National Institute for Public Health
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EU Interlaboratory comparison study food V (2011)

Detection of Salmonella in minced meat

RIVM report 330604025/2012

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Colophon

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This investigation has been performed by order and for the account of European Health and Consumer Protection Directorate-General, within the framework of V/330604/12 by the European Union Reference Laboratory, EURL for *Salmonella*

Abstract

EURL Interlaboratory comparison study on food V (2011)
Detection of *Salmonella* in minced meat

In 2011, from the 34 National Reference Laboratories (NRLs) for *Salmonella* in the European Union, 29 were able to detect both high and low levels of *Salmonella* in minced meat. Of the remaining five laboratories, one scored a moderate performance caused by an initial transcription error from the raw data to the computer. Four NRLs scored an underperformance for different reasons such as limited confirmation of *Salmonella* suspected colonies or (cross)-contamination during the test. From these four laboratories, three obtained the desired outcome in a repeat performance test. Depending on the method used, *Salmonella* was found in 95-98% of the samples tested by the laboratories.

Interlaboratory comparison study obligatory for European Member States
These are the results of the fifth food interlaboratory comparison study organised by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*). The study was conducted in September 2011, with a follow-up study in January 2012. All NRLs responsible for the detection of *Salmonella* in food samples from all European Member States, were required to participate in this study. The EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

The laboratories identify the presence of *Salmonella* by using three internationally accepted analytical methods: RVS, MKTTn and MSRV. In accordance with protocol, each laboratory received a package containing minced meat (free from *Salmonella*) and reference materials containing no or different levels of *Salmonella*. The laboratories were instructed to spike the minced meat with the reference materials and then to test the samples.

New reference material successful
During this proficiency test, lenticule discs were used as reference material for the first time in a food study. These discs require less complex preparation than the capsules that were previously used. In addition, the test samples made with this material were more like the 'normal' samples received by and analysed daily in the reference laboratories. The new procedure was so successful that it will be continued.

Keywords: *Salmonella*; EURL; NRL; proficiency test; minced meat; *Salmonella* detection methods; lenticule disc

Rapport in het kort

EU Ringonderzoek voedsel studie V (2011)
Detectie van *Salmonella* in gehakt

In 2011 waren 29 van de 34 Nationale Referentie Laboratoria (NRL's) in de Europese Unie in staat om hoge en lage concentraties van de *Salmonella*-bacterie in gehakt (varken en rund) aan te tonen. Van de vijf overige behaalde één laboratorium een matig resultaat als gevolg van een overschrijffout van ruwe data naar de computer. Vier scoorden er om uiteenlopende redenen onvoldoende, zoals doordat een te beperkt aantal tests om *Salmonella* aan te tonen werd ingezet, of mogelijk door een kruisbesmetting tijdens het onderzoek. Van deze vier behaalden drie laboratoria het gewenste resultaat alsnog tijdens de herkansing. In totaal hebben de laboratoria, afhankelijk van de gebruikte methoden, tussen de 95 en 98 procent van de (besmette) *Salmonella* aangetoond.

Ringonderzoek verplicht voor Europese lidstaten
Dit blijkt uit het vijfde voedselringonderzoek dat het Referentie-Laboratorium van de Europese Unie (EURL) voor *Salmonella* heeft georganiseerd. Het onderzoek is in september 2011 gehouden, de herkansing was in januari 2012. Deelname aan het onderzoek is verplicht voor alle NRL's van de Europese lidstaten die ervoor verantwoordelijk zijn *Salmonella* op te sporen in voedsel. Het EURL-*Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

De laboratoria tonen de *Salmonella*bacterie aan met behulp van drie internationaal erkende analysemethodes (RVS, MKTTn en MSRV). Vervolgens moeten zij de studie volgens voorschrift uitvoeren. Elk laboratorium krijgt daarvoor een pakket toegestuurd met gehakt (vrij van *Salmonella*) en referentiematerialen, die geen of verschillende besmettingsniveaus van *Salmonella* bevatten. Het gehakt en het referentiemateriaal worden vervolgens samengevoegd en onderzocht.

Nieuw referentiemateriaal succesvol
Tijdens dit ringonderzoek zijn voor het eerst de zogenoemde *lenticule discs* als referentiemateriaal gebruikt voor een voedselstudie. Deze vereisen een minder ingewikkelde voorbereiding dan de capsules die voorheen werden gebruikt. Een ander voordeel is dat de monsters die met dit materiaal worden gemaakt beter lijken op de 'gewone' monsters die in de dagelijkse praktijk bij de laboratoria binnenkomen om te worden onderzocht. De nieuwe werkwijze was dermate succesvol dat dit wordt voortgezet.

Trefwoorden: *Salmonella*; EURL; NRL; ringonderzoek; gehakt; lenticule disc; detectiemethode

Contents

Summary

In September 2011 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the fifth interlaboratory comparison study on detection of *Salmonella* in a food matrix: mixture of minced pork and beef meat. Participants were 34 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 28 NRLs from 27 EU Member States (MS), 2 candidate EU MSs and 3 NRLs from member countries of the European Free Trade Association (EFTA).

The objective of the study was to test the performance of the participating laboratories for the detection of *Salmonella* at different contamination levels in a food matrix. To do so, minced meat samples of 25 grams each were analysed in the presence of reference materials containing *Salmonella* (different species at various contamination levels). The criteria for good performance were made and the performance of the laboratories was compared to those criteria. In addition to the performance testing of the laboratories, a comparison was made between the prescribed methods (ISO 6579: Anonymous, 2002) and the requested method (Annex D of ISO 6579: Anonymous, 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* like a PCR technique.

In this food study for the first time lenticule discs were used as reference materials. The change from capsules (former studies) to lenticule discs was especially made because of the easiness of handling of the lenticules. Furthermore, with lenticule discs it was better possible to use the normal routine procedures for sample treatment and therefore to mimic the daily routine analyses better. Good experiences have been gained with the lenticule discs in the veterinary study (Kuijpers and Mooijman, 2011).

32 individually numbered lenticule discs had to be tested by the participants for the presence or absence of *Salmonella*. 25 of the lenticule discs had to be examined in combination with each 25 grams of *Salmonella* negative meat: five lenticule discs contained approximately six colony forming units (cfu) of *Salmonella* Typhimurium (STM6), five lenticule discs contained approximately 61 cfu of *S*. Typhimurium (STM61), five lenticule discs contained approximately 8 cfu of *S*. Enteritidis (SE8), five lenticule discs contained approximately 51 cfu of *S*. Enteritidis (SE51) and five lenticule discs contained no *Salmonella* at all (blank lenticule discs). The other seven lenticule discs, to which no meat had to be added, were control samples, comprising two lenticule discs SE8, one lenticule disc SE51, two lenticule discs STM6 and one blank lenticule disc.

The laboratories found *Salmonella* in 96-98% of the (contaminated) samples depending on the used selective enrichment medium. The accuracy rate for the prescribed method for food (MKTTn and RVS) gave 96% and for the method for veterinary samples (MSRV) 98%. A comparison between the different media did not show significant differences.
Longer incubation (additional 24 hours) of selective enrichment media gave more positive results (5-13%) which was most clear for the low level SE contaminated samples with selective enrichment on MSRV or MKTTn.
PCR was used as an own method by nine participants. Eight of them found the same results as when using the bacteriological culture methods.

29 out of 34 laboratories achieved the level of good performance on the first attempt. Two laboratories had difficulties with the detection of *Salmonella* with matrix and three laboratories found false positive results. One of the NRLs with false positive results scored a moderate performance; they made a transcription error during the transfer of raw data to the digital test report. For the remaining four a follow up study was organized in January 2012; three laboratories reached the desired level and one laboratory (non-EU) did not return the results.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in the Commission Regulation EC No 882/2004 (EC, 2004), is the organisation of interlaboratory comparison studies to test the performances of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the interlaboratory comparison studies on the detection of *Salmonella*, as organised by EURL-*Salmonella* (formerly called CRL-*Salmonella*) since 1995 is summarised in Annex 1. The objective of the current study, organised by the EURL for *Salmonella* in September 2011, was to see whether the participating laboratories could detect *Salmonella* at different contamination levels in minced meat. This information is important to know whether the examination of samples in the EU Member States (MS) is carried out uniformly and whether comparable results can be obtained by NRLs-*Salmonella*. Additionally, the different methods for the detection of *Salmonella* in minced meat were compared.

The prescribed method for detection of *Salmonella* in a food matrix is ISO 6579 (Anonymous, 2002). However, as good experiences have been gained with selective enrichment on Modified Semi-solid Rappaport Vassiliadis (MSRV) for the detection of *Salmonella* spp. in animal faeces (Annex D of ISO 6579: Anonymous, 2007) but also in food and animal feed samples, participating laboratories were requested also to use MSRV for testing the meat.

In this study, for the first time lenticule discs were used as reference materials in combination with a food matrix. The change from capsules (former studies) to lenticule discs was especially made because of the easiness of handling of the lenticule discs. Furthermore, with lenticule discs it was better possible to use the normal routine procedures for sample treatment and therefore to mimic the daily routine analyses better. Good experiences have been gained with the lenticule discs in the veterinary study organized in March 2011 (Kuijpers and Mooijman, 2011).

The set-up of this study was comparable to earlier interlaboratory comparison studies on the detection of *Salmonella* spp. in veterinary samples, animal feed and food samples. The contamination level of the low-level reference material was close to the detection limit of the method; the level of the high-level samples was approximately five to ten times above the detection limit. Seven control samples, comprising of different reference materials, had to be tested without the addition of meat. These control samples consisted of two lenticule discs containing approximately 8 cfu of *Salmonella* Enteritides (SE8), one lenticule disc containing approximately 51 cfu of *S.* Enteritides (SE51), two lenticule discs containing approximately 6 cfu of *Salmonella* Typhimurium (STM6) and two blank lenticule discs. 52 samples of *Salmonella* negative minced meat (25 g each) spiked with five different reference materials had to be examined. For the latter samples, the different reference materials consisted of two levels of *Salmonella* Enteritides (SE8 and SE51), two levels of *Salmonella* Typhimurium (STM6 and STM61) and blank reference materials.

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES) Department of food microbiology
Belgium	Brussels	Scientific Institute of Public Health (WIV) Food Pathogens
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	Esjberg	Danish Veterinary and Food Administration Region West Laboratory
Estonia	Tartu	Estonian Veterinary and Food Laboratory
Finland	Helsinki	Finnish Food Safety Authority Evira Research Department, Food and Feed Microbiology Unit
France	Ploufragan	Anses Laboratoire de Ploufragan, Laboratoire d'Etudes et de Recherches Avicoles, Porcines et Piscicoles Unite HQPAP
Germany	Berlin	Federal Institute for Risk Assessment (BFR) National Reference Laboratory for *Salmonella*
Greece	Halkis	Veterinary Laboratory of Chalkis Hellenic Republic Ministry of Rural Development and Food
Hungary	Budapest	Central Agricultural Office, Food and Feed Safety Directorate Food Microbiological Diagnostic Laboratory
Iceland	Reykjavik	University of Iceland, Keldur Institute for Experimental Pathology
Ireland	Kildare	Central Veterinary Research Laboratory CVRL/DAF Department of Agriculture, Food and Fishery
Italy	Legnaro (PD)	Istituto Zooprofilattico Sperimentale delle Venezie, OIE National Reference Laboratory for *Salmonella*
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Animal Disease Diagnostic Laboratory
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, LMVE
Malta	Valletta	Public Health Laboratory (PHL) Evans Buildings
Macedonia Republic of	Skopje	Food Institute Faculty of veterinary medicine-Skopje
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
Netherlands, the	Zutphen	nVWA Laboratorium Voeder en Voedselveiligheid, Microbiologie R&B
Norway	Oslo	National Veterinary Institute, Section of Bacteriology

Country	City	Institute
Poland	Pulawy	National Veterinary Research Institute (NVRI) Department of Hygiene of Food of animal Origin
Portugal	Lisboa	Laboratório Nacional de Investigação Veterinária (LNIV)
Romania	Bucharest	Hygiene and Veterinary Public Health Institute (IISPV)
Serbia	Beograd Novi	Scientific Veterinary Institute of Serbia Dept. testing food of animal origin
Slovak Republic	Bratislava	State Veterinary and Food Institute Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Majadahonda	Centro Nacional de Alimentacion, Agencia Espanola de Seguridad Alimantaria y Nutricion (AESAN)
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Berne	Institute of veterinary bacteriology, Vetsuisse National Centre for Zoonoses (ZOBA)
United Kingdom	Leeds	Health Protection Agency HPA Microbiology Services; Food, Water & Environmental Laboratory, Leeds Laboratory
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Reference materials

3.1.1 Batches of lenticule discs

The reference material consisted of lenticule discs obtained from the Health Protection Agency (HPA) in Newcastle, United Kingdom. Lenticule discs are microbiological reference materials, which are plano-convex discs containing microorganisms at a defined number in a solid water soluble matrix (HPA, 2011). They are supplied as a single unit supported on a silica gel insert in a small airtight plastic tube (see Annex 5). The discs are lens-shaped and coloured and therefore easily seen on top of the filter insert. The *Salmonella* strains used for the preparation of the lenticule discs were originally from the National Collection of Type Cultures (NCTC) of HPA.
Five batches of lenticule discs were prepared by HPA:

- *S.* Typhimurium (STM) at a level of approximately 6 cfu per lenticule disc: NCTC 12023 batch 323-101021;
- *S.* Typhimurium (STM) at a level of approximately 61 cfu per lenticule disc: NCTC 12023 batch 523-100927;
- *S.* Enteritidis (SE) at a level of approximately 8 cfu per lenticule disc: batch NCTC 6676 batch 414-110615A;
- *S.* Enteritidis (SE) at a level of approximately 51 cfu per lenticule disc: batch NCTC 6676 batch 814-110615;
- Blank lenticule disc, containing no microorganisms: batch 000-100111.

3.1.2 Homogeneity of the lenticule discs

The mean number of organisms of each batch was counted by HPA before the lenticule discs were sent to the EURL-*Salmonella*. For this, the HPA tested thirty lenticules per batch. The data were reported on the insert of the batch of lenticules and subjected to a homogeneity test at the EURL *Salmonella*. For this the same homogeneity test was used as was formerly used for the capsules. It was tested whether the variation in counts between the lenticule discs was less than two times a Poisson distribution, using the following formula: $T_2 / (I-1) \leq 2$. Where T_2 is a measure for the variation between lenticule discs of one batch and I is the number of lenticule discs (see Annex 2).

3.1.3 Test on the stability of lenticule discs and a new procedure

In literature, information can be found on the stability of several types of lenticule discs during storage and transport (Boyd et al., 2006 and Desai et al., 2006), but there is no specific information for *Salmonella*. Therefore, some additional stability tests were performed on the *Salmonella* lenticule discs at the EURL-*Salmonella* laboratory.
A limited test on the long-term stability was performed on lenticule discs containing *S.* Enteritidis (SE) and *S.* Typhimurium (STM), which were ordered by the EURL in 2005. This concerned SE at an original level of 92 cfu/lenticule disc (SE92 batch 214-050615) and STM at an original level of 93 cfu/lenticule disc (STM93 batch 523-050615). The lenticule discs were stored at -20 °C for almost 5.5 years and the mean contamination level of five lenticule discs of each batch were compared to the mean contamination level originally indicated on the insert of the batch of lenticule discs (tested with thirty lenticule discs).

Furthermore the (long-term) stability of lenticules containing SE at a low level of 7 cfu/lenticule disc (SE7 batch 414-100514) was tested after five months of storage at -20 °C.
To test the stability of the lenticule discs at elevated temperatures (as may occur during transport), a so-called challenge test was performed. For this, five lenticule discs of SE92, STM93 and SE7 were tested at day 0, after three days and after seven days of storage at 5 °C , 22 °C and at 30 °C .

For the counting of the lenticule discs in the different stability tests, each lenticule disc was placed onto Colombia agar plates with sheep blood (OXOID PB5008A, Germany). After ten minutes of rehydration of the lenticule disc, the resultant 'drop' was spread over the plate and incubated at 37 °C for 20 to 24 hours. This method is also used by HPA to count the mean number of organisms of each batch of lenticule discs.

3.1.4 Pre-tests for the interlaboratory comparison study

To check the 'robustness' of the lenticule discs, it was tested whether *Salmonella* could still be detected after mixing a *Salmonella* lenticule disc with different matrices. Lenticule discs used for the experiment were: STM10 (batch 223-050615) and SE7 (batch 414-100514). Matrices (free of *Salmonella*) tested in this experiment were: minced beef, minced mixed meat (pork/beef) and chicken faeces.
To ten portions of each 25 grams of minced mixed meat a lenticule disc (STM10) was added. Five samples were placed at 3 °C and five were placed at -20 °C for three days. Five additional portions of each 25 grams of minced meat (no lenticule added) were stored at 5 °C and after three days of storage, a lenticule disc (STM10) was added to each portion and immediately tested. To each minced meat sample (with lenticule disc) 225 ml of Buffered Pepton Water (BPW) was added and mixed for one minute in a stomacher.
To ten portions of each 25 grams of minced beef, a lenticule disc (SE7) was added. Five samples were stored at 5 °C for three days and five samples were tested immediately. To all minced beef samples (stored for three days and freshly prepared) 225 ml of BPW was added and mixed for one minute in a pulsifier.
To ten portions of each 25 grams of chicken faeces, a lenticule disc (STM10) was added. Next, 225 ml of BPW was added. Five samples were mixed in a pulsifier for one minute and five samples were not mixed.
To ten portions of each 25 grams of chicken faeces, a lenticule disc (SE7) was added. Next 225 ml of BPW was added. Five portions were mixed in a pulsifier for one minute and five portions were not mixed.

All meat samples were tested for the presence of *Salmonella* according to ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007) with selective enrichment in RVS, MKTTn and on MSRV. The faeces samples were tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007) only, with selective enrichment on MSRV.

Because of the introduction of lenticule discs, the Standard Operating Procedure (SOP) for the analysis of the samples in the interlaboratory comparison study was amended (see Annex 5). The applicability of this SOP was tested at the laboratory of the EURL by following the full protocol of the interlaboratory comparison study with the same type but a limited number of samples (SE8 and STM6).

