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

EURL-*Salmonella* Proficiency Test Primary Production, 2019

Detection of *Salmonella* in chicken faeces samples

RIVM report 2019-0137

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Colophon

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Synopsis

EURL-*Salmonella* Proficiency Test Primary Production, 2019
Detection of *Salmonella* in chicken faeces

The National Reference Laboratories (NRLs) of the European Union were able to detect *Salmonella* in chicken faeces in the yearly Proficiency Test. All laboratories were successful in finding *Salmonella* in high and low concentrations in the contaminated chicken faeces samples. All but one laboratory scored good results. This one laboratory mislabelled the negative and the positive control sample and scored a moderate performance.

This was the outcome of the Proficiency Test for detection of *Salmonella* in samples of the primary production stage organised by the coordinating EURL-*Salmonella* in October 2019.

Since 1992, participation is obligatory for all EU Member State National Reference Laboratories (NRLs) responsible for analysing *Salmonella* in animal production samples. In total, 35 NRLs participated in this study: 29 participants originated from 28 EU Member States (MS), five were based in third European countries, and one was based in a non-European country.

The EURL-*Salmonella* is located at the Dutch National Institute for Public Health and the Environment (RIVM). An important task of the EURL-*Salmonella* is to monitor and improve the performance of the National Reference Laboratories in Europe.

Keywords: *Salmonella*, EURL, NRL, Proficiency Test, *Salmonella*

Publiekssamenvatting

Het EURL-*Salmonella* ringonderzoek productiedieren (2019)

Detectie van *Salmonella* in kippenmest

De Nationale Referentie Laboratoria (NRL's) van de Europese lidstaten waren in 2019 in staat om *Salmonella* aan te tonen in kippenmest. Alle deelnemers konden hoge en lage concentraties van *Salmonella* aantonen. Op één na hebben alle laboratoria een goede score behaald. Dat ene laboratorium had de controlemonsters verwisseld en haalde daarom een matige score. Dit blijkt uit het ringonderzoek dat het overkoepelende EURL-*Salmonella* in oktober 2019 organiseerde.

Sinds 1992 zijn de NRL's van de Europese lidstaten verplicht om deel te nemen aan jaarlijkse kwaliteitstoetsen die bestaan uit zogeheten ringonderzoeken voor *Salmonella*. Elke lidstaat wijst voor de kwaliteitstoets een laboratorium aan, het Nationale Referentie Laboratorium. Deze laboratoria zijn er namens dat land voor verantwoordelijk *Salmonella* aan te tonen in de leefomgeving van dieren die voor de voedselproductie worden gehouden. In totaal hebben 35 NRL's aan dit ringonderzoek deelgenomen: 29 NRL's afkomstig uit alle 28 EU-lidstaten, vijf NRL's uit andere Europese landen en een NRL uit een niet-Europees land.

Het Europese Referentielaboratorium (EURL) *Salmonella* is gevestigd bij het Nederlandse Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Een belangrijke taak van het EURL-*Salmonella* is toezien op de kwaliteit van de nationale referentielaboratoria voor deze bacterie in Europa.

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

Contents

Summary

In October 2019, the EURL-*Salmonella* Proficiency Test on the detection of *Salmonella* in primary production stage samples was organised. A total of 35 National Reference Laboratories (NRLs) participated in this study: 29 NRLs originating from 28 EU-Member States (MS), five from third European countries (EU candidate or potential EU candidate MS and members of the European Free Trade Association (EFTA)), and one from a non-European country. Participation was obligatory for all EU Member State NRLs responsible for the detection of *Salmonella* in primary production stage samples.

Chicken faeces from a pathogen free (SPF) farm was used in this study. The chicken faeces samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium at the EURL laboratory.

Each NRL received sixteen blindly coded samples consisting of ten chicken faeces samples artificially contaminated with two different levels of *Salmonella* Typhimurium: six low (MPN concentration: 13 cfu/sample), and four high contaminated samples (MPN concentration: 35 cfu/sample). Additionally, four negative chicken faeces samples (no *Salmonella* added) and two control samples had to be analysed. The control samples consisted of a procedure control blank and a control sample to be inoculated by the participants using their own positive control strain. The samples were stored at 5 °C until the day of transport. On Monday 23 September 2019, the contaminated chicken faeces samples were packed and sent to the NRLs. On arrival, the NRLs were asked to store the samples at 5 °C until the start of the analysis.

Method
Most laboratories used the prescribed method EN-ISO 6579-1:2017, one laboratory used EN-ISO 6579:20072/Amd.1:2007 (Annex D), and one laboratory used another method.

Results control samples
Almost all laboratories scored well, analysing both the procedure control as well as their own positive control sample correctly. One laboratory mislabelled the control samples. This laboratory scored a moderate performance

Results artificially contaminated chicken faeces samples
All laboratories detected *Salmonella* in the chicken faeces samples contaminated with a low level of *Salmonella*. One laboratory (lab code 22) found one of the six samples negative for *Salmonella*, another laboratory (lab code 23) found two of the six samples negative for *Salmonella*. These results are still within the criteria for good performance, which permit three negative samples.

Almost all laboratories detected *Salmonella* in all four high level samples. One laboratory (lab code 23) scored one of the four high-level samples negative. This is still within the criteria for good performance

which permit one negative sample. The sensitivity score was 98,6% for these samples.

All negative samples were scored correctly negative, resulting in a specificity of 100%.

Overall, the laboratories scored well in this Proficiency Test with an accuracy of 99,2%. Thirty-four laboratories fulfilled the criteria of good performance. The results of one laboratory were scored moderate due to a labelling error.

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) and its successor No 625/2017 (EC 2017), is the organisation of Proficiency Tests (PT) to evaluate the performance of the National Reference Laboratories (NRLs) for *Salmonella*. The history of the PTs organised by EURL-*Salmonella* from 1995 onwards is summarised on the EURL-*Salmonella* website (EURL-*Salmonella*, 2019).

