RIVM report 330300010/2007

Improvements in the method for detection of *Salmonella* ssp. in animal faeces

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This investigation has been performed by order and for the account of the European Commission, Legislation Vétérinaire et Zootechnique and the Microbiological Laboratory for Health Protection within the framework of RIVM project E/330300/04/CS by the Community Reference Laboratory for *Salmonella*.

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Abstract

Improvements in the method for detection of *Salmonella* spp. in animal faeces.

The international standard method (ISO 6579) for the detection of *Salmonella* in food and animal feed is less applicable to faecal samples of poultry and pigs. RIVM's investigation on how to adapt the method for application to the detection of *Salmonella* in animal faeces has resulted in a new annex (Annex D) to the existing international culture method (ISO6579).

RIVM advises against the mixing of faecal samples with glycerol, a substance that stabilises bacteria when stored in a freezer, as a means to stabilising *Salmonella*. However, since research has shown that glycerol inhibits the growth of *Salmonella*, it is advisable to store the faecal samples in the refrigerator and analyse them as soon as possible for the presence of *Salmonella*.

Research was also preformed to further improve two steps of the detection method. In the first step, faecal samples are incubated in buffered peptone water (BPW) at a standard incubation time of 18 h. However, shortening of the incubation time did not improve the method. Another test was performed to ascertain whether a higher dilution of the sample in the BPW would bring forth more positive results. However, this was not the case.

In the second step of the method, a small volume of the incubated BPW is further incubated on a more selective medium for *Salmonella*, called Modified Semi-solid Rappaport Vassiliadis (MSRV). Our investigation showed that a lower concentration of the antibiotic novobiocin in MSRV resulted in better growth of *Salmonella*. For faecal samples originating from pigs even more positive results were found at this lower concentration.

Key words: *Salmonella*, animal faeces, glycerol, ISO6579, MSRV

Rapport-in-het-kort

Verbeteringen in de methode voor de detectie van *Salmonella* spp. in dierlijke faeces

De internationale standaardmethode (ISO 6579) om *Salmonella* te meten in voeding is minder geschikt voor uitwerpselen (faeces) van kippen en varkens. Het RIVM heeft onderzocht hoe de methode kan worden aangepast om *Salmonella* goed in dierlijke uitwerpselen te kunnen meten. Dit heeft geresulteerd in een nieuwe bijlage (Annex) bij de bestaande internationale meetmethode (ISO 6579).

Voor het stabiliseren van *Salmonella* in dierlijke uitwerpselen raadt het RIVM af om de uitwerpselen te mengen met glycerol. Glycerol is een middel om bacteriën bij invriezen stabiel te houden. Uit onderzoek blijkt echter dat glycerol de groei van salmonellabacteriën remt. Het is beter om de uitwerpselen te bewaren in de koelkast en ze zo snel mogelijk te onderzoeken op de aanwezigheid van *Salmonella*.

Het onderzoek ging ook in op andere aspecten om twee stappen van de meetmethode te verbeteren. Bij de eerste stap worden de uitwerpselen gekweekt in gebufferd peptonwater (BPW). De standaard kweektijd is achttien uur. Het verkorten van deze kweektijd bleek geen zin te hebben. Ook werd getest of een verdunning in een oplossing met BPW meer positieve resultaten opleverde. Dit bleek echter niet het geval.

Bij de tweede stap van de methode wordt een klein volume van de gekweekte BPW verder gekweekt op een voor *Salmonella* meer selectief medium: Modified Semi-solid Rappaport Vassiliadis (MSRV). Een lager gehalte van het antibioticum novobiocine in MSRV bleek betere groei van *Salmonella* op te leveren. Voor uitwerpselen van varkens bleek dit zelfs tot meer positieve resultaten te leiden.

Trefwoorden: Salmonella, dierlijke faeces, glycerol, ISO 6579, MSRV

Contents

Summary

The Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organizes every year a bacteriological interlaboratory comparison study on the detection of *Salmonella* in animal faeces. The national reference laboratories (NRLs) for *Salmonella* of the EU member states participate in these studies. The aim of these studies is to test the performance of the NRLs-*Salmonella* for the detection of *Salmonella* in the presence of a food or veterinary matrix and background flora. The method for detection of *Salmonella* in food and animal feed is described in ISO 6579 (2002). However, this ISO procedure is less applicable to the detection of *Salmonella* in animal faeces. For this latter matrix another selective enrichment medium was selected: Modified Semi-solid Rappaport Vassiliadis (MSRV). The full procedure for the use of this new medium will be described in a new annex of ISO 6579: Annex D. Several research activities were performed at CRL-*Salmonella* in relation with draft Annex D and/or in relation with the samples as used in the interlaboratory comparison studies.

It was found out that two concentrations of novobiocin in MSRV were used by different laboratories, being 0.01 g/L and 0.02 g/L. To find out the 'optimal' novobiocin concentration in MSRV, artificially contaminated faeces and naturally contaminated faeces were tested on MSRV with the two novobiocin concentrations. A higher percentage of positive *Salmonella* samples was found when pig faeces was analysed with MSRV containing 0.01 g/L novobiocin. The results with chicken faeces, cattle faeces and dust were less pronounced, although the migration of *Salmonella* was larger on MSRV containing 0.01 g/L novobiocin than on MSRV containing 0.02 g/L novobiocin.

For testing the stability of bacteria in chicken faeces under different storage conditions, chicken faeces mixed with peptone/glycerol as well as unmixed faeces samples were stored at different temperatures. *Salmonella* positive chicken faeces mixed with peptone/glycerol (30% $^{v}/_{v}$) showed stable results for *Salmonella* Enteritidis when stored at (-20 ± 5) ºC for at least 14 days. After 7 days of storage at (5 ± 3) ºC a $2\log_{10}$ decrease was seen and at (20 ± 5) ºC the number of *Salmonella* came below the detection limit after 2 days of storage. The number of aerobic bacteria remained stable for at least 14 days at (-20 ± 5) ºC, (5 ± 3) ºC and (20 ± 5) ºC, independent whether the faeces were mixed with peptone/glycerol or not.

For testing the stability of the micro-organisms under long-term storage conditions, samples of unmixed faeces (*Salmonella* positive as well as *Salmonella* negative) were stored at (5 ± 3) ºC and samples of mixed *Salmonella* negative faeces were stored at (-20 ± 5) ºC for at least one month.

