 10 ^{o}C.
For twelve NRLs the time of transport recorded on the test report did not correspond with the time reported by the courier. For two NRLs (labcode 6 and 21) this was due to the fact that the parcel was forwarded to another institute. Presumably the other parcels arrived at the time reported by the courier at the Institute, but due to internal logistics at the Institute the parcel arrived later at the laboratory of the NRL. In general this delay was only short (1-4 h). Only in one laboratory the delay was longer (20 h, labcode 17). However, no negative effect of this delay was expected as the parcel had been stored at – 4 ^{o}C.

Table 5 Overview of the transport time and of the temperatures during shipment of the parcels to the NRLs.

		Time (h) at					
Labcode	Transport[1] time in hours	0 °C - 5 °C	5 °C - 10 °C	10 °C - 15 °C	15 °C - 20 °C	20 °C - 25 °C	Additional Storage[2]
1	101	48	13	15	22	3	-
2	18	18					
3	24					Temperature recorder broken	
4	45	45					
5	21	20	1				
6	20	15	5			5 h transport to another institute 3 h between -15 °C and - 19 °C 2 h at 23 °C	
7	100	48	31	21			-
8	22	22					1 h at 24 °C
9	43	37	6				
10	20	18	2				
11	19	19					1 h at 4 °C
12	42	25	16			1	2 h at 24 °C
13	25	13	12				16 h between -10 °C and -15 °C
14	19	19					
15	22	21	1				
16	19	16	3				1 h at 19 °C
17	22	15	7				20 h at – 4 °C
18	22	22					
19	22	22					
20	20	16	4				
21	21	21				2 days delay transport to another institute at 1 °C and 2 h at 22 °C	
22	22	22					
23	21	19	2				3 h at 6 °C
24	25	25					
25	40	39	1				4 h at 21 °C
26	23	21				2	
27	22	20				2	
28	23	21	2				1 h at 5 °C
29	97	30	37	20			
30	43	5	34	4			1 h 26 °C
Average[3]	25	21	4				

[1] transport time according the courier

[2] storage time of the samples at the institute before arriving at the laboratory of the NRL

[3] average time without lab 1, 7 and 29

Table 6 Media combinations used per laboratory.

Labcode	Selective enrichment media	Plating-out Media
1	RVS MKTTn MSRV	XLD Hektoen Rambach
2	RVS MKTTn MSRV	XLD BGA^{mod}
3	RVS MKTTn MSRV	XLD BGA^{mod}
4	RVS MKTTn MSRV	XLD Rambach
5	RVS MKTTn MSRV	XLD SM2
6	RVS MKTTn MSRV	XLD SM2
7	RVS MKTTn MSRV	XLD Rambasch
8	RVS MKTTn MSRV	XLD BGA^{mod}
9	RVS MKTTn MSRV	XLD Rambach
10	RVS MKTTn MSRV	XLD XLT4
11	RVS MKTTn MSRV	XLD BGA^{mod}
12	RVS MKTTn MSRV	XLD BPLS Rambach
13	RVS MKTTn MSRV	XLD BPLS
14	RVS MKTTn MSRV	XLD BGA^{mod} Rapid *Salmonella*
15	RVS MKTTn MSRV	XLD BPLS Blaskal
16	RVS MKTTn MSRV	XLD BGA^{mod}
17	RVS MKTTn MSRV	XLD BPLSA
18	RVS MKTTn MSRV	XLD BPLS Rambach
19	RVS MKTTn MSRV	XLD Rambach
20	RVS MKTTn MSRV	XLD XLT4 BGA*
21**	RVS MKTTn MSRV	XLD BGA^{mod} Rambach
22	RVS MKTTn MSRV	XLD Rambach
23	RVS MKTTn MSRV	XLD BGA^{mod} Rambach
24	RVS MKTTn MSRV	XLD BGA^{mod} MLCB
25**	RVS MKTTn MSRV	XLD BGA^{mod}
26	RVS MKTTn MSRV	XLD BGA
27	RVS MKTTn MSRV	XLD Rambach
28	RVS MKTTn MSRV	XLD BGA^{mod}
29	RVS MKTTn MSRV	XLD BGA+^{mod}
30	RVS MKTTn MSRV	XLD BGA^{mod}

Explanations of the abbreviations are given in the 'List of abbreviations'

Compositions of the media not described in ISO 6579 are given in Annex 3

+ Sulphamenalate supplement

* laboratory 20 used BGA only in combination with MSRV

**laboratory 21 and 25 did confirm *Salmonella* suspected colonies only from one plating out medium

4.3.4 Media

Each laboratory was asked to test the samples with the prescribed (ISO 6579) and the requested (AnnexD of ISO 6579) methods. All laboratories used the selective enrichment media RVS, MKTTn and MSRV with the plating out medium XLD and a second plating out medium of own choice. The media used per laboratory are shown in Table 6. Eight NRLs (labcodes 1, 14, 15, 18, 20, 21, 23 and 24) used a third plating out medium. Two NRLs did not confirm *Salmonella* suspected colonies from all used plating out media (labcodes 21 and 25).
Details on the media which are not described in ISO 6579 are given in Annex 3.
In Tables 7-13 information is given on the composition of the media which were prescribed and 'requested' and on incubation temperatures. In these tables only the laboratories are indicated who reported deviations. Laboratory 6 and 25 did not mention the pH for most of the used media. Four laboratories (labcodes 1, 18, 22 and 25) did not mention de composition of all the media used. Two laboratories incubated the selective enrichment media at deviating temperatures (labcodes 15 and 22).

A second plating-out medium for choice was obligatory. Seventeen laboratories used BGA (modified) or BPLS (ISO 6579, 1993) as a second plating-out medium and one laboratory used BPLSA (Merck) this is almost similar to BGA. Eleven laboratories used Rambach, two laboratories used SM2 agar and two laboratories used XLT4. The following media were used only by one laboratory: MLCB, Hektoen, Rapid *Salmonella,* BGA and Blaskal.

The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. A total of 16 laboratories performed this extra culture step on many different media (e.g. Nutrient agar (ISO 6579, 2002), Mac Conkey, Imuna and Bromcresol purpur agar).

Table 7 Incubation time and temperature of BPW.

	Prewarming BPW		Dissolving capsules In BPW		Pre-enrichment in BPW	
Labcode	**Time (h:min)**	**Incubation temperature in ^{o}C (min-max)**	**Time (min)**	**Incubation temperature in ^{o}C (min-max)**	**Time (h:min)**	**Incubation temperature in ^{o}C (min-max)**
SOP & ISO 6579	**Overnight**	**36-38**	**45**	**36-38**	**16 – 20**	**36-38**
7	Overnight	37.2-37.7	50	36.2-36.8	17:30	36.9-37.4
14	Overnight	37.5	15	36.7-37.6	22:15	36.7- -
30	Overnight	37	45	37	23:40	37

Grey cell : deviating times and temperatures
- = no information

Table 8 Composition (in g/L) and pH of BPW medium.

Labcode	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Disodium hydrogen Phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	Potassium dihydrogen phosphate (KH_2PO_4)	pH
ISO 6579	**10**	**5**	**9**	**1.5**	**6.8 – 7.2**
2	10	5	9	1.5	-
6	10	5	9	1.5	-
10	10	5	3.5*	1.5	-
11	10	5	3.5*	1.5	7.3
14	10	5	3.5*	1.5	-
24	10	5	3.6*	1.5	7.1
25	-	-	-	-	7
28	10	5	3.5*	1.5	7.4

Grey cell: deviating from ISO 6579

- = no information

* = 3.5 g Disodium hydrogen phosphate (anhydrous) is equivalent to 9 g disodium hydrogen phosphate dodecahydrate

Table 9 Incubation temperatures of selective enrichment medium RVS, MKTTn and MSRV.

	RVS	MKTTn	MSRV
Labcode	**Incubation temperature in ^oC (min-max)**	**Incubation temperature in ^oC (min-max)**	**Incubation temperature in ^oC (min-max)**
ISO 6579 & Annex D	**40.5 – 42.5**	**36-38**	**40.5 – 42.5**
15	40.5-41.1*	37.0	40.5-41.0*
22	41.5-41.9	41.5-41.9	41.5-41.9

Grey cell: deviating times and temperatures

*Laboratory 15 had power failure in the laboratory and both incubators had problems with the temperature.

Table 10 Composition (in g/L) and pH of RVS.

Labcode	Enzymatic digest of soya (Peptone)	Sodium Chloride (NaCl)	Potassium Dihydrogen Phosphate* (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)**	Malachite green oxalate	pH
ISO 6579	**4.5**	**7.2**	**1.44**	**13.4**	**0.036**	**5.0 - 5.4**
1	-	-	-	-	-	5.2
5	4.5	7.2	1.44	28.6**	0.037	-
6	4.5	7.2	1.44	28.6**	0.036	-
8	5	8	1.4 + 0.2*	13.4	0.036	5.2
10	4.5	7.2	1.44	28.6**	0.036	-
13	4.5	8	0.6 + 0.4*	29**	0.036	5.1
14	4.5	7.2	1.3 + 0.2 *	13.4	0.036	-
16	5	8	1.6*	40***	0.04	5.3
18	5	8	1.4 + 0.2*	400***	0.4	5.2
21	4.5	8	0.4 + 0.6 *	8** + 29***	0.036	5.4
23	4.5	7.2	1.3 + 0.2 *	13.6	0.036	5.6
25	-	-	-	-	-	5.2
30	5	8	0.8*	40**	0.12	6.0

Grey cell: deviating from ISO 6579

- = no information

*= 1.4 g/L Potassium dihydrogen phosphate (KH_2PO_4) + 0.2 g/L Di-potassium hydrogen phosphate (K_2HPO_4) gives a final concentration of 1.44 g/L KH_2PO_4 K_2HPO_4

** = 13.4 g $MgCl_2$ (anhydrous) is equivalent to 28.6 g $MgCl_2$ hexahydrate.

*** Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)

Table 11 Composition (in g/L) and pH of MKTTn.

Labcode	Meat extract	Enzymatic digest of casein (Peptone)	Sodium chloride (NaCl)	Calcium Carbonate ($CaCO_3$)	Sodium Thiosulfate Penta hydrate ($Na_2S_2O_3.5H_2O$)	Oxbile	Brilliant green	Iodine	Potassium iodide (KI)	Novo-biocin	pH
ISO 6579	**4.3**	**8.6**	**2.6**	**38.7**	**47.8**	**4.8**	**0.0096 (9.6 mg)**	**4**	**5**	**0.04**	**8.0 – 8.4**
1	-	-	-	-	-	-	-	-	-	2 ml	8.2
3	4.3	8.6	2.6	38.7	30.5*	4.8	0.0096	20	25	0.04	5.2
5	4.2	8.5	2.5	38.0	30.3*	4.8	0.0095	4	5	0.05	-
6	4.2	8.5	2.5	38.0	30.3*	4.3	0.0095	4	5	0.04	-
10	4.2	8.5	2.5	38.0	30.3*	4.8	0.0095	4	5	0.05	-
11	4.3	8.6	2.6	38.7	30.5*	4.8	0.0096	4	5	0.04	7.9
13	4.3	8.6	2.6	38.7	47.8	4.8	0.0095	20	25	0.04	8.3
14	4.2	8.4	2.5	37.8	46.6	4.7	0.0094	3.9	4.9	0.04	-
17	4.3	8.6	2.6	38.7	30.5*	4.8	0.009	4	5	0.05	8.2
18	4.3	8.6	2.6	38.7	47.8	4.8	9.6	20	25	0.04	8.2
19	4.2	8.5	2.5	38.0	30.3*	4.8	0.0095	4	5	0.04	7.9
23	4.3	8.6	2.6	38.7	30.5*	4.8	0.0096	4	5	0.04	9
25	-	-	-	-	-	-	-	-	-	-	7.9
27	4.3	8.6	2.6	38.7	30.5*	4.8	0.0096	3.9	4.9	0.039	7.0
28	7.0 + 2.3**		2.4	25.0	40.7	4.8	0.0095	3.8	4.75	0.04	-
29	7.0 + 2.3**		2.3	25.0	40.7	4.8	0.0095	3.8	4.75	-	7.8
30	7.0	2.3	2.3	25.0	40.7	4.8	0.0095	20	25	0.04	7.8

Grey cell: deviating from ISO 6579

- = no information

* 30.5 g Sodium thiosulphate (anhydrous) is equivalent to 47.8 g Sodium thiosulphate pentahydrate

** 7.0 g Tryptone + 2.3 g Soya Peptone

Table 12 Composition (in g/L) and pH of MSRV.

Labcode	Enzymatic digest of casein (Tryptose)	Casein hydro-lysate	Sodium chloride (NaCl)	Potassium Dihydrogen Phosphate (KH_2PO_4 K_2HPO_4)	Magnesium chloride anhydrous ($MgCl_2$)	Malachite green oxalate	Agar	Novo biocin	pH
Annex D ISO 6579	**4.6**	**4.6**	**7.3**	**1.5**	**10.9**	**0.04**	**2.7**	**0.01 (10mg/L)**	**5.1-5.4**
1	-	-	-	-	-	-	-	2 ml	5.2
3	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	5.5
6	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	-
7	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.4
8	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.1
11	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.2
13	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.05	5.7
14	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	-
18	-	-	-	-	-	-	-	-	5.2
21	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.02	5.3
22	-	-	-	-	-	-	-	-	5.4
24	2.3+2.3*	4.6	7.3	1.5	10.9	0.04	2.5	0.01	5.4
25	-	-	-	-	-	-	-	-	5.6
27	4.6	4.65	7.3	1.5	10.9	0.04	2.5	0.01	5.2
28	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0.01	5.5
29	4.6	4.6	7.3	1.5	10.9	0.04	2.7	0	5.2

Grey cell: deviating from Annex D of ISO 6579

- = no information

* 2.3 g Tryptone + 2.3 g Enzymatic digest of Casein

Table 13 Composition (in g/L) and pH of XLD.

