National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

EU interlaboratory comparison study primary production XVIII (2015)

Detection of *Salmonella* in pig faeces

RIVM Report 2015-0082

I.E. Pol-Hofstad | K.A. Mooijman

National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

EU interlaboratory comparison study primary production XVIII (2015)

Detection of *Salmonella* in pig faeces

RIVM Report 2015-0082

Colophon

I.E. Pol-Hofstad (auteur), RIVM
K.A. Mooijman (auteur), RIVM

Contact:
Irene Pol-Hofstad
cZ&O Centre for Zoonoses & Environmental Microbiology
Irene.Pol@rivm.nl

This investigation has been performed by order and for the account of the European Commission, Directorate-General for Health and Consumer Protection (DG-Santé), within the framework of RIVM project number E/114506/15/RO European Union Reference Laboratory for *Salmonella* 2015.

This is a publication of:
National Institute for Public Health and the Environment
P.O. Box 1 | 3720 BA Bilthoven
The Netherlands
www.rivm.nl/en

Synopsis

EU interlaboratory comparison study primary production XVIII (2015)
Detection of *Salmonella* in pig faeces

In March 2015, the EURL-*Salmonella* organised the 18th interlaboratory comparison study on the detection of *Salmonella* in samples from the primary production stage. Participation was obligatory for all EU Member State NRLs that are responsible for the detection of *Salmonella* in samples from primary production. In total, 36 NRLs participated in this study: 29 NRLs from the 28 EU-Member States (MS), 6 NRLs from other countries in Europe (EU candidate MS or potential EU candidate MS and members of the European Free Trade Association (EFTA)) and, at the request of DG-Santé, one NRL from a non-European country. EURL-*Salmonella* is part of the Dutch National Institute for Public Health and the Environment (RIVM).

Due to the avian influenza outbreak in the Netherlands, it was not possible to transport chicken faeces. Therefore, the EURL chose pig faeces as an alternative matrix for this detection study. Pre-studies showed that pig faeces were susceptible to the growth of yeast and moulds during storage at 5 °C or 10 °C. Therefore, storage at -20 °C was tested as an alternative. *Salmonella* was found to be sensitive to freezing, but by using a higher starting inoculation levels, it was expected that *Salmonella* would still be found after freezing of the pig faeces.

Unfortunately, *Salmonella* survival in the frozen pig faeces samples was not stable. The results varied tremendously amongst the participants. Therefore, the performance of the participants could not be evaluated in this study.

Keywords: *Salmonella*, EURL, NRL, interlaboratory comparison study, *Salmonella* detection method, pig faeces

Publiekssamenvatting

EU-ringonderzoek primaire productie XVIII (2015)

Detectie van *Salmonella* in varkensmest

In maart 2015 vond het achttiende EURL-ringonderzoek naar detectie van *Salmonella* plaats. Deelname aan deze kwaliteitstoets is verplicht voor alle Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten die verantwoordelijk zijn voor het aantonen van *Salmonella* in dierlijke mest. Voor dit ringonderzoek is een ander type mest gebruikt, dat dit keer geen geschikt alternatief bleek. Daardoor waren de resultaten van de deelnemers niet onderling te vergelijken.

In totaal hebben 36 NRL's deelgenomen aan dit ringonderzoek:
29 NRL's afkomstig van 28 lidstaten in de EU, 6 NRL's afkomstig uit kandidaatlanden voor het EU-lidmaatschap of lidstaten van de European Free Trade Association (EFTA) status en 1 niet-Europees NRL op verzoek van de Europese Unie.

Werkwijze

Er is varkensmest gebruikt omdat het vanwege de vogelgriep in de herfst van 2014 niet was toegestaan om kippenmest te transporteren. Varkensmest staat bekend als een geschikt alternatief. Wel moeten de monsters bij een lagere temperatuur (-20 °C in plaats van 5 of 10 °C) worden bewaard om te voorkomen dat er gisten en schimmels in gaan groeien. In de testfase bleek onder deze omstandigheden een gedeelte van de toegevoegde *Salmonella* dood te gaan. Door extra veel *Salmonella* toe te voegen, zouden er genoeg bacteriën in leven moeten blijven. Tijdens de analyses van de varkensmestmonsters door de laboratoria bleek echter dat *Salmonella* het invriezen niet goed had overleefd. De hoeveelheid *Salmonella* in de aangeleverde monsters verschilde daardoor per laboratorium, zodat de resultaten niet met elkaar konden worden vergeleken.

De laboratoria hebben de monsters geanalyseerd met behulp van de internationaal voorgeschreven analysemethode (MSRV). Het overkoepelend referentielaboratorium van de Europese Unie voor *Salmonella* (EURL-*Salmonella*) is gevestigd bij het RIVM.

Kernwoorden: *Salmonella*, EURL, NRL, ringonderzoek, varkensmest, *Salmonella*-detectiemethode

Contents

Summary

In March 2015 the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised the 18th interlaboratory comparison study on the detection of *Salmonella* in samples from the primary production stage. The matrix of study was pig faeces.

The participants were 36 National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*): 29 NRLs from the 28 EU Member States (EU-MS), 6 NRLs from other countries within Europe (EU candidate MS or potential EU candidate MS, and members of the European Free Trade Association (EFTA)) and, at the request of DG-Santé, one NRL from a non-European country.

The aim of the study was to evaluate the performance of the participating laboratories in the detection of *Salmonella* at different contamination levels in a matrix from the primary production stage. Due to an outbreak of avian influenza in the Netherlands at the end of 2014, chicken faeces were not available for study and pig faeces were selected as an alternative matrix. The prescribed method of analysis was Annex D of ISO 6579 (Anonymous, 2007), using selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar.