It was also tested whether the negative influence of the background flora on the growth of *Salmonella* would be less when the samples were more diluted in BPW. A ten fold dilution was made out of the normal 1/10 dilution in BPW. For faeces not mixed with peptone/glycerol, no differences were found in the two dilutions. For chicken faeces mixed with peptone/glycerol (30% $^{v}/_{v}$) the results were variable. More positive results were found in

the 1/10 if the BPW was incubated for only 4 h. If the same BPW was incubated for 18 h the 1/100 dilution gave more positive results.
Experiments of Heuvelman and In 't Veld (1998) showed more positive isolations of *Salmonella* in artificially contaminated chicken faeces if the incubation time of BPW was shortened to 4-6 h. To test the influence of the incubation time of BPW, an incubation time of 4 h was used beside the 18 h of incubation of BPW. Control capsules and artificially contaminated chicken faeces mixed with peptone/glycerol (30% $^{v}/_{v}$) gave, in general, more positive results after 18 h of incubation of BPW than after 4 h of incubation. Naturally contaminated chicken faeces mixed with peptone/glycerol (30% $^{v}/_{v}$) showed opposite results and gave more positive results after 4 h of incubation of BPW than after 18 h of incubation.
To test the effect of preservation media on the detection of *Salmonella*, chicken faeces samples were mixed with different preservation media and compared with unmixed faeces. The preservation media used were peptone/glycerol 30%, peptone/glycerol 15%, TSB/glycerol 30%, TSB/glycerol 15% and double strength skim milk. All *Salmonella* negative faeces samples were artificially contaminated with *Salmonella* reference materials. A higher glycerol concentration resulted in less positive isolations after 18 h of incubation of BPW, whereas all unmixed faeces samples were found positive for *Salmonella* after 18 h of incubation of BPW.
To determine the effect of glycerol on the growth of *Salmonella*, capsules containing *S.* Enteritidis or *S.* Typhimurium were incubated for 24 h in BPW with different glycerol concentrations or no glycerol at all. Till 8 h of incubation of BPW at (37 ± 1) ºC, no differences were found between the different BPW solutions. After 24 h of incubation the number of *S.* Typhimurium in BPW containing 1.5% ($^{v}/_{v}$) glycerol was 2 $\log_{10}$ less than the number of *S.* Typhimurium in BPW without glycerol. For *S.* Enteritidis the difference in the number of colony forming particles was 1 $\log_{10}$. This experiment showed that glycerol had a negative influence on the detection of *Salmonella*.

List of abbreviations

BGA	Brilliant Green Agar
BPW	Buffered Peptone Water
cfp	colony forming particles
CRL	Community Reference Laboratory
dPCA	Double concentrated Plate Count Agar
dVRBG	Double concentrated Violet Red Bile Glucose agar
hcmp	highly contaminated milk powder
ISO	International Organization for Standardization
LDC	Lysine Decarboxylase
LIS	Diagnostic Laboratory for Infectious Diseases and Perinatal Screening
MSRV	Modified Semi-solid Rappaport Vassiliadis
NRL	National Reference Laboratory
RIVM	National Institute for Public Health and the Environment
RM	Reference material
SE	*Salmonella* Enteritidis
SOP	Standard Operation Procedure
SPan	*Salmonella* Panama
STM	*Salmonella* Typhimurium
TSB	Tryptone Soya Broth
TSI	Triple Sugar Iron agar
UA	Urea Agar
XLD	Xylose Lysine Desoxycholate agar

1. Introduction

In pursuance of the Directive 2003/99/EC, which replaced the Council Directive 92/117/EEC, the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) organizes bacteriological interlaboratory comparison studies on the detection of *Salmonella* in animal faeces. The studies have the following objectives: that the examination of samples in the EU Member States is carried out uniformly and that comparable results are obtained by all National Reference Laboratories for *Salmonella* (NRLs-*Salmonella*).
The 2002 version of ISO 6579 is mainly intended for the detection of *Salmonella* spp. in food and feeding stuff and is less appropriate for the detection of *Salmonella* spp. in animal faeces. It was therefore requested at ISO/TC34/SC9 (subcommittee dealing with microbiology under Technical Committee Food and Feeding stuff) to standardize the detection of *Salmonella* spp. in animal faeces. A draft proposal including Modified Semi-solid Rappaport Vassiliadis (MSRV) as selective enrichment was sent to the secretariat of ISO/TC34/SC9 in 2004. It was proposed to prepare a new annex to ISO 6579 (annex D) which would describe the procedure for detection of *Salmonella* spp. in animal faeces. Several research activities were performed at CRL-*Salmonella* in relation to draft Annex D and/or in relation to the samples as used in the interlaboratory comparison studies.
The research activities described in this report are:

- testing the optimal concentration of novobiocin in MSRV;
- using a 1:100 diluted sample for the pre-enrichment in Buffered Peptone Water (BPW) instead of 1:10;
- shortening of the incubation time of BPW from 18 h to 4 h;
- testing of the influence of different preservation media in animal faeces on the detection of *Salmonella* spp.;
- testing stability of *Salmonella* spp. and ‘background flora’ in chicken faeces when stored at different temperatures for several weeks.

2. Materials and Methods

2.1 Materials

2.1.1 Reference materials

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

- 5 colony forming particles (cfp) per capsule for *Salmonella* Panama (SPan5);
- 10 and 100 colony forming particles (cfp) per capsule for *Salmonella* Typhimurium (STM10 and STM100);
- 100 and 500 colony forming particles (cfp) per capsule for *Salmonella* Enteritidis (SE100 and SE500).

Immediately after mixing a powder, a test batch of 60 capsules was prepared to determine the mean number of cfp per capsule and the homogeneity of the mixture. The remaining mixed powders were stored at -20 ^{o}C. When the test batch fulfilled the pre-set criteria for contamination level and homogeneity, the relevant mixed powders were filled into gelatin capsules and stored at -20 ^{o}C. For the preparation of the STM 10 and STM 100 capsules, the remaining of the mixed powder of the interlaboratory comparison study of 2003 were used (Korver et al., 2005).
The pre-set criteria were:

- Mean contamination levels should lie between target level minus 30% and target level plus 50% (e.g. between 70 and 150 cfp if the target level is 100 cfp);
- The variation between capsules of one batch should fulfill: $T_2/(I-1) \leq 2$. Where T2 is a measure for the variation between capsules of one batch (see formula in Annex 1) and I is the number of capsules.

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

- reconstitution of each capsule in 5 ml peptone saline solution in a Petri dish at (38.5 ± 1) ^{o}C for (45 ± 5) minutes;
- repair of *Salmonella* by the addition of 5 ml molten double concentrated Plate Count Agar (dPCA) to the reconstituted capsule solution, and after solidification incubation at (37 ± 1) ^{o}C for (4 ± $^{1}/_{2}$) h;

- after incubation, 10 ml of molten double concentrated Violet Red Bile Glucose agar (dVRBG) was added as an overlayer and after solidification the plates were incubated for (20 ± 2) h at (37 ± 1) ^{o}C.