Labcode	Xylose	L-lysine	Lactose	Sucrose (Saccharose)	Sodium chloride (NaCl)	Yeast Extract	Phenol red	Agar	Sodium desoxy-cholate ($C_{24}H_{39}NaO_4$)	Sodium thio-sulphate ($Na_2S_2O_3$)	Iron (III) Ammonium Citrate ($C_6H_8O_7 \cdot nFe \cdot nH_3N$)	pH
ISO 6579	**3.75**	**5**	**7.5**	**7.5**	**5**	**3**	**0.08**	**9-18**	**1**	**6.8**	**0.8**	**7.2 – 7.6**
1	-	-	-	-	-	-	-	-	-	-	-	7.4
3	3.75	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.6
4	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.4
6	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
10	3.75	5	7.5	7.5	5	3	0.08	12.5	1	6.8	0.8	-
14	3.75	5	7.5	7.5	5	3	0.08	13.5	1	6.8	0.8	-
18	-	-	-	-	-	-	-	-	-	-	-	7.4
23	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.3
25	-	-	-	-	-		-	-	-	-	-	7.4
30	3.5	5	7.5	7.5	5	3	0.08	15	2.5	6.8	0.8	7.4

Grey cell: deviating from ISO 6579

- = no information

Most of the laboratories used both biochemical and serological tests for confirmation of *Salmonella.* Three laboratories (labcode 17, 23 and 24) did not use a biochemical test but used serological tests only. Seven laboratories did not use a serological test (labcodes 2, 3, 5, 6, 12, 26, 28) but used biochemical tests only. The used confirmation media or tests are summarised in Tables 14 and 15.

Table 14 Biochemical confirmation of *Salmonella.*

Labcode	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	+	+	+	-	+	+	-	-
2, 5, 12, 29	+	+	+	-	-	-	-	-
3, 8	+	+	+	-	-	+	-	-
4	+	+	+	-	-	-	Api (Rapid) 20E BBL BD Crystal E/NF	PCR*
6	-	-	-	-	-	-	Api ID32E, Api 32GN	Vidas SLM
7, 9, 20, 26	+	+	+	+	+	+	-	-
10	-	-	-	-	-	-	Api 20E	PCR
11	-	+	-	-	-	-	-	-
13	+	+	+	-	-	+	-	Mini Vidas
14	+	-	+	-	-	-	-	Sorbitol Imobility Agar
15, 22	+	+	+	-	-	-	Api 20E	-
16, 18	+	+	+	+	-	+	-	-
17	-	-	-	-	-	-	-	PCR
19	-	-	+	-	-	-	Api 10S	-
21	+	-	-	-	-	-	Api 20E	Lysine iron agar Oxidase Elisa Tecra
23, 24	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	ID 32E Biomerieux	-
27	+	+	-	-	-	-	Api 20E Api Rapid 20E	ID 0571 MOBIS *Salmonella* test cards
28	+	+	+	-	-	-	-	PCR
30	+	-	-	-	-	-	GN-ID A Panel Microgen Bioproducts	-

- = Not done/mentioned Explanations of the abbreviations are given in the 'List of abbreviations'

* Results are not reported in testreport

Table 15 Serological confirmation of *Salmonella.*

Labcode	Serological			Other
	O antigens	**Vi antigens**	**H antigens**	
1, 7, 9, 14, 16, 18, 19, 30	+	-	-	
2, 3, 5, 6, 12, 26, 28	-	-	-	
4, 8, 10, 11, 13, 17, 20, 22, 23, 24, 25, 29	+	+	-	
15, 27	-	-	-	*Salmonella* Omnivalent A-67
21	-	-	-	Anti serum Poly A-I&VI

- = Not done/mentioned

4.4 Control samples

4.4.1 General

None of the laboratories isolated *Salmonella* from the procedure control (C11: no capsule/no feed) nor from the feed control (C12: no capsule/negative feed). Thirteen laboratories scored correct results for all the control capsules containing *Salmonella*. In Annex 6 the results are given of all control samples (capsules without feed) per laboratory and per selective enrichment medium in combination with the isolation medium that gives the highest number of positives. In Table 15 the highest number of positive isolations found with all combinations of selective enrichment media and isolation media are given per laboratory.

Blank capsules without addition of feed (n=2)
The blank capsules contained only sterile milk powder. For the analyses no feed was added.
Laboratory 6 found both blank capsules positive on all media used by this laboratory. Possible causes for finding a blank sample positive may be cross-contamination, mixing up positive and negative samples or limited confirmation or misinterpretation of confirmation results. This laboratory is advised to check their procedures.

Salmonella Panama 5 capsules (SPan5) without addition of feed (n=2)
Twenty-nine laboratories isolated *Salmonella* from both Span5 capsules. One Laboratory (labcode 15) could not detect *Salmonella* Panama (SPan5) in one control capsule. When using the selective enrichment media RVS and MKTTn they missed both the capsules but they found one positive with the selective enrichment on MSRV inoculated from the same BPW.

Salmonella Typhimurium 5 capsules (STM5) without addition of feed (n=3)
Twenty-eight laboratories tested all the three capsules containing STM5 positive. Two laboratories (1 and 19) could not detect *Salmonella* (STM5) in one control capsule with all the three selective enrichment media. These capsules contained STM at a low level (approximately 5 cfp/ capsule). Due to the variation between capsules one out of two capsules containing STM5 may occasionally be negative.

Salmonella Enteritidis 20 capsules (SE20) without addition of feed (n=2)
All laboratories isolated *Salmonella* Enteritidis from both SE20 capsules.

Salmonella Enteritidis 100 capsules (SE100) without addition of feed (n=1)
All participating laboratories tested the capsule containing SE100 positive.

The results of all control samples were compared with the definition of ‘good performance’ (see section 3.6). The score for the control samples was below these criteria for one laboratory (labcode 6).

Table 16 Total number of positive results of the control samples (capsule without feed) per laboratory.

Labcode	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1
Good Performance	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**
1	0	2	**2**	2	1
2	0	2	3	2	1
3	0	2	3	2	1
4	0	2	3	2	1
5	0	2	3	2	1
6	**2**	2	3	2	1
7	0	2	3	2	1
8	0	2	3	2	1
9	0	2	3	2	1
10	0	2	3	2	1
11	0	2	3	2	1
12	0	2	3	2	1
13	0	2	3	2	1
14	0	2	3	2	1
15	0	**1**	3	2	1
16	0	2	3	2	1
17	0	2	3	2	1
18	0	2	3	2	1
19	0	2	**2**	2	1
20	0	2	3	2	1
21	0	2	3	2	1
22	0	2	3	2	1
23	0	2	3	2	1
24	0	2	3	2	1
25	0	2	3	2	1
26	0	2	3	2	1
27	0	2	3	2	1
28	0	2	3	2	1
29	0	2	3	2	1
30	0	2	3	2	1

Bold numbers: deviating results
Grey cell: results are below good performance

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

In Table 17 the specificity, sensitivity and accuracy rates for the control capsules without the addition of feed are shown. The rates are calculated for the different selective enrichment media (RVS, MKTTn and MSRV) and plating-out medium XLD. These rates were as expected or even better. For the low and high level SE capsules the rates were 100 %. For the low level materials SPan5 and STM5 the rates were close to 97 %. There was no difference between rates of EU members and the two non-EU members. In Table 17 the rates of all participants are given.

Table 17 **Specificity, sensitivity and accuracy rates of the control samples (capsules without the addition of feed) n=29 *.**

Control capsules		RVS/XLD	MKTTn/XLD	MSRV/XLD
Blank	No. of samples	58	58	58
	No. of negative samples	56	56	56
	Specificity in %	**96.6**	**96.6**	**96.6**
SPan5	No. of samples	58	58	58
	No. of positive samples	56	56	57
	Sensitivity in %	**96.6**	**96.6**	**98.3**
STM5	No. of samples	87	87	87
	No. of positive samples	85	85	85
	Sensitivity in %	**97.7**	**97.7**	**97.7**
SE20	No. of samples	58	58	58
	No. of positive samples	58	58	58
	Sensitivity in %	**100**	**100**	**100**
SE100	No. of samples	29	29	29
	No. of positive samples	29	29	29
	Sensitivity in %	**100**	**100**	**100**
All capsules with *Salmonella*	No. of samples	232	232	232
	No. of positive samples	228	228	229
	Sensitivity in %	**98.3**	**98.3**	**98.7**
All capsules	No. of samples	290	290	290
	No. of correct samples	284	284	285
	Accuracy in %	**97.9**	**97.9**	**98.3**

* Laboratory 21 did not confirm the suspected colonies from XLD, therefore their results were not taken into account.

4.5 Results feed samples artificially contaminated with *Salmonella* spp.

4.5.1 Results per type of capsule and per laboratory

General

The results of the *Salmonella* negative chicken feed samples artificially contaminated with capsules per selective enrichment medium in combination with the isolation medium giving the highest number of positives are summarised in Annex 7. In Table 18 the highest number of positive isolations found with all combinations of selective enrichment media and isolation media are given per laboratory.

Blank capsules with negative chicken feed (n=5)

Twenty-eigth laboratories correctly did not isolate *Salmonella* from these blank capsules with the addition of negative feed. Two laboratories (6 and 21) found 1 positive blank with the addition of negative chicken feed.

Laboratory 6 had found one blank capsule positive on all media. Laboratory 21 found only one positive blank after selective enrichment on MSRV. With the other media RVS and MKTTn they correctly found no positive blanks.

All blanks should be tested negative. However, as no 100 % guaranty about the *Salmonella* negativity of the matrix can be given, 1 positive out of 5 blank samples (80 % negative) is still considered acceptable.

S. Typhimurium 5 capsules (STM5) with negative chicken feed (n=5)

Twenty-five laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Typhimurium at a level of approximately 5 cfp/ capsule in combination with chicken feed when using RVS, MKTTn or MSRV. Five laboratories 2, 13, 21, 29 and 30 found one capsules negative with all three selective enrichment media. This is well acceptable as due to change some of the capsules may be negative in a batch of capsules at such low mean contamination level of 5cfp/capsule.

S. Typhimurium 50 capsules (STM50) with negative chicken feed (n=5)

All laboratories isolated *Salmonella* from all five capsules containing *Salmonella* Typhimurium at a level of approximately 50 cfp/ capsule in combination with chicken feed with the selective enrichment medium MSRV. Six laboratories 8, 15, 23, 24, 25 and 27 found one or more capsules negative when using MKTTn and laboratory 21 found one capsule negative when using RVS.

S. Enteritidis 20 capsules (SE20) with negative chicken feed (n=5)

Twenty-nine laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of approximately 20 cfp/ capsule in combination with chicken feed at least with one of the used selective enrichment media RVS, MKTTn or MSRV. Laboratory 1 found one capsule negative when using RVS and MKTTn and they found two capsules negative when using MSRV.

Table 18 Total number of positive results of the artificially contaminated chicken feed samples per laboratory.

Labcode	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5
Good performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
1	0	5	5	**4**	5
2	0	**4**	5	5	5
3	0	5	5	5	5
4	0	5	5	5	5
5	0	5	5	5	5
6	**1**	5	5	5	5
7	0	5	5	5	5
8	0	5	5	5	5
9	0	5	5	5	5
10	0	5	5	5	5
11	0	5	5	5	5
12	0	5	5	5	5
13	0	**4**	5	5	5
14	0	5	5	5	5
15	0	5	5	5	5
16	0	5	5	5	5
17	0	5	5	5	5
18	0	5	5	5	5
19	0	5	5	5	5
20	0	5	5	5	5
21	**1**	**4**	5	5	5
22	0	5	5	5	5
23	0	5	5	5	5
24	0	5	5	5	5
25	0	5	5	5	5
26	0	5	5	5	5
27	0	5	5	5	5
28	0	5	5	5	5
29	0	**4**	5	5	5
30	0	**4**	5	5	5

Bold numbers: deviating results

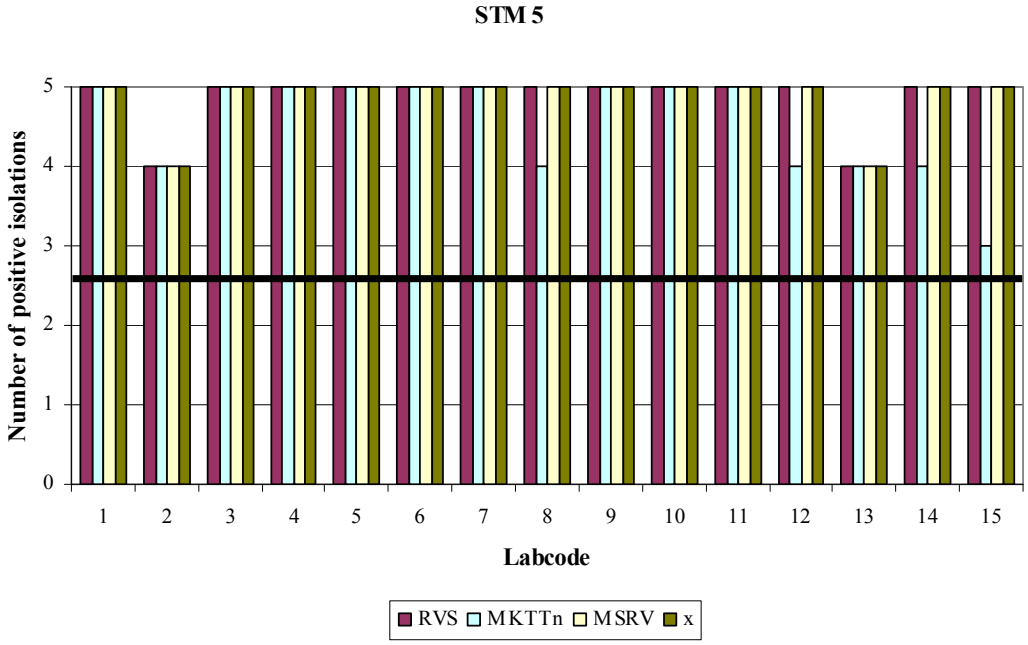
S. Enteritidis 100 capsules (SE100) with negative chicken feed (n=5)

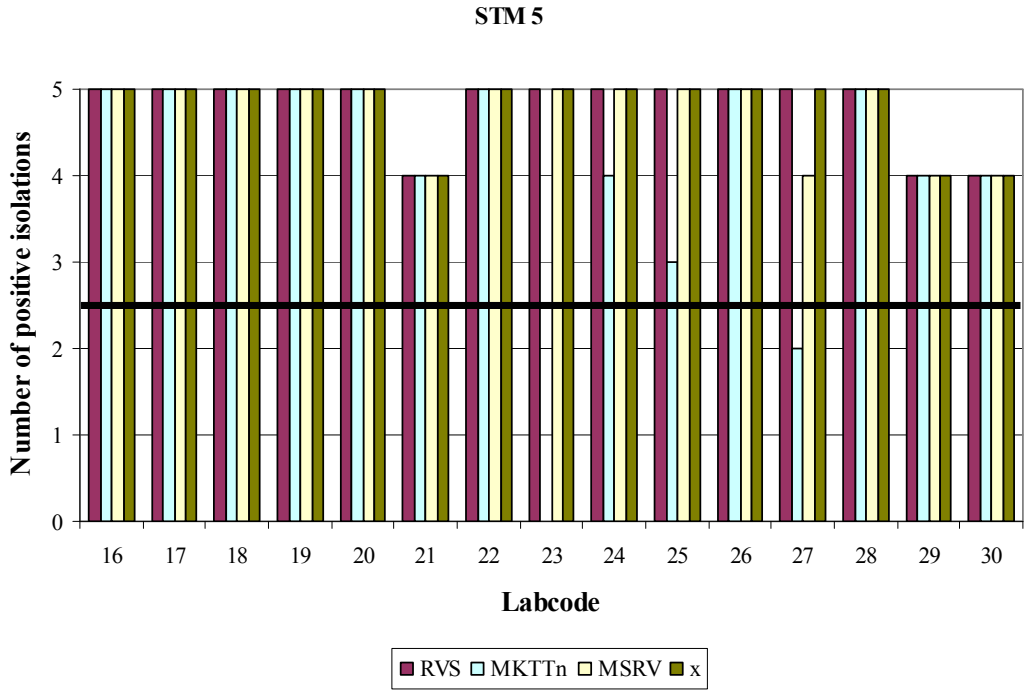
All laboratories isolated *Salmonella* from all the five capsules containing *Salmonella* Enteritidis at a level of approximately 100 cfp/ capsule in combination with chicken feed with the selective enrichment medium MSRV. Laboratory 25 found one capsule SE100 negative with selective enrichment medium RVS. Seven laboratories (labcode 1, 7, 14, 24, 27 and 28) found one or two capsules negative with selective enrichment medium MKTTn.