In October 2019, the EURL-*Salmonella* organised a PT to evaluate whether the NRLs responsible for the detection of *Salmonella* in samples from the Primary Production stage (PPS) could detect *Salmonella* at different contamination levels in chicken faeces samples. The results from PTs like this show whether the examination of samples in the EU Member States (EU-MS) is carried out uniformly and whether comparable results can be obtained by all NRLs-*Salmonella*.

The method prescribed for the detection of *Salmonella* spp. is set out in EN-ISO 6579-1:2017.

The design of this study was comparable to previous PTs organised by *EURL-Salmonella* (Diddens & Mooijman, 2019; Pol-Hofstad & Mooijman, 2019). For the current study, chicken faeces was artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STm) at the EURL-*Salmonella* laboratory.

In total, fourteen chicken faeces samples had to be tested: four high contaminated chicken faeces samples, six low contaminated chicken faeces samples, and four negative chicken faeces samples (no *Salmonella* added). Additionally, two control samples had to be tested: one procedure control and one positive control. The number of samples as well as the contamination levels were based on information described in EN-ISO 22117:2019.

2 Participants

Country	City	Institute
Austria	Graz	Austrian Agency for Health and Food Safety (AGES IMED/VEMI)
Belgium	Brussels	Sciensano
Bosnia and Herzegovina	Sarajevo	Veterinary faculty Sarajevo, department Health care of Poultry
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVMI), National Reference Centre of Food Safety
Croatia	Zagreb	Croatian Veterinary Institute, Laboratory for General Bacteriology and Microbiology
Cyprus	Nicosia	Cyprus Veterinary Services Pathology, Bacteriology, Parasitology Laboratory
Czech Republic	Praha	State Veterinary Institute
Denmark	Ringsted	Danish Veterinary and Food administration
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Bacteriology-Pathology Department
Finland	Kuopio	Finnish Food Authority, Research and Laboratory Services Department
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) Biological Safety Department
Greece	Chalkida	Veterinary Laboratory of Chalkis
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate
Iceland	Reykjavik	Matís ohf, Analysis and Consulting
Israel	Kiryat Malachi	Southern Poultry Health Laboratory (Beer Tuvia)
Ireland, Republic of	Kildare	Central Veterinary Research Laboratory (CVRL/DAFFM) Laboratories Backweston, Department of Agriculture, Food and the Marine, Bacteriology
Italy	Padova Legnaro	Istituto Zooprofilattico Sperimentale delle Venezie, OIE

Country	City	Institute
Latvia	Riga	Institute of Food Safety, Animal Health and Environment BIOR Bacteriology and Parasitology Division
Lithuania	Vilnius	National Food and Veterinary Risk Assessment Institute, Laboratory of Microbiology and Pathology, Bacteriology Group
Luxembourg, Grand-Duchy of	Diddeléng	Laboratoire de Médicine Vétérinaire de l"Etat, Bacteriologie
Malta	Valletta	Malta 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 Zoonosis and Environmental Microbiology (Z&O)
Norway	Oslo	Norwegian Veterinary Institute, Section of Microbiology
Poland	Pulawy	National Veterinary Research Institute, department of microbiology
Portugal	Vairão	Instituto Nacional de Investigação Agrária e Veterinária , Food Microbiology Laboratory
Romania	Bucharest	Institute for Diagnosis and Animal Health
Serbia	Belgrade	NIVS-Scientific Veterinary Institute of Serbia
Slovak Republic	Bratislava	State Veterinary and Food Institute
Slovenia	Ljubljana	National Veterinary Institute, Veterinary Faculty (UL, NVI)
Spain	Madrid Algete	Laboratorio Central de Veterinaria
Sweden	Uppsala	National Veterinary Institute
Switzerland	Zurich	National reference Centre for Poultry and Rabbit Disease
United Kingdom	Addlestone	Animal and Plant Health Agency (APHA), Bacteriology Department
United Kingdom	Belfast	Agri-Food and Bioscience Institute (AFBI) Veterinary Sciences Division Bacteriology

3 Materials and methods

3.1 Preparation of artificially contaminated chicken faeces samples

3.1.1 General

The matrix used for this PT was chicken faeces from a broiler breeder flock. The chicken faeces samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium at the EURL-*Salmonella* laboratory.

3.1.2 Pre-tests for the preparation of chicken faeces samples

The batch of faeces was collected from a *Salmonella* free broiler breeder flock by the Animal Health Service (GD, Deventer). The batch of faeces (2 kg) for the pre-tests arrived at the EURL on 24 June 2019. Because of the hot weather, the chicken faeces contained small flies which were inactivated by storing the faeces at -20 °C for 1 day. The next day, five samples of 25 g of the defrosted chicken faeces were taken randomly from the batch and tested for the absence of *Salmonella* according to EN-ISO 6579-1:2017.
To test the stability of proficiency test samples during transport and storage, chicken faeces was artificially contaminated with *Salmonella* and stored at 5 °C and 10 °C for a period up to three weeks. Samples consisting of 25 g chicken faeces each were contaminated with two low concentrations (5 and 10 cfu) of a diluted culture of *Salmonella* Enteritidis (Salm 532 from *EURL-Salmonella*'s own collection).
Five samples for each concentration were tested for the presence of *Salmonella* after zero, one, two, and three weeks of storage at 5 °C and 10 °C. In addition, one non-contaminated chicken faeces sample was tested each week for the concentration of background flora according to EN-ISO 21528-2:2017 for the number of *Enterobacteriaceae*, and EN-ISO 4833-1:2013 for the total aerobic count. Because of low stability of *Salmonella* Enteritidis, the same test was repeated with *Salmonella* Typhimurium ATCC 14028 in concentrations of 5 and 9 cfu per sample. For this purpose, a fresh batch of chicken faeces (5 kg) was collected from the same broiler breeder flock on 12 August 2019. After storage at -20 °C for 1 day to inactivate the flies present, the faeces was tested for presence of *Salmonella* as described.