The pig faeces samples were artificially contaminated at two levels with a diluted culture of monophasic *Salmonella* Typhimurium (mono-STM) at the laboratory of the EURL. To prevent growth of yeast and moulds, the pig faeces samples were stored at -20 °C. Pre-tests showed that *Salmonella* was susceptible to freezing, but by using higher-than-usual starting inoculation levels, it was expected that the majority of the samples would still be tested positive for *Salmonella*.

Each laboratory received 18 individually numbered blind samples to be tested for the presence or absence of *Salmonella*. These samples consisted of six blank samples, six samples with a low level of mono-STM and six samples with a high level of mono-STM. In addition, three control samples were included: two blank control samples (procedure control (BPW) and matrix control sample (pig faeces)) and one positive control, for which the participants used their own positive control.

In contrast to the good results obtained in the pre-tests, the results of the participants varied considerably. Of the low-level contaminated pig faeces samples, only 21% were found positive for *Salmonella*, and for the high-level contaminated samples, where 100% identification was expected, only 58% were found positive. Due to the large variation in results it was not possible to evaluate the performance of the laboratories.

For the positive control, the majority of the participants used a diluted culture of *Salmonella*. The *Salmonella* serovars used for the positive control sample were mostly *S*. Enteritidis and *S*. Typhimurium. However, the use of rarer serovars is recommended so that cross-contamination is easier to detect. The concentration of the positive control varied between 8 and 10^6 cfu/sample, whereas levels just above the detection limit are advised.

1 Introduction

An important task of the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), as laid down in Commission Regulation No. 882/2004 (EC, 2004), is the organisation of interlaboratory comparison studies to test the performance of the National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*). The history of the interlaboratory comparison studies organised by the EURL-*Salmonella* (formerly CRL-*Salmonella*) since 1995 is summarised on the EURL-*Salmonella* website (http://www.eurlsalmonella.eu).

In March 2015, the EURL-*Salmonella* organised an interlaboratory study to test whether the participating laboratories could detect *Salmonella* at different contamination levels in pig faeces. This information is important in order to ascertain whether the examination of samples in the EU Member States (EU-MS) is carried out uniformly and whether comparable results are obtained by all NRLs-*Salmonella*.

The method prescribed for the detection of *Salmonella* spp. in animal faeces, with selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV), is set out in NPR-CEN-ISO/TR 6579:2002/Amd 1:2007 (Anonymous, 2007).

The set-up of this study was comparable to the interlaboratory comparison study organised in 2014 on the detection of *Salmonella* spp. in samples from the primary production stage (PPS) (Kuijpers and Mooijman, 2014b). In this study, the samples (pig faeces) were artificially contaminated with a diluted culture of monophasic *Salmonella* Typhimurium (STM-mono) at the laboratory of the EURL-*Salmonella.*

In total, 18 pig faeces samples were tested: six samples per contamination level (blank, low-level and high-level), the last two containing one *Salmonella* serovar (monophasic *Salmonella* Typhimurium). Additionally, three control samples (two blank control samples and one positive control sample) were tested. The number and level of samples tested were in accordance with CEN ISO/TS 22117 (Anonymous, 2010).

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IMED/VEMI)
Belgium	Brussels	Veterinary and Agrochemical Research Centre (VAR), CODA-CERVA
Bosnia-Herzegovina	Sarajevo	Veterinary Faculty of Sarajevo, Laboratory for Bacterial Disease in Poultry
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute Poultry Centre, Laboratory for General Bacteriology and Microbiology
Cyprus	Nicosia	Cyprus Veterinary Services, Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Prague	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food Administration
Estonia	Tartu	Estonia Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Safety Authority Evira, Research Department Veterinary Bacteriology
France	Ploufragan	Anses, Laboratoire de Ploufragan-Plouzané Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
Germany	Berlin	Federal Institute for Risk Assessment (BfR), National Veterinary Reference Laboratory for *Salmonella*
Greece	Chalkida	Veterinary Laboratory of Chalkida
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate
Iceland	Reykjavik	Matís ohf, Icelandic Food and Biotech R&D
Ireland, Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFM), Laboratories Backweston, Department of Agriculture, Food and the Marine, Bacteriology
Israel	Kiryat Malachi	Southern Poultry Health Laboratory (Beer Tuvia)
Italy	Padova Legnaro	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

Country	City	Institute
Luxembourg	Luxembourg	Laboratoire de Médecine Vétérinaire de l'Etat, Animal Zoonosis
Malta	Valletta	Public Health Laboratory (PHL), Evans Building
Netherlands, the	Bilthoven	National Institute for Public Health and the Environment (RIVM/Cib), Centre for Infectious Diseases Control Centre for Zoonoses and Environmental Microbiology (cZ&O)
Norway	Oslo	Norwegian Veterinary Institute, Section of Bacteriology
Poland	Pulawy	National Veterinary Research Institute (NVRI), Department of Microbiology
Portugal	Lisbon	Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Unidade de Produção e Saúde Animal Laboratorio de Bacteriologia e Mycologica
Romania	Bucharest	Institute for Diagnosis and Animal Health, Bacteriology
Serbia	Belgrade	Institute of Veterinary Medicine of Serbia
Slovak Republic	Bratislava	State Veterinary and Food Institute, Reference Laboratory for *Salmonella*
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Switzerland	Bern	National Centre for Zoonoses, Bacterial Animal Diseases and Antimicrobial Resistance (ZOBA), Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Berne
Turkey	Ankara	Etlik Veterinary Control Central Research Institute, Bacteriological Diagnosis Laboratory
United Kingdom	Addlestone	Animal Plant Health Agency (ApHA), Bacteriology Department
United Kingdom	Belfast	Agri-Food Biosciences Institute Northern Ireland (AFBINI), Veterinary Sciences Division

3 Materials and methods

3.1 Preparation of artificially contaminated pig faeces samples

3.1.1 Matrix selection

Due to an outbreak of avian influenza in the Netherlands in autumn 2014, the EURL had to select pig faeces as the matrix for this EURL interlaboratory comparison study on the detection of *Salmonella* in samples from the primary production stage (PPS).