The results of all artificially contaminated chicken feed samples were compared with the definition of 'good performance' (see section 3.6) and all laboratories full filled these criteria.

4.5.2 Results per selective enrichment medium, capsule and per laboratory

In the Figures 1, 2, 3 and 4 the number of positive isolations per type of artificially contaminated feed sample is given per laboratory after pre-enrichment in BPW, selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on selective plating agar XLD. For determining good performance per laboratory, all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. In the figures the combination of selective enrichment medium and isolation medium giving the highest number of positives is indicated as 'x'. Laboratory 21 did not confirm the isolations from XLD, therefore in the figures the results are given from Rambach.
The results of all artificially contaminated chicken feed samples were compared with the definition of 'good performance' (see section 3.6). In the Figures 1-4 the border of good performance is indicated with a black horizontal line.
The majority of the laboratories found the highest number of positive isolations when XLD was used as isolation medium. Five laboratories found differently when using MKTTn as selective enrichment medium. The laboratories 1, 7, 14, 15 and 27 found one sample more positive after isolation on either Rambach, Hektoen Enteric, Rapid *Salmonella* or Blaskal agar, when compared to isolation on XLD, after selective enrichment in MKTTn.





Lab 21 isolation results on Rambach instead of XLD

= border of good performance

Figure 1 Results of chicken feed samples artificially contaminated with STM5 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.

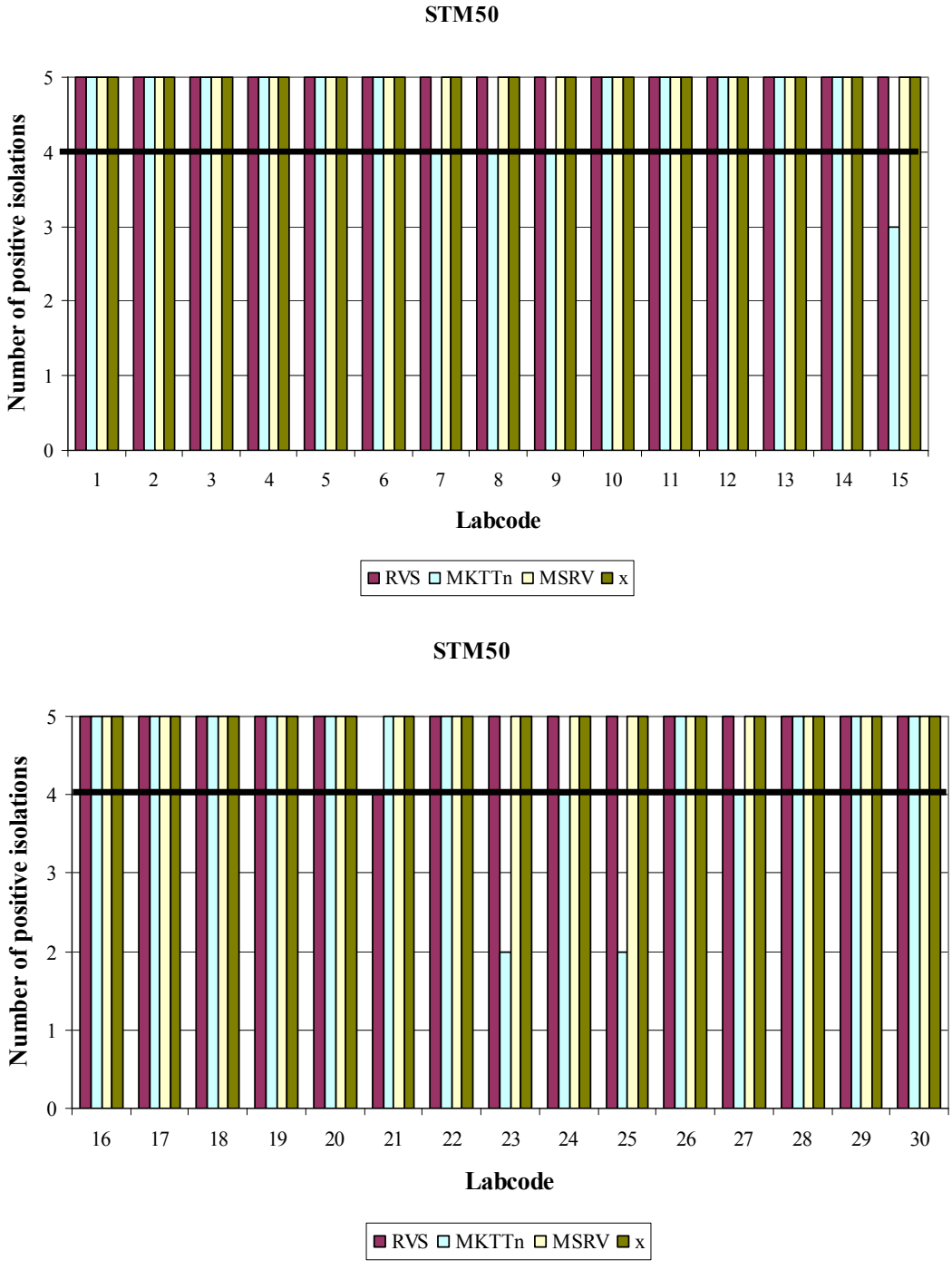
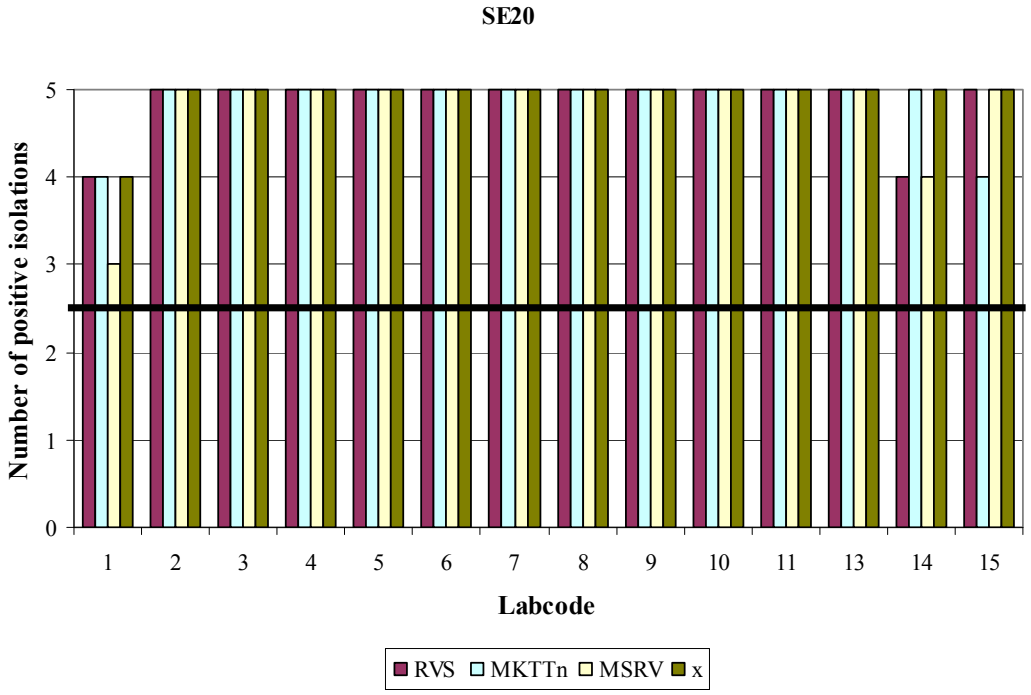
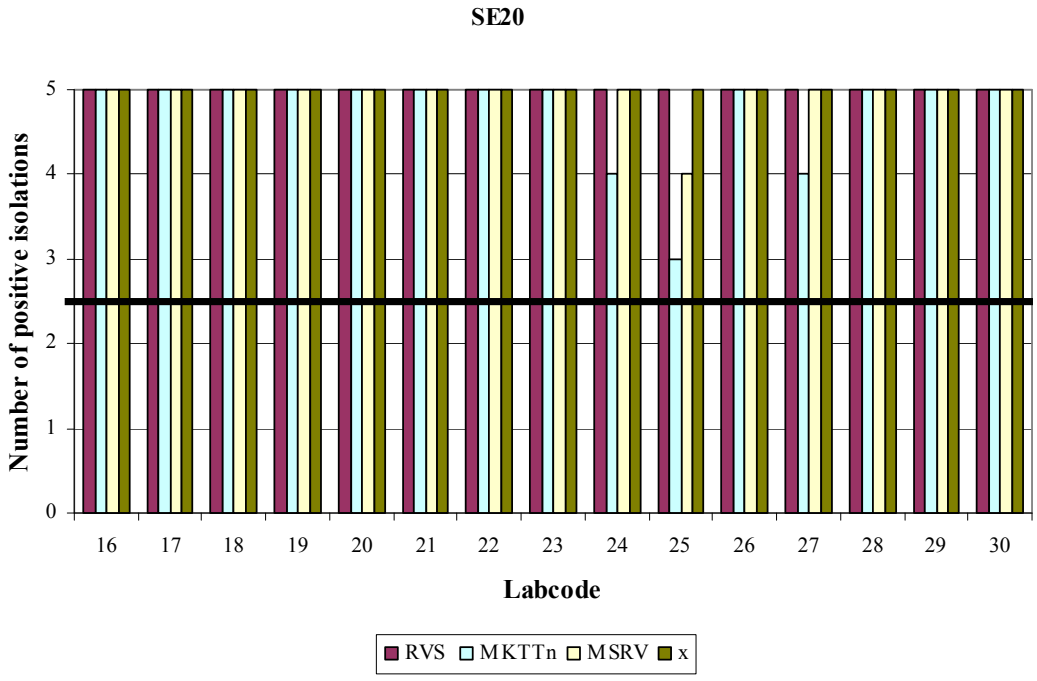


Figure 2 Results of chicken feed samples artificially contaminated with STM50 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.

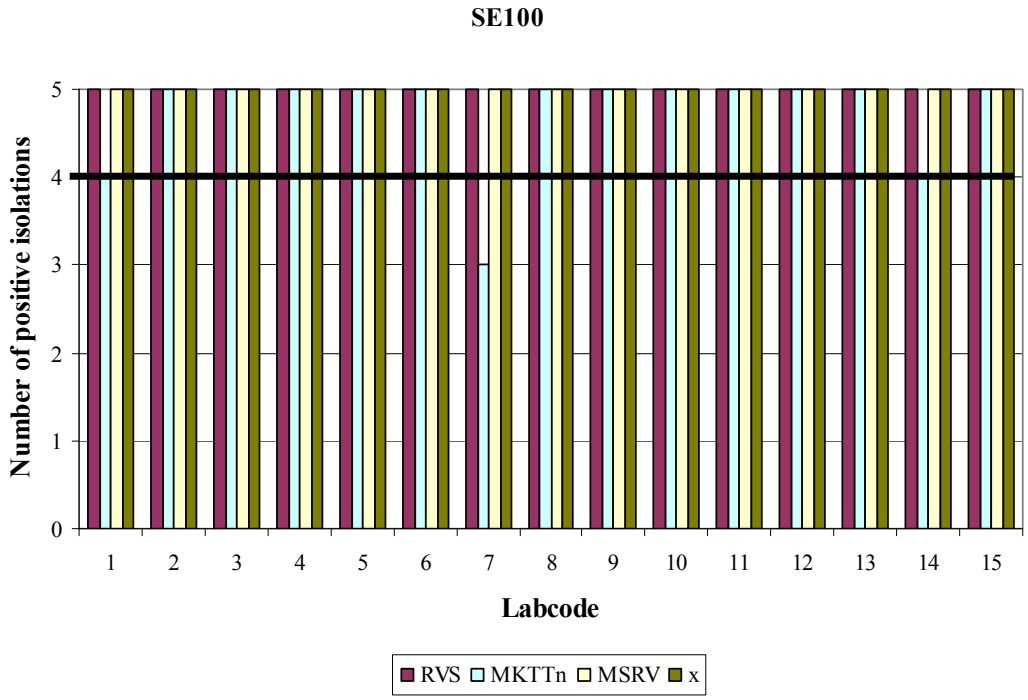


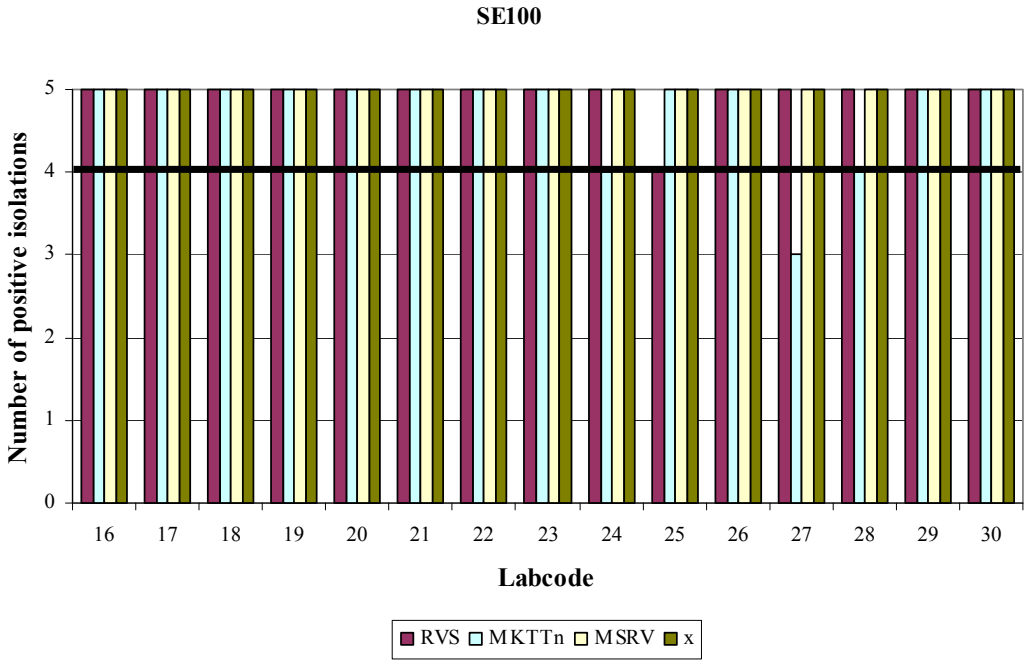


Lab 21 isolation results on Rambach instead of XLD

— = border of good performance

Figure 3 Results of chicken feed samples artificially contaminated with SE20 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.