2.1.2 Faeces samples

Chicken faeces was obtained from poultry laying flocks. The faeces samples were tested for the presence or absence of *Salmonella* spp. For this purpose ten portions of 10 g each were added to 90 ml BPW. After pre-enrichment at 37 ºC for 16-18 h, selective enrichment was carried out on MSRV. Furthermore, the cultures were plated-out on BGA (ISO 6579, 1993) and confirmed biochemically and serologically when necessary (see subsection 2.2.4). The suspected colonies of the positive faeces were isolated on TSI agar and serotyped. All *Salmonella* cultures of the positive faeces were typed as *Salmonella* Enteritidis.
From three poultry laying flocks, which were found negative for *Salmonella*, three batches of faeces (batch A, B and C) were used.

Beside chicken faeces, also pig faeces samples were used for several experiments. *Salmonella* negative pig faeces samples as well as *Salmonella* positive pig faeces samples were obtained from a national surveillance study.

The chicken faeces as used for the interlaboratory comparison studies on detection of *Salmonella* (up to 2004) were mixed with a sterile peptone/glycerol solution, containing 30% (v/v) glycerol. The mixing ratio faeces: peptone/glycerol was always 1:1.
In this experiment batches of chicken faeces samples (*Salmonella* negative batch B as well as *Salmonella* positive faeces) were mixed and homogenised with two different sterilised peptone/glycerol solutions containing 30% (v/v) or 15% (v/v) glycerol and two different TSB/glycerol solutions also containing 30% (v/v) or 15% (v/v) glycerol (mixing ratio 1:1). One liter peptone/glycerol 30% solution consisted of 300 ml glycerol, 7 g of peptone and 700 ml distilled water. One liter peptone/glycerol 15% solution consisted of 150 ml glycerol, 7 g of peptone and 850 ml distilled water. Tryptone Soya Broth (TSB) consisted of 17 g casein, 3 g soybean meal, 5 g sodium chloride, 2.5 g di-basic potassium phosphate and 2.5 g glucose in one liter distilled water.
A part of the negative chicken faeces (batch C) was mixed and homogenised with sterilised peptone/glycerol 15% solution or with sterilised double strength skim milk (mixing ratio 1:1). Double strength skim milk consisted of 20 g skim milk powder in one liter distilled water. After mixing the faeces samples with the preservation media, they were again analysed for the presence or absence of *Salmonella*. Chicken faeces batch A was not mixed with any preservation media. All mixed faeces samples were stored at (-20 ± 5) ºC and all non-mixed faeces were stored at (5 ± 3) ºC, unless described differently when used for several experiments.

2.2 Methods

2.2.1 MPN of *Salmonella* in naturally contaminated faeces

To semi-quantify the number of Salmonellae in the *Salmonella* positive chicken faeces, a Most Probable Number (MPN) method was used. For this purpose, ten grams of faeces were each added to 90 ml of buffered peptone water (BPW) in a plastic bag and mixed by using a Stomacher (60 seconds for each sample). Next tenfold dilutions were prepared in BPW until a concentration of 0.01 mg faeces per 100 ml BPW. This procedure was repeated five times. The BPW jars were incubated and handled according to the same standard procedure as described in 2.2.4. After completion of the test the MPN was calculated using a complementary log-log link in SAS.Proc logistic (SAS Institute Inc, 2004) and/or by using a MPN table (e.g. ISO 16649-3).

2.2.2 Total bacterial count in faeces

The naturally contaminated faeces with *Salmonella* as well as the negative faeces without *Salmonella* were tested for the total number of aerobic bacteria. For this the procedure of ISO 4833 was followed. In short: portions of 20 g chicken faeces were homogenised into 180 ml peptone saline solution in a plastic bag. The content was mixed by using a stomacher (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into two empty Petri dishes (diameter 9 cm). To each dish, 25 ml of molten Plate Count Agar (PCA) was added. After solidification, the plates were incubated at (30 ± 1) ºC for (72 ± 3) h.

2.2.3 Enterobacteriaceae in faeces

The faeces samples were also tested for the number of Enterobacteriaceae. For this ISO 21528-2 was followed. In short: portions of 20 g chicken faeces were homogenised into 180 ml peptone saline solution in a plastic bag by using a stomacher (60 sec). Next tenfold dilutions were prepared in peptone saline solution. Two times one ml of each dilution was brought into two empty Petri dishes (diameter 9 cm). To each dish 25 ml of molten Violet Red Bile Glucose agar (VRBG) was added. After solidification, the plates were incubated at (37 ± 1) ºC for (24 ± 2) h.

2.2.4 Detection of *Salmonella* spp.

2.2.4.1 Media

The composition of the media is described in ISO 6579 (Anonymous, 2002) and in Draft Annex D of ISO 6579 (see Annex 2).

Non selective pre-enrichment medium : Buffered Peptone water (BPW)

Selective enrichment medium : Modified Semi solid Rappaport Vassiliadis (MSRV) with novobiocin (0.01 g/L)

Solid selective media for first and second isolation

- Xylose-Lysine-Desoxycholate
- Brilliant Green Agar (BGA)

Confirmation media

Biochemical confirmation

- Triple sugar/iron agar (TSI agar)
- Urea agar
- 1-Lysine decarboxylation medium (LDC medium)

2.2.4.2 Procedure

Prewarming BPW and thawing faeces

Frozen faeces was taken out of the freezer at the end of the day before the start of the test and thawed in the closed container overnight at (5 ± 3) ºC. Sufficient jars containing 90 ml BPW were placed overnight at (37 ± 1) ºC.

Pre-enrichment

The *Salmonella* capsules and the control capsules were taken out of the freezer for one hour before they were added to the BPW, to allow them to equilibrate to room temperature. Shortly before adding the capsules, the jars with BPW were taken from the (37 ± 1) ºC incubator and inspected for visual growth. Infected jars were discarded.
The gelatin capsules were added to the jars (without mixing) and placed in the (37 ± 1) ºC incubator for 45 minutes to dissolve the capsules. After 45 minutes, 10 g of (thawed) faeces was added to the jars.
All jars were returned to the 37 ºC incubator for a first incubation of 4 h. After transferring a volume of 0.1 ml from each jar to the selective enrichment medium, the jars were further incubated for a total of (18 ± 2) h at (37 ± 1) ºC.

Selective enrichment
Each MSRV plate was inoculated with three drops of a BPW culture, with a total volume of 0.1 ml. The plates (not inverted) were incubated at (41.5 ± 1) ºC for (24 ± 3) h and if negative another (24 ± 3) h.

First isolation after 24 h
Suspect MSRV plates were further plated out on Xylose Lysine Desoxycholate agar (XLD) and on Brilliant Green Agar (BGA). XLD and BGA plates were incubated at (37 ± 1) ºC for (24 ± 3) h.

Second isolation after 48 h
After a total incubation time of two times 24 h of the MSRV plates, the procedure described above was repeated (first isolation after 24 h).