3.1.2 Pre-tests for the preparation of pig faeces samples

The matrix in this interlaboratory comparison study was pig faeces obtained from a *Salmonella*-free farm (Van Beek SPF Varkens BV, Lelystad, The Netherlands). Each batch was tested for the presence of *Salmonella* prior to preparation of the test samples (Anonymous, 2007). Pre-studies showed that pig faeces were susceptible to growth of yeast and moulds during storage at 5 °C and 10 °C. Therefore, storage at -20 °C was tested as an alternative. Samples of 25 g of pig faeces were artificially contaminated with monophasic *Salmonella* Typhimurium of pig origin (H82-1A) at a concentration of 17 and 70 cfu/25 g. The samples were stored at -20 °C. The stability of the samples after long-term storage at -20 °C (5 weeks) was tested as well as the effect of freezing and thawing such as may occur during transport of the samples. For the latter case, the effects of the following were tested:

- storage of the samples at -20 °C for 4 weeks, followed by storage at 5 °C for 6 days;
- storage of the samples at -20 °C for 4 weeks followed by storage at 5 °C for 3 days and storage at -20 °C for 3 days.

3.1.3 Preparation of the pig faeces samples

A large batch (30 kg) of pig faeces arrived at the EURL-*Salmonella* laboratory on Monday 9 February 2015. Ten samples, each of 25 g, were tested for the presence of *Salmonella* according to Annex D of ISO 6579 (Anonymous, 2007) with negative results. The pig faeces were repacked in portions of 25 g in sterile Whirl-Pak plastic bags and directly artificially contaminated with monophasic *Salmonella* Typhimurium (H82-1A, pig origin) by adding 0.1 ml of a diluted overnight culture. Three concentration levels were used; blank, low and high. The concentration of the inoculum used to contaminate the pig faeces samples was confirmed by testing on XLD agar plates. Directly after artificial contamination, the samples were stored at -20 °C until transport to the participating laboratories on Monday 9 March.

To determine the level of contamination in the final pig faeces samples, a five-tube most probable number (MPN) test was performed. The presence of *Salmonella* was determined in each dilution by following Annex D of ISO 6579 (Anonymous, 2007). From the number of confirmed positive dilutions, the MPN of *Salmonella* in the original sample was calculated using an MPN calculation program in Excel (Jarvis et al., 2010).

3.1.4 *Determination of background flora*

To obtain an indication of the amount of background flora in the samples, the number of aerobic bacteria and *Enterobacteriaceae* were determined in the blank pig faeces samples using ISO 4833 (Anonymous, 2003) and ISO 21528-2 (Anonymous, 2004), respectively. A sample of 20 g of pig faeces was homogenised in peptone saline solution. Serial dilutions were analysed on PCA (Plate Count Agar) and VRBG (Violet, Red Bile Glucose Agar) to obtain the total number of aerobic bacteria and *Enterobacteriaceae* present in the samples.

3.2 Design of the interlaboratory comparison study

Each participant received 18 artificially contaminated pig faeces samples numbered from B1 to B18. In addition, the laboratories had to test three control samples (C1–C3). Table 1 gives an overview of the number and type of samples tested by the participants.

For the control samples, the laboratories were asked to use their own positive *Salmonella* control (C3) which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this positive control, controls of the BPW (C2) and of the matrix (C1) had to be analysed. The protocol, SOP and test report used during the study can be found on the EURL-*Salmonella* website or obtained from the author of this report (EURL-Salmonella 2015a, 2015b and 2015c).

Table 1. Overview of the number and type of samples tested per laboratory in the interlaboratory comparison study

Contamination level	Test samples pig faeces (n=18)
STM mono low-level in pig faeces	6
STM mono high-level in pig faeces	6
Blank (BL) pig faeces	6
	Control samples (n=3)
Uncontaminated pig faeces (C1)	1
BPW only (C2)	1
Own control with *Salmonella* (C3)	1

3.2.1 *Sample packaging and temperature recording during shipment*

Each NRL received 21 coded Whirl-Pak plastic bags containing the artificially contaminated pig faeces samples, the blank samples and the control samples. The 21 bags were packed in one safety bag. Each safety bag was placed in one large shipping box, together with two frozen cooling devices. The shipping boxes were sent to the participants as ‘biological substances category B (UN3373)’ using a door-to-door courier service. Laboratories were asked to store the samples at -20 °C until the start of the analyses. To monitor exposure to abusive temperatures during shipment and/or storage, micro temperature loggers were used to record the temperature during transport.

3.3 Methods

The method prescribed for this interlaboratory comparison study was Annex D of ISO 6579 (Anonymous, 2007), which consists of a pre-enrichment in Buffered Peptone Water (BPW) and selective enrichment on Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar, followed by plating-out on Xylose Lysine Deoxycholate agar (XLD) and a second medium of choice. Confirmation is performed using the appropriate biochemical tests (ISO 6579; Anonymous, 2002) or using reliable, commercially available identification kits and/or serological tests. In addition, the NRLs are free to use their own method, such as a Polymerase Chain Reaction (PCR) procedure.

3.4 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated according to the following formulae:

Specificity rate:

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

Sensitivity rate:

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

Accuracy rate:

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

4 Results

4.1 Artificial contamination of pig faeces samples

4.1.1 *Pre-tests prior to preparation of pig faeces samples*

Storage of the artificially contaminated pig faeces at 5 or 10 °C resulted in growth of fungi at the surface of the faeces. Therefore, it was necessary to store the samples at -20 °C. In Table 2, the effect of storage at -20 °C on the survival of *Salmonella* is shown. After five weeks of storage at -20 °C, only one of the six high-level contaminated samples and none of the low-level contaminated samples was found to be positive. When the transport of the samples was mimicked by storing the samples at 5 °C for six days, five of the six high-level contaminated samples and two of the six low-level contaminated samples were found to be positive. When testing subsequent storage at -20 °C as would have been the case upon receipt at the participating laboratories, all six high-level contaminated samples were found positive for *Salmonella*. Of the low-level contaminated samples, three out of six were found positive.