Lab 21 isolation results on Rambach instead of XLD

= border of good performance

Figure 4 Results of chicken feed samples artificially contaminated with SE100 capsules (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar XLD. The highest number of positive isolations found with all combinations of selective enrichment media and isolation media used by a laboratory is given as x.

The differences in the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment media are given in Table 19. XLD showed the highest number of positive isolations compared to other plating out media, independent on the selective enrichment medium used. The majority of the laboratories used BGA as the second plating out medium (see Table 5).
The choice of plating out medium does not seem to have a large effect on the number of positive isolations. Only when MKTTn is used for selective enrichment, XLD gave 6 % more positive results than other plating-out media.
The difference in the number of positive isolations after 24 h and 48 h of incubation of the selective enrichment media was the highest for MKTTn (Table 17), 6-8 % more positive isolations were found after 48 h of incubation. For RVS and MSRV the difference between the two incubation times was only 2-3 %.

Table 19 Mean percentages of positive results of all participating laboratories after selective enrichment in RVS, MKTTn and on MSRV, incubated for 24 and 48 hours and followed by incubation on different plating out media, when analyzing the artificially contaminated chicken feed samples.

Plating-out medium	Selective enrichment medium		
	RVS	MKTTn	MSRV
	24 / 48 h	24 / 48 h	24 / 48 h
XLD	97 / 99 %	86 / 92 %	95 / 98 %
Other (most often BGA)	95 / 98 %	78 / 86 %	95 / 98 %

In Tables 20 and 21 differences between selective enrichment media and isolation media per capsule are shown as odds ratios (OR). In addition the 95 % confidence intervals and p-values are given. An odds ratio equal to "∞" means that it is very large. Usually this occurs when the odds of detecting *Salmonella* for the reference medium is zero or near zero. An odds ratio of zero means that the odds of detecting *Salmonella* for the other medium is zero. Laboratory 21 did not confirm isolations from XLD, so that these results could not be taken into account for the calculations.

The interpretation of Table 20 is as follows: given a selective enrichment medium the odds of finding a positive isolation with the different plating out media are compared. For instance the odds of finding *Salmonella* from the SE20 samples after selective enrichment in RVS is for XLD as isolation medium a factor 5.6 (OR) higher than for media other than XLD. If MKTTn is used as selective enrichment medium, XLD shows significantly more positive results. In general for MSRV and RVS the odds are also higher, but not significant. Laboratory 25 did not confirm isolation media other than XLD, so that these results could not be taken into account for the calculations.

The interpretation of Table 21 is similar to that of Table 20, except that selective enrichment media are mutually compared given XLD as isolation medium. If MSRV or RVS are used as selective enrichment media, the odds of finding *Salmonella* are significantly higher than in case of MKTTn is used. RVS compared to MSRV results in lower odds, although this is not significant.

Table 20 **Number of positive isolations found with XLD compared to the number of positive isolations found with other isolation media, given a selective enrichment medium.**
Samples : chicken feed, artificially contaminated with *Salmonella* positive capsules.

Selective enrichment medium	Compared isolation media	Capsule	Odds Ratios	95 % lower	95 % upper	p-value
RVS	XLD compared to other than XLD	STM5	1.054	0.204	5.445	0.950
		STM50	∞	0	∞	0.998
		SE20	5.610	0.864	36.401	0.071
		SE100	1.540	0.010	234.420	0.866
		all SE	3.66	0.795	16.86	0.096
		all STM	1.365	0.304	6.117	0.684
		all capsules	2.232	0.845	5.896	0.105
MKTTn	XLD compared to other than XLD	STM5	1.969	0.981	3.952	0.057
		STM50	2.333	0.988	5.513	0.053
		SE20	2.843	0.998	8.097	0.050
		SE100	1.941	0.735	5.125	0.180
		all SE	2.385	1.185	4.800	0.015
		all STM	2.136	1.240	3.681	0.006
		all capsules	2.070	1.384	3.096	0.0004
MSRV	XLD compared to other than XLD	STM5	1.247	0.315	4.930	0.753
		STM50	1	0	∞	1
		SE20	0.881	0.142	5.468	0.891
		SE100	1	0	∞	1
		all SE	0.892	0.170	4.687	0.893
		all STM	1.223	0.320	4.675	0.769
		all capsules	1.083	0.407	2.881	0.873
All enrichment media	XLD compared to other than XLD	STM5	1.490	0.8978	2.474	0.123
		STM50	1.999	0.9514	4.199	0.067
		SE20	2.662	1.243	5.700	0.012
		SE100	1.704	0.708	4.099	0.234
		all SE	2.220	1.265	3.897	0.005
		all STM	1.636	1.081	2.477	0.019
		all capsules	1.828	1.316	2.539	0.0004

Grey cells: significant difference $p < 0.05$

Table 21 **Number of positive isolations found with a selective enrichment medium compared to the number of positive isolations found with another selective enrichment medium, given that the isolation is on XLD.**
Samples : chicken feed, artificially contaminated with *Salmonella* positive capsules.

Compared selective enrichment media	Isolation medium	Capsule	Odds Ratios	95 % lower	95 % upper	p-value
RVS compared to MKTTn	XLD	STM5	0.141	0.041	0.487	0.002
		STM50	2E-08	0	∞	0.992
		SE20	0.310	0.046	2.090	0.229
		SE100	0.113	0.010	1.259	0.076
		all SE	0.187	0.046	0.760	0.019
		all STM	0.083	0.026	0.265	2E-05
		all capsules	0.123	0.053	0.287	1E-06
RVS compared to MSRV	XLD	STM5	0.766	0.157	3.748	0.743
		STM50	1	0	∞	1
		SE20	0.402	0.051	3.153	0.386
		SE100	∞	0	∞	0.998
		all SE	0.718	0.132	3.901	0.701
		all STM	0.785	0.167	3.679	0.759
		all capsules	0.767	0.255	2.306	0.636
MSRV compared to MKTTn	XLD	STM5	0.176	0.056	0.551	0.003
		STM50	2E-08	0	∞	0.994
		SE20	0.619	0.132	2.90	0.543
		SE100	3E-08	0	∞	0.995
		all SE	0.249	0.071	0.871	0.030
		all STM	0.106	0.037	0.302	3E-05
		all capsules	0.160	0.075	0.342	2E-06

Grey cells: significant difference $p < 0.05$

Figure 5 shows the performance of each laboratory as odds ratios compared to the mean of all laboratories for the artificially contaminated samples. The blank capsules are not used in this calculation. The mean (= 1) is defined as the odds of detecting *Salmonella* based on the fixed effects only (capsule, enrichment medium and isolation medium). Laboratories below the mean have a lower odds to detect *Salmonella*. Ten laboratories 1, 2, 3, 9, 13, 15, 23, 24, 25 and 27 found significant lower number of positive results compared to the mean of all laboratories.

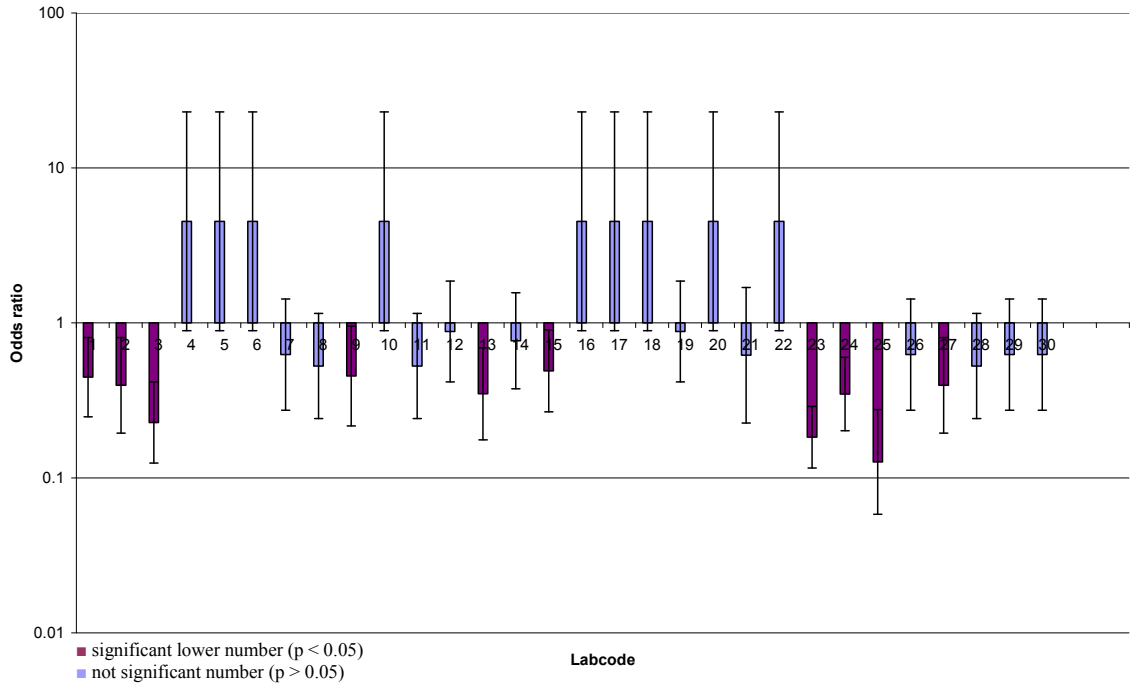


Figure 5 **Performance of each laboratory compared to the mean of all laboratories for the artificially contaminated feed samples (without blanks).**

4.5.3 Specificity, sensitivity and accuracy rates of the artificially contaminated feed samples

The specificity, sensitivity and accuracy rates for all types of capsules added to the chicken feed are shown in Table 22. The results are given for the different medium combinations: BPW followed by selective enrichment in RVS, MKTTn and on MSRV and isolation on selective plating agar XLD. It was expected that the blank capsules and the high level materials scored close to 100 % and the low level materials scored a minimum of 75-80 %. The rates showed the expected or even better results. The specificity rates (of the blank capsules) were for all three selective enrichment media 99 %. For all capsules containing *Salmonella,* the sensitivity rates found with MSRV and RVS were higher than the rates of MKTTn. The sensitivity rates of the high level materials scored close to 100 % and the low level materials close to 97 % (found with MSRV and RVS).
There was no difference between the rates of EU Member States and the two non-EU Member States. In Table 22 the rates of all participants are given.

Table 22 **Specificity, sensitivity and accuracy rates for all participating laboratories (n=29)* of the artificially contaminated chicken feed samples (each capsule added to 25 g chicken feed) for the selective enrichment in RVS, MKTTn and on MSRV and plating out medium XLD.**

Capsules with chicken feed		RVS/XLD	MKTTn/XLD	MSRV/XLD
Blank (n=5)	No. of samples	145	145	145
	No. of negative samples	144	144	144
	Specificity in %	**99.3**	**99.3**	**99.3**
STM5 (n=5)	No. of samples	145	145	145
	No. of positive samples	141	125	140
	Sensitivity in %	**97.2**	**86.2**	**96.6**
STM50 (n=5)	No. of samples	145	145	145
	No. of positive samples	145	132	145
	Sensitivity in %	**100**	**91.03**	**100**
SE20 (n=5)	No. of samples	145	145	145
	No. of positive samples	143	139	141
	Sensitivity in %	**98.6**	**95.9**	**97.2**
SE100 (n=5)	No. of samples	145	145	145
	No. of positive samples	144	137	145
	Sensitivity in %	**99.3**	**94.5**	**100**
All capsules with *Salmonella*	No. of samples	580	580	580
	No. of positive samples	573	533	571
	Sensitivity in %	**98.8**	**91.9**	**98.5**
All capsules	No. of samples	725	725	725
	No. of correct samples	717	677	715
	Accuracy in %	**98.9**	**93.4**	**98.6**

* Laboratory 21 did not confirm the suspected colonies from XLD, therefore their results were not taken into account.

4.6 Own method

PCR
Three laboratories (labcodes 10, 17, and 28) applied a PCR method as additional detection technique. These laboratories tested the samples after pre-enrichment in BPW and used a real time PCR. In Table 23 further details on the volumes used in the PCR techniques are summarized.

Table 23 **Details on real time Polymerase Chain Reaction procedures, used as own method during the interlaboratory comparison study by three laboratories.**

Labcode	Volume of BPW (µl)	Volume of DNA sample (µl)	Volume of DNA / PCR mix (µl)
10	50	100	3/?
17	1000	100	12/30
28	1000	300	5/25

Laboratory 10 (Hein et al., 2006) and 17 (Malorny et al., 2004) used a non-commercially available PCR technique validated for the matrices: chicken rinse or meat, minced meat and raw milk. Laboratory 28 used Applied Biosystems Taqman *Salmonella* enterica detection kit and this PCR is validated for food and feed by AFNOR (AFNOR, 2007).