3.1.3 Preparation of chicken faeces samples for Proficiency Test

A large batch (20 kg) of chicken faeces from the same flock as the pre-tests arrived at the EURL-*Salmonella* laboratory on Tuesday 26 August 2019 and was stored at -20 °C for 1 day to inactivate the flies present. Ten samples of 25 g each were tested for the absence of *Salmonella* according to EN-ISO 6579-1:2017. After testing negative, 25 grams of chicken faeces was weighed into the coded sample bags and stored at -20 °C for 3 weeks. In the week of 16 September, the chicken faeces samples were defrosted and artificially contaminated with *Salmonella* Typhimurium by adding no more than 0.5 ml of the appropriate dilution of an overnight culture. Two concentration levels were used: low (5-10 cfu/sample) and high (50-100 cfu/sample). The concentration of the inoculum used to contaminate the chicken faeces samples was determined by streaking the inoculum on XLD agar plates. Immediately

after artificial contamination, the high, low, and negative samples were stored at 5 °C until transport to the participating laboratories on Monday 23 September 2019.

3.1.4 Determination of the level of background flora in chicken faeces

To obtain information on the level of background flora in the samples, the number of aerobic bacteria and the number of *Enterobacteriaceae* were determined in the chicken faeces samples using EN-ISO 4833-1:2013 and EN-ISO 21528-2:2017, respectively. Peptone saline solution (225ml) was added to each chicken faeces sample of 25g. After mixing by hand (kneading), serial dilutions were prepared in peptone saline and analysed on PCA (Plate Count Agar) and VRBG (Violet Red Bile Glucose Agar) to obtain the total number of aerobic bacteria and *Enterobacteriaceae*.

3.1.5 Determination of the number of Salmonella *in chicken faeces by MPN*

The level of contamination of *Salmonella* in the artificially contaminated chicken faeces samples was determined using a five-tube most probable number (MPN) technique. For this, ten-fold dilutions of five artificially contaminated chicken faeces samples at each contamination level were tested representing 25 g, 2,5 g, and 0,25 g of the original sample. The presence of *Salmonella* was determined in each dilution following EN-ISO 6579-1:2017. The MPN of *Salmonella* in the original sample was calculated from the number of confirmed positive dilutions, using freely available Excel-Based MPN software (Jarvis et al., 2010).

3.2 Design of the Proficiency Test

3.2.1 Number and type of samples

Each participant received fourteen artificially contaminated chicken faeces samples numbered B1 to B14. In addition, the laboratories had to test two control samples (C1 and C2). 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 strain which they normally use when analysing routine samples for the detection of *Salmonella*. In addition to this positive control (C2), a procedure control (C1) consisting only of Buffered Peptone Water (BPW), had to be analysed. The protocol and test report can be found in Annex I and II respectively.

3.2.2 Shipment of parcels and temperature recording during shipment

The sixteen coded samples containing the contaminated and the negative chicken faeces samples and the control samples were packed in two safety bags. These were placed in one large shipping box together with four frozen (-20 °C) cooling devices. The shipping boxes were sent to the participants as biological substances category B (UN3373) via a door-to-door courier service. The participants were asked to store the samples at 5 °C on receipt. To monitor exposure to abusive temperatures during shipment and storage, a micro temperature logger was placed in between the samples to record the temperature.

Table 1. Overview of the number and type of samples tested per laboratory in the Proficiency Test PPS 2019

Contamination level	**Chicken faeces (n=14)**
***S*. Typhimurium low level**	6
***S*. Typhimurium high level**	4
Negative (no *Salmonella* added)	4
	Control samples (n=2)
Blank procedure control (BPW only)	1
Positive control (own control with *Salmonella*)	1

3.3 Methods

The method prescribed for this PT was EN-ISO 6579-1:2017 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 was performed using the appropriate biochemical and serological tests as prescribed in EN-ISO 6579-1:2017 or using reliable, validated identification kits. In addition to the EN-ISO method, the NRLs were free to use their own method, such as a Polymerase Chain Reaction (PCR) procedure. Only the results obtained with the prescribed EN-ISO 6579-1:2017 were used to assess the performance of the participant.

3.4 Statistical analysis of the data

The specificity, sensitivity and accuracy rates were calculated for the artificially contaminated chicken faeces samples. For the control samples, only the accuracy rates were calculated. The rates were calculated with the following formulae:

Specificity rate:

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

Sensitivity rate:

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

Accuracy rate:

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

3.5 Criteria for good performance

For the determination of 'good performance', the criteria indicated in Table 2 were used.

Table 2. Criteria for testing good performance in the PT PPS 2019

Contamination level	% positive	# positive samples/ total # samples
Chicken faeces samples		
***S*. Typhimurium high-level**	Min. 80 %	Min. 3/4
***S*. Typhimurium low-level**	Min. 50 %	Min. 3/6
Negative (no *Salmonella* added)[1]	Max. 25 %[1]	Max. 1/4[1]
Control samples		
Procedure control (BPW only)	0 %	0 /1
Positive control with *Salmonella*	100 %	1 /1

1. All should be negative. However, as no 100% guarantee of the *Salmonella* negativity of the matrix can be given, 1 positive out of 4 negative samples (25% positive) is considered acceptable.

4 Results and discussion

4.1 Preparation of artificially contaminated chicken faeces samples

4.1.1 Pre-tests for the preparation of chicken faeces samples

The study set-up was based on the study-design used in 2017 by the *EURL-Salmonella* (Pol-Hofstad and Mooijman, 2017). To test if the chicken faeces samples were stable during transport and storage, the samples were contaminated with a high and a low concentration of *Salmonella* Enteritidis as described in 3.1.2.

The pre-test samples were stored at 5 °C to mimic storage conditions and at 10 °C to test the effect of temperature abuse during transport. The pre-test samples were stored for up to three weeks and analysed for presence of *Salmonella* using EN-ISO 6579:1-2017. Results are presented in Figure 1.