Table 2. Number of artificially contaminated pig faeces samples tested positive for Salmonella *out of the total number of samples, after storage under different conditions*

Storage conditions	Pig faeces – low contamination (17 cfu / 25 g)	Pig faeces – high contamination (70 cfu / 25 g)
-20 °C (5 weeks)	0/6	1/6
-20°C (4 weeks) plus 5 °C (6 days)	2/6	5/6
-20 °C (4 weeks) plus 5 °C (3 days) plus -20 °C (3 days)	3/6	6/6

4.1.2 *Contamination level of the artificially contaminated pig faeces samples*

Immediately after arrival of 30 kg of pig faeces at the laboratory of the EURL, the batch was checked for the presence of *Salmonella* and tested negative. In addition, the presence of background flora was determined by analysing the number of aerobic bacteria and *Enterobacteriaceae* per gram. The results are presented in Table 3. The total aerobic count was 1.5×10^9 cfu/g and the number of *Enterobacteriaceae* was 4.7×10^6 cfu/g in the fresh pig faeces. Storage at -20 °C caused a decrease in background flora of about 2–3 log units.

Table 3. Number of aerobic bacteria and Enterobacteriaceae *per gram pig faeces*

Date of testing	Aerobic bacteria cfu / g	*Enterobacteriaceae* cfu / g
9 Feb 2015	1.5×10^9	4.7×10^6
16 March 2015, after storage at -20 °C	1.2×10^7	1.3×10^3

On 10 February 2015, the batch of pig faeces was divided into 25 g samples, packed in Whirl-Pak plastic bags and artificially contaminated before storage at -20 °C. As freezing of the samples affected the contamination level of *Salmonella*, the initial contamination level of the samples was increased compared to the pre-tests.
The low-level contaminated pig faeces samples were inoculated with 84 cfu/25 g, the high-level contaminated samples with 530 cfu/25 g. The samples were stored at -20 °C for four weeks until transport to the participants.
Samples were sent to the NRLs on Monday 9 March 2015. The NRLs were asked to store the samples upon arrival at -20 °C.

Unfortunately, in this study *Salmonella* proved to be extremely sensitive to freezing. The MPN results showed that in the low-level faeces samples, no *Salmonella* could be detected, while in the high-level samples only 0.7 and 0.2 cfu/25 g could be found (Table 4). In spite of the results of the pre-tests, *Salmonella* was not stable in the pig faeces after storage at -20 °C and subsequent transport conditions. This was confirmed by the results obtained by the participants and will be discussed later in this report.

Table 4. Monophasic Salmonella *Typhimurium (mono STM) concentration in inoculum culture and in test samples of inoculated pig faeces under different storage conditions.*

Date of testing	Low-level mono STM cfu / 25 g pig faeces	High-level mono STM cfu / 25 g pig faeces
12 February 2015 (inoculum-level diluted culture)	84	530
16 March 2015 (after 1 day at 5 °C and 6 days at -20 °C) MPN of inoculated pig faeces (95 % confidence limit)	0 (0–0.7)	0.7 (0.2–2.2)
16 March 2015 (after 4 days at 5 °C and 3 days at -20 °C) MPN of inoculated pig faeces (95 % confidence limit)	0 (0–0.7)	0.2 (0.03–1.4)

4.2 Technical data: interlaboratory comparison study

4.2.1 General

Thirty-six NRLs-*Salmonella* participated in this study: 29 NRLs from the 28 EU-MS and 6 NRLs from other countries within Europe (EU candidate MS or potential EU candidate MS and members of the European Free Trade Association (EFTA)) and, at the request of DG-Santé, 1 NRL from a non-European country.

4.2.2 Accreditation/certification

Thirty-four laboratories are accredited for their quality system according to ISO/IEC 17025 (Anonymous, 2005); two EU-MS laboratories (lab codes 22 and 28) were in the process of accreditation in 2015.

4.2.3 Transport of samples

Twenty-six participants received the samples within one day of dispatch, eight participants within two days; one participant received the parcel after three days and one participant after four days of transport. For all parcels, the temperature did not exceed 4 °C during transport to the NRLs. Most NRLs stored the samples at -20 °C upon arrival at the laboratory. Temperatures of the samples during storage at the laboratories ranged from -34 °C to -14 °C. One laboratory (lab code 34) overlooked this request and stored the samples at 4 °C for three days until this mistake was discovered and samples were placed at -20 °C. The temperature of this package reached a maximum temperature of -1 °C. For one laboratory (lab code 2), the temperature profile of the samples during transport and storage at the laboratory was not available because of a defective temperature recorder.

4.2.4 Media

Each laboratory was asked to test the samples using the prescribed method (Annex D of ISO 6579; Anonymous, 2007). All laboratories used the selective enrichment medium MSRV, the plating-out medium XLD and a second plating-out medium of their own choice.

Table 5 provides information on the pH, the concentration of Novobiocin, the incubation time and temperature that are prescribed for BPW and MSRV. The table lists only the deviations from the prescribed method that were reported.
One laboratory (lab code 5) reported a larger incubation time for the pre-enrichment in BPW. Three laboratories (lab code 6, 14 and 15) reported a higher pH than the prescribed maximum pH of 7.2 for BPW, and one laboratory reported a lower pH (lab code 5).
Six laboratories (lab code 11, 12, 16, 17, 20 and 37) used MSRV with a higher or lower concentration of Novobiocin than the prescribed 10 mg/L. Four laboratories (lab code 2, 6, 18, and 24) reported a higher pH for the MSRV than the prescribed maximum pH of 5.4; one laboratory (lab code 1) reported a lower pH. Laboratories 10 and 33 did not supply the requested information on the media used.