The laboratories found the same results with the PCR-technique as with the bacteriological culture methods (MSRV and RVS). All samples were scored correctly.

VIDAS
One laboratory (labcode 6) applied VIDAS SLM (an ELISA based method) as additional method. This method is validated for animal and human food by AFNOR (AFNOR 1994).The laboratory tested the samples after pre-enrichment in BPW according to the Vidas SLM protocol of Biomerieux. The laboratory found all *Salmonella* samples positive with the VIDAS method. However also blank samples were tested positive for *Salmonella*. With the VIDAS three out of five blank samples (with chicken feed) and both blank control capsules (without matrix) were found positive for *Salmonella*.
This laboratory (labcode 6) also found blank samples positive with the bacteriological culture methods.

4.7 Performance of the NRLs

4.7.1 General

Twenty-nine NRLs fulfilled the criteria of good performance. One laboratory scored below the criteria of good performance with the prescribed and requested method. When only the prescribed method (ISO 6579) is taken into account the score of another laboratory was also below the criteria of good performance. Laboratory 15 could not detect both SPan5 control capsules with RVS and MKTTn but they found one SPan5 positive with the requested method MSRV and so they scored a good performance.

Laboratory 6 found both blank control samples (without matrix) and one blank sample with matrix positive for *Salmonella* with all the methods. This laboratory was contacted by the CRL-*Salmonella* in November 2008 to ask for any explanations for the deviating results. The NRL investigated possible reasons and checked different equipments. They found *Salmonella* in the aspiration system and on the working bench. The following corrective actions were taken: autoclaving of the aspiration system, changing of the filter, cleaning of all working benches, incubators and refrigerators. The NRL modified their procedures to minimise future contamination risks.
The positive blank samples, therefore, were most likely caused by cross contamination.
To check whether the actions taken to prevent cross contamination have been successful, laboratory 6 participated in a follow up study organised by the CRL-S in March 2009.

Two laboratories (labcodes 21 and 25) used a deviating procedure. They confirmed the suspected colonies from only one isolation medium. According to ISO 6579 (2002) at least one suspect/typical colony of each selective isolation medium should be confirmed. Both laboratories were contacted by the CRL-*Salmonella* in November 2008 to ask for further information on their procedures.
Laboratory 21 indicated that they routinely use only RVS for selective enrichment of *Salmonella* from animal feed samples and have no experiences with MKTTn or MSRV. For this study they used all prescribed and requested selective enrichment media, but due to lack of capacity they were not able to confirm all isolation media.
Laboratory 25 did not further explain why they only confirmed suspected colonies from XLD, while they found suspect colonies on both isolation media XLD and BGA.
Both laboratories fulfilled the criteria of good performance and no further action was taken.

4.7.2 Follow-up study

The set-up of the follow-up study was the same as the full interlaboratory comparison study in October 2008, but with other batches of capsules (see section 4.1 'Reference materials'). In this follow-up study more blank capsules were tested, as these samples were causing most of the problems. An overview is given in Table 24. The number of aerobic bacteria ($3*10^5$ cfu/g) and Enterobacteriaceae ($9.2*10^2$ cfu/g) were tested on 10 February 2009 after the chicken feed was stored at 5 ^oC for 5 months (since the study in October 2008).
On Monday 9 March 2009 one parcel with two plastic container was send to the laboratory 6 containing: 7 control capsules (numbered C1 – C7), 15 capsules (numbered 1 – l5), 480 g chicken feed and 1 temperature recorder.
The performance of this follow up study was on 16 March 2009. The laboratory had to follow the same SOP and Protocol as in the study of October 2008 (see Annexes 4 and 5). The test report was different from the October study (see Annex 8). For the media only the differences with the October study needed to be indicated.

Table 24 **Overview of the types and the number of capsules tested by laboratory 6 in the follow-up of the interlaboratory comparison study.**

Capsules	Control capsules (n=7) no feed added	Test samples (n=15) with 25 g *Salmonella* negative chicken feed
S. Enteritidis 20 (SE20)	3	4
S. Enteritidis 100 (SE100)	-	1
S. Typhimurium 5 (STM5)	-	4
S. Typhimurium 50 (STM50)	1	-
Blank	3	6

For the media compositions, incubation times and temperatures no differences were observed in comparison with the full study.
Laboratory 6 scored again an under performance. They scored all the 15 artificially contaminated samples correctly. For the prescribed method (RVS and MKTTn) they also scored all the control capsules correctly but with the requested method (MSRV) they found one of the three blank capsules (without matrix) positive.
The CRL-*Salmonella* contacted this laboratory in April 2009 to ask for any explanation for the deviating results. The laboratory never used MSRV method for analysing feed samples but has used it on request in the ringtrial. The most plausible reason for the false positive result is a contamination on the working bench. Till now they could not find the contamination source for the false positive *Salmonella*. They will control the absence of *Salmonella* on working benches, incubators and pipette systems and they will review their cleaning procedure.
We advise the NRL to make sure that the laboratory equipment is well cleaned and disinfected and to analyse blank samples with each series of analyses to check for any possible future cross contamination.

5 Discussion

Transport of the samples
Nor transport time, nor transport temperature seem to have negatively affected the results. The laboratories with the longest transport times and/or the highest transport temperatures (labcodes 1, 7 and 29) still found good results.

Performance of the laboratories
The prescribed method (ISO 6579: RVS and MKTTn) and the requested method (Annex D of ISO 6579: MSRV) were used by all the laboratories. Two laboratories (labcodes 21 and 25) used a deviating procedure they confirmed only one isolation medium with suspected colonies of *Salmonella* instead of confirmation of all media used by the laboratory. Although the result of both laboratories was still within the limits of good performance, laboratory 25 found significant lower mean number of positives than all the other laboratories. It would be interesting to know whether the deviating confirmation procedure caused this low number of positives.
For determining 'good performance' per laboratory all combinations of selective enrichment media and isolation media used by each laboratory were taken into account. Twenty-eight out of 30 laboratories scored a 'good performance' for the prescribed method (RVS and MKTTn). One NRL (labcode 15) achieved this level when also the requested method (MSRV) was taken into account. One laboratory (labcode 6) was unable to fulfil the criteria in the full study, and neither in a follow up test. The under performance of this laboratory (positive blanks with all methods) was most probably caused by cross contamination as they found out after checking their procedures after the study in October 2008. Despite their activities the problem still does not seem to be completely solved in the follow-up study in March 2009. They found again one positive blank control sample, this time only for the requested method (MSRV). They scored all samples correctly with the prescribed method (RVS and MKTTn) inoculated from the same BPW (pre-enrichment medium). The reason for this failure in the follow up study is most probably again cross contamination. A possible other explanation for this one mistake with the requested method (MSRV) is their inexperience with this medium.

The results found with the MSRV and RVS media were very good. Although the scope of Annex D of ISO 6579 is detection of *Salmonella* spp. in samples of the primary production, it showed, in this study, better results when compared to the results found with the prescribed method for food analyses (ISO 6579). The selective enrichment medium MKTTn of ISO 6579 showed the lowest number of positive results.
The majority of the ten laboratories which found a significant lower number of positive results for the artificially contaminated samples with *Salmonella* compared to the mean of all laboratories (Figure 5), had problems with finding positives with MKTTn.

Specificity, sensitivity and accuracy rates
The rates of the control samples were good. These rates were as expected or even better. For the low and high level SE capsules the rates were 100 %. For the low level materials SPan5 and STM5 the rates were close to 97 % while at least 75-80 % was expected.
For the artificially contaminated chicken feed samples the highest rates were found with MSRV and RVS. The rates found with MKTTn were approximately 10 % lower than the rates of the two other selective enrichment media. The specificity rates (of the blank capsules) were for all three selective enrichment media 99 %. The sensitivity rates of the high level materials were close to 100 % and the low level materials close to 97 % (found with MSRV and RVS).
Although the mean contamination level of the SE100 capsules was decreased under the minimum target level, the sensitivity was still 99.3-100 % (found with RVS and MSRV). All laboratories were able to find all SE100 capsules positive after selective enrichment in MSRV.

Media
According to Annex D of ISO 6579 (Anonymous, 2007) the concentration of novobiocin in MSRV should be 10 mg/L and the pH between 5.1-5.4. Four laboratories reported the use of a higher concentration of novobiocin and four laboratories did not mention the use of novobiocin. Six laboratories reported a higher/lower pH or did not mention the pH. The eight laboratories (labcode 4, 5, 10, 16, 17, 18, 20 and 22) with a significant higher number of positive results (Figure 5) used MSRV with the prescribed novobiocin concentration and with the correct pH, two of them did not give all the details but they mentioned no deviations. A higher concentration of novobiocin in the MSRV can negatively influence the motility of *Salmonella* and may result in less positive results. A higher pH of MSRV may stimulate the growth of disturbing background flora which can negatively influence the growth of *Salmonella*.
Deviations in media compositions or incubation temperatures were reported but no clear effects were found on the results.
The selective enrichment media MSRV and RVS showed high percentages of positive results already after 24 hours of incubation. These media showed only 2-3 % more positives after 48 h compared to 24 h of incubation. With the selective enrichment medium MKTTn 6-8 % more positive results were found after 48 hours of incubation than after 24 hours.

In general XLD showed (slightly) more positive results than any of the other isolation media used. This effect was most striking when XLD was used after selective enrichment in MKTTn.

PCR
Only three laboratories used a PCR technique additional to the prescribed and requested methods. The results found with the PCR methods were comparable to the results found with the bacteriological detection methods.

VIDAS
One laboratory used VIDAS (an ELISA based method) additional to the prescribed and requested methods. This laboratory found with both the VIDAS and with the bacteriological detection methods blank samples positive. Due to the false positive results it was not possible to compare the different methods.

Evaluation of this study

The contamination levels of the capsules used in this first animal feed study were comparable with the latest veterinary and food studies (Kuijpers et al. 2008a and 2008b). However, the contamination level of the low level *Salmonella* Enteritidis reference material used during this feed study was slightly higher (15-20 cfp/capsule) than of the materials used in the former studies (approximately 10 cfp/capsule). This higher level was chosen as during the former studies it was shown that the low level *S*. Enteritides materials were just at or below the detection limit of the method. With the SE20 capsules all NRLs found good results. In the present feed study the high level SE reference materials were, unintendedly lower (approximately 50 cfp/capsule) than in the former studies (approximately 80-100 cfp/capsule). This did not seem to have affected the results of the NRLs. All participants were able to find all feed samples artificially contaminated with high level SE positive. Although the chosen animal feed matrix contained the highest amount of background flora compared to the other tested feed matrices, it was by far lower than the number of Enterobacteriaceae and aerobic bacteria found in the matrices used in the latest food and veterinary studies. The lower disturbance of background flora in the present feed study may have positively influenced the detection of *Salmonella* and thus the outcome of this study.

6 Conclusions

- Twenty nine of the thirty NRLs for *Salmonella* achieved the level of 'good performance' for the detection of *Salmonella* in chicken feed at once. One laboratory was unable to achieve this level neither in a follow up study.
- The accuracy, specificity and sensitivity rates for the control samples (without feed) found after selective enrichment in RVS, MKTTn and on MSRV were between 97-100 %.
- The specificity rate of the chicken feed samples artificially 'contaminated' with blank capsules was 99 %.
- For all artificially contaminated chicken feed samples with *Salmonella* the rates found with MKTTn were lower than the rates of MSRV and RVS.
- The sensitivity rates for artificially contaminated chicken feed samples with *Salmonella* were higher than 97 % after selective enrichment on MSRV and in RVS. After selective enrichment in MKTTn the sensitivity rate was 86-91 % for *Salmonella* Typhimurium and approximately 95 % for *Salmonella* Enteritidis.
- The low level materials of *S*. Enteritidis (SE20) were easier to detect than the low level materials of *S*. Typhimurium (STM5).
- The accuracy rates for the artificially contaminated chicken feed samples were higher than 98 % for RVS and MSRV and was 93 % for MKTTn.
- Significantly more positive results were found on XLD when compared to other plating-out media after selective enrichment in MKTTn.
- When MSRV or RVS are used as selective enrichment media, the odds of finding *Salmonella* are significantly higher than when using MKTTn (all in combination with isolation medium XLD).
- XLD showed slightly more positive results than other plating-out media independent on the selective enrichment medium used.
- The number of positive isolations is more influenced by the choice of the selective enrichment medium than by the choice of the plating-out medium.
- A longer incubation time than 24 hours is more important (more positive results after 48 h) for selective enrichment in MKTTn than for MSRV, for the matrix used (chicken feed).
- MSRV and RVS are good selective enrichment media for the detection of *Salmonella* in the matrix used (chicken feed).
- MKTTn is not the optimal selective enrichment medium for the detection of *Salmonella* in the matrix used (chicken feed).
- This first animal feed study showed to have been successful; all participants found good results.

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Annex 1 History of CRL-*Salmonella* interlaboratory comparison studies on the detection of *Salmonella*

Table A.1 History of CRL-*Salmonella* interlaboratory comparison studies on detection of *Salmonella* in veterinary samples.