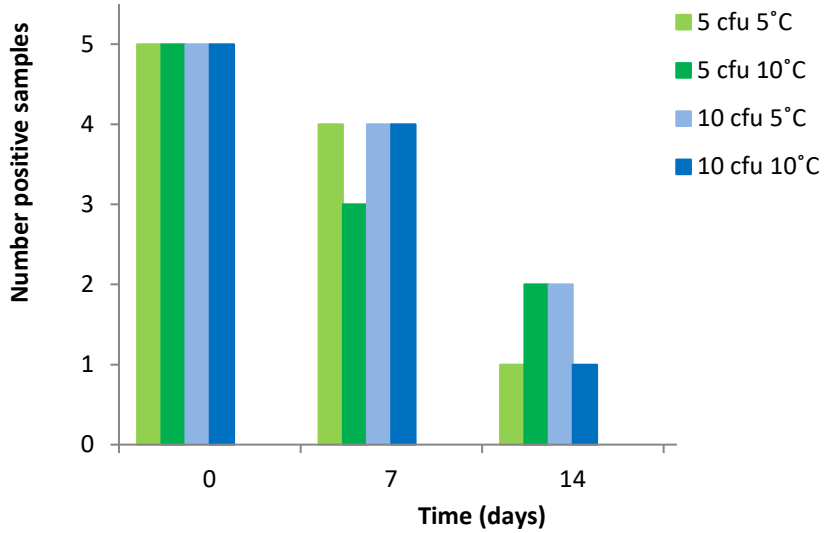

Figure 1. Stability tests of chicken faeces samples artificially contaminated with Salmonella *Enteritidis after storage for two weeks at 5 °C and 10 °C. Different colours indicate different concentrations of* Salmonella *Enteritidis.*

Figure 1 shows that the storage of the pre-test samples at 5 °C or 10 °C for two weeks had a relatively large effect on the survival of *Salmonella* Enteritidis. When low contamination levels were used (5,5 cfu), one to two of the five samples tested negative for *Salmonella* after 1 week of storage. After two weeks, almost all samples were negative. Therefore, a more stable strain of *Salmonella* was chosen as test organism. The pre-tests were repeated with *Salmonella* Typhimurium in two concentrations (5 and 9 cfu).

Results of the second pre-test using *Salmonella* Typhimurium are shown in Figure 2. *Salmonella* Typhimurium survived for a longer period in chicken faeces. After two weeks at 5 °C, all 5 samples were still positive for *Salmonella*. After three weeks of storage, the number of *Salmonella* positive samples at 5 °C decreased to 4 or 2 samples.

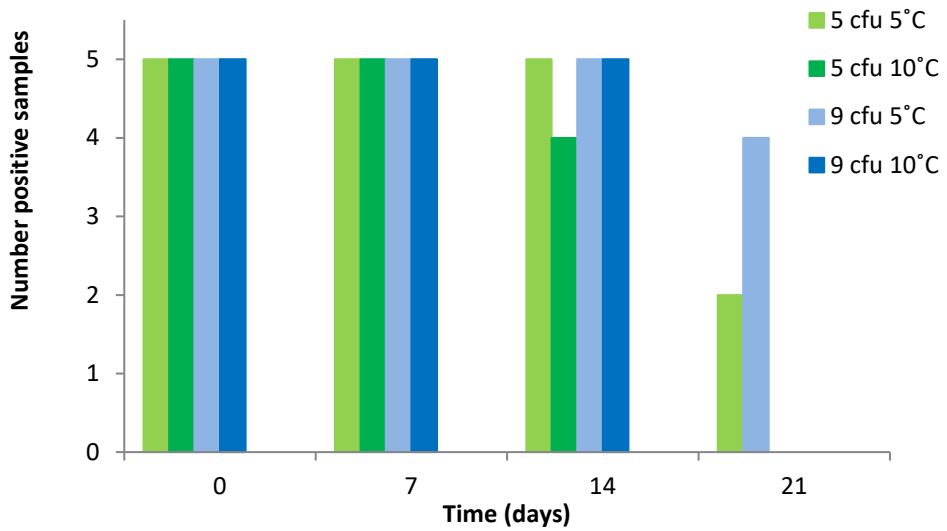

Figure 2. Stability tests of chicken faeces samples artificially contaminated with Salmonella *Typhimurium after storage for three weeks at 5 °C and two weeks at 10 °C. Different colours indicate different concentrations of* Salmonella *Typhimurium.*

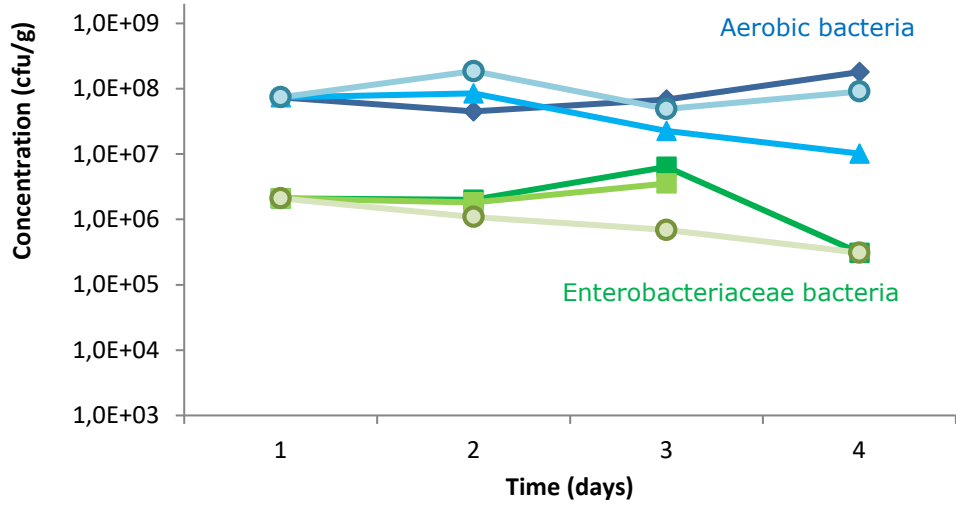

Figure 3. The effect of temperature and storage time on the number of aerobic bacteria and Enterobacteriaceae in chicken faeces samples in the second pre-test (dark colour = 5 °C, light colour = 10 °C, open circles = -20 °C).