3.2 Minced meat samples

3.2.1 General

The minced meat was obtained from 'groothandel Makro', Nieuwegein, The Netherlands. A batch of 33 kg minced meat (a mixture of beef and pork) arrived at EURL-*Salmonella* on 16 August 2011 in portions of 1 – 3 kg. The meat was repacked in portions of approximately 800 grams and stored at –20 °C. The meat was checked for the absence of *Salmonella* by testing ten times 25 g randomly picked from the different portions. For the testing for *Salmonella* ISO 6579 (Anonymous, 2002) and Annex D of ISO 6579 (Anonymous, 2007) was followed. For this purpose, each sample of 25 g was added to 225 ml Buffered Peptone Water (BPW). After pre-enrichment at 37 (± 1) °C for 16-18 hours, selective enrichment was carried out in Rappaport Vassiliadis Soya broth (RVS), Mueller Kaufmann Tetrathionate novobiocin broth (MKTTn) and on Modified Semi-solid Rappaport Vassiliadis (MSRV). Next, the suspect plates were plated-out on Xylose Lysine Deoxycholate agar (XLD) and Brilliance *Salmonella* agar (BSA) and confirmed biochemically.

3.2.2 Total bacterial count in minced meat

The total number of aerobic bacteria in the meat was investigated. The procedure of ISO 4833 (Anonymous, 2003a) was followed for this purpose. Portions of 20 grams of meat were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a stomacher (sixty seconds). Next tenfold dilutions were prepared in a peptone saline solution. Two times 1 ml of each dilution was brought into two 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) °C for 72 (± 3) hours and the total number of aerobic bacteria was counted after incubation.

3.2.3 Number of Enterobacteriaceae in meat

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. Portions of 20 grams of meat were homogenized into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a stomacher (sixty seconds). Next tenfold dilutions were prepared in peptone saline solution. Two times 1 ml of each dilution was brought into two 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. These plates were incubated at 37 (± 1) °C for 24 (± 2) hours 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: lenticule discs and minced meat

On 19 September 2011 (one week before the study) the reference materials (35 individually numbered lenticule discs) and 800 grams of *Salmonella* negative minced meat were packed with cooling devices as biological substance category

B (UN 3373) and sent by door-to-door courier service to the participants. After arrival at the laboratory, the lenticule discs had to be stored at -20 °C and the meat had to be stored at +5 °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 EURL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/prof_testing/detection_stud/ or can be obtained through the corresponding author of this report.

Seven control lenticule discs had to be tested without meat (numbered C1-C7). 25 lenticule discs (numbered B1-B25) were each tested in combination with 25 grams of meat (negative for *Salmonella*). Table 1 shows the types and the number of lenticule discs and meat samples which had to be tested.

Table 1 Overview of the types and the number of lenticule discs tested per laboratory in the interlaboratory comparison study

Lenticule discs	Control lenticule discs (n=7) No matrix added	Test samples (n=25) with 25 grams *Salmonella* negative minced meat
S. Enteritidis 8 (SE8)	2	5
S. Enteritidis 51 (SE51)	1	5
S. Typhimurium 6 (STM6)	2	5
S. Typhimurium 61 (STM61)	-	5
Blank	2	5

3.3.2 Pre-treatment of the samples

In this food-study for the first time lenticule discs were used. As these lenticule discs were easier to dissolve and more robust than the formerly used capsules, the NRLs could use pre-treatment procedures of the samples as they normally use in daily routine analyses. To gain information on the different pre-treatment procedures (e.g. pre-warming of BPW, different ways of mixing the samples in BPW) and to check whether the different procedures did not influence the results, some additional questions were added to the test report.

3.3.3 Sample packaging and temperature recording during shipment

The lenticule discs and the minced meat were packed in two plastic containers firmly closed with screw caps (biopacks). Both biopacks were placed in one large shipping box, together with three frozen (-20 °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 EURL-*Salmonella* to measure the temperature every hour. Each NRL had to return the temperature recorder, immediately after receipt of the parcel, to the EURL. At the EURL-*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 graph 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). In addition 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 (PCR)-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 (ISO 6579: Anonymous, 2002) or by reliable, commercially available identification kits and serological tests.

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 minced meat (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 was used for modelling the binary outcomes as a function of a fixed effect part, consisting of the lenticule discs, enrichment media and isolation media, and a random effect part, consisting of the different laboratories. Mutual differences between media and lenticule discs are shown as odds ratios (OR) stratified by medium. The odds of detecting *Salmonella* is the probability of detecting *Salmonella* divided by the probability of not detecting it. An odds ratio is the ratio of the odds of detecting *Salmonella* in one group to the odds of detecting it in another group and can be interpreted as an effect size. Groups are, for instance, two different media.

A Bayesian approach was adopted to prevent spurious odds ratios, i.e. zero or infinite odds ratios. This was done by putting vague prior information on the odds of detecting *Salmonella*. A priori, the odds were set to 1 with a 95% confidence interval of 0.025 - 40. As a result, the eventual odds and odds ratios will be 'shrunken' towards one, and values equal to zero or infinity are made impossible.

Results were analysed using the statistical software R (R Development Core Team, 2012).

3.6 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 STM6 lenticule discs with matrix 4/5 positive with RVS/XLD but no positives with MKTTn or any other selective enrichment or isolation medium, this was still considered a good result. For the blank lenticule discs, all combinations of media used per laboratory were also taken into account. If, for example a laboratory found 2/5 blank lenticule discs positive with MKTTn/BGA but no positives with the other media, this was still considered a 'no-good' result.

Table 2 Criteria for testing good performance in the Food-V study (2011)

Control samples (lenticules, no matrix)	Minimum result	
	Percentage positive	No. of positive samples/ total No. of samples
SE51	100%	1/1
STM6 and SE8	50%	1/2
Blank control lenticules	0%	0/2

Samples: artificially contaminated meat (lenticules with matrix)	Minimum result	
	Percentage positive	No. of positive samples/ total No. of samples
Blank[1]	20% at max[1]	1/5 at max[1]
STM61 and SE51	80%	4/5
STM6 and SE8	50%	3/5

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

4 Results

4.1 Reference materials

4.1.1 Contamination level and homogeneity of the lenticule discs

Table 3 summarises the information on the contamination level of each batch of lenticule discs as tested by HPA. The mean levels, as well as the lowest and highest counts (in cfu) found per batch are indicated. Additionally, the results of the homogeneity test of each batch as performed by the EURL *Salmonella* are indicated The results of the homogeneity test show that each batch fulfilled well the criteria as originally set for the capsule reference materials (T_2 / (I-1)) ≤ 2).

Table 3 Level of contamination and homogeneity of SE and STM lenticule discs

	SE8	SE51	STM6	STM61
Batch number	414-100515A	814-110615	323-101021	523-100927
Date testing lenticules*	01.07.2011	01.07.2011	5.11.2010	11.10.2010
Number of lenticules tested	30	30	30	30
Mean cfu per lenticule	8	51	6	61
Min-max cfu per lenticule	4-13	41-65	1-10	48-77
T_2 / (I-1)**	0.68	0.87	0.86	0.89

cfu=colony forming units.
min-max=enumerated minimum and maximum cfu.
* Tested by HPA.
** Calculated by EURL-*Salmonella.*
formula T_2 see Annex 2; I is number of lenticule discs; Demand for homogeneity $T_2/(I-1) \leq 2$

Table 4 Level of contamination of SE and STM lenticule discs before and after storage

	SE7	SE92	STM93
Batch number	414-100514	214-050615	523-050615
Storage at -20 °C since date of testing	27.05.2010	01.05.2005	01.05.2005
Number of lenticules tested*	30	30	30
Mean cfu per lenticule	7	92	93
Min-max cfu per lenticule	3-13	70-110	73-121
Date testing lenticules	25.10.2010	25.10.2010	25.10.2010
Number of lenticules tested**	5	5	5
Mean cfu per lenticule disc	3.4	75.2	64.8
Min-max cfu per lenticule	1-5	73-86	58-76

cfu=colony forming units.
min-max=enumerated minimum and maximum cfu.
* Tested by HPA.
** Tested by EURL-*Salmonella.*

4.1.2 Testing stability of lenticule discs

Table 4 summarises the results of the (limited) test on the long-term stability of the lenticule discs. All batches showed a (small) decrease in the mean cfu after storage at -20 °C.

Figure 1 shows the results of the challenge test. No effect on the mean number of cfu was seen after storage of the batches SE7 at 22 °C and STM93 at 5 °C for one week. The decrease in the mean number of cfu was more obvious when the batches were stored at 30 °C for three to seven days. The storage of the lenticule discs at elevated temperatures seem to have a larger effect to the lenticule discs containing SE than to the materials containing STM.

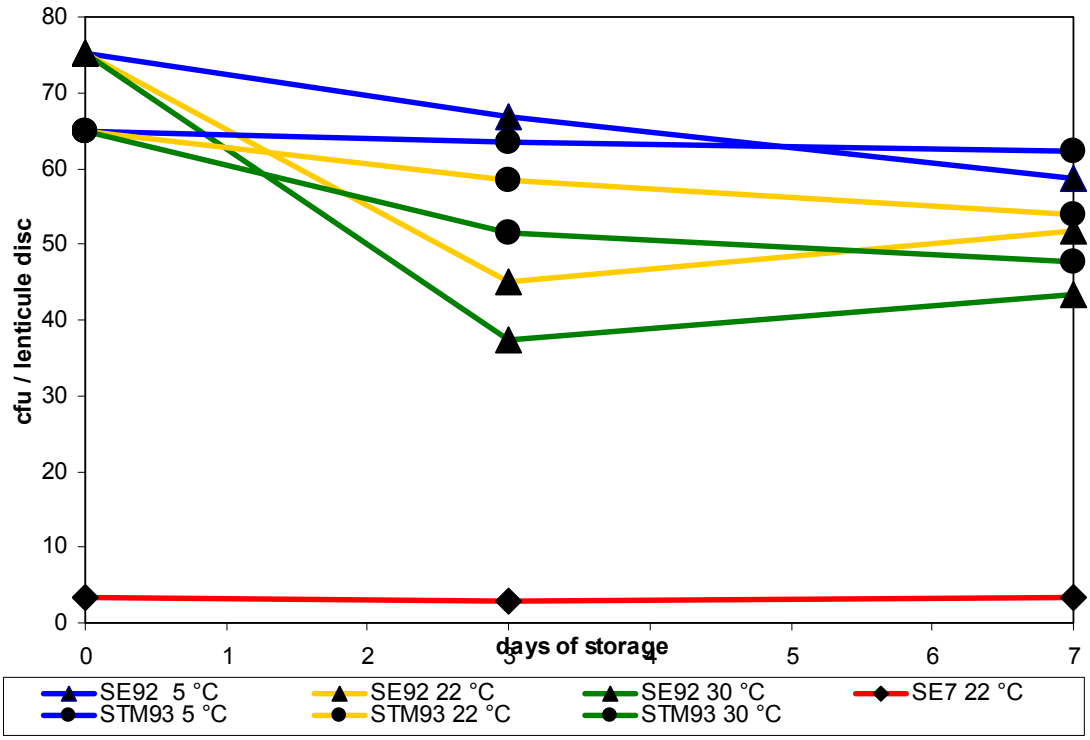

Figure 1 Challenge test of lenticule discs stored at different temperatures Mean results of five lenticule discs per test are indicated.

4.1.3 Pre-test for the interlaboratory comparison study

Table 5 summarises the results of the 'robustness test' of lenticule discs. The different combinations of matrices, lenticule discs, storage and mixing did not show any effect on the results, that is, all samples were tested positive for *Salmonella*.
The pre-test of the full (new) procedure of the interlaboratory comparison study performed at the EURL-*Salmonella* showed good results. All samples of artificially contaminated minced meat (with SE8 and STM6) were scored correctly with MKTTn, RVS and MSRV.

Table 5 Results robustness test of the lenticule discs

Matrix (25 g/sample)	Lenticule disc	Combination lenticule disc in matrix		Mixing 1 min Stomacher/pulsifier	No of positive results/ Total no of samples
		Storage temperature	Storage time		
Minced meat (pork/beef)	STM10	3 °C	3 days	yes	5/5
	STM10	-20 °C	3 days	yes	5/5
	STM10	3 °C (only meat)	3 days	yes	5/5
Minced beef	SE7	5 °C	3 days	yes	5/5
	SE7	-	-	yes	5/5
Chicken faeces	STM10	-	-	yes	5/5
	STM10	-	-	no	5/5
	SE7	-	-	yes	5/5
	SE7	-	-	no	5/5

4.2 Minced meat samples

The batch of minced meat was tested negative for *Salmonella* and stored at -20 °C on 16 August 2011. On Monday 19 September 2011 the meat was mailed to the NRLs. After receipt, the NRLs had to store the meat at 5 °C. The number of aerobic bacteria and the number of *Enterobacteriaceae* were tested twice; firstly at the day the meat arrived at the EURL (16/08/2011) and secondly, after storage -20 °C since 16 August 2011 and at 5 °C for one week, close to the planned date of the interlaboratory comparison study (26 September 2011). Table 6 shows the results.

Table 6 Number of aerobic bacteria and the number of Enterobacteriaceae per gram of minced meat

Date	*Enterobacteriaceae* cfu/g	Aerobic bacteria cfu/g
16 August 2011	$3.2*10^2$	$1.3*10^5$
3 October 2011 (stored at –20 °C until 26 September and placed at 5 °C for one week)	$2.2*10^2$	$5.6*10^6$

4.3 Technical data interlaboratory comparison study

4.3.1 General

In this study 34 NRLs participated: 29 NRLs from 27 EU-MS, three NRLs from members of the EFTA and two EU candidate MSs. 32 laboratories performed the study on the planned date (week 39 starting on 26 September 2011). One laboratory (lab code 3) performed the study one week later. Laboratory 22 made a mistake with the treatment of all tubes with reference materials. They received a new parcel and performed the study immediately after arrival of the parcel at the institute on 4 October.

4.3.2 Accreditation/certification

All laboratories with the exception of laboratory 19 indicated to be accredited according ISO/IEC 17025 (Anonymous, 2005). 28 laboratories are accredited for ISO 6579 for the detection of *Salmonella* in food and animal feeding stuffs and 22 of them are also accredited for Annex D of ISO 6579 for different matrices. Two laboratories (lab codes 4 and 16) are accredited for the detection of *Salmonella* in animal faeces and veterinary samples by using MSRV (Annex D of ISO 6579) and RVS. One laboratory (lab code 8) is accredited for the detection of *Salmonella* in food and animal feeding stuffs by using MSRV. One laboratory (lab code 15) is accredited for the detection of *Salmonella* in food and animal feeding stuffs by using RVS and is in the process for an accreditation for MSRV. Two laboratories (lab codes 7 and 19) are planning to become accredited in 2011.

4.3.3 Transport of samples

Table 7 gives an overview of the transport times and the temperatures during transport of the parcels. The NRLs returned the temperature recorders immediately after receipt to the EURL-*Salmonella.* The average transport time to the EU-MS was 27 hours. 28 of the laboratories received the materials within one day. Two parcels (lab codes 2 and 19) were delayed and the latest parcel arrived after four days at the institute. The parcel of laboratory 19 arrived at the institute within three days but needed extra transport time to the laboratory of the NRL and was therefore delayed for five days. Unfortunately, the temperature of the parcel was not longer recorded during these additional five days of transport, due to the fact that the temperature recorder was separated from the parcel. For the majority of the parcels the transport temperature did not exceed 5 °C. Two laboratories (lab codes 2 and 9) reported a temperature above 5 °C for more than one day. For four NRLs the time of transport recorded on the test report did not correspond with the time reported by the courier. Presumably, the parcel arrived at the time reported by the courier at the institute, but due to internal logistics at the institute, the parcel arrived one to five hours later at the laboratory of the NRL.

Table 7 Overview of transport time and temperatures during shipment of the parcels to the NRLs

		Time in hours (h) at				
Lab code	Transport time[1] total in hours (h)	-20 °C - 0 °C	0 °C - 5 °C	5 °C - 10 °C	> 10 °C	Additional Storage[2] time in hours (h)
1	21	9	12			
2	104	4	67	33		
3	1	1				
4	25	9	16			
5	26	8	18			
6	20	9	11			4 h 4 °C
7	27	9	18			
8	25	11	14			
9	71	5	27	29	10	
10	25	9	16			
11	24	9	15			
12	20	15	5			
13	46	7	39			
14	27	8	19			
15	24	8	16			
16	22	9	13			2 h 5 °C
17	24	9	15			
18	23	15	8			5 h 3 °C
19*	78	49	29			5 days
20	27	8	19			
21	46	9	33	4		
22	25	15	10			
23	22	9	13			
24	23	9	14			
25	25	15	9		1	
26	26	9	17			
27	25	16	9			
28	25	10	15			
29	25	10	15			2 h at 1 °C
30	23	8	15			
31	47	11	34	1	1	
32	23	8	15			
33	23	8	14		1	
34	23	8	15			
Average	31					
Average EU[3]	27					

1=Transport time according to the courier.
2=Storage time of the samples at the institute before arriving at the laboratory of the NRL.
3=Average Transport time to the countries of EU Member-States.

*The parcel of laboratory 19 needed extra transport time (five days) to the laboratory of the institute. During this additional transport time the temperature was no longer recorded.

4.3.4 Pre-treatment of the samples

For testing the samples, the laboratories were asked to use as much as possible the procedures and materials as normally used for routine samples (see Annex 5, SOP of this study).
13 laboratories used plastic bags, 13 laboratories used plastic bottles and 8 laboratories used jars. Twenty laboratories used containers pre-filled with BPW. The majority of the laboratories pre-warmed the BPW at room temperature (65%) the others at 37 °C. The samples (BPW, lenticule disc and matrix) were mixed in the laboratories by stomacher (41%), shaking (35%), kneading (6%), swirling (6%), mixing with a spoon (6%) or by magnetic stirring (3%). None of the laboratories used a pulsifier or vortex to mix. The time of mixing varied between five seconds and one minute for most of the laboratories (82%). Laboratory 32 mixed for two minutes, laboratory 10 for five minutes and laboratory 19 for twenty minutes per sample. Two laboratories (lab code 2 and 15) did not mention the time of mixing. Only one laboratory (lab code 13) did not mix the samples.