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces[1] added	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
I	1995	26	STM5	6	No	RV and SC	BGA and own	Voogt et al., 1996 (report 284500003)
		4	Blank	0	No			
II	1996	15	STM100	116	1 gram	RV, SC and own	BGA and own	Voogt et al., 1997 (report 284500007)
		15	STM1000	930	1 gram			
		2	SPan5	5	No			
		1	STM100	116	No			
		1	Blank	0	No			
III	1998	14	STM10	11	1 gram	RV and own	BGA and own	Raes et al., 1998 (report 284500011)
		14	STM100	94	1 gram			
		7	STM100	94	1 gram*			
		14	SE100	95	1 gram			
		4	STM10	11	No			
		2	SPan5	5	No			
		5	Blank	0	No			
IV	1999	5	STM10	4	10 gram	RV or RVS, MSRV and own	BGA and own	Raes et al., 2000 (report 284500014)
		5	STM100	210	10 gram			
		5	SE100	60	10 gram			
		5	SE500	220	10 gram			
		5	Blank	0	10 gram			
		3	STM10	5	No			
		3	SE100	60	No			
		2	SPan5	5	No			
		2	Blank	0	No			
V	2000	5	STM10	4	10 gram	RV or RVS, MSRV and own	BGA and XLD	Raes et al., 2001 (report 284500018)
		5	STM100	47	10 gram			
		5	SE100	63	10 gram			
		5	SE500	450	10 gram			
		5	Blank	0	10 gram			
		3	STM10	4	No			
		3	SE100	63	No			
		2	SPan5	5	No			
		2	Blank	0	No			
		20	None	-	25 gram**			

Table A.1 (continued)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces[1] added	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
VI	2002	5	STM10	11	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2003 (report 330300001)
		5	STM100	139	10 gram			
		5	SE100	92	10 gram			
		5	SE500	389	10 gram			
		5	Blank	0	10 gram			
		3	STM10	11	No			
		3	SE100	92	No			
		2	SPan5	5	No			
		2	Blank	0	No			
		20	None	-	25 gram**			
VII	2003	5	STM10	12	10 gram	RVS, MSRV, MKTTn and own	BGA, XLD and own	Korver et al., 2005 (report 330300004)
		5	STM100	96	10 gram			
		5	SE100	127	10 gram			
		5	SE500	595	10 gram			
		5	Blank	0	10 gram			
		3	STM10	12	No			
		3	SE100	127	No			
		2	SPan5	9	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
VIII	2004	7	STM10	13	10 gram	MSRV and own	XLD and own	Korver et al., 2005 (report 330300008)
		4	STM100	81	10 gram			
		7	SE100	74	10 gram			
		4	SE500	434	10 gram			
		3	Blank	0	10 gram			
		3	STM10	13	No			
		2	SE100	74	No			
		1	SE500	434	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		20	None	-	10 gram**			
IX	2005	5	STM10	9	10 gram[2]	MSRV and own	XLD and own	Berk et al., 2006 (report 330300011)
		5	STM100	86	10 gram			
		5	SE100	122	10 gram			
		5	SE500	441	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	86	No			
		1	SE500	441	No			
		2	SPan5	7	No			
		2	Blank	0	No			
		10	None	-	10 gram***			

Table A.1 (continued)

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative faeces added[2]	Selective enrichment medium	Plating-out medium	Reference [3] (RIVM report)
X	2006	5	STM10	9	10 gram	MSRV and own	XLD and own	Kuijpers et al., 2007 (Report 330604004)
		5	STM100	98	10 gram			
		5	SE100	74	10 gram			
		5	SE500	519	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	98	No			
		1	SE500	519	No			
		2	SPan5	5	No			
		2	Blank	0	No			
XI	2008	5	STM5	6	10 gram	MSRV and own	XLD and own	Kuijpers et al., 2008 (Report 330604011)
		5	STM50	47	10 gram			
		5	SE10	9	10 gram			
		5	SE100	90	10 gram			
		5	Blank	0	10 gram			
		3	STM5	6	No			
		2	SE10	9	No			
		1	SE100	90	No			
		2	SPan5	5	No			
		2	Blank	0	No			

[1]Faeces mixed (1:1) with a solution of peptone/glycerol. Final concentration glycerol in the faeces mixture was 15 %(v/v)

[2] Faeces not mixed with any preservation medium

[3] The report of each study can be found at the CRL-*Salmonella* website:
http://www.rivm.nl/crlsalmonella/publication/ or can be obtained through the corresponding author of this report.

* = with antibiotics

** = Naturally contaminated chicken faeces with *Salmonella*

*** = Naturally contaminated dust with *Salmonella*

Table A.2 CRL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in food samples.

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative meat	Selective enrichment medium	Plating-out medium	Reference [1] (RIVM report)
I	2006	5	STM10	9	10 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al.,. 2007 (Report 330604003)
		5	STM100	98	10 gram			
		5	SE100	74	10 gram			
		5	SE500	519	10 gram			
		5	Blank	0	10 gram			
		3	STM10	9	No			
		2	SE100	98	No			
		1	SE500	519	No			
		2	SPan5	5	No			
		2	Blank	0	No			
II	2007	5	STM5	4	10 gram	RVS, MKTTn, MSRV and own	XLD and own	Kuijpers et al., 2008 (Report 330604010)
		5	STM50	40	10 gram			
		5	SE10	7	10 gram			
		5	SE100	71	10 gram			
		5	Blank	0	10 gram			
		3	STM5	4	No			
		2	SE10	7	No			
		1	SE100	71	No			
		2	SPan5	7	No			
		2	Blank	0	No			

Table A.3 CRL-*Salmonella* interlaboratory comparison study on the detection of *Salmonella* in animal feed samples.

Study	Year	Number of samples	Capsules	Actual number of cfp/capsule	*Salmonella* negative feed	Selective enrichment medium	Plating-out medium	Reference [1] (RIVM report)
I	2008	5	STM5	5	25 gram	RVS, MKTTn, MSRV and own	XLD and own	This report
		5	STM50	43	25 gram			
		5	SE20	15	25 gram			
		5	SE100	48	25 gram			
		5	Blank	0	25 gram			
		3	STM5	5	No			
		2	SE20	15	No			
		1	SE100	48	No			
		2	SPan5	5	No			
		2	Blank	0	No			

[1] The report of each study can be found at the CRL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/ or can be obtained through the corresponding author of this report.

Annex 2 Calculation of T_2

The variation between capsules of one batch of reference materials is calculated by means of the so-called T_2 statistic (Heisterkamp et al., 1993)*.

$$T_2 = \sum_i [(z_i - z_+ / I)^2 / (z_+ / I)]$$

where, z_i = count of one capsule (i)
z_+ = sum of counts of all capsules
I = total number of capsules analysed

In case of a Poisson distribution, T_2 follows a χ^2 -distribution with (I-1) degrees of freedom. In this case, the expected T_2-value is the same as the number of degrees of freedom and thus $T_2/(I-1)$ is expected to be equal to one. For the variation between capsules of one batch, the Poisson distribution is the theoretical smallest possible variation which could be achieved. However, over-dispersion is expected and $T_2/(I-1)$ will mostly be larger than 1 (Heisterkamp et al., 1993). An acceptable variation for a batch of capsules will be $T_2/(I-1) \leq 2$.

*Heisterkamp SH, Hoekstra JA, van Strijp-Lockefeer NGWM, Havelaar A, Mooijman KA, In ‘t Veld PH, Notermans SHW, 1993. Statistical analysis of certification trials for microbiological reference materials. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg. EUR Report; EUR 15008 EN.

Annex 3 Information on the media used

BGA modified (Oxoid CM 0329, Hampshire, United Kingdom) (BPLS Merck 1.10747 Darmstadt, Germany) (Lab M Ltd., lab 34 Bury, United Kingdom) (Biomark Laboratories B439, Maharashtra, India) (Himedia Laboratories M971-500G, Mumbai, India)
Watson and Walker 1978 A modification of brilliant green agar for improved isolation of *Salmonella*. J. Appl.Bact. 45 195-204
Composition of BGA modified: according ISO 6579, 1993

BGA (Oxoid CM 0263, Hampshire, United Kingdom)
Composition of BGA medium: the concentration of the compounds in g/L water: Proteose peptone 10.0, Yeast extract 3.0, Lactose 10.0, Sucrose 10.0, Sodium chloride 5.0, Phenol red 0.08, Brilliant green 0.0125, Agar 12.0, pH 6.9

BGA (Conda laboratories, Madrid, Spain)
Composition of BGA medium: the concentration of the compounds in g/L water: Yeast extract 3 Tryptone 5, Peptic digest of animal tissue 5, Lactose 10 Saccharose 10, Sodium chloride 5, Phenol red 0.08, Sulfadiazine 0.08, Agar 20.0, pH 6.9

BPLSA (Merck 107237.0500, Darmstadt, Germany)
Composition of BPLSA medium: the concentration of the compounds in g/L water: Peptone from meat 5.0, Peptone from casein 5.0, Meat extract 5.0, Sodium chloride 3.0, di-sodium hydrogen phosphate 2.0, Lactose 10, Sucrose 10, Phenol red 0.08, Brilliant green 0.0125, Agar agar 12.0, pH 7

Blaskal Bromthymol blue agar
Composition of Blaskal medium : the concentration of the compounds in g/L water:
Beef Heart Infusion 10, Tryptose 10, Sodium Chloride 5, Agar 15, Laktose-Saccarose solution 60 ml (60 ml contains : Lactose 13.3, Saccarose 13.3, Sodium thisulfate.5H_2O 1.33, Bromthymolblue 0.2 % 53.3 ml, Crystal violet 0.1 %, 6.67 ml) pH 7.5

Hektoen Enteric (Biokar Diagnostics BK 067HA, Beauvais, France)
King, S. a. Metzger, W.J.: A new plating medium for the isolation of enteric pathogens. I. Hektoen Enteric Agar. - Appl. Mikrobiol.,1968: 16; 557-578.
Composition is not provided, pH 7.5

MLCB (Lab M. Ltd. lab 116, Bury, United Kingdom)
Inoue T, Takagi S, Ohnishi A, et al. Foodborne disease *Salmonella* isolation medium (MLCB). Japanese Journal of Veterinary Science 1968;30 (suppl):26.
Composition of MLCB medium: the concentration of the compounds in g/L water: Yeast Extract 5.0, Tryptone 5.0, Meat Peptones 7.0, Sodium Chloride 4.0, Mannitol 3.0, L-Lysine HCL 5.0, Sodium Thiosulphate 4.0, Ferric Ammonium Citrate Green 1.0, Brilliant Green 0.012, Crystal Violet 0.01, Agar No.2 15.0, pH 6.8

Rambach (Merck 107500.0001/2/3 or 1.15999.0001, Darmstadt, Germany) (Chromagar RR701 CHROMagar Company, Paris, France)
Rambach, A.: New Plate Medium far Facilitated Differentiation of *Salmonella* spp. from Proteus sac. and Other Enteric Bacteria». - Appl. Environm. Microbiol., 56; 301-303 (1990).
Composition of Rambach medium: the concentration of the compounds in g/L water: Peptone 8.0, NaCl 5.0, sodium deoxycholate 1.0, Cromogenic mix 1.5, propylene glycol 10.5, agar-agar 15, Rambach agar supplement 10 ml, pH 7.0-7.3

Rapid *Salmonella* agar (Biorad 3563961, Marnes-La-Coquette, France)
Casein Peptone 5, Meat extract 5, Selective agents (Sodium citrate, sodium deoxycholate, ferrammonium citrate) 14, Chromogenic mixture 310 mg, Agar 12, pH 7.2

SM2 = SM ID2 (bioMérieux SM2 43621, Marcy l' Etoile, France)
Pignato, S., G. Giammanco, and G. Giammanco. 1995 Rambach agar and SM-ID medium sensitivity for presumptive identification of *Salmonella* subspecies I to VI. J. Med. Microbiol., Vol 43, Issue 1 68-71
Composition of SM ID2 medium: the concentration of the compounds in g/L water: Peptones (swine and bovine) 6.25, Tris 0.16, Lactose 6.0, Ox bile (bovine and swine) 1.5, Cromogenic mix 9.63, Sodium chloride 5.0, Selective mix 0.03, Agar 14 pH not mentioned

XLT4 (Oxoid PO5116A, Hampshire, United Kingdom)
Miller, R.G., C.R. Tate. 1990. XLT4: A highly selective plating medium for the isolation of *Salmonella*. The Maryland Poultryman, April: 2-7 (1990).
Composition of XLT4 medium: the concentration of the compounds in g/L water: Peptone 1.6, Yeast Extract 3, L-Lysine 5, Lactose 7.5, Saccharose 7.5, Xylose 3.75, Sodium Chloride 5, Sodium Thiosulphate 6.8, Ferric Ammonium Citratus 0.8, 7-ethyl-2 methyl-4-undecanol hydrogen 4.6 ml (or niaproof 4 4.6 g), Phenol Red 0.08, Agar 18 pH 7.4

Annex 4 Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN ANIMAL FEED organised by CRL-*Salmonella*
FEED STUDY I - 2008

Introduction

This protocol describes the procedures for the first interlaboratory comparison study on the detection of *Salmonella* spp. in a feed matrix amongst the National Reference Laboratories (NRLs for *Salmonella*) in the EU. In earlier studies the detection of *Salmonella* spp. in veterinary and food samples were studied. Additional to these matrices the detection of *Salmonella* spp. in animal feed is also an important task for the CRL-*Salmonella*, as well as for the NRLs-*Salmonella*. This is described in Commission Regulations EC No 882/2004 on official controls. The organisation of this interlaboratory comparison study was discussed with the NRLs for *Salmonella* at the annual CRL-*Salmonella* workshop in May 2008.

This first study will have a comparable set-up as the earlier studies on the detection of *Salmonella* spp. in food and veterinary samples.
The prescribed method is the procedure as described in ISO 6579 (Microbiology of food and feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Fourth edition, 2002.) Beside ISO 6579 it is requested also to use Annex D of ISO 6579 (EN-ISO 6579:2002/Amd1: 2007: Amendment 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage). The method in this annex is especially intended for the detection of *Salmonella* spp. in animal faeces and environmental samples from the primary production stage, but is also likely to be applicable for the analyses of feed samples. Furthermore, laboratories are allowed to additionally analyse the samples with other methods, like molecular methods and/or other methods (routinely) used in their laboratories.

The samples will consist of chicken feed (*Salmonella* negative) artificially contaminated with reference materials. The reference materials (RMs) consist of gelatine capsules containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) at different contamination levels. Each laboratory will examine 25 feed samples (**25 g each**) in combination with a capsule containing STM or SE and 10 control samples (capsules only).

The samples will be packed in 2 plastic containers in one large box together with cooling elements. One container will contain the capsules the other container will contain the chicken feed. The container with the capsules will also contain a temperature recorder to measure the temperature during transport of the samples. The recorder will be packed in a plastic bag, which will also contain your lab code. **You are urgently requested to return this complete plastic bag with recorder and lab code to the CRL-*Salmonella*, immediately after receipt of the parcel**. For this purpose a return envelope with a preprinted address label of the CRL-*Salmonella* has been included. Do not forget to note your labcode before returning it to the CRL.

Each box with samples will be sent as biological substance category B (UN3373) by door-to-door courier service. Please contact CRL-*Salmonella* when the parcel has not arrived at your laboratory at 25 of September 2008 (this is 4 working days after the day of mailing).