The effect of storage and temperature on the background flora in the second pre-test is shown in Figure 3. Little difference can be seen in the number of aerobic bacteria when the samples are stored at 5 °C or 10 °C. The number of aerobic bacteria remained just above (5 °C) or below (10 °C) the initial level (10^8 cfu/g) for up to three weeks. Furthermore, the *Enterobacteriaceae* level remained around the starting level (10^6 cfu/g) at both 5 °C or 10 °C.

Because of the high environment temperatures during the hot summer months, the chicken faeces contained flies. To inactivate the flies, the faeces was stored at -20 °C for 1 day to three weeks. The effect of freezing on the background flora is shown in Figure 3 (open, round

symbols). For the aerobic count, no effect of freezing the samples was recorded. For *Enterobacteriaceae*, the number of cfu decreased by approximately 1 log unit after three weeks of storage.

4.1.2 Preparation of chicken faeces samples for the Proficiency Test

Samples for the PT were prepared as described in 3.1.3.

4.1.3 Background flora in the chicken faeces samples

The concentration of the background flora of the study samples was determined according to EN-ISO 21528-2:2017 and EN-ISO 4833-1:2013 as described in 3.1.4; results are shown in Table 3. The number of *Enterobacteriaceae* varied between $4{,}3x10^6$ cfu/g on the day of preparation (t = 0) to $1{,}1x10^6$ cfu/g after five weeks of storage (4 weeks at -20 °C and 1 week at 5 °C (t = 34 days)). The number of aerobic bacteria remained constant at approximately 10^8 cfu/g during the five weeks of storage.

Table 3. Number of aerobic bacteria and Enterobacteriaceae per gram of chicken faeces at t = 0 and t = 34 days (28 days at -20 °C and 7 days at 5 °C)

Date of testing	**t = 0 days (27 Aug 2019)**	**t = 34 days (30 Sept 2019)**
***Enterobacteriaceae* cfu/g**	$4{,}3x10^6$	$1{,}1x10^6$
Aerobic bacteria cfu/g	$5{,}3x10^8$	$7{,}4x10^8$

4.1.4 Number of Salmonella *in chicken faeces samples*

The chicken faeces samples were artificially contaminated at the EURL-*Salmonella* laboratory by adding the appropriate volume of a diluted *Salmonella* culture. Table 4 shows the contamination level of the diluted culture of *Salmonella* Typhimurium used as inoculum to contaminate the chicken faeces. The results show that the intended levels of approximately 10 cfu for the low-level samples and 50 cfu for the high-level samples were not reached; the low-level samples were inoculated with only 3 cfu. This inoculum was considered too low to ensure stable, *Salmonella* positive samples after storage and transport to the participants. Therefore, it was decided to increase the contamination of the low-level samples by an extra addition of two times the volume of the first inoculum. The concentration of the second inoculum appeared to be somewhat higher than anticipated (16 cfu instead of 6 cfu); the inoculation level of the high-level samples was considered sufficient.

Table 4. Number of Salmonella *Typhimurium (STm) in the inoculums and in the chicken faeces samples*

Date of testing	Low level STm (cfu/sample)	High level STm (cfu/sample)
18 Sept 2019 (first inoculum level diluted culture)	3	30
19 Sept 2019 (second inoculum level diluted culture)	16	n/a
30 Sept 2019 MPN contaminated chicken faeces (95 % confidence limit)	13 (4,5-37,5)	35 (11-110)

After inoculation, the samples were stored at 5 °C for almost two weeks until transport to the participants on 30 September 2019. The final contamination level of *Salmonella* in the chicken faeces was determined by performing a five-tube Most Probable Number (MPN) test in the week of the PT study (see Table 4).

4.2 Technical data Proficiency Test

4.2.1 General

A total of 35 NRLs *Salmonella* participated in this study: 29 originated from 28 EU-MS, five from third European countries (EU candidate or potential EU candidate MS and members of the EFTA countries), and one from a non-European country.

4.2.2 Accreditation

Almost all laboratories (34) were accredited according to EN-ISO/IEC 17025:2005 for EN-ISO 6579-1:2017. One laboratory was also accredited for EN-ISO 6579:2002, one laboratory for Annex D ISO 6579:2007, one laboratory for iQ-Check *Salmonella* II RT-PCR and one laboratory was only accredited for another method (OIE manual). For the samples in this PT, 33 laboratories used EN-ISO 6579-1:2017, one laboratory used Annex D ISO 6579:2007, and one laboratory used OIE manual 3.9.8.

4.2.3 Transport of samples

The samples were transported using a door-to-door courier on Monday 23 September 2019. Twenty-seven laboratories received the parcel within one day after dispatch, six participants within two days, and one laboratory within three days. One parcel took almost a week to arrive due to customs transport problems.
The temperature during transport and storage was recorded using a temperature recorder placed between the samples in the sample bag. The temperature of the samples during transport was predominantly between -4 °C and +4 °C.
The participants were asked to store the parcel at 5 °C on arrival at their laboratories. The storage temperature at the receiving laboratories ranged from 0 – 10 °C. The start date of the analysis for almost all laboratories was 30 September 2019. Laboratory 28 received its parcel

late and only started analysis on 1 October 2019. The temperature of this parcel during transport stayed below 5°C to 25 September, but increased rapidly to 10 °C on 27 September and 16 °C on 29 September. The parcel arrived at the laboratory on 30 September and was placed at 5 °C until the start of the analyses the following day.
In addition, two laboratories (lab codes 7 and 21) started the analysis on the day of arrival (24 September 2019) because of national holidays in the starting week.