4.3.5 Media

Each laboratory was asked to test the samples with the prescribed method (ISO 6579) and the requested (Annex D of ISO 6579) method. Table 8 shows the media used per laboratory. Details on the media which are not described in ISO 6579 (Anonymous, 1993 and 2002) are given in Annex 3. 32 laboratories used the selective enrichment media RV(S), MKTTn and MSRV with plating-out medium XLD and a second plating-out medium of their own choice.
Laboratory 15 did not use the prescribed medium MKTTn and laboratory 19 did not use the requested mediumd MSRV. Two laboratories (lab codes 21 and 28) used more than two isolation media. The Tables 9-15 give information on the composition of the media that were prescribed and requested and on the incubation temperatures and times. These tables only indicate the laboratories who reported deviations. Five laboratories (lab codes 14, 16, 23, 26 and 31) reported a longer incubation time or a lower temperature of the pre-enrichment in BPW. Two laboratories (lab codes 19 and 34) incubated the selective enrichment medium RVS at a lower temperature, deviating from the prescribed temperature of 41.5 °C. The laboratories 3, 6, 7, 12, 15, 23, 24, 29 and 31 did not always mention the pH of the media. Five laboratories (lab codes 6, 8, 24, 26 and 30) did not always mention the composition of the media used. Three laboratories (lab code 20, 23 and 30) used RV instead of the prescribed RVS. Four laboratories (lab codes 8, 26, 30 and 34) used MSRV without novobiocin and seven laboratories used MSRV with a higher concentration of novobiocin than the prescribed 0.01 g/L. Two laboratories (lab codes 8 and 29) reported a higher pH for the MSRV than the described maximum pH of 5.4. Laboratory 15 used dehydrated MSRV after the expired date (2010/02) but they performed a quality control, which showed still good quality. Laboratory 29 used XLD with the addition of novobiocin (1 ml of a 1.5% novobiocin solution).

A second plating-out medium for choice was obligatory. Fifteen laboratories used BGA modified (ISO 6579, 1993) or BPLS as a second plating-out medium. Eight laboratories used Rambach, three laboratories SM2 agar, three laboratories lused BSA and three laboratories used Rapid Salmonella (RS). The following media were used only by one laboratory: BGA, BPLSA, MLCB and Compass.

Table 8 Media combinations used per laboratory

Lab Code	Selective Enrichment media	Plating-out media
1	RVS MKTTn MSRV	XLD BGA^{mod}
2	RVS MKTTn MSRV	XLD Rambach
3	RVS MKTTn MSRV	XLD BSA
4	RVS MKTTn MSRV	XLD BPLS(BGA^{mod})
5	RVS MKTTn MSRV	XLD BGA^{mod}
6	RVS MKTTn MSRV	XLD BGA^{mod}
7	RVS MKTTn MSRV	XLD RS
8	RVS MKTTn MSRV	XLD BGA^{mod}
9	RVS MKTTn MSRV	XLD BGA^{mod}
10	RVS MKTTn MSRV	XLD RS
11	RVS MKTTn MSRV	XLD Rambach
12	RVS MKTTn MSRV	XLD SM2
13	RVS MKTTn MSRV	XLD Rambach
14	RVS MKTTn MSRV	XLD BGA^{mod}
15	RVS MSRV	XLD BSA
16	RVS MKTTn MSRV	XLD BGA
17	RVS MKTTn MSRV	XLD BGA^{mod}

Lab code	Selective Enrichment media	Plating-out Media
18	RVS MKTTn MSRV	XLD BGA^{mod}
19	RVS MKTTn	XLD BGA^{mod}
20	RV MKTTn MSRV	XLD BPLSA
21	RVS MKTTn MSRV	XLD BPLS(BGA^{mod}) Rambach
22	RVS MKTTn MSRV	XLD BGA^{mod}
23	RV MKTTn MSRV	XLD BSA
24	RVS MKTTn MSRV	XLD SM2
25	RVS MKTTn MSRV	XLD BGA^{mod}
26	RVS MKTTn MSRV	XLD SM2
27	RVS MKTTn MSRV	XLD Rambach
28	RVS MKTTn MSRV	XLD Compass MLCB
29	RVS MKTTn MSRV	XLD +novobiocin BGA^{mod}
30	RV MKTTn MSRV	XLD Rambach
31	RVS MKTTn MSRV	XLD BGA^{mod}
32	RVS MKTTn MSRV	XLD Rambach
33	RVS MKTTn MSRV	XLD Rambach
34	RVS MKTTn MSRV	XLD RS

Explanations of the abbreviations are given in the 'List of abbreviations'.
Compositions of the media not described in ISO 6579 (Anonymous, 1993 and 2002) are given in Annex 3.

Table 9 Incubation time and temperature of BPW

	Pre-enrichment in BPW	
Lab code	**Time (h:min)**	**Incubation temperature in °C (min-max)**
SOP & ISO 6579	**16 – 20**	**36-38**
14	21:45	37.5
16	18:24	35.5-36.6-37.1*
23	20:30	37
26	21:00	37-37.2
31	22:00	37

Grey cell: deviating times and temperatures.
**Laboratory 16 The incubator remained for four hours < 36°C the samples were moved to another incubator.*

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

Lab code	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Disodium hydrogen Phosphate dode-cahydrate* ($Na_2HPO_4.12H_2O$)	Potassium dihydrogen phosphate (KH_2PO_4)	pH
ISO 6579	**10.0**	**5.0**	**9.0**	**1.5**	**6.8 – 7.2**
3	10	5	9	1.5	-
4, 34	10	5	3.5*	1.5	7.3
6	-	-	-	-	-
7	12	5	3.5*	1.5	-
12	10	5	9	1.5	-
14	10	5	9	1.5	7.3
18	10	5	3.5*	-	7.3
26	-	-	-	-	7.0

Grey cell: deviating from ISO 6579. -=No information
*=3.5 grams Disodium hydrogen phosphate (anhydrous) is equivalent to 9 grams disodium hydrogen phosphate dodecahydrate.

Table 11 Incubation temperatures of selective enrichment medium RVS, MKTTn and MSRV

	RVS	MKTTn	MSRV
Lab code	**Incubation temperature in °C (min-max)**	**Incubation temperature in °C (min-max)**	**Incubation temperature in °C (min-max)**
ISO 6579 & Annex D	**40.5 – 42.5**	**36–38**	**40.5 – 42.5**
2			39.9-41
19	37 - 41.5	37	
34	36.4-42.1	36.7-37.1	41.4-42.1

Grey cell: deviating times and temperatures.

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

Lab code	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**
3	4.5	7.2	1.26 + 0.18*	13.6	0.036	-
6, 24	-	-	-	-	-	-
12	4.5	7.2	1.26 + 0.18*	28.6**	0.036	-
19	4.5	8	0.6 + 0.4	29**	0.036	5.2
20, 30(RV)	5	8	1.6	40	0.04	5.2
23 (RV)	5	8	1.6	40	0.04	-
31	4.5	7.2	1.44	28.6**	0.036	-

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.

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

Lab code	Meat Extract	Enzymatic digest of casein (Peptone)	Sodium Chloride (NaCl)	Calcium Carbonate ($CaCO_3$)	Sodium Thiosulfate Penta hydrate ($Na_2S_2O_3$. $5H_2O$)	Ox bile	Brilliant green	Iodine	Potassium iodide (KI)	Novo-Biocin	pH
ISO 6579	**4.3**	**8.6**	**2.6**	**38.7**	**47.8**	**4.8**	**9.6 mg)**	**4**	**5**	**40 mg**	**7.8 – 8.2**
3, 23	4.3	8.6	2.6	38.7	30.5*	4.8	9.6	4	5	40	-
6	-	-	-	-	-	-	-	-	-	-	-
7	4.2	8.5	2.5	38.0	30.3*	4.8	9.5	4	5	39	8.1
9	7	2.3	2.3	25	40.7	4.8	10	4	5	40	8.1
10	4.3	8.6	2.6	38.7	30.5*	4.8	9.6	-	-	-	6.7
12	4.2	8.5	2.5	38.0	30.3*	4.8	9.5	4	5	39	-
19	4.3	8.6	2.6	38.7	47.8	4.8	9.5	4	5	-	8.1
24	-	-	-	-	-	-	-	-	-	-	-
27, 31	4.3	8.6	2.6	38.7	30.5*	4.8	9.6	4	5	10	8.0
29	7	2.3	2.3	25	40.7	4.8	9.5	4	4.8	40	-
33	4.3	8.6	2.6	38.7	30.5*	4.8	9.6	3.9	4.9	39	7.4

Grey cell: deviating from ISO 6579. -=no information.

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

Table 14 Composition (in g/L) and pH of MSRV

Lab code	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**	**10 mg**	**5.1- 5.4**
2	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.3
5	4.6	4.6	7.3	1.5	10.9	0.04	2.7	50	5.2
8	4.6	4.6	7.3	1.5	10.9	0.04	2.7	-	5.6
9	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.2
10	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.2
12	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	-
15***	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	-
17	2.3	4.7	7.3	1.5	10.9	0.04	2.5	10	5.1
20	4.6	4.6	7.3	1.5	10.9	0.04	2.7	20	5.4
22	4.6	4.6	7.3	1.5	10.9	0.04	2.7	50	5.2
23	4.6	4.6	7.3	1.5	10.9	0.04	2.7	5	5.4
26, 30	-	-	-	-	-	-	-	-	5.2
29	4.6	4.6	7.3	1.5	10.9	0.04	2.7	10	5.5
34	4.6	4.6	7.3	1.5	10.9	0.04	2.7	-	5.2

Grey cell: deviating from Annex D of ISO 6579 -=No information
*Pepton mixture **Yeast extract.
*** Laboratory 15 used MSRV after the expire date (2010/02) but they did a quality control.*

Table 15 Composition (in g/L) and pH of XLD

Lab code	Xylose	L-lysine	Lactose	Sucrose (Saccharose)	Sodium Chloride (NaCl)	Yeast extract	Phenol red	Agar	Sodium deoxy-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**	**5.0**	**7.5**	**7.5**	**5.0**	**3.0**	**0.08**	**9-18**	**1.0**	**6.8**	**0.8**	**7.2 – 7.6**
6	-	-	-	-	-		-	-	-	-	-	-
8, 30	-	-	-	-	-	-	-	-	-	-	-	7.4
9	-	5	3.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.3
12, 31	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	-
14, 32	3.5	5	7.5	7.5	5	3	0.08	13.5	2.5	6.8	0.8	7.4
19	3.5	5	7.5	7.5	5	3	0.08	15	2.5	6.8	0.8	7.4
23	3.75	5.3	7.5	7.5	5	3	0.08	12.5	1	6.8	0.8	-
24	-	-	-	-	-		-	-	-	-	-	-

Grey cell: deviating from ISO 6579 -=No information

Table 16 Biochemical confirmation of Salmonella

Lab code	TSI	UA	LDC	Gal	VP	Indole	Kit	Other
1	+	+	+	+	-	+		
2, 14, 21, 27, 32	+	+	+	+	+	+		
3	+	+	+	-	-	+		Oxidase
4	+	+	+	-	-	-	API20E	
5	+	+	+	-	-	+		PCR
6	-	+	+	-	-	-	API20E	PCR
7	+	-	-	-	-	+		BBL Crystal BBL ENT/NF
8	-	-	-	-	-	-	API20E	Real time PCR
9	+	+	+	-	-	+		
10	-	-	-	-	-	-	ID32E	
11	+	+	+	-	-	-	Enterotest 24 PL	MALDI-TOF
12	-	-	-	-	-	-	Microbact 12A	
13	+	-	+	+	+	+		Urea/indole
15	+	+	-	-	-	-		Lysin Iron agar
16	+	+	+	+	+	+		RealTime PCR
17	-	-	-	-	-	-		Chromogenic agar
18	-	-	-	-	-	-		Kohns No 1 medium
19	+	+	+	-	+	+		
20	-	-	-	-	-	-		PCR, Dulcit ,ONPG, Malonat, Salicin
22	+	+	+	-	-	+	API 20E	
23	+	-	-	-	-	-		H_2S, Oxidase
24	-	-	-	-	-	-	API Rapid ID 32E	Kigler
25, 28	+	+	+	+	+	+		PCR
26	-	-	-	-	-	-	VITEK 2 GN, MUCAP Test	PCR
29	+	+	+	-	-	-		
30	+	+	+	-	-	+		Semi-solid glucose
31	+	-	-	-	-	-	Microbact	
33	+	+	+	-	-	-	Rapid20E (API 20E)	Brolacin
34	-	-	-	-	-	-	Enterotube II	PCR, Kovacs

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

Table 17 Serological confirmation of Salmonella

Lab code	Serological			
	O antigens	**H antigens**	**Vi Antigens**	**Other**
1, 2, 5, 17, 26, 32	+	-	-	
4, 10, 21, 23, 24, 25, 29, 33	-	-	-	-
6, 9, 11, 13, 14, 16, 18, 19, 20, 27, 30	+	+	-	
3,7,8, 12, 15, 22, 34	-	-	-	Agglutination test anti-Salmonella
28	+	-	+	
31	+	+	+	

-=Not done/not mentioned.

The use of an extra plating agar between the 'isolation' and the 'confirmation' steps was optional. A total of 25 laboratories performed this extra culture step on many different media (e.g. Nutrient agar: ISO 6579, 2002).

All participating laboratories performed confirmation tests for *Salmonella*: most of them biochemical, serological or both. The Tables 16 and 17 summarises the confirmation media and tests. Of a few laboratories the confirmation seem to have been limited (lab codes 17, 18 and 23).

4.4 Control samples

4.4.1 General

Thirty laboratories scored correct results for all the control lenticule discs. Table 18 summarises the highest number of positive isolations found with all combinations of selective enrichment media and isolation media per laboratory (lenticule discs without meat). Annex 6 Table A.6.1 gives the results found with the selective enrichment media RVS, MKTTn and MSRV in combination with the used isolation media per laboratory.

Procedure control without lenticule disc (n=2)
33 laboratories correctly analysed the procedure controls: one meat control sample (25 g of minced meat/no lenticule disc) and one control of BPW only (no meat/no lenticule disc). Laboratory 31 scored both control samples positive on all used media (RVS, MKTTn and MSRV).

Blank lenticule discs without addition of meat (n=2)
33 laboratories correctly analysed the two blank lenticule discs negative for *Salmonella* with all used media. Laboratory 15 found one blank control lenticule disc positive on all used media (RVS and MSRV). All blanks should have been tested negative. A possible cause for finding a blank sample positive may be cross-contamination.

Table 18 Total number of positive results of the control samples (lenticule disc without meat) per laboratory

Lab code	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	SE8 n=2	SE51 n=1	STM6 n=2	Blank n=2	Procedure Control n=2*
Good Performance	**≥ 1**	**1**	**≥ 1**	**0**	**0**
15	2	1	2	**1**	0
17	2	1	**1**	0	0
31	2	1	2	0	**2**
Other laboratories 1 – 14; 16; 18 – 30; 32 – 34	2	1	2	0	0

* one meat control (25 g of minced meat/no lenticule disc) and one BPW control (only BPW/ no lenticule disc / no meat)
Bold numbers: deviating results
Grey cell: results are below good performance.

S. Enteritidis 8 lenticule discs (SE8) without addition of meat (n=2)
All participating laboratories tested both control lenticule discs containing SE8 positive.

S. Enteritidis 51 lenticule discs (SE51) without addition of meat (n=1)
All participating laboratories tested the one control lenticule disc containing SE51 positive.

S. Typhimurium 6 lenticule discs (STM6) without addition of faeces (n=2)
33 laboratories isolated *Salmonella* Typhimurium at a mean level of approximately 6 cfu/lenticule disc from both lenticule discs. Only laboratory 17 could not detect *Salmonella* in one out of two STM6 lenticule discs. These lenticule discs contained STM at a low level (approx 6 cfu/lenticule). Due to change, one out of two lenticule discs containing STM6 may be negative.

The results of all control samples were compared with the definition of 'good performance' (see section 3.6). Two laboratories (lab code 15 and 31) scored below these criteria.

4.4.2 Specificity, sensitivity and accuracy rates of the control samples

Table 19 shows the specificity, sensitivity and accuracy rates for the control lenticule discs without the addition of meat. The rates are calculated for the different selective enrichment media (RVS, MKTTn and MSRV) with plating-out medium XLD. The calculations were performed on the results of all participants and on the results of only the EU-MS (without the results of the EFTA States and candidate EU-MSs). No differences were found between these groups.
The maximum possible rates (100%) were found for the SE control samples. The sensitivity rates of STM6 were close to 98.5% and the specificity rates of the blank lenticule discs were 100%.

Table 19 Specificity. sensitivity and accuracy rates found with the control samples (lenticule discs without the addition of meat)

Control lenticule disc		RVS/XLD		MKTTn/XLD*		MSRV/XLD**	
	Laboratories	All n=34	EU n=29	All n=33	EU n=29	All n=33	EU n=29
Blank (n=2)	No. of samples	68	58	66	58	66	58
	No. of negative samples	67	58	66	58	65	58
	Specificity in%	**98.5**	**100**	**100**	**100**	**98.5**	**100**
SE8 (n=2)	No. of samples	68	58	66	58	66	58
	No. of positive samples	68	58	66	58	66	58
	Sensitivity in%	**100**	**100**	**100**	**100**	**100**	**100**
SE51 (n=1)	No. of samples	34	29	33	29	33	29
	No. of positive samples	34	29	33	29	33	29
	Sensitivity in%	**100**	**100**	**100**	**100**	**100**	**100**
STM6 (n=2)	No. of samples	68	58	66	58	66	58
	No. of positive samples	67	57	65	57	65	57
	Sensitivity in%	**98.5**	**98.3**	**98.5**	**98.3**	**98.5**	**98.3**
All lenticule discs with *Salmonella*	No. of samples	170	145	165	145	165	145
	No. of positive samples	169	144	164	144	164	144
	Sensitivity in%	**99.4**	**99.3**	**99.4**	**99.3**	**99.4**	**99.3**
All lenticule discs	No. of samples	238	203	231	203	231	203
	No. of correct samples	236	202	230	202	229	202
	Accuracy in%	**99.2**	**99.5**	**99.6**	**99.5**	**99.1**	**99.5**

* Results without Laboratory 15 (non-EU): they did not use MKTTn.

**Results without Laboratory 19 (non-EU): they did not use MSRV.

4.5 Results minced meat samples artificially contaminated with *Salmonella*

4.5.1 Results per type of lenticule disc and per laboratory

General

Table 20 gives the results of the *Salmonella* negative minced meat samples artificially contaminated with lenticule discs. This table gives the highest number of positive isolations found with the different selective enrichment media (RVS, MKTTn and MSRV) in combination with any isolation medium per laboratory. Annex 7, Table A.7.1 gives the results found with the selective enrichment media RVS, MKTTn and MSRV in combination with the used isolation media per laboratory.