The medium for the second plating-out was a free choice. Most laboratories used BGA (Anonymous, 1993) or a Chromogenic medium as the second plating-out medium (Table 6).
The use of an extra non-selective plating agar between the ‘isolation’ and ‘confirmation’ steps was optional. A total of 24 laboratories performed this extra step (e.g. by using Nutrient agar ISO 6579; Anonymous, 2002).

Table 5. Reported technical deviations from the prescribed procedure

Lab code	BPW		MSRV		
	Incubation time (h)	pH	pH	Novobiocin	Incubation temperature (min–max ^{0}C)
Prescribed in ISO 6579 Annex D	**16 – 20 h**	**6.8 – 7.2**	**5.1 – 5.4**	**10 mg/L**	**40.5 – 42.5**
1			5.0		
2			5.5		
5	24	6.2			
6		7.3	5.5		
10		-	-		
11				0.05	
12				20	
14		7.3			
15		7.3			
16				1	
17				20	
18			5.5		
20				25	
24			5.6		
33		-	-		
37				5	

- No information

Table 6. Media used for second plating-out

Medium	Number of users	Lab code
ASAP (BioMerieux)	1	4
BGA^{mod} (ISO 6579, 1993)	5	21, 28, 29, 32, 34
BGA	9	2, 10, 13, 14, 18, 20, 26, 30, 37
BPLS (Merck, Biolife)	4	9, 11, 12, 17
BSA (Oxoid)	2	1, 15
BxLH (Home-made)	1	3
MAC (Oxoid)	1	5
Rambach (Merck)	7	7, 8, 16, 19, 24, 31, 36
RS (Bio-rad)	4	6, 23, 33, 35
SM(ID)2 (Biomerieux)	2	22, 27

Explanations of the abbreviations are given in the ‘List of abbreviations’.

4.3 Control samples

4.3.1 General

Table 7 gives the results for the control samples. The results given in the table are the highest number of positive isolations found with MSRV in combination with any isolation medium (MSRV/x). There was no difference between the scores of the different isolation media used: XLD or an alternative medium.

Table 7. Summary of results of the laboratories for the control samples

	Number of labs with compliant results (MSRV in combination with two isolation media)		
	compliant	non-compliant	lab code
Positive control own *Salmonella* n=1	36	0	
Procedure control BPW n=1	36	0	
Matrix control n=1	35	1	2

Positive control with Salmonella

All laboratories scored good results with their own *Salmonella* positive control sample and detected *Salmonella* with all used media.

For the positive control, the majority of participants used a diluted culture of *Salmonella* (24 laboratories). Others used a lenticule disc (6), a freeze-dried ampoule (2) or a capsule (2) with *Salmonella*. The *Salmonella* serovars most often used were *Salmonella* Enteritidis (14) and *Salmonella* Typhimurium (11). *Salmonella* Nottingham and *S*. Alachua were used by three and two laboratories, respectively. *S*. Goldcoast, *S*. Infantis, *S*. Tenessee, S. Dublin, *S*. Bongori, *S*. Abony and *S*. Abaetetuba were each used by one laboratory. The concentration of *Salmonella* in the positive control samples used by the different participants varied between 8 and 10^6 cfu/sample.

Table 8. Specificity, sensitivity and accuracy rates of the control samples

Control samples		MSRV / X	
		All labs n=36	EU-NRL labs n=29
Procedure control blank (BPW) n=1	No. of samples	36	29
	No. of negative samples	36	29
	Correct score in %	**100**	**100**
Matrix control blank (pig faeces) n=1	No. of samples	36	29
	No. of negative samples	35	29
	Correct score in %	**97**	**100**
Positive control (own *Salmonella*) n=1	No. of samples	36	29
	No. of positive samples	36	29
	Correct score in %	**100**	**100**
All control samples	No. of samples	108	87
	No. of correct samples	107	87
	Accuracy in %	**99**	**100**

X = isolation medium (XLD or non-XLD) that gave the highest number of positives.

Procedure control blank (only BPW)
All laboratories correctly analysed the procedure control sample (no matrix, only BPW) correctly as negative for Salmonella.

Matrix control blank (pig faeces)
All but one laboratory (lab code 2) correctly analysed the matrix control sample (25 g of pig faeces) as negative for Salmonella.

4.3.2 Specificity, sensitivity and accuracy rates of the control samples
Table 8 shows the specificity, sensitivity and accuracy rates for the control samples with selective enrichment on MSRV in combination with the isolation medium that gave the highest number of positive samples for *Salmonella* (MSRV/x). The laboratories scored an excellent result for the control samples with an accuracy rate of 99 % for MSRV/x.

4.4 Results for pig faeces samples artificially contaminated with *Salmonella*

4.4.1 Results per level of Salmonella *and per laboratory*
General
Although in the pre-test good results were found with frozen pig faeces, the MPN test at the EURL laboratory with the ring trial samples revealed almost no survival of *Salmonella* after storage of the contaminated pig faeces at -20 °C (Table 4). These results were confirmed by the results of the participating laboratories, which showed wide variation between the artificially contaminated samples.

Blank pig faeces samples
All but two laboratories (lab code 2 and 35) correctly scored all six blank pig faeces samples negative for *Salmonella* with all used media. These two laboratories scored respectively three and two blank samples as positive.

High-level contaminated monophasic Salmonella *Typhimurium samples*
Five laboratories (lab code 3, 7, 8, 18 and 27) detected *Salmonella* in all six high-level contaminated pig faeces samples with the prescribed method (MSRV). Five laboratories (lab code 9, 20, 28, 32 and 36) detected *Salmonella* in five of the six high-level samples, and seven laboratories (lab code 2, 10, 11, 15, 16, 21 and 30) detected *Salmonella* in four of the six high-level samples. Ten laboratories (lab code 6, 12, 17, 19, 22, 24, 26, 29, 31 and 37) were able to detect *Salmonella* in three of the six high-level samples. Four laboratories (lab code 1, 4, 14 and 23) found only two of the six high-level samples positive for *Salmonella.* And five laboratories (lab code 5, 13, 33, 34 and 35) could detect *Salmonella* in only one of the six high-level samples.