Table 5. Second plating-out media used by the NRLs

Media	No. of users
ASAP	1
BGA	7
BGA mod	8
BPLS	3
BSA	2
BxLH	1
Smi(ID)2	1
Rambach	7
Chromo *Salmonella*	1
RAPID'*Salmonella*	4

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

The prescribed method was EN-ISO 6579-1:2017 for which MSRV agar had to be used as selective enrichment medium and XLD agar and a second medium free of choice for plating out. Table 5 shows which second plating-out media were chosen by the participants.
Technical details on the method which deviated from the prescribed ISO method (EN-ISO 6579-1:2017) are listed in Table 6 (grey-shaded cells); five laboratories reported details of deviations. Four laboratories (lab codes 12, 13, 27 and 28) used MSRV with a pH higher or lower than prescribed. In addition, one laboratory (lab code 32) used MSRV with a four times higher concentration of Novobiocin than the prescribed 10 mg/l. One laboratory did not report the novobiocin concentration at all (lab code 28).

Table 6. Reported technical deviations from the prescribed EN-ISO 6579-1:2017

Lab code	BPW	MSRV	
	Incubation time (h:min)	pH	Novobiocin
EN-ISO 6579-1	16–20 h	5,1–5,4	10 mg/l
12	20	5,5	10 mg/L
13	19:15	4,85	10 mg/L
27	19	5,6	10 mg/L
28	20	5,52	mg/L
32	18	5,1	40 mg/L

All participating laboratories performed one or several confirmation tests for *Salmonella*. Table 7 summarises all reported combinations. Twenty-seven laboratories performed a biochemical test. Twelve laboratories used only one confirmation test; most laboratories used a combination

of two or more confirmation methods. Other methods used were: Maldi-tof and Chromogenic agar method.

Table 7. Number of laboratories using the different confirmation methods

Number of labs	Biochemical	Serological	Serotyping	PCR	Other
5	x				
6	x	x			
1	x	x			x
2	x	x	x		
1	x	x	x	X	
6	x		x		
1	x		x	X	
4	x				x
1		x			x
3			x		
2			x		x
4					x

4.3 Control samples

4.3.1 General

Two control samples were sent to the laboratories. One was used as a procedure control. The other was used as a positive control to which the laboratories had to add their own positive control strain normally used in their routine analysis for *Salmonella* detection.

Procedure control (BPW only)

Thirty-four laboratories analysed the procedure control correctly negative for *Salmonella* and scored good results for this control sample. One laboratory (lab code 6) reported this samples as positive for *Salmonella*. This was caused by an error when labelling the samples. After inquiries by the EURL for a possible explanation, this laboratory could demonstrate the labelling error with their raw data showing that the procedure control was treated as the positive control and the positive control was treated as the procedure control. This laboratory scored a moderate performance.

Positive control with Salmonella

All laboratories correctly scored their own *Salmonella* positive control sample as positive. Laboratory 6 reported this samples as negative as a result of their labelling mistake as shown by their raw data.
The *Salmonella* serovars used for the positive control sample are shown in Table 8. The majority of the NRLs-*Salmonella* use *S*. Enteritidis or *S*. Typhimurium for their positive control samples. However, the use of a less common *Salmonella* serovar in routine samples may be advisable in order to make the detection of possible cross contamination easier.

Table 8. Salmonella *serovars used by participants for the positive control samples*

***Salmonella* serovar**	**Number of users**
S. Enteritidis	12
S. Typhimurium	9
S. Nottingham	6
S. Alachua, S. Blegdam, S. Bongori, S. Harleystreet, *S.* Regent, *S.* Tranaroa*, S.* Tennessee*, S.* Abaetetuba	1 (per serovar)

4.3.2 *Correct scores of the control samples*

Table 9 shows the number of correctly analysed control samples for all participants and for the EU-MS only. The data have been corrected for the mistake in labelling of the samples. No differences were found between these two groups. All laboratories showed correct results, resulting in accuracy rates of 100%.

Table 9. Correct scores found with the control samples by all participants and by the laboratories of the EU-MS only

Control samples		**All labs n=35**	**EU-MS n = 29**
Procedure control n=1	No. of samples	35	29
	No. of negative samples	35	29
	Specificity in %	**100%**	**100%**
Positive control (own *Salmonella*) n=1	No. of samples	35	29
	No. of positive samples	35	29
	Sensitivity in %	**100%**	**100%**
All control samples n=2	No. of samples	70	58
	No. of correct samples	70	58
	Accuracy in %	**100%**	**100%**

Note: Laboratory 6 mislabelled the procedure control and the positive control. The correct scores and accuracy in this table were calculated on basis of the corrected data.

4.4 Artificially contaminated chicken faeces samples

4.4.1 *General*

Chicken faeces samples artificially contaminated with two different levels of *Salmonella* Typhimurium, low (MPN concentration 13 cfu/sample) and high (MPN concentration 35 cfu/sample) as well as negative samples, were analysed for the presence of *Salmonella* by the participants. Table 10 shows the overall results found by the participants.

Table 10. Number of positive results found with the artificially contaminated chicken faeces samples at each laboratory

	Number of positive isolations		
	Negative n=4	**STm low n=6**	**STm high n=4**
Criteria good performance	≤1	≥3	≥3
Lab code 21	0	5	4
Lab code 23	0	4	3
All other NRLs	0	6	4

Negative chicken faeces samples
All laboratories correctly analysed the negative samples negative for *Salmonella*.

Chicken faeces contaminated with low level of Salmonella *Typhimurium*
Almost all laboratories were able to detect *Salmonella* in all six chicken faeces samples contaminated with a low inoculum level of approximately 13 cfu *S.* Typhimurium. Two laboratories (lab codes 21 and 23) reported one and two of the six samples negative for *Salmonella*. In respect of low level samples, a negative score for a maximum of three of six samples is regarded acceptable hence these laboratories scored well above the criteria for good performance. The results of all participants are shown in Figure 4.