Blank lenticule discs with negative minced meat (n=5)
26 laboratories correctly did not isolate *Salmonella* from the blank lenticule discs with the addition of negative minced meat. Seven laboratories found one blank sample added to negative meat positive for *Salmonella* and one laboratory found two blank samples positive. Laboratory 33 found two positive blank results with RVS in combination with isolation on Rambach. With the other used media this laboratory correctly found the blank samples negative. Three laboratories (lab codes 15, 29 and 31) found one positive blank result with all used media (selective enrichment media RVS, MKTTn and MSRV in combination with isolation on XLD and second isolation medium). Three laboratories (lab codes 5, 12 and 22) found one positive blank result after selective enrichment on MSRV. With the other used selective enrichment media, these laboratories scored this sample, inoculated from the same BPW, correctly negative. Laboratory 6 found one positive blank result after selective enrichment in RVS and on MSRV, but scored the same sample correctly negative with MKTTn.
In theory, all blanks should be tested negative. However, as no 100 % guaranty about the *Salmonella* negativity of the minced meat can be given, one positive out of five blank samples (80% negative) will still be considered as acceptable. Finding more than one blank sample positive is not very likely. A false positive result for a blank sample may have been caused by cross-contamination, limited confirmation or by misinterpretation of the results.

S. Enteritidis 8 lenticule discs (SE8) with negative minced meat (n=5)
33 laboratories were able to isolate *Salmonella* from all the five lenticule discs containing *Salmonella* Enteritidis at a level of approximately 8 cfu/lenticule disc in combination with minced meat. Laboratory 19 and 27 could not detect *Salmonella* Enteritidis in respectively four and three out of five SE8 samples with all the used media.
These lenticule discs contained SE at a low level (approximately 8 cfu/lenticule). Due to change one out of five lenticule discs containing SE8 may be negative. However, it is not very likely to find more than one SE8 lenticule disc negative.

S. Enteritidis 51 lenticule discs (SE51) with negative minced meat (n=5)
31 laboratories isolated *Salmonella* from all the five lenticule discs containing *Salmonella* Enteritidis at a level of approximately 51 cfu/ lenticule disc in combination with minced meat. One laboratory (lab code 7) found one lenticule disc negative on all used media. Laboratory 19 and 27 could not detect *Salmonella* Enteritidis in respectively three and two out of five SE51 samples with all the used media.

S. Typhimurium 6 lenticule discs (STM6) with mince meat(n=5)
32 laboratories isolated *Salmonella* from all the five lenticule discs containing *Salmonella* Typhimurium at a level of approximately 6 cfu/ lenticule disc in combination with mince meat. One laboratory (lab code 7) found one lenticule disc negative with all media used. Laboratory 19 could not detect *Salmonella* Typhimurium in respectively four out of five STM6 samples with all media used. These lenticule discs contained STM at a low level (approximately 6 cfu/lenticule disc). Due to the variation between lenticule discs, one out of five lenticule discs containing STM6 may be negative. However, it is not very likely to find more than one STM6 lenticule disc negative.

S. Typhimurium 61 lenticule discs (STM61) with negative minced meat (n=5)
All laboratories with the exception of laboratory 19 isolated *Salmonella* from all five lenticule discs containing *Salmonella* Typhimurium at a level of approximately 61 cfu/lenticule disc in combination with mince meat.

Laboratory 19 could not detect *Salmonella* Typhimurium in any of the five STM61 samples with all media used.

The results of all artificially contaminated minced meat samples were compared with the definition of 'good performance' (see section 3.6). Three laboratories (lab code 19, 27 and 33) scored below these criteria.

Table 20 Number of positive results found with the artificially contaminated minced meat samples per laboratory

Lab code	The highest number of positive isolations found with all combinations of selective enrichment media and isolation media				
	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5
Good Performance	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤ 1**
5	5	5	5	5	**1**
6	5	5	5	5	**1**
7	5	**4**	**4**	5	0
12	5	5	5	5	**1**
15	5	5	5	5	**1**
19	**1**	**2**	**1**	**0**	0
22	5	5	5	5	**1**
27	**2**	**3**	5	5	0
29	5	5	5	5	**1**
31	5	5	5	5	**1**
33	5	5	5	5	**2**
Other laboratories 1-4; 8-11; 13; 14; 16-18; 20; 21; 23–26; 28; 30; 32; 34	5	5	5	5	0

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

4.5.1 Results per selective enrichment medium, lenticule disc and per laboratory

Figures 2-5 show the number of positive isolations per artificially contaminated minced meat sample and per laboratory after pre-enrichment in BPW, selective enrichment in RVS, MKTTn and on MSRV, followed by isolation on a selective plating agar. To determine good performance per laboratory, all combinations of selective enrichment media and isolation media used by the laboratory were taken into account. The results of all artificially contaminated minced meat samples were compared with the definition of 'good performance' (see section 3.6). The black horizontal line in Figures 2–5 indicates the border of good performance.

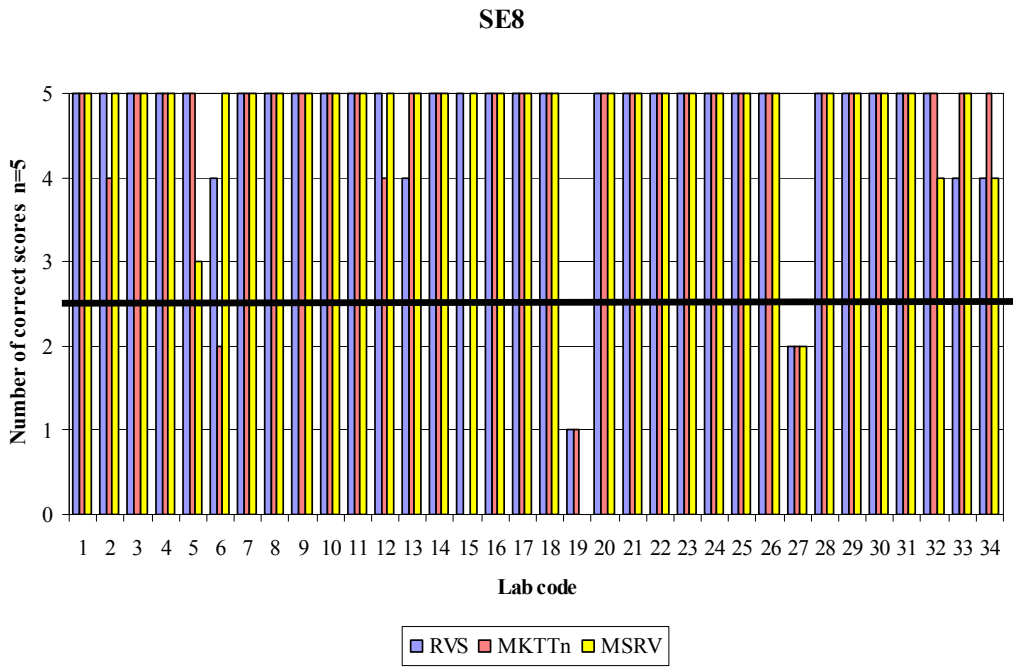

Figure 2 Results per laboratory for the detection of Salmonella in minced meat samples artificially contaminated with SE8 lenticule discs (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar

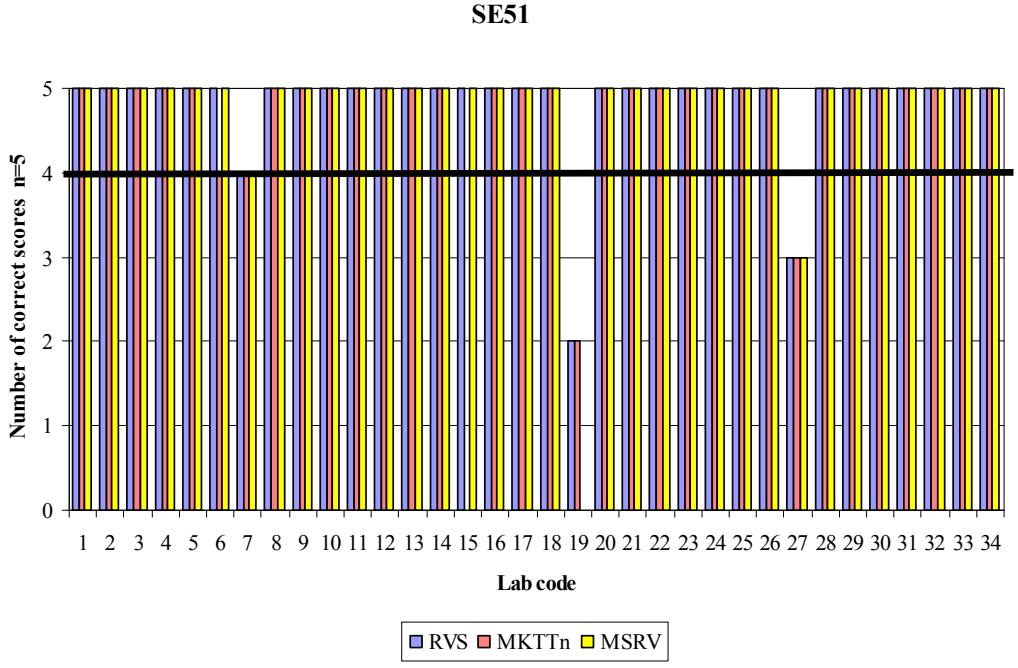

Figure 3 Results per laboratory for the detection of Salmonella in minced meat samples artificially contaminated with SE51 lenticule discs (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar

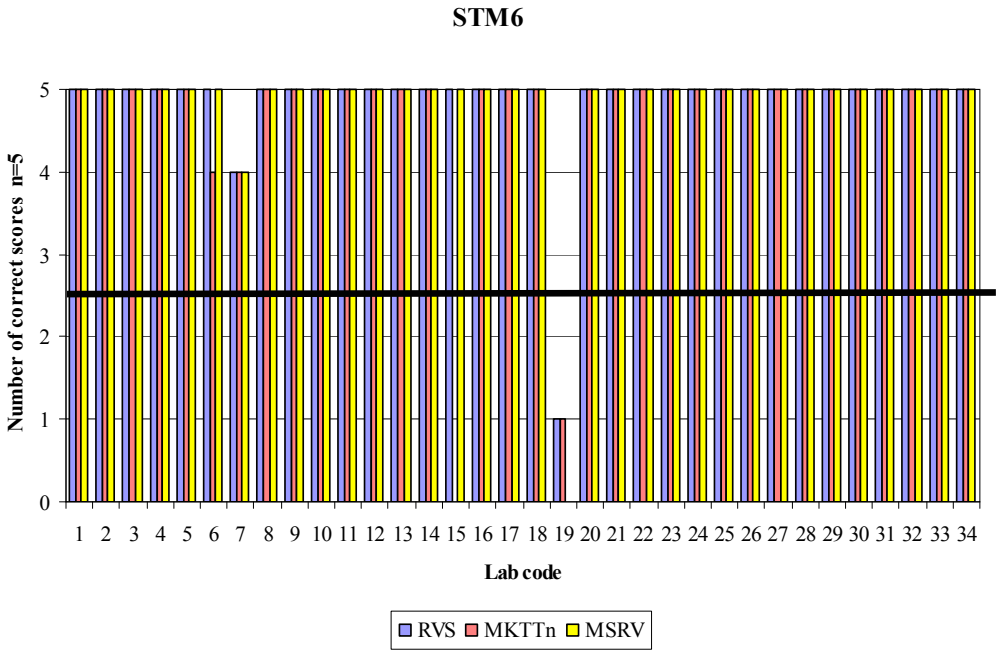

Figure 4 Results per laboratory for the detection of Salmonella in minced meat samples artificially contaminated with STM6 lenticule discs (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar

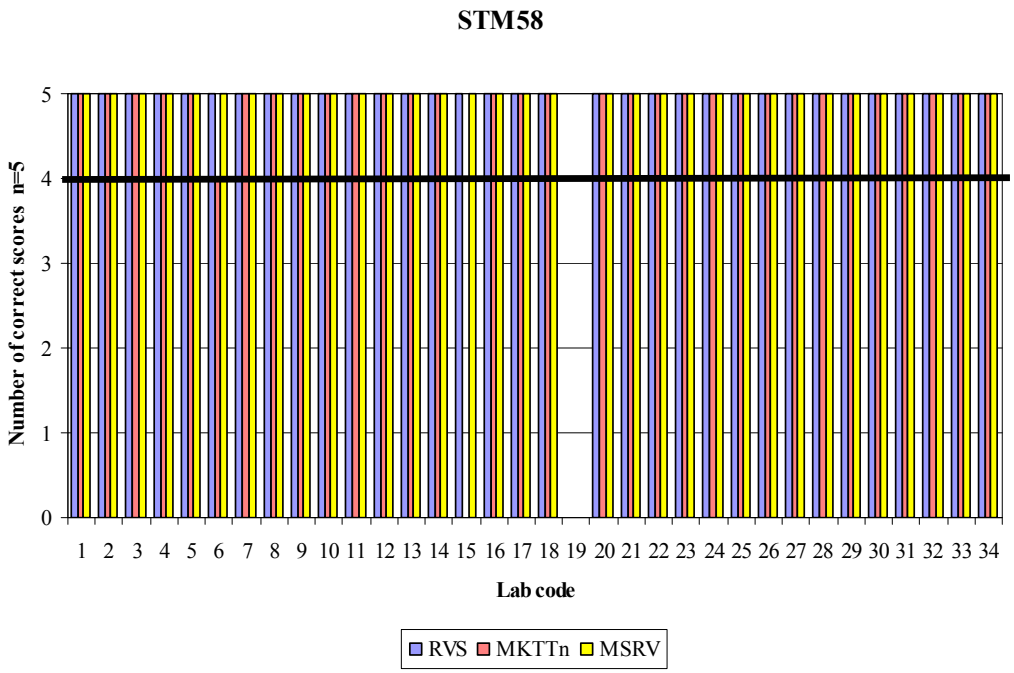

Figure 5 Results per laboratory for the detection of Salmonella in minced meat samples artificially contaminated with STM58 lenticule discs (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar

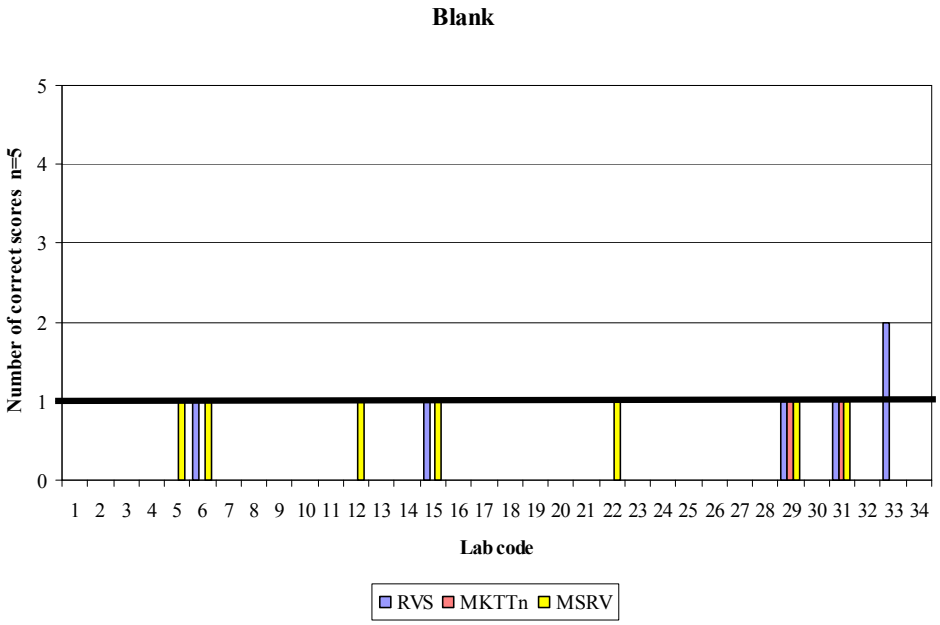

Figure 6 Results per laboratory for the detection of Salmonella in minced meat samples artificially contaminated with Blank lenticule discs (n=5) after selective enrichment in RVS, MKTTn and on MSRV followed by isolation on selective plating agar

Table 21 presents the results of the number of positive isolations after 24 and 48 hours of incubation of the selective enrichment media. The choice of plating-out medium does not seem to have a large effect on the number of positive isolations. When MKTTn is used for selective enrichment, XLD gave 2 - 3% more positive results than other plating-out media. The majority of the laboratories used BGA as the second plating-out medium (see Table 8).

Table 21 Mean percentages of positive results for the detection of Salmonella in the artificially contaminated minced meat samples after selective enrichment in RVS, MKTTn and on MSRV, incubated for 24 hours and for 48 hours and followed by isolation on different plating out media

Plating out medium	**Selective enrichment medium**		
	RVS	**MKTTn**	**MSRV**
	24/48 h	24/48 h	24/48 h
XLD	94/96%	91/95%	94 / 98%
Other (most often BGA)	92/95%	88/93%	94 / 98%
Difference XLD/other	2/1%	3/2%	0%

The difference in the number of positive isolations after 24 hours and after 48 hours of incubation of the selective enrichment media was the highest for MKTTn: 4 - 5% more positive isolations were found after 48 hours of incubation. Table 22 shows the additional positive results for the different lenticule discs after the extra 24 hours of incubation in/on the different selective enrichment media. Most clear are the extra positive results for the low level contaminated SE samples: 9 – 10% with MKTTn and 11 – 13% with MSRV.

Table 22 Percentages of positive results found after an additional 24 hours of incubation in/on the selective enrichment media (RVS, MKTTn, MSRV) for the artificially contaminated (with SE and STM lenticule discs) minced meat samples

Lenticule disc	**Difference between 24 h and 48 h of incubation in %**		
	Selective enrichment medium		
	RVS	**MKTTn**	**MSRV**
SE8	5 - 7%	9 - 10%	11 – 13%
SE51	5%	2 – 6%	3%
STM6 & STM61	< 1%	1 – 2%	< 1%

Tables 23 and 24 show the differences between selective enrichment media and isolation media per lenticule as odds ratios (OR). In addition, the 95% confidence intervals and p-values are given.