Objectives

The main objective of this first interlaboratory comparison study on the detection of *Salmonella* in a feed matrix is to evaluate the results of the detection of different contamination levels of *Salmonella* in the presence of competitive micro-organisms in a feed matrix, using different methods, among and within the NRLs.

Outline of the study

Each participant will receive (in week 39 of 2008) one box containing 2 plastic containers, packed with cooling elements. The containers contain:

Container 1:

- 25 numbered vials; each containing one *Salmonella* Typhimurium, one *Salmonella* Enteritidis or a blank capsule (numbered 1-25);
- 10 control vials; each containing one capsule with or without *Salmonella* (numbered C1-C10).

This container will also contain the small electronic temperature recorder in a plastic bag with your lab code. **This recorder (in the plastic bag) should be returned to the CRL-*Salmonella* as soon as possible.**
Store container 1 at (-20 ± 5) ºC immediately after receipt.

Container 2:

- Approximately 700 g of chicken feed (free from *Salmonella*).

Store container 2 at (5 ± 3) ºC immediately after receipt.

The performance of the study will be in week 41 (starting on 6 October 2008).

The documents necessary for performing the study are:

- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in feed I (2008);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in feed I (2008);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in feed I (2008);
- ISO 6579 (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.;
- Amendment ISO 6579:2002/Amd 1: 2007 Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage.

The media used for the collaborative study will not be supplied by the CRL.

All data have to be reported in the test report and sent to the CRL-*Salmonella* before **24 October 2008.** The CRL will prepare a summary report soon after the study to inform all NRLs on the overall results.
Results which will be received after the deadline can not be used in the analyses for the short report.

If you have questions or remarks about the interlaboratory comparison study please contact:

Angelina Kuijpers (Tel. number: + 31 30 274 2093) or
Kirsten Mooijman (Tel. number: + 31 30 274 3537)
RIVM / LZO Pb 63
P.O. Box 1
3720 BA Bilthoven
The Netherlands
Fax. number: + 31 30 274 4434
E-mail : Angelina.Kuijpers@rivm.nl or Kirsten.Mooijman@rivm.nl

Time table of interlaboratory comparison study FEED I (2008)

Week	Date	Topic
37	8 – 12 September	Mailing of the protocol, standard operating procedure and test report to the NRLs-*Salmonella*
39	22 – 26 September	Mailing of the parcels to the NRLs as Biological Substance Category B (UN3373) by door-to-door courier service. Immediately after arrival of the parcels at the laboratory: - Check for any serious damages (**do not accept damaged packages)**; - Check for completeness; - Remove the electronic temperature recorder from the parcel (leave it in the plastic bag with lab code) and return it to CRL-*Salmonella* using the return envelope; - **Store the capsules at -20 ± 5 °C** - **Store the feed at +5 ± 3 °C** **If you did not receive the parcel at 25 September, do contact the CRL immediately.**
40	29 September – 3 October	Preparation of: 1. Non selective pre-enrichment medium (see SOP 6.1) 2. Selective enrichment media (see SOP 6.2) 3. Solid selective plating media (see SOP 6.3) 4. Confirmation media (see SOP 6.4)
41	6 – 10 October	Performance of the study, following the instructions as given in the protocol and the SOP of study Feed I (2008).
43	Before 24 October	Completion of the test report. Send the test report, preferably by e-mail to the CRL Salmonella (Angelina.kuijpers@rivm.nl)*.
44	27 – 31 October	Checking the results by the National Reference Laboratories.
	December 2008	Sending of the final results to the NRLs together with a short report. As a follow-up, actions will be undertaken for those NRLs which scored below the average results of all NRLs.

* If the test report is e-mailed to the CRL it is not longer necessary to sent the original test report as well, unless it is not legible (to be indicated by CRL-*Salmonella*)

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Annex 5 Standard Operating Procedure (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN ANIMAL FEED organised by CRL-*Salmonella* FEED STUDY I - 2008

1 Scope and field of application

This standard operating procedure (SOP) describes the procedure for the detection of *Salmonella* in the presence of competitive micro-organisms in a feed matrix. For this purpose Reference Materials (RMs), containing sublethally injured *Salmonella* Typhimurium (STM), *Salmonella* Enteritidis (SE) or *Salmonella* Panama (SPan) as prepared by the Community Reference Laboratory (CRL) for *Salmonella,* are used. As matrix, chicken feed (negative for *Salmonella*) is used. The application of this SOP is limited to the interlaboratory comparison study for *Salmonella* described in this SOP.

2 References

International Standard – ISO 6579: 2002(E)
Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

Amendment ISO 6579:2002/Amd 1 2007. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage.

3 Definitions

For the purpose of this SOP, the following definitions apply:

- *Salmonella*: micro-organisms which form typical colonies on isolation media for *Salmonella* and which display the serological and/or biochemical reactions described when tests are carried out in accordance with this SOP.
- *Reference Material*: a gelatine capsule containing a quantified amount of a test organism in spray dried milk.

4 Principle

The detection of *Salmonella* involves the following stages:
a) Pre-enrichment
b) Selective enrichment
c) Isolation
d) Confirmation of typical colonies as *Salmonella*.

5 List of abbreviations

BPW	Buffered Peptone Water
MKTTn	Muller Kaufmann Tetrathionate novobiocin broth
MSRV	Modified semi-solid Rappaport Vassiliadis medium
RM	Reference Material
RVS	Rappaport Vassiliadis medium with Soya
SOP	Standard Operating Procedure
XLD	Xylose Lysine Deoxycholate agar

6 Culture media

For this study the prescribed method is ISO 6579, with an extra incubation step of 24 h of the selective enrichment media. Additional to ISO 6579 it is requested also to apply Annex D of ISO 6579.

Non selective pre-enrichment medium	BPW
Selective enrichment media	MKTTn & RVS (prescribed)
	MSRV (requested)

Selective plating media for first and second isolation XLD and a second medium for choice (obligatory!)

Composition and preparation of the media and reagents are described in Annex B, and in Annex D of the ISO 6579: 2002(E). In the list of media given in 6.1 up to 6.4, reference is made to the relevant part of ISO 6579. Complete ready-to-use media or dehydrated media are also allowed to be used, as long as the composition is in accordance with the information given below. Control the quality of the media before use.

Beside the prescribed method (ISO 6579) and the requested method (Annex D of ISO 6579) it is allowed to use other methods, e.g. the one(s) routinely used in your laboratory ['Own' method(s)]. Prepare media for the 'own' method(s) according to the relevant instructions. Note all relevant information in the test report.

6.1 Non selective pre-enrichment medium

- Buffered Peptone water (BPW) (ISO6579 Annex B.1)

Mind to distribute the BPW in portions of **225 ml** into suitable flasks before sterilisation.

6.2 Selective enrichment medium

- Rappaport Vassiliadis medium with soya (RVS broth) (ISO6579 Annex B.2)
- Muller Kauffmann tetrathionate-novobiocin broth (MKTTn) (ISO6579 Annex B.3)
- Modified Semi solid Rappaport Vassiliadis (MSRV) (requested) (ISO6579 Annex D)
- Own selective enrichment medium routinely used in your laboratory (optionally)

6.3 Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate (140 mm and 90 mm plates) (ISO6579 Annex B.4)
- Second isolation medium for choice (obligatory)
- Own isolation medium routinely used in your laboratory (optionally)

6.4 Confirmation media

Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercial available identification kits.

- Nutrient agar (optionally) (ISO6579 Annex B.5)

7 Apparatus and glassware

The usual used microbiological laboratory equipment. If requested, note specifications of the apparatus and glassware on the test report.

7.1 Apparatus

- Oven (for dry sterilisation) or autoclave (for wet sterilisation);
- Water bath or incubator, capable of operating at 37 °C ± 1 °C ;
- Water bath or incubator, capable of operating at 41,5 °C ± 1 °C
- Sterile loops of 1 µl and of 10 µl;
- pH-meter; having an accuracy of calibration of ± 0.1 pH unit at 25 °C.

7.2 Glassware

- Culture bottles or jars with nominal capacity of 300 ml;
- Culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
- (Micro-) pipettes; nominal capacity 0,1 ml and 1 ml;
- Petri dishes; standard size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

8 Procedure

Below the prescribed and the requested method for the first interlaboratory comparison study in a feed matrix of CRL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. Beside these methods it is also allowed to use one or more own methods. Please record all relevant data in the test report. Details of

the prescribed method can be found in ISO 6579. Details of the requested method can be found in Annex D of ISO 6579 (2007).

8.1 Prewarming BPW (day 0)

Label 25 jars containing **225 ml of BPW** from 1 to 25. Also label 12 jars of BPW from C1 to C12 (control capsules). One jar is a procedure control (= C11) to which no capsule or feed is added and one jar is a negative feed control to which only 25 g chicken feed is added (= C12). These control jars should further be handled in the same way as the other jars. Place all jars (at least) **overnight** at 37 °C (± 1 °C). Also place some extra non-labelled jars containing 225 ml of BPW at 37 °C in case some jars might have been contaminated. Record in the test report (page 2 & 3) the requested data of BPW.

8.2 Pre-enrichment (day 1)

Take the numbered vials with the *Salmonella* capsules and the control capsules out of the freezer one hour before they are added to the BPW, to allow them to equilibrate to room temperature.
Shortly before adding the capsules, take the jars with BPW from the 37 °C incubator and inspect them for visual growth. Discard infected jars.
Add to 35 labelled jars a gelatine capsule from the vial with the corresponding label number. Do not open the gelatine capsule and do not shake the BPW to dissolve the capsule more rapidly. Place the jars with the capsules in the 37 °C incubator for **45 minutes** for dissolving of the capsules. Record the temperature and time at the start and at the end of this period in the test report (page 3). After 45 minutes add the chicken feed to the jars as follows :

- **Add 25 g of chicken feed to jars labelled 1-25 and C12,**
- **Add no feed to jars labelled C1 - C11,**

Do not shake the jars after adding the chicken feed.

Place all jars in the 37 °C (± 1 °C) incubator for 18 h ± 2 h. Record the temperature and time at the start and at the end of the incubation period and other requested data on page 3 of the test report.

If PCR is performed, fill in all requested data on page 20 & 29 of the test report.

8.3 Selective enrichment (day 2)

Allow the selective enrichment broths RVS and MKTTn (prescribed method) to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the MSRV plates (requested method) in a Laminair Air Flow cabinet if necessary. Record (page 4-11) the requested data of the selective enrichment broths (RVS and MKTTn), MSRV plates and own selective enrichment media (if used) in the test report. Label 25 jars/tubes/plates of each selective enrichment medium from 1 to 25. Also label 12 jars/tubes/plates from C1 to C12. All selective media are incubated for 24 h and later on for another 24 h.

If own selective enrichment media are used, label them in the same way as described above.

After equilibration of the media to room temperature:
Prescribed method:

- Transfer 0.1 ml of each BPW culture to each tube with a corresponding label containing 10 ml RVS medium. Incubate at 41,5 °C ± 1 °C for 24 h ± 3 h and later on for another 24 h ± 3 h;
- Transfer 1 ml of BPW culture to each tube with a corresponding label containing 10 ml MKTTn medium. Incubate at 37 °C ± 1 °C for 24 h ± 3 h and later on for another 24 h ± 3 h;

Requested method:

- Inoculate each MSRV plate with three drops of each BPW culture with a corresponding label. Inoculate a MSRV plate with a total volume of 0.1 ml. Incubate **(not upside down)** at 41,5 °C ± 1 °C for 24 h ± 3 h and if negative for another 24 h ± 3 h;

Optional method:

- Inoculate the routinely used selective medium/media (other than those mentioned above), with the corresponding BPW culture (note the inoculation volume of BPW used and the volume of the selective medium/media on test report). Incubate at the temperature and for the time period routinely used.

Place the jars/tubes/plates in the appropriate incubator(s)/water bath(s) and record the temperature and time for the different enrichment media at the start and at the end of the incubation period and other requested data in the test report (page 4-11).

8.4 Isolation media (first and second isolation) (day 3 and 4)

Record in the test report (page 12-17) the requested data of the isolation media used. Label two times 25 large size Petri dishes and 25 standard size Petri dishes of the isolation media from 1 to 25 and label two times 12 large size Petri dishes and 12 standard size Petri dishes from C1 to C12.
Note:
In the case that you do not have large dishes (140 mm) at your disposal use two standard size (90-100 mm) dishes. Inoculate one after the other, using the same loop.

6.1.1.1 First isolation after 24 h

Inoculation:
Inoculate, by means of a 10 µl loop, from MKTTn and RVS cultures the surface of isolation media in large size Petri dishes (or two standard size Petri dishes) with the corresponding label numbers. Use a 1 µl loop to inoculate from suspect MSRV plates, the surface of isolation media in one standard size Petri dish with the corresponding label number. The following isolation media will be used:

1) Xylose Lysine Desoxycholate agar (XLD)
   Place the inoculated plates with the bottom up in the incubator set at 37 ± 1 °C (record temperature and time and other requested data in test report, page 12-13).
2) Second isolation medium. Follow the instructions of the manufacturer (record temperature and time and other requested data in test report, page 14-15).
3) Optionally: selective isolation medium/media routinely used in your laboratory. Incubate the medium/media at the temperature and for the time period routinely used (record temperature and time and other requested data in test report, page 16-17).

After incubation for 24 h ± 3 h, examine the Petri dishes for the presence of typical colonies of *Salmonella*.

6.1.1.2 Second isolation after 48 h

After a total incubation time of 48 h ± 3 h of the selective enrichment media, repeat the procedure described above (**First isolation after 24 h**). Repeat the full procedure only when the First isolation after 24 h on selective enrichment media is negative.

8.5 Confirmation of colonies from first and second isolation (day 4 and day 5)

For confirmation take from each Petri dish of each selective medium at least 1 colony considered to be typical or suspect (use only well isolated colonies). Store the plates at
5 °C ± 3 °C.
Before confirmation (see below), optionally, streak the typical colonies onto the surface of nutrient agar plates with the corresponding label numbers, in a manner which allows to develop well isolated colonies. Record on the test report (page 18) the requested data of the nutrient agar. Incubate the inoculated plates at 37 °C ± 1 °C for 24 h ± 3 h.