Confirmation
For confirmation, at least one colony considered to be typical or suspect was taken from each Petri dish of each selective medium and inoculated on:

- TSI agar
- Urea agar
- 1-Lysine decarboxylation medium

2.3 Novobiocin concentration in MSRV

While drafting Annex D of ISO 6579 (see Annex 2) it was found out that two concentrations novobiocin in MSRV were used by different laboratories, being 0.01 g/L and 0.02 g/L. To find out the ‘optimal’ novobiocin concentration in MSRV, artificially contaminated chicken and cattle faeces as well as naturally contaminated chicken faeces, naturally contaminated dust and also naturally contaminated pig faeces were tested on MSRV with the two novobiocin concentrations. The plating-out media were XLD and BGA. The number and type of tested samples, as well as the contamination levels are given in Table 1.

Table 1 Types and the number of samples used to compare two novobiocin concentrations (0.01 g/L and 0.02 g/L) in MSRV

Capsules	**Test samples (n=25) with 10 g mixed [1]*Salmonella*-negative chicken faeces**	**Test samples (n=27) with 10 g unmixed *Salmonella*-negative chicken faeces**	**Test samples (n=17) with 25 g unmixed *Salmonella*-negative chicken faeces**
S. Panama 5	---	5	3
S. Enteritidis 100	7	5	3
S. Enteritidis 500	4	5	3
S. Typhimurium 10	7	5	3
S. Typhimurium 100	4	5	3
Blank	3	2	2
Capsules	**Control capsules (n = 10) No faeces added**	**Test samples (n=20) with 10 g mixed [1]*Salmonella*-positive chicken faeces**	**Test samples (n=255) with 25 g unmixed pig faeces**
S. Panama 5	2	---	---
S. Enteritidis 100	2	---	---
S. Enteritidis 500	1	---	---
S. Typhimurium 10	3	---	---
S. Typhimurium 100	---	---	---
Blank	2	---	---
No capsules	---	20	255
Capsules or culture	**Test samples (n=10) with 10 g *Salmonella* positive dust**	**Test samples (n=7) with 25 g unmixed cattle faeces**	**Test samples (n=15) with 25 g unmixed cattle faeces**
S. Dublin 40	---	---	5
S. Dublin 400	---	---	5
S. Dublin 1600	---	---	5
S. Typhimurium 10	---	5	---
S. Typhimurium 100	---	5	---
Blank	---	2	---
No capsules	10	---	---

[1]: faeces was mixed (1:1) with a peptone/glycerol solution, containing 30% (v/v) glycerol

2.4 Stability of Salmonella and aerobic total count in chicken faeces

For testing the stability of the micro-organisms in the faeces under different short-term storage conditions, samples of the *Salmonella* positive chicken faeces, mixed with peptone/glycerol 30% as well as unmixed, were stored at (-20 ± 5) ºC, (5 ± 3) ºC and at (20 ± 5) ºC. The number of *Salmonella* and the total bacterial count were determined during two weeks on day 0, 2, 7 and 14.

For testing stability under long-term storage conditions, the following experiments were performed. Unmixed negative faeces batches A and B were stored for four moths at (5 ± 3) ºC and the negative faeces batch B mixed with peptone/glycerol 30% were stored for four months at (-20 ± 5) ºC. The unmixed positive faeces were stored for one month at (5 ± 3) ºC. The total bacterial count was investigated at the beginning and the end of these periods.
To quantify the number of *Salmonella,* the MPN method described in subsection 2.2.1 was used. For the enumeration of the total number of aerobic bacteria the procedure as described in subsection 2.2.2 was followed.

2.5 Incubation of chicken and pig faeces in dilution steps of 1/10 or 1/100 in BPW

It was tested whether the negative influence of background flora on the growth of *Salmonella* would be less when the sample would be more diluted in BPW. For this purpose artificially contaminated *Salmonella*-free chicken faeces, naturally contaminated chicken faeces and naturally contaminated pig faeces were tested.
The capsules (for artificial contamination of negative faeces), the negative (batch B) and the positive chicken faeces, both mixed with peptone/glycerol 30%, were stored at (-20 ± 5) ºC until the start of the study. Unmixed positive chicken faeces were stored at (5 ± 3) ºC. Details about the handling of the samples can be found in subsection 2.2.4. The medium combination used was MSRV/BGA.
Ten control capsules were tested without faeces (see Table 2). Ten capsules were tested in combination with each 10 g of mixed (peptone/glycerol 30%) chicken faeces (negative for *Salmonell*a, batch B). Furthermore, 35 samples of each 10 g of naturally contaminated faeces (mixed with peptone/glycerol 30%) and 5 samples of each 10 g unmixed naturally contaminated faeces (with *Salmonella* Enteritidis) were analysed. Finally, 98 pig faeces samples (*Salmonella* positive as well as *Salmonella* negative) of each 25 g were tested. For all samples a tenfold dilution was made out of the (normal) 1/10 dilution in BPW. For both dilutions (1/10 and 1/100 in BPW) the procedure as described in subsection 2.2.4 was followed. The BPW containing the chicken faeces was incubated for 4 h and for 18 h (see subsection 2.2.4.2). The BPW containing the pig faeces was incubated for only 18 h.
A summary of the types and the number of capsules and faeces samples is given in Table 2.

Table 2 Types and number of capsules and faeces samples as tested in the 1/10 and 1/100 dilution in BPW study

Capsules	Control capsules (n = 10) No faeces added	Test samples (n=10) with 10 g mixed *Salmonella*-negative faeces[1]	Test samples (n=35) with 10 g mixed *Salmonella*-positive faeces[1]	Test samples (n=5) with 10 g unmixed *Salmonella*-positive faeces[1]	Test samples (n=98) with 25 g unmixed pig faeces
S. Typhimurium 100	5	5	---	---	---
S. Enteritidis 500	5	5	---	---	---
No capsules	---	---	35	5	98

[1]*: chicken faeces*

2.6 Incubation of chicken faeces and pig faeces in BPW for 4h and for 18h

Experiments of Heuvelman and In 't Veld (1998) showed more positive isolations of *Salmonella* in artificially contaminated chicken faeces if the incubation time of BPW was shortened to 4-6 h. To test the influence of the incubation time of BPW the following experiments were carried out.
Capsules, negative (batch B) and positive chicken faeces mixed with peptone/glycerol 30% were stored at (-20 ± 5) ºC until the start of the study. Unmixed negative faeces (batch B) were stored at (5 ± 3) ºC. Details about the analyses of the samples can be found in subsection 2.2.4. The medium combination used was MSRV/XLD.
Ten control capsules were tested without faeces (see Table 3). Twenty-five capsules were tested in combination with each 10 g of mixed (peptone/glycerol 30%) chicken faeces (negative for *Salmonella*, batch B). Also twenty samples of each 10 g of naturally contaminated mixed (peptone/glycerol 30%) chicken faeces samples (with *Salmonella* Enteritidis) were analysed. The control capsules and the naturally contaminated mixed faeces were tested twice and the artificially contaminated mixed faeces was tested three times.
Furthermore, 25 capsules were tested twice in combination with each 10 g of unmixed faeces (negative for *Salmonella*, batch B). All samples were incubated for (4 ± $^1/_2$) h and (18 ± 2) h in BPW.
Finally, 306 unmixed pig faeces samples (*Salmonella* positive as well as *Salmonella* negative) of each 25 g were analysed after (4 ± $^1/_2$) h of incubation in BPW and after (18 ± 2) h in BPW. For pig faeces the medium combination MSRV/BGA was used.
A summary of the types and number of capsules and faeces samples is given in Table 3.