In Table 23, the odds of finding a positive isolation with the different plating-out media are compared, given a selective enrichment medium. For instance, the odds of finding *Salmonella* from the STM6 samples after selective enrichment in MKTTn is a factor of 3.69 higher when XLD is used as isolation medium compared to an isolation medium other than XLD. In general, if MKTTn is used as selective enrichment medium, the ORs are larger than the ORs of RVS and MSRV. In other words when MKTTn is used for selective enrichment it is easier to detect *Salmonella* if XLD is used compared to other isolation media. This is significant for all lenticules. For the other selective enrichment media RVS and MSRV, there is no significant difference for the detection of *Salmonella* after plating out on XLD or on other isolation media.

The interpretation of Table 24 is similar to that of Table 23, except that selective enrichment media are mutually compared, given XLD as isolation medium. For instance, the odds of finding *Salmonella* from all STM samples after selective enrichment in MKTTn is a factor of 0.35 lower than with MSRV. In general, if RVS is used as selective enrichment media, the chance of finding *Salmonella* is larger than when MKTTn is used, but RVS compared with MSRV gives a smaller chance. However, none of the differences are significant.

Table 23 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: minced meat, artificially contaminated with Salmonella positive lenticule discs

Selective enrichment medium	Compared isolation media	Lenticule disc	Odds Ratios	95% lower	95% upper	p-value*
RVS	XLD compared to other than XLD	SE8	1.53	0.6	3.98	0.37
		SE51	1.71	0.39	8.12	0.49
		STM6	1.37	0.25	7.49	0.71
		STM61	1.38	0.27	7.65	0.71
		all SE	1.62	0.66	3.98	0.28
		all STM	1.37	0.42	4.56	0.6
		all lenticules	1.49	0.72	3.17	0.29
MKTTn	XLD compared to other than XLD	SE8	1.21	0.49	3.21	0.68
		SE51	3.21	0.91	11.86	0.07
		STM6	3.69	1	15.53	0.05
		STM61	1.7	0.38	7.78	0.48
		all SE	1.97	0.89	4.46	0.09
		all STM	2.51	0.93	6.89	0.07
		all lenticules	2.22	1.18	4.33	0.01
MSRV	XLD compared to other than XLD	SE8	1.29	0.44	3.68	0.64
		SE51	1.67	0.41	7.24	0.49
		STM6	2.4	0.39	19.22	0.37
		STM61	1.03	0.03	41.04	0.98
		all SE	1.47	0.58	3.57	0.41
		all STM	1.58	0.2	12.46	0.65
		all lenticules	1.52	0.51	4.62	0.44
All enrichment media	XLD compared to other than XLD	SE8	1.34	0.77	2.41	0.31
		SE51	2.09	0.94	4.75	0.08
		STM6	2.29	0.89	6.22	0.09
		STM61	1.35	0.33	5.36	0.65
		all SE	1.67	1.01	2.74	0.05
		all STM	1.75	0.77	4.11	0.18
		all lenticules	1.71	1.06	2.82	0.03

* significant difference in case $p < 0.05$.

Table 24 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: minced meat, artificially contaminated with Salmonella positive lenticule discs

Compared selective enrichment media	Isolation medium	Lenticule disc	Odds Ratios	95% lower	95% upper	p-value*
RVS compared to MKTTn	XLD	SE8	1.17	0.43	3.15	0.77
		SE51	1.36	0.3	6.5	0.69
		STM6	1.44	0.26	7.84	0.67
		STM61	1.45	0.28	7.84	0.66
		all SE	1.26	0.51	3.18	0.62
		all STM	1.44	0.47	4.6	0.55
		all lenticules	1.35	0.65	2.87	0.42
RVS compared to MSRV	XLD	SE8	0.73	0.25	2.06	0.55
		SE51	1.47	0.29	7.34	0.63
		STM6	0.86	0.1	5.82	0.91
		STM61	0.3	0.01	3.18	0.38
		all SE	1.03	0.4	2.72	0.95
		all STM	0.51	0.07	2.42	0.45
		all lenticules	0.73	0.24	1.84	0.53
MKTTn compared to MSRV	XLD	SE8	0.62	0.21	1.76	0.37
		SE51	1.08	0.21	5.05	0.91
		STM6	0.6	0.07	3.88	0.63
		STM61	0.21	0.01	2.22	0.23
		all SE	0.82	0.32	2.12	0.68
		all STM	0.35	0.05	1.65	0.21
		all lenticules	0.54	0.19	1.35	0.2

Significant difference in case $p < 0.05$.

Figure 7 shows the performance of each laboratory as odds ratios compared to the mean of all laboratories for the artificially contaminated samples. In this calculation, the blank lenticules are not used. The mean (OR=1) is defined as the odds of detecting *Salmonella* based on the fixed effects only (lenticule, enrichment medium and isolation medium). Laboratories below the mean have a lower probability to detect *Salmonella*. Thirteen laboratories 3, 4, 8, 9, 10, 11, 16, 20, 23, 24, 25, 26 and 28 found a higher number of positive results compared to the mean of all laboratories; they scored all samples correctly for all used media. In general, the laboratories performed very well. There is only a small difference between the performance of the laboratories just above the mean (e.g. laboratory 20) or just below the mean (e.g. laboratory 32). Laboratory 20 missed only one lenticule on one isolation medium and laboratory 32 missed only one lenticule with MSRV on both used isolation media. Four laboratories scored a significant lower performance. Laboratory 19 showed the lowest performance. The performance of the laboratories 6 and 7 were significantly lower but they still scored within the limits of good performance. Laboratory 6 scored low for the selective enrichment in MKTTn but performed well with MSRV and RVS. Laboratory 7 missed two samples with all used selective enrichment media in combination with all used isolation media.

As for determining the performance of a laboratory, all used media were taken into account.

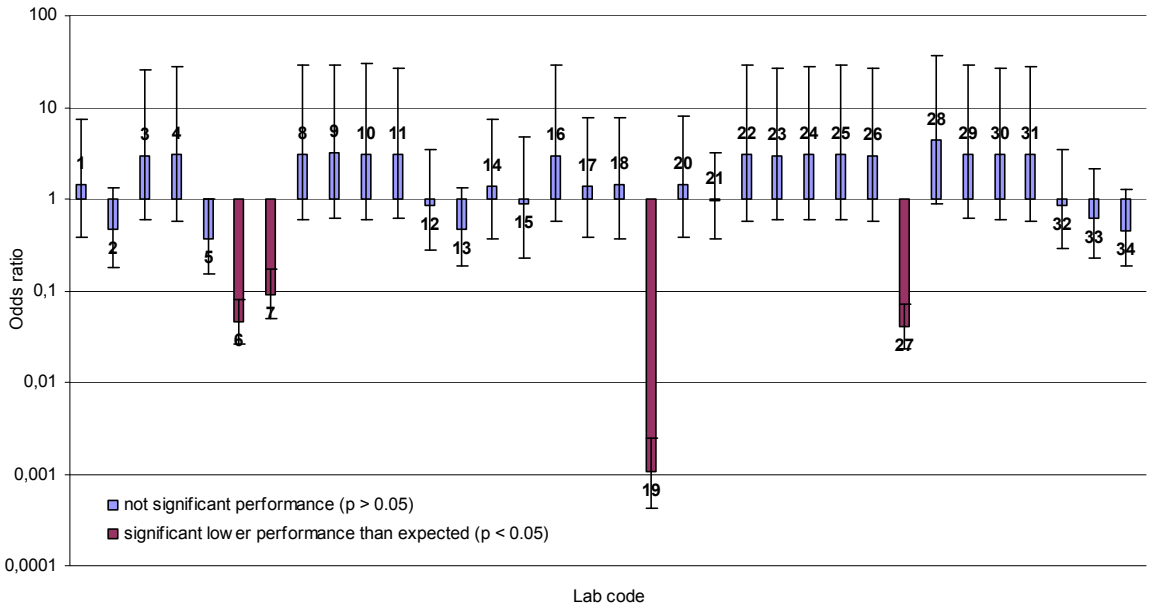

Figure 7 Performance of each laboratory compared to the mean of all laboratories for the artificially contaminated minced meat samples (without blanks)

4.5.2 Specificity, sensitivity and accuracy rates of the artificially contaminated samples

Table 25 shows the specificity, sensitivity and accuracy rates for all types of lenticule discs added to the minced meat. The rates are calculated for the different selective enrichment media (RVS, MKTTn and MSRV) with plating-out medium XLD. The calculations were performed on the results of all participants and on the results of only the EU-MS (without the results of the European Free Trade Association States and candidate EU-MS. Only small differences were found between these groups, the rates of the EU-MS were in general 2% higher. The specificity rates (of the blank lenticule discs) were 96 -99%. The highest rates were found with MSRV. The lowest sensitivity rates were found with the SE8 lenticule discs with MKTTn (91 – 93%). The accuracy rates were comparable between the different selective enrichment media (96 – 98%).

Table 25 Specificity, sensitivity and accuracy rates for all participating laboratories of the artificially contaminated meat samples (each lenticule disc added to 25 g minced meat) for the selective enrichment in RVS, MKTTn and on MSRV and plating out medium XLD

Lenticule disc with minced meat		**RVS/XLD**		**MKTTn/XLD***		**MSRV/XLD****	
	Laboratories	All n=34	EU n=29	All n=33	EU n=29	All n=33	EU n=29
Blank (n=5)	No. of samples	170	145	165	145	165	145
	No. of negative samples	166	142	163	143	159	140
	Specificity in %	**97.7**	**97.9**	**98.8**	**98.6**	**96.4**	**96.6**
SE8 (n=5)	No. of samples	170	145	165	145	165	145
	No. of positive samples	156	136	150	135	158	138
	Sensitivity in %	**91.8**	**93.8**	**90.9**	**93.1**	**95.8**	**95.2**
SE51 (n=5)	No. of samples	170	145	165	145	165	145
	No. of positive samples	164	142	158	141	162	142
	Sensitivity in %	**96.5**	**97.9**	**95.8**	**97.2**	**98.2**	**97.9**
STM6 (n=5)	No. of samples	170	145	165	145	165	145
	No. of positive samples	165	144	159	143	164	144
	Sensitivity in %	**97.1**	**99.3**	**96.4**	**98.6**	**99.4**	**99.3**
STM61 (n=5)	No. of samples	170	145	165	145	165	145
	No. of positive samples	165	145	159	144	165	145
	Sensitivity in %	**97.1**	**100**	**96.4**	**99.3**	**100**	**100**
All lenticule discs with *Salmonella*	No. of samples	680	580	660	580	660	580
	No. of positive samples	650	567	626	563	649	569
	Sensitivity in %	**95.6**	**97.8**	**94.9**	**97.1**	**98.3**	**98.1**
All lenticule Discs	No. of samples	850	725	825	725	825	725
	No. of correct samples	816	709	789	706	808	709
	Accuracy in %	**96**	**97.8**	**95.6**	**97.4**	**97.9**	**97.8**

* Results without Laboratory 15 (non-EU): they did not use MKTTn

**Results without Laboratory 19 (non-EU): they did not use MSRV

4.6 Own method: PCR

Nine laboratories (lab codes 5, 6, 8, 16, 20, 25, 26, 28 and 34) applied a Polymerase Chain Reaction (PCR) method as an additional detection technique. All laboratories tested the samples after pre-enrichment in BPW. Four PCR methods were validated. Five laboratories (lab code 8, 20, 25, 28 and 34) used the PCR routinely. Table 26 gives further details on the used PCR methods.

Table 26 Details on Polymerase Chain Reaction procedures, used as own method during the interlaboratory comparison study by nine participants

Lab code	PCR method: Reference
5	Conventional PCR, Aabo et al. (1993), Not validated, Commercial
6	Real time PCR, Not validated, Commercially (Applied Biosystems Microseq *Salmonella*)
8	Real time PCR, in house validation* Non commercial
16	PCR no further information, Not Validated Non commercial
20	Real time PCR, Malorny et al., (2004), Not validated, Non commercial
25	PCR no further information, Validated*, Non commercial
26	Real time PCR, Malorny et al., (2004), Not validated, Non commercial
28	PCR BAX system, Validated:AFNOR QUA-18/3-11/02 (2009), Commercial
34	PCR end time, Validated: Nordval 030 (2009), Commercial

*Participants indicated the PCR method to be validated. However, it is not clear whether the method has been validated in accordance with ISO 16140 (Anonymous, 2003b) and no information on certificate number was given.

Table 27 gives the results of both the PCR and the bacteriological cultivation (BAC). Seven laboratories found the same results with the PCR technique as with the bacteriological culture method; all samples were scored correctly. One laboratory (lab code 6) found a deviating result with a (false) positive blank for both methods PCR and cultivation in RVS and on MSRV. One laboratory (lab code 5) found three samples negative with PCR while they correctly found these samples positive with cultivation.

Table 27 Number of positive results found for the control samples and for the artificially contaminated minced meat samples by using a PCR technique and the bacteriological culture technique (BAC, given the highest number of positive results with RVS/MKTTn or MSRV)

Lenticule disc	Lab 5		Lab 6		Lab 8, Lab 16, Lab 20, Lab 25 Lab 26, Lab 28 Lab 34	
	BAC	PCR	BAC	PCR	BAC	PCR
Control samples without matrix (n=7)						
SE8 (n=2)	2	2	2	2	2	2
SE51 (n=1)	1	1	1	1	1	1
STM6 (n=2)	2	2	2	2	2	2
Blank (n=2)	0	0	0	0	0	0
Test samples with minced meat (n=25)						
SE8 (n=5)	5	**3**	5	5	5	5
SE51 (n=5)	5	5	5	5	5	5
STM6 (n=5)	5	**4**	5	5	5	5
STM61 (n=5)	5	5	5	5	5	5
Blank (n=5)	0	0	**1**	**1**	0	0

Bold numbers: unexpected results
Grey cells: different results found with the BAC or with PCR.

4.7 Performance of the NRLs

4.7.1 General

29 NRLs fulfilled the criteria of good performance and five laboratories scored below these criteria. As for the determination of good performance the results of all media were taken into account it may occur that some laboratories did not score well with one medium, but overall still scored a good performance. For example laboratory 6 found only few positive results with the samples artificially contaminated with SE when using MKTTn (2/5 of SE8 and 4/5 of SE51), but scored good results with RVS and MSRV. Of the laboratories which did not score a good performance two laboratories (lab codes 19 and 27) showed to have a problem with the detection of *Salmonella* with minced meat. Three laboratories (lab codes 15, 31 and 33) reported false positive blank samples.

The five laboratories were contacted by the EURL-*Salmonella* in November 2011 to ask for possible explanations for their deviating results and some NRLs were asked to perform some additional test (e.g. biochemical tests, serotyping and phage typing).

Laboratory 15 found one blank control sample (lenticule discs without meat) positive on all used media (RVS and on MSRV). All blanks should have been tested negative. The laboratory also found one blank sample with minced meat positive on all used media. However, one (false) positive blank result with matrix is still accepted. The laboratory did go through all procedures, but did not find a clarification for the (false) positive blanks. The PCR performed as own method also tested both samples positive. The inoculums for selective enrichment (RVS and MSRV) and PCR were taken from the same pre-enriched culture in BPW. Quality control on the BPW had shown the medium to be correctly sterile. The laboratory performed serotyping on the (false) positive samples and both were typed as *S*. Typhimurium. It was not possible to perform phage typing. The laboratory indicated to find in general only few routine samples positive for *Salmonella*. At the time of the interlaboratory comparison study the only samples positive for *S*. Typhimurium happen to be those of the study. Therefore, the most likely clarification for their deviating results is cross contamination at the first step in the procedure: the pre-enrichment in BPW.

Laboratory 31 found one matrix control sample (with meat/no lenticule disc) and one procedure control sample (only BPW) positive with all used selective enrichment media (RVS, MKTTn and on MSRV). Both control samples should have been tested negative. After the laboratory received the final results they immediately observed the mistake and contacted the EURL to explain the mistake and showed the raw laboratory data with the correct results.
The laboratory made an initial transcription error. The mistake occurred when the raw data were transferred into the digital version of the test report in the computer. The laboratory observed the mistake in the digital test report and changed this but most probably this was not saved in the computer and the wrong data were send to the EURL. Also a transcription error is an incorrect result but a follow-up study for this mistake was not considered appropriate. Therefore, the results of laboratory 31 were indicated as 'moderate performance'.

Laboratory 33 found two blank samples (lenticule discs with meat) positive after 48 hours of selective enrichment in RVS in combination with isolation on Rambach agar. The same blank samples were correctly tested negative after selective enrichment in MKTTn and on MSRV. All blank samples should have

been tested negative. However, as no 100% guarantee on the negativity of the matrix can be given, one (false) positive blank with matrix is still accepted. However, finding more than one blank sample positive for *Salmonella* but two positive blanks is considered as no good performance. The laboratory indicated that the false positive samples showed slightly different results on Rambach agar, but were still likely S*almonella.* The confirmation tests (TSI, UA, LDC, Omnivalent, OBIS reactions and API profiles) gave 'normal' *Salmonella* reactions. In the test report the laboratory mentioned to have used API20E for confirmation but in fact they had used Rapid20E. For routine samples, they perform API20E followed by serotyping, but because of the amount of positive samples in this ringtest they decided to skip the serotyping which is performed in another department of the institute. The laboratory performed several additional tests with the two false positive isolates together with some other positive samples from the ringtest. They typed the strains with PCR (Inva), API20E, RapiD20E, inoculation on MSRV and on Brolacin. The false positive strains both gave negative results for *Salmonella* with the PCR, API20E and cultivation on MSRV and on Brolacin. The laboratory found a discrepancy between the results of API20E (no *Salmonella*) and Rapid20E, which gave a result of *Salmonella* spp ID 95%. The laboratory contacted the manufacturer to discuss the incorrect profile. In conclusion, the false positive results were caused by the incorrect profile given by Rapid20E in combination with omitting serotyping of the samples.
As in daily routine the laboratory always analyses the samples with further confirmation by serotyping no problems will the analysis of routine samples are expected. The laboratory will consider the same confirmation for at least of some samples of the ringtest.