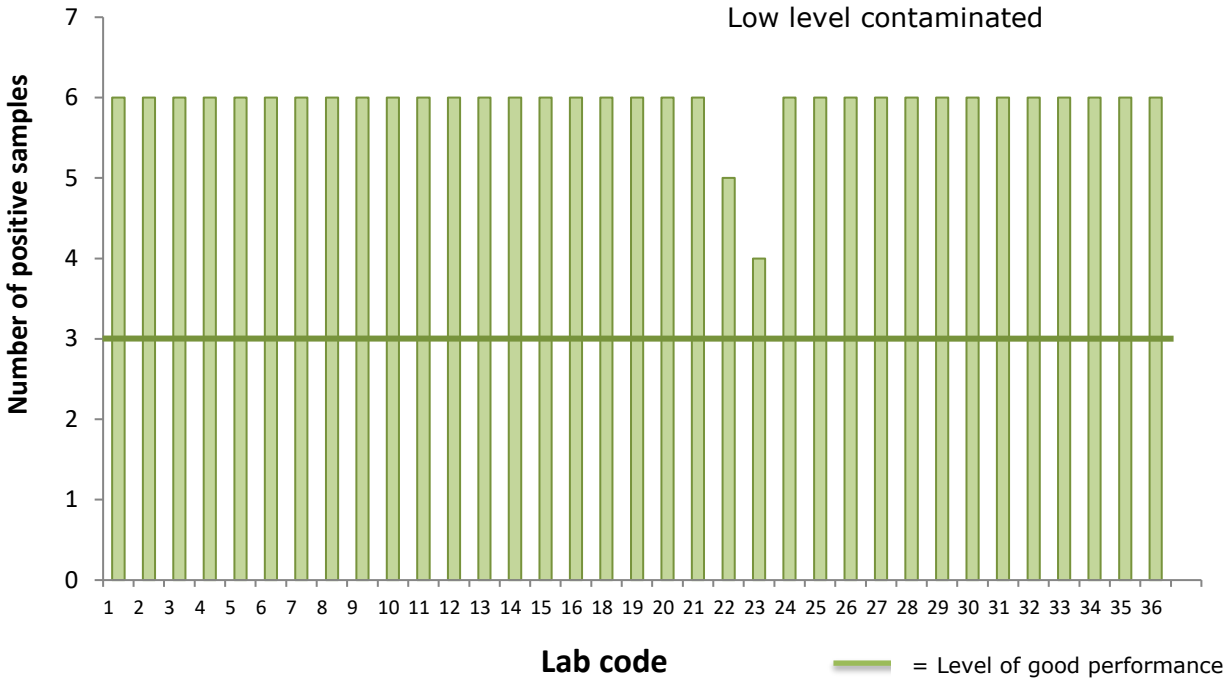

Figure 4. Number of positive Salmonella *isolations per laboratory found in the chicken faeces samples contaminated with low level* Salmonella *Typhimurium (n=6).*

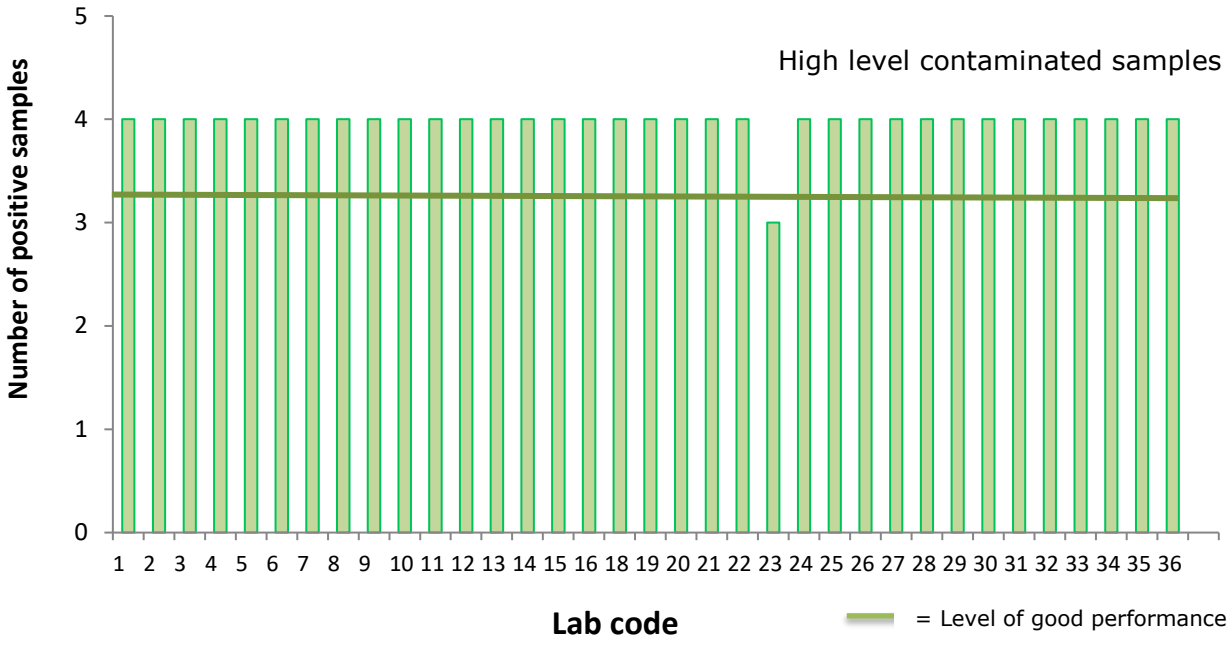

Figure 5. Number of positive Salmonella *isolations per laboratory found in the chicken faeces samples contaminated with high level* Salmonella *Typhimurium (n=4).*

Chicken faeces contaminated with high level of Salmonella *Typhimurium*
Almost all laboratories were able to detect *Salmonella* in all four samples inoculated with a high concentration of *S.* Typhimurium. Laboratory 23 found one high-level sample negative for *Salmonella*. This is still above the criteria for good performance. The results are shown in Figure 5.