Table 3 Types and number of capsules and faeces samples as tested in experiments with 4 h and 18 h of incubation of BPW

Capsules	Control capsules (n = 10) No faeces added	Test samples (n=25) with 10 g mixed *Salmonella*-negative faeces[1]	Test samples (n=25) with 10 g unmixed *Salmonella*-negative faeces[1]	Test samples (n=20) with 10 g mixed *Salmonella*-positive faeces[1]	Test samples (n=306) with 25 g unmixed pig faeces
S. Panama 5	2	---	---	---	---
S. Enteritidis 100	2	7	7	---	---
S. Enteritidis 500	1	4	4	---	---
S. Typhimurium 10	3	7	7	---	---
S. Typhimurium 100	---	4	4	---	---
Blank	2	3	3	---	---
No capsules	---	---	---	20	306

[1]: *chicken faeces*

2.7 Influence of glycerol on the detection of *Salmonella*

By Chun et al. (1972) a negative influence of glycerol on the growth of *Salmonella* was described. Up to 2004 all faeces samples used for the CRL-*Salmonella* interlaboratory comparison studies were mixed with a solution of peptone/glycerol (30% ($^{v}/_{v}$) glycerol). In the following experiment *Salmonella*-negative chicken faeces artificially contaminated with capsules were tested for the number of positive isolations. For this purpose unmixed faeces (batch A) was used, as well as faeces (batch A) mixed with peptone/glycerol 30%, peptone/glycerol 15%, TSB/glycerol 30% or TSB/glycerol 15%. Faeces mixed with a glycerol solution were stored at (-20 ± 5) ºC until the start of the study. Unmixed faeces were stored at (5 ± 3) ºC. With each type of faeces, 25 capsules were tested as described in subsection 2.2.4 (4 h and 18 h of incubation in BPW, followed by MSRV/XLD). Details on the types and number of capsules used in the experiments are given in Table 4.

Table 4 Types and number of capsules tested in the experiments for testing the influence of preservation solutions in chicken faeces

Capsules	Test samples (n=25) with 10 g *Salmonella*- negative chicken faeces (mixed or unmixed)
S. Enteritidis 100	7
S. Enteritidis 500	4
S. Typhimurium 10	7
S. Typhimurium 100	4
Blank	3

2.8 Comparison between skim milk and peptone/glycerol as preservation media for storage of faeces samples

In 1991, Opara et al. described the use of double strength skim milk as preservation medium for storage of faeces samples as a reliable alternative for peptone/glycerol solutions. In the following experiment *Salmonella*-negative chicken faeces, unmixed or mixed with different preservation media and artificially contaminated with capsules, were tested for the number of positive isolations.
For testing the stability of the micro-organisms in the faeces under different long-term storage conditions, the faeces samples were stored at different temperatures for 77 days. The total bacterial count and the number of Enterobacteriaceae were investigated on day 0, 7, 14, 28, 56 and 77.
For this purpose unmixed faeces, faeces mixed 1:1 with peptone/glycerol 15% ($^{v}/_{v}$) and faeces mixed 1:1 with double strength skim milk (faeces batch C) were used. The mixed faeces samples were stored at (-20 ± 5) ºC and (5 ± 3) ºC and the unmixed faeces samples were stored at (5 ± 3) ºC. Twenty-five capsules were tested in combination with each 10 g of *Salmonella*-negative faeces (mixed or unmixed) as described in subsection 2.2.4. The BPW cultures were incubated for 4 h and 18 h, followed by culturing on MSRV/XLD. Details on the types and number of capsules used in the experiments are given in Table 4. For the enumeration of the total number of aerobic bacteria and the number of Enterobacteriaceae the procedures as described in subsections 2.2.2 and 2.2.3 were followed.

2.9 Influence of glycerol on the growth of *Salmonella*

Earlier studies showed that the number of positive isolations of *Salmonella* was variable when chicken faeces (naturally or artificially contaminated) mixed with a glycerol solution were analysed. In contrast with the mixed faeces, almost all unmixed faeces samples (naturally or artificially contaminated) were found positive for *Salmonella* after 18 h of incubation of BPW. This was an indication that glycerol solutions may have a negative effect on the detection of *Salmonella.* To determine the effect of glycerol on the growth of *Salmonella* the following experiment was carried out.
Capsules containing *S.* Typhimurium or *S.* Enteritidis at a level of 100 cfp/capsule were tested each without faeces in the pre-enrichment medium BPW. Glycerol was added to the BPW to determine the effect on the growth of *Salmonella*. The capsules were taken out of the freezer one hour before they were added to the BPW, to allow them to equilibrate to room temperature. The capsules were each incubated for 24 h at (37 ± 1) ^{o}C in 100 ml prewarmed BPW containing 1.5% ($^{v}/_{v}$) glycerol, 0.75% ($^{v}/_{v}$) glycerol or no glycerol at all. The final concentration of 1.5% ($^{v}/_{v}$) glycerol in BPW was the same concentration as used in the experiments where 10 g faeces 1:1 mixed with peptone/glycerol 30% or TSB/glycerol 30% was added to 90 ml BPW. The number of Salmonellae was investigated after 0, 2, 4, 6, 8

and 24 h of incubation of BPW. For enumeration of the number of Salmonellae tenfold dilutions of the pre-enrichment medium BPW were prepared in peptone saline solution. Two times one ml of each dilution was brought into two empty Petri dishes (diameter 9 cm). To each dish, 10 ml of molten Plate Count Agar (PCA) was added. After solidification, 10 ml of molten double concentrated Violet Red Bile Glucose agar (dVRBG) was added to each dish. The plates were incubated at (37 ± 1) ºC for (24 ± 2) h.

3. Results

3.1 Reference materials

The level of contamination and the homogeneity of the test batches as well as of the final batches of capsules are presented in Table 5. The final batches were tested on two dates and are presented in Table 5 as final batch 1 and final batch 2. All batches met the preset criteria as stated under subsection 2.1.1. The enumerated minimum and maximum levels within each batch of capsules are also given. For the preparation of the STM 10, STM 100 and the SPan 5 capsules the remaining of the mixed powders of the interlaboratory comparison study on bacteriological detection *Salmonella* spp. of 2003 was used. Therefore no information of the test batch is given here.