Laboratory 19 could only detect *Salmonella* in four out of twenty *Salmonella* lenticule discs with matrix (minced meat) while the scores of the control lenticule discs (without matrix) SE8, STM6 and SE51 were correctly positive. The laboratory (non-EU) participated for the first time and there were different possible clarifications for their difficulties with the detection of *Salmonella* in the minced meat samples:
-The laboratory mentioned not to be accredited according ISO 17025, so that no information on the quality system of the laboratory was available.
-The parcel for the ringtest was sent to the address of the contact person. Next the parcel was forwarded to the laboratory which took five additional days. The temperature of the parcel during the additional shipment time is not known because the temperature recorder was separated from the parcel after arriving at the contact.
-The confirmation tests were not performed completely as the laboratory was afraid not to be able finish before the deadline of the ringtest. Some deviations were observed in the media and the incubation temperature used (see Tables 9 -15), like a lower incubation temperature at the start of the incubation of RVS (37 ºC instead of 41.5 ºC) and the absence of novobiocin in MKTTn.
-A very long stomacher time of the samples was (twenty minutes, where one minute is normal).
-The isolation medium BGA did not give any positive results for the samples with minced meat.

Laboratory 27 could only detect *Salmonella* Enteritidis in three out of five SE51 and two out of five SE8 lenticule discs with matrix (minced meat) while the results with the control lenticule discs, Blank, SE8, STM6 and SE51, as well as with the artificially contaminated meat samples with STM6, STM61 and Blank lenticule discs, were all scored correctly.

The laboratory did perform an internal audit on the deviating results in the ringtest. The laboratory is not using MSRV as a routine method. The results of MSRV and the prescribed method (RVS and MKTTn) were in discrepancy and they were confused about those different results. The laboratory repeated the analyses of the (false) negative samples from the BPW cultures stored at the refrigerator and found positive results with RVS and MKTTn, but still negative results with MSRV. A possible clarification for this may have been a problem with the preparation of one batch of MSRV used for those samples. To prevent problems in future the laboratory will reconsider the way of preparing MSRV and will add additional quality controls.

To check whether the actions taken have been successful, laboratory 15, 19, 27 and 33 participated in a follow-up study organised by the EURL-*Salmonella* in February 2012.

4.7.2 Follow-up study

The set-up of the follow-up study was the same as the full interlaboratory comparison study as organised in September 2011, but with a lower number of samples (see section 4.1 'Reference materials'). In this follow-up study, blank samples and low level samples containing *S.* Enteritidis were tested, as these types of samples caused most of the problems. Table 28 gives an overview of the samples used in the follow-up study.

Table 28 Overview of the types and the number of lenticule discs tested in the follow-up interlaboratory comparison study

Lenticule discs	**Control lenticule discs (n=6) No matrix added**	**Test samples (n=15) with 25 grams *Salmonella* negative minced meat**
S. Enteritidis 8 (SE8)	2	5
S. Enteritidis 51 (SE51)	1	3
S. Typhimurium 6 (STM6)	-	-
S. Typhimurium 61 (STM61)	1	2
Blank	2	5

On Monday 16 January 2012, one parcel with two plastic containers was sent to the laboratories 15, 19, 27 and 33 containing: six control lenticule discs (numbered C1 – C6), 15 lenticule discs (numbered B1 – B15), 400 grams of minced meat and one temperature recorder.
On 10 January 2012, the number of aerobic bacteria ($6.5 * 10^7$ cfu/g) and *Enterobacteriaceae* ($4.8 * 10^3$ cfu/g) in the minced meat was tested after it was stored at 5 °C for one week. These numbers were one log higher than the numbers found in the minced meat used in the full study (see Table 6).
The performance of this follow-up study started in week 4 (23 January 2012). Each laboratory had to follow the same SOP and protocol as in the study of September 2011 (see Annexes 4 and 5). The test report was different from the September study (see Annex 8). For the media used, only the differences with the March study needed to be indicated.

For the media compositions, incubation times and temperatures, no or minor differences were observed in comparison to the full study.

Laboratory 15 performed the follow up study with a new batch of MSRV (not expired) with a pH of 5.3. Additionally, they performed the follow up study with their own methods: PCR and a *Salmonella* Tecra test (combination of culture, immunocapture on sticks and ELISA).
They scored all samples correctly with the prescribed method RVS, requested method MSRV and their own methods.

Laboratory 27 pre-treated the samples differently in the follow up study. They did use pre-filled BPW bottles instead of plastic bags and they shook the samples for two minutes instead of using a stomacher for thirty seconds. They used a third isolation medium additional to XLD and Rambach, being Hektoen agar.
They scored all samples correct.

Laboratory 33 followed their routine procedure for the confirmation of the suspected *Salmonella* colonies. For this they tested suspect colonies biochemically (TSI, UA, LDC), identification kit API20E and by serotyping (O and H antigens).
They scored all samples correctly.

Laboratory 19 did not return their results, nor did they inform the EURL in other way. This laboratory is a non EU MS and is not obliged to participate in the studies. However, the EURL is obliged to inform EC DG-Sanco in case of problems with NRLs (under performance).

With these results, the laboratories 15, 27 and 33 fulfilled the criteria of good performance (see section 3.6) in this follow-up study.

5 Discussion

Reference materials
After many years of using 'capsule-reference materials' to artificially contaminate the matrix in interlaboratory comparison studies of the EURL-*Salmonella*, it was decided to change to lenticule discs. The main reason for this change was the easiness of handling and the robustness of the lenticule discs, which could help to better mimic 'real-life' routine samples. The different tests performed at the EURL confirmed this. The original criterion for the variation in counts between capsules within one batch was that it should be less than twice a Poisson distribution. The same homogeneity test was applied to the batches of lenticule discs as ordered for the interlaboratory comparison study. The test showed that the variation between lenticule discs of all four batches was even less than once a Poisson distribution. It could therefore be concluded that the batches of lenticule discs were well homogeneous.

A limited long-term stability test was performed on three batches of lenticule discs stored at -20 ºC. For all batches a decrease in the mean number of cfu was seen. However, several factors may have influenced the tests. The analyses at the start and at the end of the study were performed in two different laboratories (HPA and the EURL-*Salmonella*). Furthermore, the mean level of the batches of lenticule discs was determined by HPA before they were shipped to the EURL. Shipping may have had a (slight) negative effect on the mean level of the lenticules. Therefore, it may have been the case that the mean levels were somewhat lower when received at the EURL, but this was not tested. Additionally, the HPA determined the mean levels on thirty lenticule discs per batch, the EURL on only 5 lenticule discs. This also hampers the comparison of the results because of the differences in variation in results around the mean level. However, for the interlaboratory comparison studies it is more important to know the stability of the reference materials after a short time of storage, especially under 'abuse circumstances', which may be the case during transport. The results of the challenge test show little or no effect on the mean contamination levels of the batches of lenticule discs with SE and STM after one week of storage at 5 ºC. When stored at higher temperatures like 22 ºC or 30 ºC a decrease in the mean number of cfu was seen after three to seven days. Still a well countable mean level remained in the batches. In the worst situation, the mean level after storage was half the mean level before storage (SE92 stored at 30 ºC).

To prevent the batches of lenticule discs for a decrease in the mean level during transport, the materials were packed with frozen cooling devices and transported by courier service. From the information of the temperature recorders, which were included in the parcels, it can be seen that the temperature in the parcel remained below 5 ºC for most of the transport time. It can therefore be assumed that transport would not have negatively affected the mean level of the batches. This was confirmed by the fact that the laboratories (lab codes 2 and 9) with the longest transport times and with the highest transport temperature still found good results.

Performance of the laboratories
According to EC Regulations (EC, 2004), each NRL should have been accredited for their relevant work field before 31 December 2009 (EC, 2005). All

laboratories are accredited with the exception of two participants (one non-EU country) who are in the process to become accredited.
32 laboratories used the prescribed method (ISO 6579: RVS & MKTTn) and the requested method (Annex D of ISO 6579: MSRV. One laboratory did use the prescribed methods (RVS and MKTTn) but not the requested method (MSRV). Another laboratory did use RVS and MSRV, but not the prescribed selective enrichment in MKTTn. Nine laboratories used additionally an 'own' method (PCR).

For determining 'good performance' per laboratory, the best performing isolation medium after selective enrichment in RVS, MKTTn or MSRV was taken into account (being the medium with the highest number of positive isolations).
29 out of in total 34 laboratories scored 'good performance'. Three laboratories (lab codes 15, 27 and 33) scored an underperformance and participated in a follow-up study. Laboratory 19 (non-EU) scored an 'underperformance' in the full study but did not return their results from the follow-up study. A likely explanation (among others) for the problems of laboratory 19 may be inexperience with the ringtest. In earlier studies it has been observed that laboratories participating for the first time often show an underperformance, but improve during the course of the studies. For this, good contact between the EURL and the NRL is important (Kuijpers and Mooijman, 2012). One laboratory (lab code 31) scored a 'moderate performance' as they made an initial transcription error from the raw data.
Laboratory 6 found deviating results with the MKTTn method, but scored good performance with RVS and the requested method MSRV.

A possible clarification for the false positive blanks of laboratory 15 may be (cross) contamination at the first step in the procedure: the pre-enrichment in BPW.
A limited confirmation of *Salmonella* suspected (atypical) colonies on Rambach after selective enrichment in RVS seems to be the most plausible clarification for the false positive blank results found by laboratory 33.
Laboratory 27 showed to have problems with the detection of S. Enteritidis; unfortunately, it was not possible to find a clarification for their problems.
The problems of the laboratories 15, 27 and 33 seemed to have been solved in the follow-up study where they scored all samples correctly.

A relatively high number of blank samples with matrix were scored positive for *Salmonella*. Seven laboratories found one blank sample added to *Salmonella* negative meat positive for *Salmonella* and one laboratory found two blank samples positive. Four participants found this false positive result only with one or two of the selective enrichment media. Three laboratories found false positive results on all used media. No 100% guaranty about the *Salmonella* negativity of the minced meat could be given, but there was only a small change that the minced meat contained *Salmonella*. In this ringtest 201 samples of 25 gram minced meat each were tested negative (99%).
False positive blank results can have different causes:
-cross contamination, which can occur at different stages of the procedure for the detection of *Salmonella;*
-limited confirmation or misinterpretation of the test results. The number of background flora in the matrix used in this study was relatively high which may have caused problems with reading of the isolation media. In combination with a limited confirmation, the *Enterobacteriaceae* present in the matrix can be misinterpreted as *Salmonella,* resulting in a false positive blank result.

Specificity, sensitivity and accuracy rates
The calculations were performed on the results of all participants and on the results of only the EU Member States (without the results of participants from the EFTA and candidate EU-MS countries). Only minor differences (if any) were found between these groups.
The accuracy rates of the control samples were at least 99%. The sensitivity rates and the specificity rates of the artificially contaminated samples were at least 96%, showing that the NRLs were well able to detect *Salmonella* at different levels. Only the sensitivity rate of the SE8-meat samples was lower (91- 96%), which is not unexpectedly at this contamination level.

Pre-treatment of the samples
In this study the participants could use their routine procedures to pre-treat the samples, especially for the mixing of the samples in BPW. In the former food studies this was not possible as the capsules needed a special pre-treatment for dissolution. The participating laboratories indeed used different methods to mix the samples in BPW, like stomacher, shaking, kneading or no mixing at all. No effect of any of the pre-treatment procedures or of no pre-treatment of the samples was seen on the results, which confirms the robustness of the lenticule disc reference materials.

Media and incubation
Deviations in media compositions or incubation temperatures were reported but no effects were seen on the results.
For most of the laboratories the results with RVS, MKTTn and MSRV in combination with all used isolation media gave the same scores.
The choice of the plating-out medium does not seem to have a large effect on the number of positive isolations. When MKTTn is used for selective enrichment medium, XLD gave 3% more positive results than other plating-out media. For MSRV the difference between XLD and another plating-out medium was nil.
The increase in the number of positive results after 48 hours of incubation, compared to 24 hours of incubation, of the selective enrichment in MKTTn and on MSRV was 4-5%. This was most clear for the low level contaminated SE samples which showed 9-13% more positive results after 48 hours of incubation. An explanation for this latter result may be the fact that the level of contamination of 8 cfu SE per lenticule disc in combination with the used matrix (minced meat) was close to the detection limit. The presence of high amounts of background flora may initially have suppressed the growth of *Salmonella* Enteritides in/on the enrichment media.

PCR
Nine laboratories used a PCR technique additional to the prescribed method and eight of them found the same results as with the bacteriological detection methods. One laboratory found more negative results with their PCR method than with the bacteriological detection method.
Compared to former interlaboratory comparison studies an increase is seen in the use of PCR as an own method. A few years ago, three to four laboratories used PCR additional to the prescribed method and currently nine laboratories.

Evaluation of this study
Overall it was concluded that the first EURL-*Salmonella* food study organised with lenticule discs was successful.
The use of lenticule discs as reference materials to artificially contaminate a matrix showed some advantages as well as some disadvantages compared to the capsule reference materials.
Advantages:

- It is possible to treat the samples in the same way as routine samples, especially in relation to mixing of the samples. For example there is no problem to use a stomacher or pulsifier for mixing of the samples with lenticule discs.
- The risk of cross-contamination is reduced compared to the situation when capsules were used, as the lenticule disc is added to BPW after addition of the matrix instead of before.

Disadvantages:

- *Salmonella* Enteritidis in the lenticule discs gave atypical colonies on Rambach isolation medium.

6 Conclusions

- 32 out of 34 participants achieved the level of 'good performance' for the detection of *Salmonella* in minced meat. Three laboratories needed a follow-up study to reach the desired level. One laboratory scored an underperformance in the full study; they did not participate in a follow up study. One laboratory scored a 'moderate performance'.

- The accuracy, specificity and sensitivity rates for the control samples (without matrix) after selective enrichment in RVS, MKTTn and on MSRV were > 98%.

- The specificity rates of the minced meat samples artificially 'contaminated' with blank lenticule discs was 96 – 99% for RVS, MKTTn and MSRV.

- For all artificially contaminated minced meat samples with *Salmonella*, the sensitivity rates after selective enrichment on MSRV (98%) were higher than the rates after selective enrichment in MKTTn and RVS (96%).

- The low-level materials of *S*. Typhimurium (STM6) were easier to detect than the low-level materials of *S*. Enteritidis (SE8).

- The accuracy rates of the artificially contaminated minced meat samples were 96% for RVS and MKTTn and 98% for MSRV.

- The different pre-treatment procedures as used by the participants to mix the matrix and lenticule disc in BPW did not influence the ability to detect *Salmonella* in the samples of this interlaboratory comparison study.

- 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.

- An additional incubation time of 24 hours gave 4-5% more positive results after selective enrichment in MKTTn and on MSRV. This is most obvious for the low level contaminated SE samples (9-13%).

- PCR was used by 9 out of 34 participants as an own method and gave mostly comparable results to the bacteriological culture methods (RVS, MKTTn and RVS).

- An increase is seen in the use of PCR as an own method compared to former interlaboratory comparison studies.

- As the interlaboratory comparison study is intended to test the ability of a laboratory to detect Salmonella in a given matrix, it is important to use the routine procedures for this as much as possible, for example full confirmation of *Salmonella* suspected colonies to prevent false positive results.

- The first EURL-*Salmonella* food- study organised with lenticule discs as reference material to artificially contaminate a matrix was successful.

List of abbreviations

BAC	Bacteriological Culture technique
BGA	Brilliant Green Agar
BPLS(A)	Brilliant green Phenol-red Lactose Sucrose (Agar)
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar
cfu	colony forming units
CRL	Community Reference Laboratory (new name EURL)
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
Gal	Galactosidase
HPA	Health Protection Agency
ISO	International Standardisation Organisation
LDC	Lysine Decarboxylase
MKTTn	Mueller Kauffmann Tetrathionate novobiocin Broth
MLCB	Mannitol Lysine Crystal Violet Brilliant Green Agar
MS	Member State
MSRV	Modified Semi-solid Rappaport Vassiliadis
NCTC	National Collection of Type Cultures (HPA)
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)
RS	Rapid *Salmonella*
RV(S)	Rappaport Vassiliadis (Soya) broth
SE	*Salmonella* Enteritidis
SM (ID)2	*Salmonella* Detection and Identification-2
SOP	Standard Operating Procedure
STM	*Salmonella* Typhimurium
TSI	Triple Sugar Iron agar
UA	Urea Agar
VP	Voges-Proskauer
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate agar

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

Table A1.1 History of EURL-Salmonella interlaboratory comparison studies on detection of Salmonella in veterinary samples

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment medium	Plating-out medium
I 1995 Voogt et al., 1996 RIVM report 284500003	26 4	STM5 Blank	6 0	No No		RV and SC	BGA and own
II 1996 Voogt et al., 1997 RIVM report 284500007	15 15 2 1 1	STM100 STM1000 SPan5 STM100 Blank	116 930 5 116 0	1 gram 1 gram No No No	Chicken faeces mixed with Glycerol[3]	RV, SC and own	BGA and own
III 1998 Raes et al, 1998 RIVM report 284500011	14 14 7 14 4 2 5	STM10 STM100 STM100S E100 STM10 SPan5 Blank	11 94 94 95 11 5 0	1 gram 1 gram 1 gram* 1 gram No No No	Chicken faeces mixed with Glycerol[3]	RV and own	BGA and own
IV 1999 Raes et al, 2000 RIVM report 284500014	5 5 5 5 5 3 3 2 2	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank	4 210 60 220 0 5 60 5 0	10 gram 10 gram 10 gram 10 gram 10 gram No No No No	Chicken faeces mixed with Glycerol[3]	RV or RVS, MSRV and own	BGA and own
V 2000 Raes et al, 2001 RIVM report 284500018	5 5 5 5 5 3 3 2 2 20	STM10 STM100 SE100 SE500 Blank STM10 SE100 SPan5 Blank None	4 47 63 450 0 4 63 5 0 -	10 gram 10 gram 10 gram 10 gram 10 gram No No No No 25 gram**	Chicken faeces mixed with Glycerol[3]	RV or RVS, MSRV and own	BGA and XLD

Table A1.1 (continued)