If the selected colony is not confirmed as *Salmonella*, test at maximum another 5 typical colonies from the original isolation medium (stored at 5 °C). Report the number of colonies tested and the number of colonies confirmed as *Salmonella* for each dish in Table 1 (isolation using RVS), Table 2 (isolation using MKTTn), Table 3 (isolation using MSRV) and Table 4 (isolation using own enrichment) on the test report pages 21-28. For the results of detection of *Salmonella* using PCR, fill in Table 5 on the test report page 29.

Confirmation of identity
The identity from the colony selected as described above (either directly from the isolation medium, or from nutrient agar) is confirmed by means of appropriate biochemical and serological tests. Follow the instructions of ISO 6579. Note in the test report (page 19) which media/tests have been used for confirmation. The interpretation of the biochemical tests is given in Table 1 of ISO 6579:2002 on page 9.

9. Test report

The test report will contain all information that might influence the results and is not mentioned in this SOP. Some incidents or deviations from the specified procedures will also be recorded. The test report will include the names of the persons, who are carrying out the work and will be signed by these persons. If the study was carried out by another laboratory than the NRL, please also give the details of this laboratory in the test report.

Scheme of Bacteriological Interlaboratory Comparison Study FEED I (2008)
On detection of *Salmonella* spp. in chicken feed (see Annex A)

Day	Topic	Description
0	**Prewarming BPW**	Place at least at the end of the day sufficient jars, each containing 225 ml BPW, at 37 °C ± 1 °C.
1	**Pre-enrichment**	Add 1 capsule to **225 ml** (prewarmed) BPW Do not shake Incubate 45 min. at 37 °C ± 1 °C Add **25 g** chicken feed to BPW Incubate 18 h ± 2 h at 37 °C ± 1 °C
2	**Selective enrichment**	0,1 ml BPW culture in 10 ml RVS, incubate at (41.5 ± 1) °C for (24 ± 3) h 1 ml BPW culture in 10 ml MKTTn, incubate at (37 ± 1) °C for (24 ± 3) h 0,1 ml BPW culture on MSRV plate, incubate at (41.5 ± 1) °C for (24 ± 3) h Own selective enrichment medi(um)(a)
3	**First isolation after 24 h**	Inoculate from RVS, MKTTn, suspect MSRV plates (24 h) and own medi(um)(a) ➢ Xylose Lysine Desoxycholate agar, incubate at (37 ± 1) °C for (24 ± 3) h ➢ Second isolation medium ➢ Own selective medi(um)(a), incubate for specified time at the specified temperature
3	**Continue selective Enrichment**	Incubate RVS, MKTTn, MSRV and own medium another 24 (± 3) hours at the relevant temperatures
4	**Second isolation after 48 h**	If the first isolation was negative, inoculate from RVS, MKTTn, suspect MSRV plates (48 h) and Own medi(um)(a) ➢ Xylose Lysine Desoxycholate agar ➢ Second isolation medium ➢ Own selective medi(um)(a)
4	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the first isolation media (day 3).
5	**Confirmation of identity**	Confirm the identity of the *Salmonella* suspect colonies from the second isolation media (day 4).

Annex 6 Number of positive results of the control samples (capsule without feed) per laboratory and per selective enrichment medium in combination with the isolation medium that gives the highest number of positive isolations.

Labcode	RVS					MKTTn					MSRV				
	Blank n=2	SPan5 N=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1
Good Performance	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**
1	0	2	**2**	2	1	0	2	**2**	2	1	0	2	**2**	2	1
2	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
3	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
4	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
5	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
6	**2**	2	3	2	1	**2**	2	3	2	1	**2**	2	3	2	1
7	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
8	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
9	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
10	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
11	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
12	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
13	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
14	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
15	0	**0**	3	2	1	0	**0**	3	2	1	0	**1**	3	2	1
16	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
17	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
18	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
19	0	2	**2**	2	1	0	2	**2**	2	1	0	2	**2**	2	1
20	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1

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Labcode	RVS					MKTTn					MSRV				
	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1	Blank n=2	SPan5 n=2	STM5 n=3	SE20 n=2	SE100 n=1
Good Performance	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**	**0**	**≥ 1**	**≥ 2**	**≥ 1**	**1**
21	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
22	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
23	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
24	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
25	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
26	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
27	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
28	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
29	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1
30	0	2	3	2	1	0	2	3	2	1	0	2	3	2	1

-: not performed

bold numbers: deviating results

grey cells : results are below the criteria of good performance

Annex 7 Number of positive results of the artificially contaminated chicken feed (with capsule) per laboratory and per selective enrichment medium in combination with the isolation medium that gives the highest number of positive isolations.

Labcode	RVS					MKTTn					MSRV				
	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5
Good Performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
1	0	5	5	**4**	5	0	5	5	**4**	5	0	5	5	**3**	5
2	0	**4**	5	5	5	0	**4**	5	5	5	0	**4**	5	5	5
3	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
4	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
5	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
6	**1**	5	5	5	5	**1**	5	5	5	5	**1**	5	5	5	5
7	0	5	5	5	5	0	5	5	5	3	0	5	5	5	5
8	0	5	5	5	5	0	**4**	**4**	5	5	0	5	5	5	5
9	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
10	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
11	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
12	0	5	5	5	5	0	**4**	5	5	5	0	5	5	5	5
13	0	**4**	5	**4**	5	0	**4**	5	5	5	0	**4**	5	5	5
14	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
15	0	5	5	5	5	0	**4**	**4**	5	5	0	5	5	5	5
16	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
17	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
18	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
19	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
20	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5

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Labcode	RVS					MKTTn					MSRV				
	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5	Blank n=5	STM5 n=5	STM50 n=5	SE20 n=5	SE100 n=5
Good Performance	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**	**≤ 1**	**≥ 2**	**≥ 4**	**≥ 2**	**≥ 4**
21	0	**4**	**4**	5	5	0	**4**	5	5	5	**1**	**4**	5	5	5
22	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
23	0	5	5	5	5	0	**0**	**2**	5	5	0	5	5	5	5
24	0	5	5	5	5	0	**4**	**4**	**4**	**4**	0	5	5	5	5
25	0	5	5	5	**4**	0	**3**	**2**	**3**	5	0	5	5	**4**	5
26	0	5	5	5	5	0	5	5	5	5	0	5	5	5	5
27	0	5	5	5	5	0	5	**4**	5	5	0	5	5	5	5
28	0	5	5	5	5	0	5	5	5	**4**	0	5	5	5	5
29	0	**4**	5	5	5	0	**4**	5	5	5	0	**4**	5	5	5
30	0	**4**	5	5	5	0	**4**	5	5	5	0	**4**	5	5	5

-: not performed

bold numbers: deviating results

grey cells : results are below the criteria of good performance

Annex 8 Test report follow-up study

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN ANIMAL FEED
organised by CRL-*Salmonella*
FEED STUDY I FOLLOW UP
March 2009

Laboratory code This is the same code as in FEED I 2008	
Laboratory name	
Address	
Country	
Date of arrival of the parcels	…… - ……. – 2009
Start time of storage at - 20 °C (capsules) Start time of storage at + 5 °C (feed)	Date:……. Time:…… Date:……. Time:……
Parcels damaged?	☐ Yes ☐ No
Starting date testing	…… - ……. – 2009

PRE-ENRICHMENT – Buffered Peptone Water (BPW)	
Medium information BPW	
Was the composition of BPW the same as used in BRO FEED I 2008 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of BPW	
Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of BPW?	☐ Yes ☐ No
Prewarming time and temperature of the BPW (at least overnight)	
At the start	Date: …… - ……. – 2009 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	Date: …… - ……. – 2009 time: …… h …… min temperature incubator: ……...…… °C
Incubation time and temperature for dissolving the capsules (45 min)	
At the start	Date: …… - ……. – 2009 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	time: …… h …… min temperature incubator: ……...…… °C
Incubation time and temperature for pre-enrichment (18 ± 2) hrs after adding the feed	
At the start	Date: …… - ……. – 2009 time: ……... h ……..…… min temperature incubator: ……..…… °C
At the end	Date: …… - ……. – 2009 time: ……... h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Rappaport Vassiliadis Soya medium (RVS)
Medium information RVS
Was the composition of RVS the same as used in BRO FEED I 2008 ?
☐ Yes

☐ No please give more details in an annex :	
Preparation of RVS	
Date of preparation	…… - ……. - 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of RVS?	☐ Yes ☐ No
Incubation time and temperature for selective enrichment	
At the start of the first period (24 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Muller Kauffmann Tetra Thionate + novobiocin (MKTTn)	
Medium information MKTTn	
Was the composition of MKTTn the same as used in BRO FEED I 2008 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of MKTTn	
Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of MKTTn?	☐ Yes ☐ No
Incubation time and temperature for selective enrichment	
At the start of the first period (24 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

SELECTIVE ENRICHMENT - Modified Semi solid Rappaport Vassiliadis medium (MSRV)	
Medium information MSRV	
Was the composition of MSRV the same as used in BRO FEED I 2008 ?	
☐ Yes	
☐ No please give more details in an annex :	
Specific data of composition of MSRV medium. What is the concentration of novobiocin in 1000 ml water:	
Novobiocin	☐ 0.01 g/L ☐ 0.02 g/L ☐ Other : …g/L
Preparation of MSRV	
Date of preparation	…… - ……. - 2009

pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of MSRV?	☐ Yes ☐ No
Incubation time and temperature for selective enrichment	
At the start of the first period (24 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

OWN SELECTIVE ENRICHMENT - Own Selective enrichment medium, routinely used in your laboratory (optional)
Name of medium :
Was the composition of the Own selective the same as used in BRO FEED I 2008 ?
☐ Yes ☐ No Please give more details in an annex :

FIRST AND SECOND ISOLATION - Xylose Lysine Desoxycholate medium (XLD)	
Medium information XLD	
Was the composition of XLD the same as used in BRO FEED I 2008 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of XLD	
Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of XLD ?	☐ Yes ☐ No
Incubation time and temperature for isolation	
At the start of the first period (24 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Second Isolation medium.
Medium information second isolation medium :
Name of second isolation medium :
Was the composition of the second medium the same as used in BRO FEED I 2008 ?
☐ Yes
☐ No please give more details in an annex :
Preparation of the second isolation medium

Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control ?	☐ Yes ☐ No
Incubation time and temperature for isolation	
At the start of the first period (24 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2009 time: …….. h ……..…… min temperature incubator: ……..…… °C

FIRST AND SECOND ISOLATION – Own Isolation medium routinely used in your lab. (optional)	
Name of medium :	
Was the composition of the Own isolation medium the same as used in BRO FEED I 2008 ?	
☐ Yes ☐ No	
Please give more details in an annex :	

CONFIRMATION – Nutrient agar	
Did you streak the colonies on Nutrient agar before starting confirmation?	
☐ Yes If yes give further information on nutrient agar below	☐ No
Medium Nutrient agar	
Name of Nutrient agar :	
Was the composition of Nutrient agar the same as used in BRO FEED I 2008 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of the nutrient agar	
Date of preparation	…… - ……. – 2009
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of agar ?	☐ Yes ☐ No

CONFIRMATION of *Salmonella* suspected colonies
What media/tests did you use for confirmation ?
☐ Biochemical: ☐TSI ☐UA ☐ LDC ☐ galactosidase ☐ Voges-Proskauer (VP) ☐ Indole ☐ Identification kit name of the kit : …….. ☐ Other : …….
☐ Serotyping: ☐ O antigen ☐ H antigen ☐ Vi antigen ☐ Other : ……..
☐ Other confirmation test : …….

DETECTION BY PCR	
General questions	
Did you use PCR ?	☐ Yes ☐ No

If yes and when different from PCR-technique used during Feed BRO 2008, please give more information in an annex .

Table 1: Results of isolation using RVS (dish numbers 1-15, C1-C7, C11 and C12)

sample no.	RVS 24 hours						RVS 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C11												
C12												

Col [a] = **number** of colonies used for confirmation
Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 2: Results of isolation using MKTTn (dish numbers 1-15, C1-C7, C11 and C12

sample no.	MKTTn 24 hours						MKTTn 48 hours					
	XLD		Second		Own		XLD		Second		Own	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												

10												
11												
12												
13												
14												
15												
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C11												
C12												

Col [a] = **number** of colonies used for confirmation
Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 3: Results of isolation using MSRV (dish numbers 1-15, C1-C7, C11 and C12)

	MSRV 24 hours						MSRV 48 hours					
sample no.	XLD		Second		Own		XLD		Second		Own	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
C1												
C2												
C3												
C4												
C5												
C6												
C7												

C11												
C12												

Col [a] = **number** of colonies used for confirmation
Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 4: Results of isolation using OWN selective enrichment medium (dish numbers 1-15, C1-C7, C11 and C12)

	OWN 24 hours						OWN 48 hours					
sample no.	XLD		Second isolation medium		Own isolation medium		XLD		Second isolation medium		Own isolation medium	
	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]	Col [a]	Sal [b]
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
C1												
C2												
C3												
C4												
C5												
C6												
C7												
C11												
C12												

Col [a] = **number** of colonies used for confirmation
Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 5: Results of detection using PCR (dish numbers 1-15, C1-C7, C11 and C12)

sample no.	PCR + or -		
		no.	
1		C1	
2		C2	
3		C3	
4		C4	
5		C5	

6		C6	
7		C7	
8		C11	
9		C12	
10			
11			
12			
13			
14			
15			

Comment(s) on operational details that might have influenced the test results:

Name of person (s) carrying out the follow up feed I interlaboratory Comparison study.	
Is the person(s) carrying out the follow up feed I interlaboratory Comparison study working in the laboratory of NRL mentioned on page 1 ?	☐ Yes ☐ No give more information of the laboratory carrying out the study : Laboratory name ……. Address ……. Is this laboratory accredited or certified for the determination of *Salmonella*. ☐ Yes ☐ No
Date and signature	

Name of person in charge of the NRL	
Date and signature	

Please send the completed test report before 7 April 2009 preferable by email to CRL-*Salmonella*. If the test report is e-mailed to the CRL it is not necessary to send the original test report as well, unless it is not legible (to be indicated by CRL-*Salmonella*).
Use the address below:

Angelina Kuijpers
RIVM / LZO internal Pb 63
P.O. Box 1
3720 BA Bilthoven
The Netherlands

E-mail : Angelina.Kuijpers@rivm.nl
Tel. number: + 31 30 274 2093
Fax. number: + 31 30 274 4434

RIVM

National Institute
for Public Health
and the Environment

P.O. Box 1
3720 BA Bilthoven
The Netherlands
www.rivm.com