Table 5 Level of contamination and homogeneity of SE, SPan and STM capsules

	SE 100	SE 500	SPan 5	STM 10	STM 100
Test batch					
Date testing capsules	27-09-04	26-08-04			
Number of capsules tested	22	44			
Mean cfp per capsule	83	711			
Min-max cfp per capsule	58–102	470–1150			
T_2 / (I-1)	0.97	1.71			
Final batch 1					
Date testing capsules	01-10-04	01-10-04	17-06-04	10-08-04	10-08-04
Number of capsules tested	25	25	46	50	50
Mean cfp per capsule	62	418	9	13	113
Min-max cfp per capsule	40 – 82	290-540	5-15	7-22	97-136
T_2 / (I-1)	1.09	0.79	0.97	0.66	0.89
Final batch 2					
Date testing capsules	11-11-04	12-11-04	16-11-04	11-11-04	11-11-04
Number of capsules tested	23	25	25	23	19
Mean cfp per capsule	74	434	7	11	81
Min-max cfp per capsule	46 – 108	280-620	3-13	7-22	52-120
T_2 / (I-1)	1.92	1.85	0.56	1.32	1.54

cfp = colony forming particles;
min-max = enumerated minimum and maximum cfp;
formula T_2 see Appendix 2; I is number of capsules;
demand for homogeneity T2/(I-1) ≤ 2
SPan = Salmonella Panama
STM = Salmonella Typhimurium
SE = Salmonella Enteritidis

3.2 Faeces samples

At 29-09-2004 the positive chicken faeces samples and the negative chicken faeces samples (batch A and B) were received at CRL-*Salmonella*. On 07-10-2004 the positive faeces were mixed with peptone/glycerol 30% and stored at (-20 ± 5) ^{o}C. On 12-10-2004 the number of *Salmonella* was determined of the unmixed faeces samples stored at (5 ± 3) ^{o}C and of the overnight thawed faeces samples mixed with peptone/glycerol (30%) using the MPN method. The MPN result of the unmixed positive faeces was 2.4 x 10^3 cfp per gram (95% confidence interval: 0.8 – 5.5 x 10^3 cfp per gram) and 8.7 x 10^3 cfp per gram (95% confidence interval: 2.2 – 20.3 x 10^3 cfp per gram) for the faeces mixed with peptone/glycerol 30%.
In Table 6 the total number of aerobic bacteria is shown of the different chicken faeces samples, being *Salmonella* positive and *Salmonella* negative, mixed and not mixed with peptone/glycerol 30%.

Table 6 Number of aerobic bacteria per gram chicken faeces

	Faeces <u>not</u> mixed with peptone/glycerol	**Faeces 1:1 mixed with peptone/glycerol 30%**
Naturally contaminated chicken faeces with *Salmonella*	6.1 x 10^9 cfp/gram (determined at 04-10-2004)	3.4 x 10^9 cfp/gram (determined at 12-10-2004)
Negative chicken faeces without *Salmonella* (batch A)	5.2 x 10^9 cfp/gram (determined at 04-10-2004)	---
Negative chicken faeces without *Salmonella* (batch B)	5.3 x 10^8 cfp/gram (determined at 04-10-2004)	3.0 x 10^8 cfp/gram (determined at 26-10-2004)

---: not tested

3.3 Novobiocin concentration in MSRV

The percentages of *Salmonella* positives results of the artificially and naturally contaminated mixed chicken faeces after 4 h and 18 h of incubation of BPW and further incubated on MSRV with two concentrations of novobiocin (0.01 g/L and 0.02 g/L) are shown in Table 7 and Figure 1.

Table 7 *Percentages of positive isolations of artificially contaminated and of naturally contaminated chicken faeces (both mixed with peptone/glycerol 30%) tested with medium combination MSRV/BGA (MSRV containing 0.01 and 0.02 g/L novobiocin) after 4 h and 18 h of incubation of BPW.*

	0.01 g/L novobiocin in MSRV		0.02 g/L novobiocin in MSRV	
Incubation BPW →	**4h**	**18h**	**4h**	**18h**
STM 10 + 10g faeces (n=7)	14	28	43	28
STM 100 + 10g faeces (n=4)	50	75	75	50
SE 100 + 10g faeces (n=7)	14	14	14	14
SE 500 + 10g faeces (n=4)	75	0	25	0
Salmonella positive faeces (n=10)	90	10	90	10

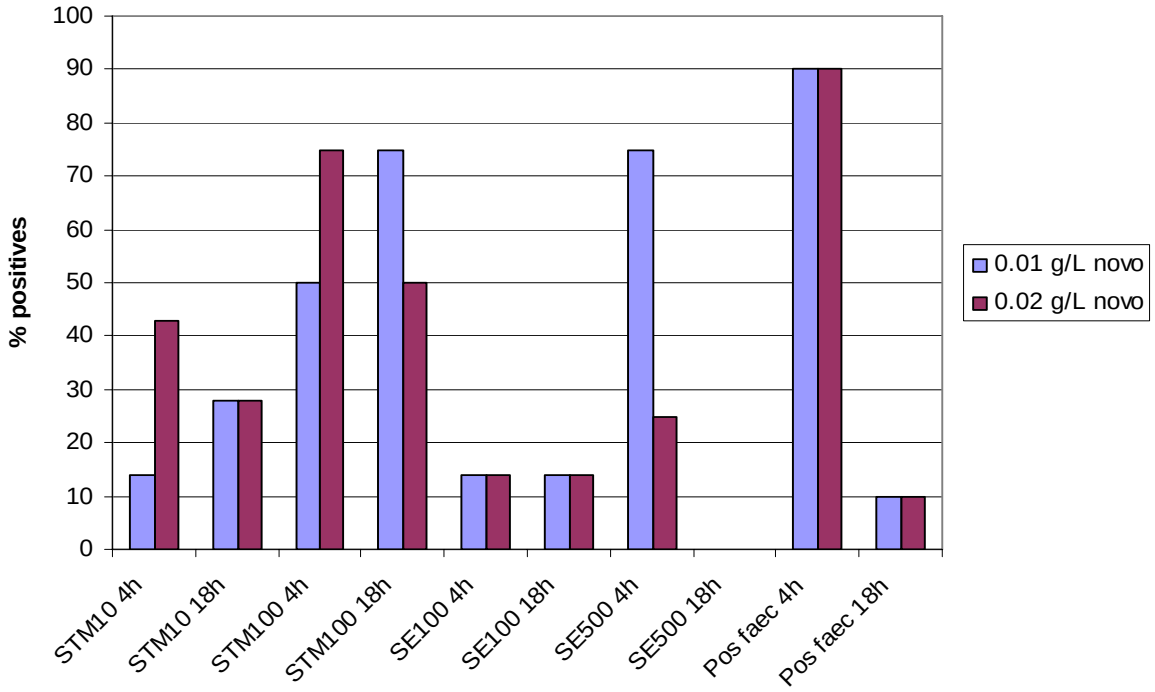

Figure 1 *Percentages of positive isolations of artificially contaminated and of naturally contaminated chicken faeces (both mixed with peptone/glycerol 30%) tested with medium combination MSRV/BGA (MSRV containing 0.01 and 0.02 g/L novobiocin) after 4 h and 18 h of incubation of BPW.*

The number and percentages of positive isolations (taking the highest number of positives as 100%) after analysing 255 pig faeces samples, using two concentrations (0.01 and 0.02 g/L) of novobiocin in MSRV, are shown in Table 8 and Figure 2.