Low-level contaminated monophasic Salmonella *Typhimurium samples*
No laboratories were able to detect *Salmonella* in all six low-level contaminated pig faeces samples with the prescribed method (MSRV). One laboratory (lab code 2) detected *Salmonella* in five of the six low-level contaminated samples. One laboratory (lab code 7) detected *Salmonella* in four of the six low-level contaminated samples. And two laboratories (lab code 27 and 32) were able to detect *Salmonella* in three

of the six low-level contaminated samples. There were ten laboratories (lab code 4, 6, 10, 12, 14, 17, 21, 22, 26 and 33) that detected *Salmonella* in only two of the six low-level contaminated samples and a further ten laboratories (lab code 3, 8, 11, 16, 19, 28, 29, 31, 34 and 36) that were able to detect *Salmonella* in only one of the six low-level contaminated samples. Twelve laboratories (lab code 1, 5, 9, 13, 15, 18, 20, 23, 24, 30, 35 and 37) scored all six low-level contaminated samples negative for *Salmonella*.

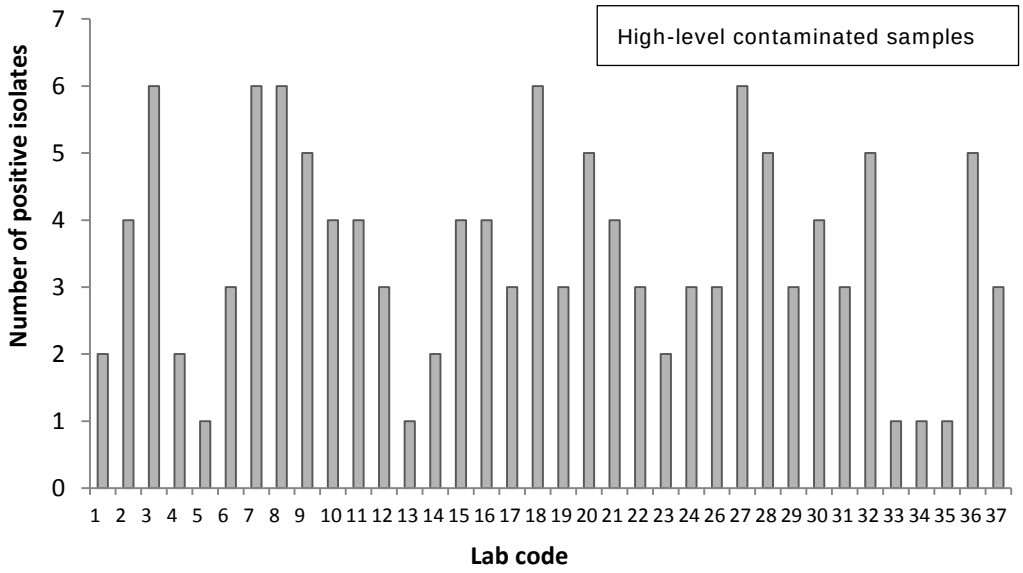

Figure 1. Number of positive isolations per laboratory for 25 g pig faeces samples artificially contaminated with high-level monophasic S. Typhimurium (n=6). The best results (highest number of positive samples) of all used isolation media after selective enrichment (MSRV) were taken into account (MSRV/x).

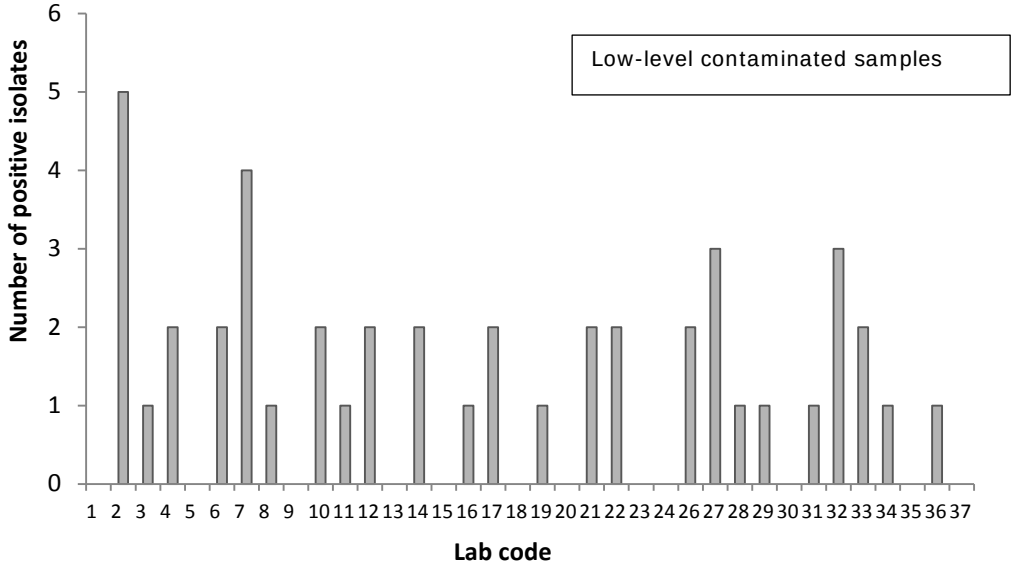

Figure 2. Number of positive isolations per laboratory for 25 g pig faeces samples artificially contaminated with low-level monophasic S. Typhimurium (n=6). The best results (highest number of positive samples) of all used isolation media after selective enrichment MSRV were taken into account (MSRV/x).

Figures 1 and 2 show the large variation between laboratories in the number of samples tested as positive. This large variation in results together with the low MPN values of the pig faeces samples indicate that *Salmonella* was not stable in these samples during storage at -20 °C and during transportation.