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

Table A1.1 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment medium	Plating-out medium
X	5	STM10	9	10 gram	Pig	MSRV	XLD
2006	5	STM100	98	10 gram	faeces[4]	and own	and own
	5	SE100	74	10 gram			
	5	SE500	519	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM10	9	No			
2007	2	SE100	98	No			
RIVM Report	1	SE500	519	No			
330604004	2	SPan5	5	No			
	2	Blank	0	No			
XI	5	STM5	6	10 gram	Chicken	MSRV	XLD
2008	5	STM50	47	10 gram	faeces[4]	and own	and own
	5	SE10	9	10 gram			
	5	SE100	90	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	6	No			
2008	2	SE10	9	No			
RIVM Report	1	SE100	90	No			
330604011	2	SPan5	5	No			
	2	Blank	0	No			
XII	5	STM5	6	10 gram	Chicken	MSRV	XLD
2009	5	STM50	53	10 gram	faeces[4]	and own	and own
	5	SE20	18	10 gram			
	5	SE100	84	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	6	No			
2009	2	SE20	18	No			
RIVM Report	1	SE100	84	No			
330604014	2	SPan5	7	No			
	2	Blank	0	No			
XIII	5	STM5	5	10 gram	Chicken	MSRV	XLD
2010	5	STM50	56	10 gram	faeces[4]	and own	and own
	5	SE20	13	10 gram			
	5	SE100	78	10 gram			
	5	Blank	0	10 gram			
Kuijpers et al.,	4	SE20	22	10 gram*			
2010	2	STM5	8	No			
RIVM Report	2	SE20	13	No			
330604018	1	SE100	78	No			
	1	Blank	0	No			

Table A1.1 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment medium	Plating-out medium
XIV	5	STM6	6	25 gram	Chicken faeces[4]	MSRV and own	XLD and own
2011	5	STM61	61	25 gram			
	5	SE6	6	25 gram			
	5	SE57	57	25 gram			
	5	Blank	0	25 gram			
Kuijpers and	2	STM6	6	No			
Mooijman 2011	2	SE6	6	No			
RIVM report	1	SE57	57	No			
330604023	1	Blank	0	No			

[1] The report of each study can be obtained through the corresponding author of this report or can be found at the EURL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/.
[2] In the studies organised from 1995 to 2010 the RMs existed of gelatine capsules containing artificially contaminated milk powder. In the studies organised from 2011 the RMs existed of lenticule discs (HPA, UK).
[3] Faeces mixed (1:1) with a solution of peptone/glycerol. Final concentration glycerol in the faeces mixture was 15%(v/v).
[4] Faeces not mixed with any preservation medium.
* =With antibiotics
** =Naturally contaminated chicken faeces with *Salmonella*
*** =Naturally contaminated dust with *Salmonella*

Table A1.2 EURL-Salmonella interlaboratory comparison study on the detection of Salmonella in food samples

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment Medium	Plating-out medium
I	5	STM10	9	10 gram	Minced beef	RVS,	XLD
2006	5	STM100	98	10 gram		MKTTn,	and own
	5	SE100	74	10 gram		MSRV	
	5	SE500	519	10 gram		and own	
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM10	9	No			
2007	2	SE100	98	No			
RIVM Report	1	SE500	519	No			
330604003	2	SPan5	5	No			
	2	Blank	0	No			
II	5	STM5	4	10 gram	Minced beef	RVS,	XLD
2007	5	STM50	40	10 gram		MKTTn,	and own
	5	SE10	7	10 gram		MSRV	
	5	SE100	71	10 gram		and own	
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	4	No			
2008	2	SE10	7	No			
RIVM Report	1	SE100	71	No			
330604010	2	SPan5	7	No			
	2	Blank	0	No			
III	5	STM5	6	10 gram	Minced	RVS,	XLD
2009	5	STM50	54	10 gram	chicken meat	MKTTn,	and own
	5	SE20	12	10 gram		MSRV	
	5	SE100	50	10 gram		and own	
	5	Blank	0	10 gram			
Kuijpers et al.,	3	STM5	6	No			
2010	2	SE20	12	No			
RIVM Report	1	SE100	50	No			
330604017	2	SPan5	6	No			
	2	Blank	0	No			
IV	8	STM5	6	25 gram	Minced	RVS,	XLD
2010	8	STM50	55	25 gram	pork/beef	MKTTn,	and own
	8	Blank	0	25 gram	meat	MSRV	
Kuijpers et al.,	3	STM5	6	No		and own	
2011	1	STM50	55	No			
RIVM Report	1	Blank	0	No			
330604020							

Table A1.2 (continued)

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/RM	Matrix amount	Matrix type	Selective enrichment Medium	Plating-out medium
V	5	STM6	6	25 gram	Minced	RVS,	XLD
2011	5	STM61	61	25 gram	pork/beef	MKTTn,	and own
	5	SE8	8	25 gram	meat	MSRV	
	5	SE51	51	25 gram		and own	
	5	Blank	0	25 gram			
	2	STM6	6	No			
	2	SE8	8	No			
This study	1	SE51	51	No			
	2	Blank	0	No			

[1] The report of each study can be obtained through the corresponding author of this report or can be found at the EURL-*Salmonella* website: http://www.rivm.nl/crlsalmonella/publication/.
[2] In the studies organised from 1995 to 2010 the RMs existed of gelatine capsules containing artificially contaminated milk powder. In the studies organised from 2011 the RMs existed of lenticule discs (HPA, UK).

Table A1.3 EURL-Salmonella interlaboratory comparison study on the detection of Salmonella in animal feed samples

Study Year Reference[1]	Number of samples	RM[2]	Actual number of cfu/capsule	Matrix amount	Matrix type	Selective enrichment medium	Plating-out medium
I	5	STM5	5	25 gram	Chicken feed (mixed grains)	RVS, MKTTn, MSRV and own	XLD and own
2008	5	STM50	43	25 gram			
	5	SE20	15	25 gram			
	5	SE100	48	25 gram			
	5	Blank	0	25 gram			
Kuijpers et al.,	3	STM5	5	No			
2009	2	SE20	15	No			
RIVM Report	1	SE100	48	No			
330604012	2	SPan5	5	No			
	2	Blank	0	No			

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

[2] In the studies organised from 1995 to 2010 the RMs existed of gelatine capsules containing artificially contaminated milk powder. In the studies organised from 2011 the RMs existed of lenticule discs (HPA, UK).

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 1. 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

RV (Oxoid CM 0699/TV 5017E, Hampshire, United Kingdom)
Vassiliadis P, Pateraki E, Papaiconomou N, Papadakis JA and Trichopoulos D, 1976. Annales de Microbiologie (Institut Pasteur) 127B. 195-200.
Composition of RV medium: the concentration of the compounds in g/L water: Soya Peptone 5, Sodium Chloride 8, Potassium dihydrogen phosphate 1.6, Magnesium Chloride 40, Malachiet green 0.04 pH 5.2- 5.4.

BGA modified (Oxoid CM 0329/PO0171A; Hampshire, United Kingdom) (BPLS, Merck 1.10747, Darmstadt, Germany) (Lab M, lab 34 Bury, United Kingdom) (HImedia Laboratories M971, Mumbai, India) (Biolife 4012562, Milan, Italy)
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: Edel and Kampelmacher; according ISO 6579, 1993.

BGA (Conda laboratories 136600, 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, pH 7.4.

BGA (Torlak 300413, Belgrade. Serbia)
Composition of BGA medium: the concentration of the compounds in g/L water: Pepton 13, Ekstarkt kvasca 3, Lactose 10, Saccharose 10, Sodium chloride 5, Phenol red 0.08, Brilliant green 0.0125, Agar 15, pH 6.9.

BPLSA (Merck 107237.0500, Darmstadt, Germany)
Adam D, Zusatz von Natriumdesoxycholat zum Brilliantgrün-Phenolrot-Agar nach Kristensen-Kauffmann zur Hemmung des Schwärmvermögens von Proteuskeimen, 1966 Ärztl. Lab. 12, 245.
Composition of BPLSA medium: the concentration of the compounds in g/L water: Peptone from meat 5, Peptone from casein 5, Meat extract 5, Sodium chloride 3, di-sodium hydrogen phosphate 2, Lactose 10, Sucrose 10, Phenol red 0.08, brilliant green 0.0125, Agar agar 12, pH 7.

Brilliance *Salmonella* Agar BSA (previous OSCM) (Oxoid CM 1092; PO 5098A, Hampshire, United Kingdom)
Schönenbrücher V, Mallinson ET, Bülte M. A comparison of standard cultural methods for the detection of foodborne *Salmonella* species including three new chromogenic plating media. Int J Food Microbiol. 2008 Mar 31;123(1-2):61-6.
Composition of BSA agar: the concentration of the compounds in g/L water: Salmonella Growth mix 14, Chromogen mix 25, Agar 15, Cefsulodin 0.012, novobiocin 0.05, pH 7.2.

Compass *Salmonella*: (Biokar Diagnostics BM 06608, Beauvais, France)
Perez JM et al., Comparison of four chromogenic media and Hektoen agar for detection and presumptive identification of Salmonella strains in human stools, J Clin. Microbiol., 2003, Mar., 41(3), 1130 – 4.
Composition of Compass agar: the concentration of the compounds in g/L water: Pepton 10, Sodium chloride 5, Phosphate Buffer 7, Inhibitory agents 9, Chromogenic mixture 1.4, Bacteriological agar 15, pH 7.

MLCB (Oxoid CM 0783, Hampshire, United Kingdom)
Inoue T, Takagi S, Ohnishi A, et al. Foodborne disease S*almonella* 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, Lab-Lemco powder 2.0, Peptone 10.0, Sodium Chloride 4.0, Mannitol 3.0, L-Lysine HCL 5.0, Sodium Thiosulphate 4.0, Ferric Ammonium (III) Citrate 1.0, Brilliant Green 0.012, Crystal Violet 0.01, Agar No.2 15.0, pH 6.8.

Rambach (Merck 107500.0002, Darmstadt, Germany)
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, NaCl 5, Sodium deoxycholate 1.0, Chromogenic mix 1.5, Propylene glycol 10.5, Agar-agar 15, Rambach agar supplement 10 ml, pH 7.1-7.3.

Rapid Salmonella (RS) agar (Biorad 356-4705, Marnes-La-Coquette, France)
Composition of Rapid Salmonella agar: the concentration of the compounds in g/L water: Casein Peptone 5, Meat extract 5, Selective agents 14, Chromogenic mixture 0.31, Agar 12.7, pH 7.2.

SM(ID)2 = Chrom ID (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) 6.3, Tris 0.2, Lactose 6, Ox bile (bovine and swine) 1.5, Chromogenic mix 9.6, Sodium chloride 5, Selective mix 0.03, Agar 14, pH 6.7-7.3.

Annex 4

Protocol

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD organised by EURL-*Salmonella*

FOOD STUDY V - 2011

Introduction

This protocol describes the procedures for the fifth interlaboratory comparison study on the detection of *Salmonella* spp. in a food matrix amongst the National Reference Laboratories (NRLs for *Salmonella*) in the EU. In this study the number of samples to be tested will be comparable to earlier studies on the detection of *Salmonella* spp. but the type of reference materials will be different. The prescribed method is like in earlier studies, the procedure as described in ISO 6579 ('Microbiology of food and feeding stuffs – Horizontal method for the detection of *Salmonella* spp.' Fourth edition, 2002.) Additional to 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 applicable for the analyses of food samples. Furthermore laboratories who are interested can also perform PCR on the samples and/or use additional methods (routinely) used in their laboratories.

The samples will consist of minced meat (*Salmonella* negative) artificially contaminated with reference materials. The reference materials (RMs) consist of lenticule discs containing *Salmonella* strains at different contamination levels. Each laboratory will examine 25 meat samples (25 g each) in combination with a *Salmonella* lenticule disc and 7 control samples (lenticule discs only).
.

The samples will be packed in 2 plastic containers in one large box together with cooling elements. One container will contain the lenticule discs the other will contain the minced meat. The container with the lenticule discs 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 EURL-*Salmonella*, immediately after receipt of the parcel.** For this purpose a return envelope with a preprinted address label of the EURL-*Salmonella* has been included. Do not forget to note your lab code before returning it to the EURL.
Each box with samples will be sent as biological substance category B (UN3373) by door-to-door courier service. **Please contact the EURL-*Salmonella* when the parcel has not arrived at your laboratory on 22th of September 2011** (this is 4 working days after the day of mailing).

Objective

The main objective of the interlaboratory comparison study is to evaluate the performance of the NRLs for *Salmonella* for their ability to detect *Salmonella* spp. at different contamination levels in a food matrix using different methods.

Outline of the study

In week 38 (starting on 19 Sept) of 2011 each participant will receive one box containing 2 biopacks, packed with cooling elements.

The containers contain:

Container 1:
one plastic bag with 32 numbered vials each containing one lenticule disc with or without *Salmonella*
-25 vials numbered **B**1-**B**25;
-7 vials numbered **C**1-**C**7;
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 EURL-*Salmonella* as soon as possible.**
Store container 1, with its content, at (-20 ± 5) ºC immediately after receipt.

Container 2:
one plastic bag with approximately 750 g of minced meat (free from *Salmonella*).
Store container 2, with its content, at (5 ± 3) ºC immediately after receipt.

The performance of the study will be in week 39 (starting on 26 September 2011).

The documents necessary for performing the study are:
- Protocol Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food V (2011) (this document);
- SOP Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food V (2011);
- Test report Interlaboratory comparison study on the bacteriological detection of *Salmonella* spp. in food V (2011);
- 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 EURL.

All data have to be reported in the test report and sent to the EURL-*Salmonella* **before 14 October 2011.** The EURL will prepare a summary report soon after the study to inform all

NRLs on their own results and on the overall results. **Results which will be received after the deadline can not be used in the analyses for the interim summary 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 (internal 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 FOOD V (2011)

Week	Date	Topic
36	5 - 9 September	Mailing of the protocol, Standard Operating Procedure and test report to the NRLs-*Salmonella*
38	19 - 23 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 **EURL-*Salmonella*** using the return envelope; - **Store the lenticule discs at -20 ± 5 °C** - **Store the meat at + 5 ± 3 °C** **If you did not receive the parcel at 22 September, do contact the EURL immediately.** 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)
39	26 - 30 September	Performance of the study, following the instructions as given in the protocol and the SOP of study Food V (2011).
41	Before 14 October	Completion of the test report. Send the test report by e-mail to the EURL *Salmonella* Angelina.Kuijpers@rivm.nl *.
42	17 - 22 October	Checking the results by the National Reference Laboratories.
	December 2011	Sending of the final results to the NRLs together with an interim summary report. A follow-up will be discussed with NRLs who showed no good performance, according to pre-defined criteria.

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

Annex 5 Standard Operating Procedure (SOP)

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD organised by EURL-*Salmonella*

FOOD STUDY V- 2011

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 food matrix. For this purpose Reference Materials (RMs) containing *Salmonella* Typhimurium (STM) or *Salmonella* Enteritidis (SE), as prepared by the Health Protection Agency (HPA, United Kingdom), are used. As matrix, minced meat (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.

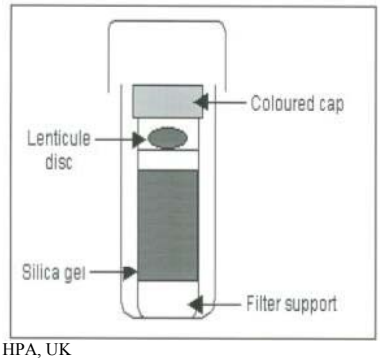
ISO 6579:2002/Amd 1 2007. Amendment 1 Annex D: Detection of *Salmonella* spp. in animal faces and in environmental samples from the primary production stage.

Lenticule Disc Handling Information. HPA Culture Collection, Salisbury, United Kingdom. More information on the reference materials (lenticule discs) as produced by the HPA can be found on: http://www.hpacultures.org.uk/products/lenticulediscs/index.jsp

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 lenticule disc containing microorganism at a defined number in a water soluble matrix.
Note: Each lenticule is individually packed in small vials as indicated in the picture below.



HPA, UK

4 Principle

The detection of *Salmonella* involves the following stages:
1) Pre-enrichment
2) Selective enrichment
3) Isolation
4) Confirmation of typical colonies as *Salmonella*.

5 List of abbreviations

BPW	Buffered Peptone Water
MKTTn	Muller Kauffmann 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 Methods and 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 (6.1)
Selective enrichment medium (prescribed)(6.2)	MKTTn & RVS
	MSRV (requested) (6.2)
Selective plating medium for first and second isolation	XLD and a second medium for choice (obligatory!)(6.3)
Confirmation media	see 6.4

Composition and preparation of the media and reagents are described in Annex B, and in Annex D of the ISO 6579: 2002. 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. Check the quality of the media before use.

In addition to the prescribed method (ISO 6579) and requested method (Annex D of ISO 6579) it is possible to use other methods, e.g. the one(s) routinely used in your laboratory ['Own' method(s)]. This can vary from another culture method to 'a PCR technique'. If necessary prepare media for the 'own' method(s) according to the relevant instructions. Record all relevant information in the test report.

6.1 Non selective pre-enrichment medium
-Buffered Peptone water (BPW) (ISO6579 Annex B.1)

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 used in your laboratory (optional)

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 medium used in your laboratory (optional)

6.4 Confirmation media
-Biochemical confirmation as described in ISO 6579 Annex B.6-B.11 or by reliable, commercially available identification kits.
-Nutrient agar (optional) (ISO6579Annex 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
-Sterile culture containers (bottles, jars or plastic bags) with nominal capacity of approximately 400 ml;
-Sterile culture bottles or jars with nominal capacity of 300 ml;

-Sterile culture tubes with approximate sizes: 8 mm in diameter and 160 mm in length;
-Sterile micro-pipettes; nominal capacity 0.1 ml and 1 ml;
-Sterile 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 of the interlaboratory comparison study in a food matrix of EURL-*Salmonella* is described. The different steps in the procedure are also summarized in Annex A of this SOP. In addition to 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).
For testing the samples use as much as possible the materials you are normally using for your routine samples. For example, either use bags or jars for the pre-enrichment in BPW depending on what you routinely use. Bottles, bags or jars for the pre-enrichment in BPW are further mentioned as containers.

8.1 Pre-enrichment (day 1)

Use BPW equilibrated to at least room temperature (follow your routine procedure).
Record in the test report (pages 2 & 3) the requested data on BPW.

Take the numbered vials with the *Salmonella* lenticules out of the freezer, 10-15 minutes before they are added to the BPW, to allow them to equilibrate to room temperature.

-Label 34 containers as follow:

- 25 containers from B1 to B25
- 9 containers from C1 to C9 (control lenticules)

-Add 25 g of meat to each container labelled B1 – B25 and C9.
-Add 225 ml BPW to each container (B1- B25 and C1-C9).
When your containers are already pre filled with 225 ml BPW, add 25 g of meat to the BPW.
Add no matrix/ meat to the containers labelled C1 – C8.
One container is a procedure control to which no lenticule or meat is added (= C8).
One container is the negative meat control to which only 25 g meat is added (= C9).
These control conainers should be handled in the same way as the other containers.