Table 8 *Number and percentages of positive isolations of 255 pig faeces samples tested with medium combination MSRV/BGA (MSRV containing 0.01 and 0.02 g/L novobiocin) after 18 h of incubation of BPW (taking the highest number of positives as 100%).*

	Number and % of positives	
	0.01 g/L novobiocin in MSRV	0.02 g/L novobiocin in MSRV
24h MSRV	25 (86%)	13 (45%)
48h MSRV	29 (100%)	22 (76%)

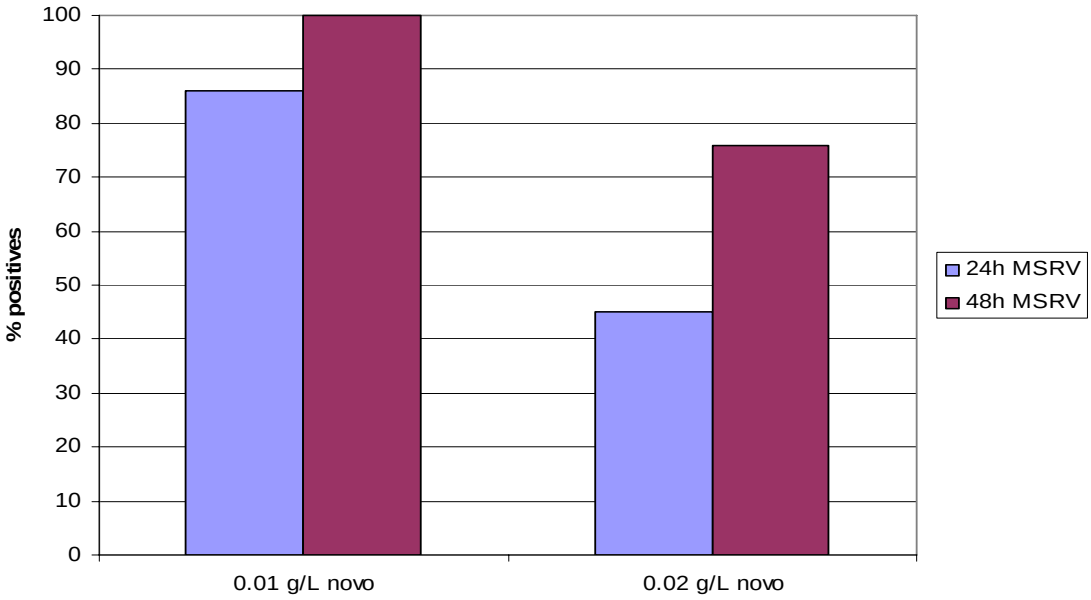

Figure 2 *Percentages of positive isolations of pig faeces (taking the highest number of positives as 100%) for medium combination MSRV/BGA (MSRV containing 0.01 and 0.02 g/L novobiocin) after 18 h of incubation of BPW.*

A higher percentage of positive *Salmonella* samples was found when pig faeces was analysed with MSRV containing 0.01 g/L novobiocin, when compared to MSRV containing 0.02 g/L novobiocin. The results with mixed poultry faeces were less pronounced. All the unmixed poultry faeces, unmixed cattle faeces and dust samples were found positive for *Salmonella* when analysed on MSRV containing 0.01 g/L novobiocin and on MSRV containing 0.02 g/L novobiocin. For all samples the migration of *Salmonella* was larger on MSRV containing 0.01 g/L novobiocin than on MSRV containing 0.02 g/L novobiocin (see an example in Figure 3). For some samples the migration of *Salmonella* on MSRV containing 0.02 g/L novobiocin was so small that the plates seemed negative after 24 h of incubation, while the MSRV plates containing 0.01 g/L novobiocin were clearly suspect for *Salmonella*.

Figure 3 *Migration of S.Typhimurium in artificially contaminated cattle faeces on MSRV containing 0.01 g/L novobiocin and 0.02 g/L novobiocin after 24 h of incubation at (41.5 ± 1) ºC.*

3.4 Stability of bacteria in chicken faeces

The MPN results for *Salmonella* of the unmixed *Salmonella* positive chicken faeces stored for one month at (5 ± 3) ºC and the total aerobic counts of the negative chicken faeces (mixed or unmixed) stored for four months are shown in Table 9. The unmixed negative faeces were stored at (5 ± 3) ºC and the negative faeces mixed with peptone/glycerol 30% were stored at (-20 ± 5) ^{o}C.

Table 9 *The MPN results for Salmonella of the unmixed positive faeces stored for one month and the total aerobic counts for the negative (mixed and unmixed) faeces stored for four months (18 h BPW / 48 h MSRV / BGA)*

Positive chicken faeces (SE) not mixed, stored at 5 ºC MPN *Salmonella* (18 h BPW, 48 h MSRV) in cfp/g (95% confidence interval)			
	October 2004	**November 2004**	
Positive faeces	2.4 x 10^3 (0.8 – 5.5)	0.04 (0.007 – 0.11)	
Negative chicken faeces stored at 5 ºC (mixed faeces stored at -20 ºC) Total aerobic colony count 30 ºC in cfp/g			
	October 2004		**February 2005**
Batch A, not mixed	5.2 x 10^9		2.0 x 10^9
Batch B, not mixed	5.3 x 10^8		1.5 x 10^8
Batch B, mixed	3.0 x 10^8		9.8 x 10^7
Mixed = mixed (1:1) with peptone/glycerol (30%)			

The MPN results for *Salmonella* of the mixed positive chicken faeces and the total aerobic counts for the mixed and unmixed positive faeces stored for two weeks at different temperatures are shown in Table 10 and in Figure 4.