4.4.2 *Specificity, sensitivity and accuracy rates of the artificially contaminated samples*

The instability of the samples is confirmed by the calculations shown in Table 9, which shows the specificity, sensitivity and accuracy rates for the artificially contaminated pig faeces samples after selective enrichment on MSRV in combination with the isolation medium that gave the highest number of positive results for *Salmonella* (MSRV/x). The calculations were performed using the results of all participants and the results of EU-MS laboratories only, but differences between the two groups of laboratories were very small. The specificity rate calculated using the blank samples was very high for this study. However, sensitivity and accuracy were found to be very low in both groups.

Because of unstable *Salmonella* concentration in the samples it is not possible to evaluate the performance of the laboratories in this study.

Table 9. Specificity, sensitivity and accuracy rates of the artificially contaminated pig faeces samples after selective enrichment on MSRV

Pig faeces samples		MSRV/X All participants n=36	MSRV/X EU-MS n=29
Blank n=6	No. of samples	216	174
	No. of negative samples	211	169
	Specificity in %	**98**	**97**
Low-level mono-STM n=6	No. of samples	216	174
	No. of positive samples	45	37
	Sensitivity in %	**21**	**21**
High-level mono-STM n=6	No. of samples	216	174
	No. of positive samples	126	104
	Sensitivity in %	**58**	**60**
All faeces samples with mono-STM	No. of samples	432	348
	No. of positive samples	171	141
	Sensitivity in %	**40**	**41**
All faeces samples (pos. and neg.)	No. of samples	648	522
	No. of correct samples	382	310
	Accuracy in %	**59**	**59**

X = Isolation medium (XLD or non-XLD), which gave the highest number of positives.

5 Discussion

Preparation of the pig faeces samples
In the light of successful experiences in earlier studies with artificial contamination of matrices with a diluted *Salmonella* culture (Detection of *Salmonella* in boot socks (Kuijpers and Mooijman, 2014a), in minced chicken meat (Kuijpers et al., 2014) and in chicken faeces (Kuijpers and Mooijman, 2014b)), the matrix samples in this study were also artificially contaminated at the laboratory of the EURL. As each matrix and *Salmonella* serovar combination may behave differently, the samples were tested prior to the study for their stability during storage and transport temperatures. Pre-tests showed growth of moulds and yeast in the pig faeces samples during storage at 5 °C. To inhibit the growth of these organisms, the pig faeces had to be stored at -20°C. *Salmonella* was susceptible to freezing but it was expected that this could be overcome by contaminating the pig faeces samples with a higher starting/inoculation concentration than in previous studies so that enough *Salmonella* would survive to ensure reliable test results. The samples were therefore inoculated with 84 and 530 cfu/25 g, respectively, compared with typical initial inoculation levels in earlier studies of 15 and 50 cfu/25 g.

Despite the precautions and the satisfactory pre-test results, *Salmonella* proved to be unexpectedly sensitive to the thawing and freezing cycle during transport and storage. The temperature during transport in the tempex box, including cooling elements, slowly approached 0 °C. This in combination with the subsequent storage at -20 °C upon arrival at the NRLs caused *Salmonella* survival to decrease dramatically. Only 21% of the low-level samples and 58% of the high-level samples were found positive for *Salmonella*, whereas for the latter samples 100% detection was expected. Results also varied greatly amongst the laboratories. For that reason, the EURL was not able to evaluate the results and the performance of the participants.

Accreditation
According to EC regulations Nos. 882/2004 (EC, 2004) and 2076/2005 (EC, 2005), each NRL should have been accredited in its relevant field before 31 December 2009. Thirty-two laboratories were accredited. Two (EU-MS) participants (lab codes 22 and 28) were still in the process of accreditation, which is quite late.

Positive control samples
The participants were asked to use the positive control strain(s) they routinely use in their laboratories. *S*. Enteritidis and *S*. Typhimurium were the most frequently used serovars and the concentration varied between 8 and 10^6 cfu/sample. A positive control sample should demonstrate that media are capable of supporting growth of low numbers of a range of organisms. Ideally, the concentration of the positive control should be just above the detection limit to test the sensitivity of the method. However, the majority of the participants used a much higher concentration. Furthermore, it may be advisable to use a

serovar rarely isolated from the routine samples analysed in the laboratory. This would help to detect cross-contamination.

Evaluation of this study
Unfortunately, *Salmonella* was less stable in the interlaboratory study samples than expected from the results of the pre-tests. It is very difficult to explain the observed differences. Perhaps the composition of the two batches of pig faeces was different as a result of differences in pig feed ingredients. It is known from the literature that certain ingredients, including acid additives and antimicrobials, are used in the pig feed industry to inactivate pathogens or regulate gut conditions, thereby inhibiting or reducing *Salmonella* numbers (Sharan et al., 2013; Faundez et al., 2004; Ibrahim et al., 2008; Sanhueza et al., 2013; Wells et al., 2010). In addition, the successive freezing, thawing and re-freezing cycles during storage and transport could have contributed to the low *Salmonella* survival in the pig faeces samples.

6 Conclusions

- Because of the instability of the samples it is not possible to evaluate the performance of the laboratories.
- The majority of the participating laboratories used a high contamination level for the positive control, which may give little information on the sensitivity of the method.
- The majority of the NRLs-*Salmonella* still use *S*. Enteritidis or *S*. Typhimurium for their positive control samples. However, the use of a more rarely found *Salmonella* serovar may facilitate the detection of cross-contamination.