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

Table 11 shows the specificity, sensitivity and accuracy rates for all artificially contaminated chicken faeces samples. The calculations were performed on the results of all participants and on the results of the EU-MS participants only. All participants performed well in this study: the specificity rate (100%), the sensitivity rates (low level: 98,6%; high level 99,3%) and the accuracy rate (99,2%) were high. Hardly any differences were found between the two groups.

Table 11. Specificity, sensitivity and accuracy rates found by the participating laboratories (all participants and EU-MS only) with the artificially contaminated chicken faeces samples

Chicken faeces samples		All participants n=35	EU-MS n=29
Negative samples n=4	No. of samples	140	116
	No. of negative samples	140	116
	Specificity in %	**100**	**100**
Low level STm n=6	No. of samples	210	174
	No. of positive samples	207	171
	Sensitivity in %	**98,6**	**98,3**
High level STm n=4	No. of samples	140	116
	No. of positive samples	139	115
	Sensitivity in %	**99,3**	**99,1**
All chicken faeces samples with STm n = 14	No. of samples	350	290
	No. of positive samples	346	286
	Sensitivity in %	**98,9**	**98,6**
All chicken faeces samples (pos. and neg.)	No. of samples	490	406
	No. of correct samples	486	402
	Accuracy in %	**99,2**	**99**

4.4.3 *Second detection method*

This year, seven laboratories (lab codes 8, 12, 20, 21, 25, 27 and 31) also used a second method to analyse the chicken faeces samples. An overview of the methods used per laboratory can be found in Table 12. Almost all laboratories used a PCR method as second method, with one laboratory using an extended variance of the ISO 6579-1:2017 (a third selective medium). Only validated methods were used. Two laboratories used this second method routinely for samples analysis.

The majority of NRLs found identical results with their second method compared to the bacteriological culture method. Two laboratories (lab codes 25 and 31) found different results. Laboratory 25 found one low level samples negative for *Salmonella* with their second method but positive with the bacteriological culture method. Laboratory 31 found one negative sample positive with the second method in contrast to the results obtained with the bacteriological culture method.

*Table 12. Details on the second detection method used by NRLs-*Salmonella *during the Proficiency Test*

Lab code	Second detection method	Validated (by)	Reference	Routinely # per year
8	Real Time PCR 7500	National Accreditation Authority	ISO 6579:2002 / Amd 1 2007. Annex D.	2755
12	Real Rime PCR	NF validation: AOAC-RI	ISO 16140	No
20	An extended ISO 6579-1:2017 (PPS)	ISO	ISO 6579-1:2017	No
21	real time PCR	National Food and Feed Code (§64)	Malorny et al.(2004) AEM 70:7046-7052	No
25	PCR	in house	Josefsen et al. (2007) Malorny et al. (2004) O.I.E Chapter 2.2.3	No
27	BAX PCR for *Salmonella* (commercial End-time PCR-system)	Nordval	NORDVAL Certificate #030	7500
30	qPCR iQ-*Salmonella* II (BIORAD)	ADRIA	BRD07/06-07/04	No

4.5 Performance of the NRLs

4.5.1 General

All laboratories were able to detect *Salmonella* in high and low concentrations in chicken faeces samples. Of the 35 laboratories, 34 fulfilled the criteria of good performance. One laboratory (lab code 6) mislabelled the control samples and added the positive control strain to the procedure control sample. This laboratory scored a moderate performance. No follow-up study was deemed necessary for this deviating result as this was an administrative deviation and not a technical deviation.

5 Conclusions

All NRLs for *Salmonella* were able to detect high and low levels of *Salmonella* in chicken faeces samples.

Thirty-four NRLs scored a 'good performance'. One laboratory (lab code 6) scored a moderate performance for mislabelling the control samples (administrative error).

The accuracy, specificity and sensitivity rates of the control samples were all 100%.

The sensitivity rate of all labs found with the chicken faeces samples artificially contaminated with a low level of *S*. Typhimurium was 98,6%.

The sensitivity rate of all labs found with the chicken faeces samples artificially contaminated with a high level of *S*. Typhimurium was 99,3%.

The accuracy rate of all NRLs for detection of *Salmonella* in the artificially contaminated chicken faeces samples was 99,2%.

Seven participants used a second method in addition to the prescribed bacteriological culture method. Five laboratories reported identical results for both methods. One laboratory found one low level sample negative for *Salmonella* in contrast to their positive result using the bacteriological culture method. Another laboratory detected *Salmonella* in a negative sample in contrast to their results using the bacterial culture method.

List of abbreviations

AOAC	Association of Official Analytical Chemists
ASAP	AES *Salmonella* Agar Plate
ATCC	American Type Culture Collection
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
BxLH	Brilliant green, Xylose, Lysine, Sulphonamide
CEN	European Committee for Standardization
cfu	Colony-forming units
DG-SANTE	Directorate-General for Health and Consumer Protection
EC	European Commission
EFTA	European Free Trade Association
EN	European Standard
EU	European Union
EURL	European Union Reference Laboratory
GD	Gezondheidsdienst voor dieren
ISO	International Organization for Standardization
MPN	Most Probable Number
MS	Member State
MSRV	Modified Semi-solid Rappaport-Vassiliadis
NRL	National Reference Laboratory
O.I.E.	World Organisation for Animal Health
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PPS	Primary Production Stage
PT	Proficiency Test
RIVM	Rijksinstituut voor Volksgezondheid en het Milieu (National Institute for Public Health and the Environment)
SM (ID)2	*Salmonella* Detection and Identification-2
SPF	Specific Pathogen Free
STm	*Salmonella* Typhimurium
VRBG	Violet Red Bile Glucose
XLD	Xylose Lysine Deoxycholate

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