-Add to the 32 labelled containers (containing BPW with or without meat) a lenticule disc from the vial with the corresponding label number (B1- B25 and C1 – C7).
No lenticules are added to C8 and C9.

- Leave all the containers for 10 – 15 minutes at room temperature to re-hydrate the lenticule. As the disc is coloured, it may be visible when it is re-hydrated. Even when it is not visible whether the lenticule is re-hydrated, proceed with the next steps of the procedure after 15 minutes standing at room temperature.

- Mix the samples by using a pulsifier or a stomacher. When this is not possible (eg when the samples are contained in a jar), shake the samples carefully.

-Incubate all samples at 37 °C ± 1 °C 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, record all requested data on pages 20-21 & 30of the test report.

8.2 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), 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 34 RVS tubes, MKTTn tubes and MSRV plates as follow:

- 25 tubes/plates from B1 to B25
- 9 tubes/plates from C1 to C9 (control)

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

After equilibration:
Prescribed methods:
-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 each 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 24h ± 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 the test report). Incubate at the temperature 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).

8.3 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.
Allow the isolation media to equilibrate to room temperature, if they were stored at a lower temperature. Dry the surface of the plates, if necessary.

-Label two times 34 large size Petri dishes and 34 standard size Petri dishes of each isolation medium from B1 to B25 and C1 to C9.

Note: In case you do not have large dishes (140 mm) at your disposal, use two standard size (90 -100 mm) dishes, one after the other, using the same loop.

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. Inoculate, by means of a 1 µl loop from suspect MSRV plates, the surface of isolation media in one standard size Petri dish with the corresponding label numbers. Inoculate the isolation media in such a way that isolated colonies will be obtained.
The following isolation media will be used:

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

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

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.

8.4 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 from RVS), Table 2 (isolation from MKTTn), Table 3 (isolation from MSRV) and Table 4 (isolation from own selective enrichment medium) on the test report pages 22-29.
For the results of detection of *Salmonella* using PCR complete in Table 5 on the test report at page 30.

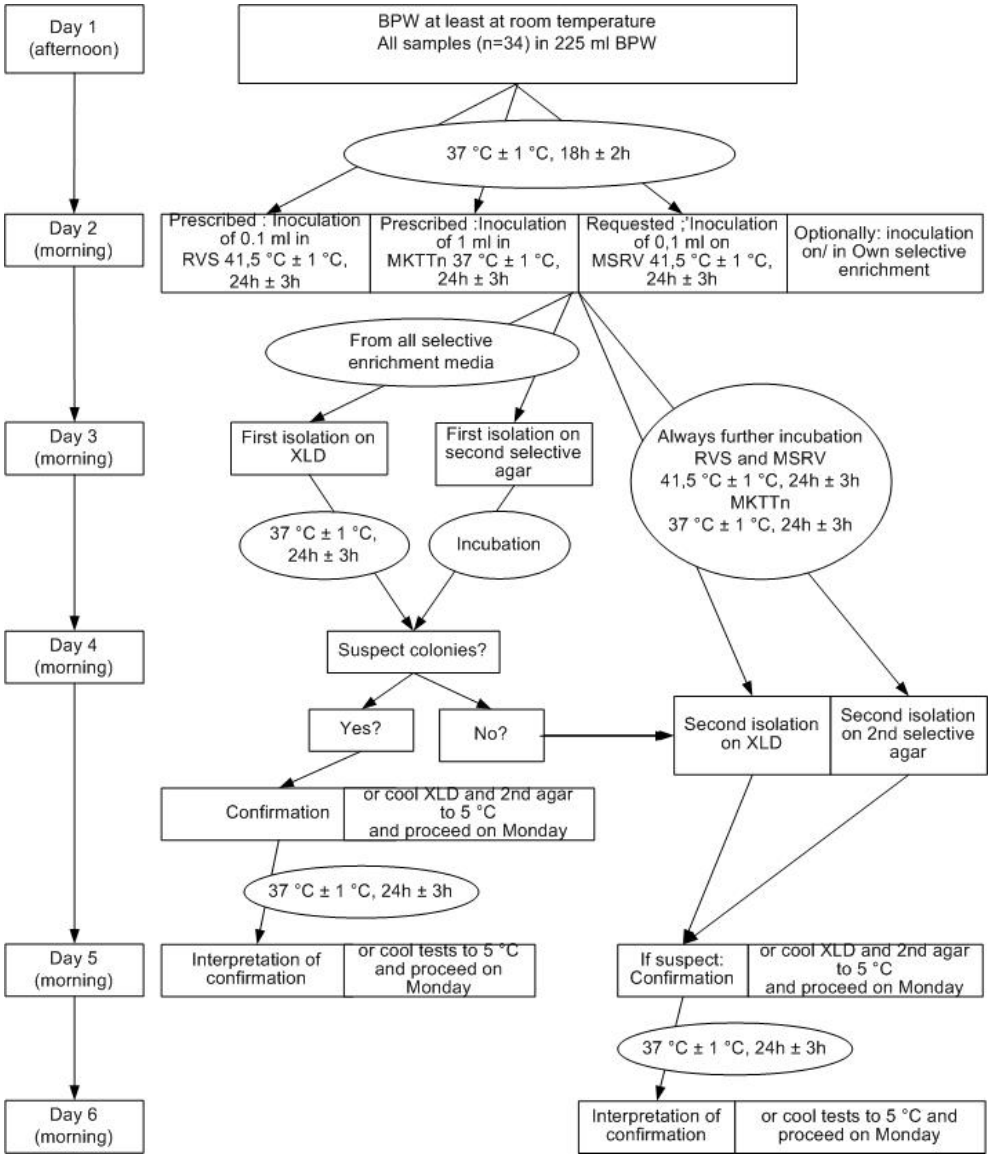
Confirmation of identity
The identity from the colony selected above (either directly from the isolation medium, or from nutrient agar) is confirmed by means of appropriate biochemical and serological tests. Follow the instructions of ISO 6579. Note in the test report (page 19) which media/tests have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

Conserve one positive isolate (*Salmonella* strain) from each sample.
After the interlaboratory comparison study it may be necessary to perform some additional testing (in case of deviating results). Therefore it is requested to conserve one *Salmonella* confirmed colony from one of the used isolation media of each of the used selective enrichment medium from each suspect sample B1-B25 and C1-C9.

9 Test report

Record in the test report all information that might influence the results and is not mentioned in this SOP. Also record incidents or deviations from the specified procedures. The test report should include the name of the person in charge for the NRL, and the names of the persons who are carrying out the work. 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 FOOD V (2011) On detection of *Salmonella* spp. in minced meat (see Annex A)		
Day	Topic	Description
1	**Pre-enrichment**	Allow the BPW to equilibrate to at least room temperature Add 25 g meat to container (jar or plastic bag) Add 225 ml BPW to meat (or add 25 meat directly to 225 ml BPW) Add 1 lenticule disc to BPW Leave 10- 15 minutes at room temperature Mix (pulsifier or stomacher) sample 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 (24h) and own medi(um)(a): • Xylose Lysine Desoxycholate agar, incubate at (37 ± 1) °C for (24 ± 3) h • Second isolation medium* (obligatory) • Own selective medi(um)(a)* (optional) *= 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, 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
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 first isolation media (day 4).


Day 1 (afternoon)
BPW at least at room temperature
All samples (n=34) in 225 ml BPW
37 °C ± 1 °C, 18h ± 2h
Day 2 (morning)
Prescribed : Inoculation of 0.1 ml in RVS 41,5 °C ± 1 °C, 24h ± 3h
Prescribed :Inoculation of 1 ml in MKTTn 37 °C ± 1 °C, 24h ± 3h
Requested ;'Inoculation of 0,1 ml on MSRV 41,5 °C ± 1 °C, 24h ± 3h
Optionally: inoculation on/ in Own selective enrichment
From all selective enrichment media
Day 3 (morning)
First isolation on XLD
First isolation on second selective agar
Always further incubation RVS and MSRV 41,5 °C ± 1 °C, 24h ± 3h MKTTn 37 °C ± 1 °C, 24h ± 3h
37 °C ± 1 °C, 24h ± 3h
Incubation
Day 4 (morning)
Suspect colonies?
Yes?
No?
Second isolation on XLD
Second isolation on 2nd selective agar
Confirmation
or cool XLD and 2nd agar to 5 °C and proceed on Monday
37 °C ± 1 °C, 24h ± 3h
Day 5 (morning)
Interpretation of confirmation
or cool tests to 5 °C and proceed on Monday
If suspect: Confirmation
or cool XLD and 2nd agar to 5 °C and proceed on Monday
37 °C ± 1 °C, 24h ± 3h
Day 6 (morning)
Interpretation of confirmation
or cool tests to 5 °C and proceed on Monday

Annex 6 Number of positive results of the control samples (lenticule without matrix)

per laboratory and per selective enrichment medium

Lab code	RVS					MKTTn					MSRV				
	SE8 n=2	SE51 N=1	STM6 n=2	Blank n=2	Control n=2*	SE8 n=2	SE51 n=1	STM6 n=2	Blank n=2	Control n=2*	SE8 n=2	SE51 n=1	STM6 n=2	Blank n=2	Control n=2*
Good Performance	**≥ 1**	**1**	**≥ 1**	**0**	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**0**
1	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
2	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
3	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
4	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
5	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
6	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
7	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
8	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
9	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
10	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
11	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
12	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
13	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
14	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
15	2	1	2	**1**	0	-	-	-	-	-	2	1	2	**1**	0
16	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
17	2	1	**1**	0	0	2	1	**1**	0	0	2	1	**1**	0	0
18	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
19	2	1	2	0	0	2	1	2	0	0	-	-	-	-	-
20	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0

Lab code	RVS					MKTTn					MSRV				
	SE8 n=2	SE51 N=1	STM6 n=2	Blank n=2	Control n=2*	SE8 n=2	SE51 n=1	STM6 n=2	Blank n=2	Control n=2*	SE8 n=2	SE51 n=1	STM6 n=2	Blank n=2	Control n=2*
Good Performance	**≥ 1**	**1**	**≥ 1**	**0**	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**0**	**≥ 1**	**1**	**≥ 1**	**0**	**0**
21	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
22	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
23	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
24	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
25	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
26	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
27	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
28	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
29	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
30	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
31	2	1	2	0	**2**	2	1	2	0	**2**	2	1	2	0	**2**
32	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
33	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0
34	2	1	2	0	0	2	1	2	0	0	2	1	2	0	0

* one meat control (25 g of minced meat/no lenticule) and one procedure control (only BPW)

-: 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 minced meat samples (with lenticule)

per laboratory and per selective enrichment medium

Lab code	RVS XLD/2nd *					MKTTn XLD/2nd *					MSRV XLD/2nd *				
	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5
Good Performance	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤1**	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤ 1**	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤ 1**
1	5/4	5	5	5	0	5	5	5	5	0	5	5	5	5	0
2	5/3	5	5	5	0	**4**	5	5	5	0	5	5	5	5	0
3	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
4	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
5	5	5	5	5	0	5/4	5	5	5	0	3	5	5	5	**1**
6	**4**	5	5	5	**1**	**2/1**	**4/1**	**4/0**	**4/3**	0	5/**4**	5/**3**	5/**3**	5	**1**
7	5/**2**	**4**	**4**	5	0	5/**4**	**4**	**4**	5	0	5/**4**	**4**	**4**	5	0
8	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
9	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
10	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
11	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
12	5	5	5	5	0	**4**	5	5	5	0	5	5	5	5	**1**
13	**4**	5	5	5	0	**3**/5	5	5	5	0	5	5	5	5	0
14	**4**/5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
15	**4**/5	5	5	5	**1**	-	-	-	-	-	5	5	5	5	**1**
16	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
17	**4**/5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
18	5	5	5	5	0	5/**4**	5	5	5	0	5	5	5	5	0
19	**1/0**	**2/0**	**1/0**	**0**	0	**1/0**	**2/0**	**1/0**	**0**	0	-	-	-	-	-
20	5	5	5	5/**4**	0	5	5	5	5	0	5	5	5	5	0

Lab code	RVS XLD/2nd *					MKTTn XLD/2nd *					MSRV XLD/2nd *				
	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5	SE8 n=5	SE51 n=5	STM6 n=5	STM61 n=5	Blank n=5
Good Performance	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤ 1**	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤ 1**	**≥ 3**	**≥ 4**	**≥ 3**	**≥ 4**	**≤ 1**
21	5	5	5	5	0	5	5/**4**	5/**4**	5/**4**	0	5	5	5	5	0
22	5	5	5	5	0	5	5	5	5	0	5	5	5	5	**1**
23	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
24	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
25	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
26	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
27	**2**	**3**	5	5	0	2	3	5	5	0	**2**	**3**	5	5	0
28	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
29	5	5	5	5	**1**	5	5	5	5	**1**	5	5	5	5	**1**
30	5	5	5	5	0	5	5	5	5	0	5	5	5	5	0
31	5	5	5	5	**1**	5	5	5	5	**1**	5	5	5	5	**1**
32	5	5	5	5	0	5	5	5	5	0	**4**	5	5	5	0
33	**4**	5	5	5	**0/2**	**4**/5	5	5	5	0	5	5	5	5	0
34	**4**	5	5	5	0	5	5	5	5	0	**4**	5	5	5	0

* When only one figure isgiven both isolation media gave the same result.

-: not performed

bold numbers: deviating results

grey cells: results are below the criteria of good performance.

Annex 8 Test report Follow-up study

FOLLOW UP
TEST REPORT

INTERLABORATORY COMPARISON STUDY ON THE DETECTION OF *SALMONELLA* spp. IN FOOD
organised by EURL-*Salmonella*
FOOD STUDY V 2011
FOLLOW UP January 2012

Laboratory code This is the same code as in FOOD V 2012	
Laboratory name	
Address	
Country	
Date of arrival of the parcels	…… - …… – 2012
Start time of storage at - 20 °C (lenticule discs) Start time of storage at + 5 °C (meat)	Date:…… Time:…… Date:…… Time:……
Parcels damaged?	☐ Yes ☐ No
Starting date testing	…… - …… – 2012

PRE-ENRICHMENT – Buffered Peptone Water (BPW)	
Medium information BPW	
Was the composition of BPW the same as used in BRO FOOD V 2011 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of BPW	
Date of preparation	…… - ……. – 2012
pH after preparation	……., measured at …… °C
pH at the day of use	……., measured at …… °C
Did you perform quality control of BPW?	☐ Yes ☐ No

Incubation time and temperature for pre-enrichment (18 ± 2) hrs after adding meat and lenticule disc	
Start at	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
End at	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C

Pre-treatment of the samples
Did you use the same containers, equilibration of the BPW and mix the samples the same way as in BRO FOOD V 2011 ?
☐ Yes continue on the page 4
☐ No please give more details in the tables on this page :

Containers with BPW	
Did you use containers with pre filled BPW ?	? Yes ? No
What kind of containers did you use for the pre-enrichment in BPW ?	? plastic bags ? jars ? bottles ? ……

Equilibration of the BPW	
At which temperature did you equilibrate the BPW ?	? at 37 °C ? at room temperature ? ……. °C
For how long did you equilibrate the BPW ?	……. h

Mix the samples (BPW, lenticule disc, meat)	
How did you mix the samples ?	? shake ? knead ? vortex ? pulsifier ? stomacher ? ……
How long did you mix the samples ?	
? did not mix the samples	

SELECTIVE ENRICHMENT - Rappaport Vassiliadis Soya medium (RVS)	
Medium information RVS	
Was the composition of RVS the same as used in BRO FOOD V 2011 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of RVS	
Date of preparation	…… - ……. - 2012
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: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2012 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 FOOD IV 2011 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of MKTTn	
Date of preparation	…… - ……. – 2012
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: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2012 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 FOOD V 2011 ?	
☐ 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	…… - ……. - 2012
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: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2012 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 FOOD V 2011 ?
☐ 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 FOOD V 2011 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of XLD	
Date of preparation	…… - ……. – 2012
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: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. - 2012 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 FOOD V 2011 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of the second isolation medium	
Date of preparation	…… - ……. – 2012
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: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the first period (24 h)	Date: …… - ……. - 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the start of the second period (48 h)	Date: …… - ……. – 2012 time: …….. h ……..…… min temperature incubator: ……..…… °C
At the end of the second period (48 h)	Date: …… - ……. – 2012 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 FOOD V 2011?
☐ 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 FOOD IV 2011 ?	
☐ Yes	
☐ No please give more details in an annex :	
Preparation of the nutrient agar	
Date of preparation	…… - ……. – 2012
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 FOOD V BRO 2011, please give more information in an annex .	

Table 1: Results of isolation using **RVS** (dish numbers B1-B15, C1-C6, C8 and C9)

	RVS 24 hours						RVS 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]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												
B13												
B14												
B15												
C1												
C2												
C3												
C4												
C5												
C6												
C8												
C9												

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 B1-B15, C1-C6, C8 and C9)

	MKTTn 24 hours						MKTTn 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]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												
B13												
B14												
B15												
C1												
C2												
C3												
C4												
C5												
C6												
C8												
C9												

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 B1-B15, C1-C6, C8 and C9)

	MSRV 24 hours						MSRV 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]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												
B13												
B14												
B15												
C1												
C2												
C3												
C4												
C5												
C6												
C8												
C9												

Col [a] = **number** of colonies used for confirmation

Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 3: Results of isolation using **OWN** (dish numbers B1-B15, C1-C6, C8 and C9)

	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]
B1												
B2												
B3												
B4												
B5												
B6												
B7												
B8												
B9												
B10												
B11												
B12												
B13												
B14												
B15												
C1												
C2												
C3												
C4												
C5												
C6												
C8												
C9												

Col [a] = **number** of colonies used for confirmation

Sal [b] = **number** of colonies confirmed as *Salmonella*

Table 5: Results of detection using PCR (sample numbers B1-B15, C1-C6, C8 and C9)

sample no.	PCR + or -		
		no.	
B1		C1	
B2		C2	
B3		C3	
B4		C4	
B5		C5	
B6		C6	
B7			
B8		C8	
B9		C9	
B10			
B11			
B12			
B13			
B14			
B15			

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

Name of person (s) carrying out the follow up FOOD V interlaboratory Comparison study.	
Is the person(s) carrying out the follow up FOOD V 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 3 February 2012 by email to EURL-*Salmonella*. If the test report is e-mailed to the EURL it is not necessary to send the original test report as well, unless it is not legible (to be indicated by EURL-*Salmonella*).
Use the address below:

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