List of abbreviations

ASAP	AES *Salmonella* agar plate
BGA	Brilliant Green Agar
BGA (mod)	Brilliant Green Agar (modified)
BPLS	Brilliant Green Phenol-red Lactose Sucrose
BPW	Buffered Peptone Water
BSA	Brilliance *Salmonella* Agar (OSCM)
BxLH	Brilliant green, Xylose, Lysine, Sulphonamide
CEN	Comité Européen de Normalisation (European Committee for Standardization)
cfu	Colony-forming units
CRL	Community Reference Laboratory
DG-Santé	Directorate-General for Health and Food Safety
EC	European Commission
EFTA	European Free Trade Association
EU	European Union
EURL	European Union Reference Laboratory
ISO	International Organization for Standardization
MAC	MacConkey Agar
Mono-STM	Monophasic *Salmonella* Typhimurium
MPN	most probable number
MS	Member State
MSRV	Modified Semi-solid Rappaport-Vassiliadis
NRL	National Reference Laboratory
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PPS	primary production stage
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
RS	Rapid *Salmonella*
SM(ID)2	*Salmonella* Detection and Identification-2
SPF	Specific Pathogen Free
SOP	Standard Operating Procedure
VRBG	Violet Red Bile Glucose agar
XLD	Xylose Lysine Deoxycholate Agar

References

Anonymous (1993). ISO 6579 (E). Microbiology – General guidance on methods for the detection of *Salmonella*. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2002). ISO 6579 (E). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2003). ISO 4833. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30 degrees C. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2004). ISO 21528-2. Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count method. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2005). ISO/IEC 17025. General requirements for the competence of testing and calibration laboratories. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2007). Amendment of ISO 6579:2002/Amd1 Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. International Organization for Standardization, Geneva, Switzerland.

Anonymous (2010). ISO 22117. Microbiology of food and animal feeding stuffs – Specific requirements and guidance for Proficiency Testing (PT) by interlaboratory comparison. International Organization for Standardization, Geneva, Switzerland.

EC (2004). Commission Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Official Journal of the European Union L 165 of 30 April 2004. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2004R0882:20060525:EN:PDF (29 September, 2015)

EC (2005). Commission Regulation (EC) No. 2076/2005 of the European Parliament and of the Council of 5 December 2005 laying down transitional arrangements for the implementation of Regulations (EC) No. 853/2004, (EC) No. 854/2004 and (EC) No. 882/2004 of the European Parliament and of the Council and amending Regulations (EC) No. 853/2004 and (EC) No. 854/2004. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0083:0088:EN:PDF (29 September, 2015)

EURL-*Salmonella* (2015a). Protocol Interlaboratory Comparison Study on the detection of *Salmonella* spp. in samples from the primary production stage organised by EURL-*Salmonella* STUDY XVIII – 2015. http://www.eurlsalmonella.eu/dsresource?type=pdf&disposition=inline&objectid=rivmp:273617&versionid=&subobjectname= (13 November, 2015)

EURL-*Salmonella* (2015b). Standard operating procedure (SOP) Interlaboratory
Comparison Study on the detection of *Salmonella* spp. in samples from the primary production stage organised by EURL-*Salmonella* STUDY XVIII – 2015.
http://www.eurlsalmonella.eu/dsresource?type=pdf&disposition=inline&objectid=rivmp:273618&versionid=&subobjectname=
(13 November, 2015)

EURL-*Salmonella* (2015c). Test report Interlaboratory Comparison Study on the detection of *Salmonella* spp. in samples from the primary production organised by EURL-*Salmonella* STUDY XVIII – 2015.
http://www.eurlsalmonella.eu/dsresource?type=pdf&disposition=inline&objectid=rivmp:273619&versionid=&subobjectname=
(13 November 2015)

Faundez, G., Troncoso, M., Navarrete, P. and Figueroa, G. (2004). Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni*. BMC microbiology, 4:19.

Ibrahim, S.A., Yang, H. and Seo, C.W. (2008). Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice. Food Chemistry 109: 137-143.

Jarvis, B., Wilrich, C. and Wilrich, P.-T. (2010) Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. J. Appl. Microbiol. 109: 1660–1667.
Link to MPN calculation programme ISO 7216:
http://standards.iso.org/iso/7218/ (13 November 2015)

Kuijpers, A.F.A. and Mooijman, K.A. (2014a). EU Interlaboratory comparison study primary production XVI (2013); Detection of *Salmonella* in chicken faeces adhering to boot socks. RIVM report 330604031, Bilthoven, the Netherlands.
http://www.rivm.nl/bibliotheek/rapporten/330604031.pdf
(13 November, 2015)

Kuijpers, A.F.A. and Mooijman, K.A. (2014b). EU Interlaboratory comparison study primary production XVII (2014); Detection of Salmonella in chicken faeces. RIVM report 2014-0011, Bilthoven, The Netherlands.
http://www.rivm.nl/dsresource?objectid=rivmp:289065&type=org&disposition=inline&ns_nc=1 (13 November 2015)

Kuijpers, A.F.A., Kassteele, van de J. and Mooijman, K.A. (2014). EU Interlaboratory comparison study food VI (2013); Detection of *Salmonella* in minced chicken meat. RIVM report 2014-0010, Bilthoven, the Netherlands.
http://www.rivm.nl/bibliotheek/rapporten/2014-0010.pdf
(13 November 2015)

Sanhueza, S.M., Alcántara, R. and Sánchez, G. (2013). Antimicrobial evaluation of copper sulphate (II) on strains of *Enterococcus faecalis*. In vitro study. Journal of Oral Research 2(3): 114–118.

Sharan, R., Chibber, S. and Reed, R.H. (2011) A murine model to study the antibacterial effect of copper on infectivity of *Salmonella Enterica* serovar Typhimurium. International Journal of Environmental Research and Public Health 8: 21–36.

Wells, J.E., Oliver, W.T. and Yen J.T. (2010). The effects of dietary additives on faecal levels of *Lactobacillus* spp., coliforms, and *Escherichia coli*, and faecal prevalence of *Salmonella* spp. and *Campylobacter* spp. in US production of nursery swine. Journal of Applied Microbiology 108(1): 306–314.

RIVM
Committed to *health and sustainability*