Table 10 *Log10 values of the MPN results for Salmonella and of the total aerobic count of Salmonella positive chicken faeces (SE) (18 h BPW / 48 h MSRV / BGA)*

Day	SE -20ºC	SE +5ºC	SE +20ºC	TC-m -20ºC	TC-m +5ºC	TC-m +20ºC	TC-nm -20ºC	TC-nm +5ºC	TC-nm +20ºC
0	4.23	4.23	4.23	9.21	9.21	9.21	9.49	9.49	9.49
2	3.97	3.4	-0.4	9.18	9.18	8.89	9.61	9.61	9.31
7	3.97	2.23	-0.35	9.2	9.05	8.89	9.63	9.43	9.08
14	3.97	1.11	---	9.2	8.98	8.61	9.6	9.39	8.56

SE: Salmonella Enteritidis
TC: Total aerobic count (30ºC)
m: mixed with a preservation medium
nm: not mixed with a preservation media
---: not tested

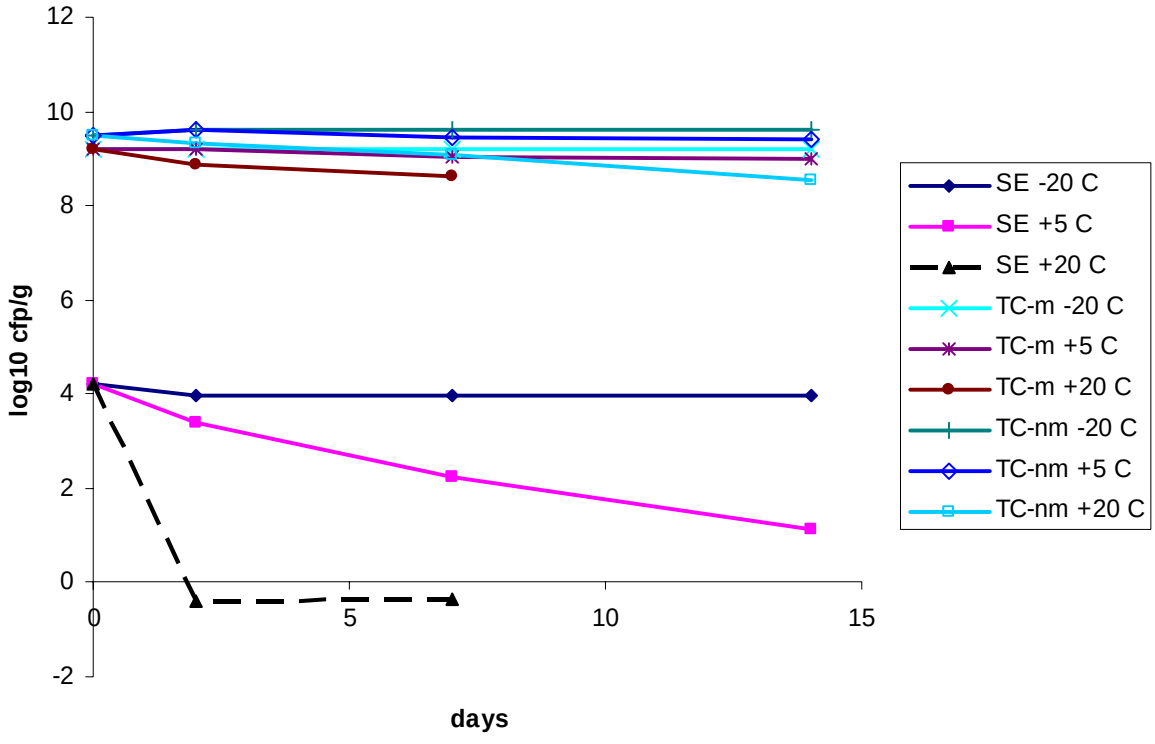

Figure 4 *Log_{10} values of the MPN results of mixed positive chicken faeces containing Salmonella Enteritidis (SE) and the total aerobic counts (TC) of the mixed (m) and unmixed (nm) positive faeces stored at different temperatures (-20 ºC, +5 ºC and +20 ºC)*

Salmonella positive chicken faeces mixed with peptone/glycerol 30% showed stable results for *Salmonella* Enteritidis (10^3-10^4 cfp/g) when stored at -20 ºC, for at least 14 days. Storage at +5 ºC showed almost 2 $\log_{10}$ decrease in the number of cfp after 7 days of storage. At +20 ºC the number of *Salmonella* came below the detection limit after 2 days of storage. The total number of aerobic bacteria (ca 10^9 cfp/g) remained stable for at least 14 days at -20 ºC, + 5 ºC, and +20 ºC, independent whether the faeces was mixed with peptone/glycerol or not.

3.5 Incubation of faeces in dilution steps of 1/10 and 1/100 in BPW

The number of positive isolations of 1/10 and 1/100 dilutions of chicken faeces and capsules in BPW after 4 h and 18 h of incubation is shown in Table 11. The medium combination used was MSRV/BGA.

Table 11 Number of positive isolations of 1/10 and 1/100 dilutions of chicken faeces and capsules after 4 h and 18 h of BPW incubation

Type and number of samples tested	4 h BPW		18 h BPW	
	1/10	1/100	1/10	1/100
***Salmonella* pos (SE) chicken faeces (not mixed); no. of pos.**				
5	5	5	5	5
***Salmonella* pos (SE) chicken faeces mixed with peptone/glycerol 30%; no. of pos.**				
35	35	28	5	33
Negative chicken faeces mixed with peptone/glycerol 30% + capsules; no. of pos.				
STM100; 5	4	3	5	5
SE500; 5	2	0	3	5
Control capsules (no faeces); no. of pos.				
STM100; 5	5	5	5	5
SE500; 5	5	2	5	5
Pig faeces (not mixed); no. of pos.				
98	Not tested	Not tested	24	24

For faeces not mixed with peptone/glycerol and originating from chicken or from pigs, no differences were found in the two dilutions. For chicken faeces mixed with peptone/glycerol 30% the results were variable. More positives results were found in a 1/10 dilution if the BPW was incubated for only 4 h. However, if the same BPW was incubated for 18 h the 1/100 dilution gave more positive results.

3.6 Incubation of chicken faeces and pig faeces in BPW for 4 h and for 18 h

The percentages of positive isolations of control capsules after 4 h and 18 h of incubation of BPW are shown in Table 12 and Figure 5 (tested twice). The medium combination used was MSRV/XLD.

Table 12 Percentages of positive isolations of control capsules (no faeces added) after 4 h and 18 h of incubation of BPW (tested twice)

	Percentages of positives							
Capsules →	**Span 5 (n=2)**		**STM10 (n=3)**		**SE 100 (n=2)**		**SE 500 (n=1)**	
Incubation BPW →	**4h**	**18h**	**4h**	**18h**	**4h**	**18h**	**4h**	**18h**
November 2004	50	100	66	100	50	100	100	100
January 2005	0	100	33	100	0	100	100	100

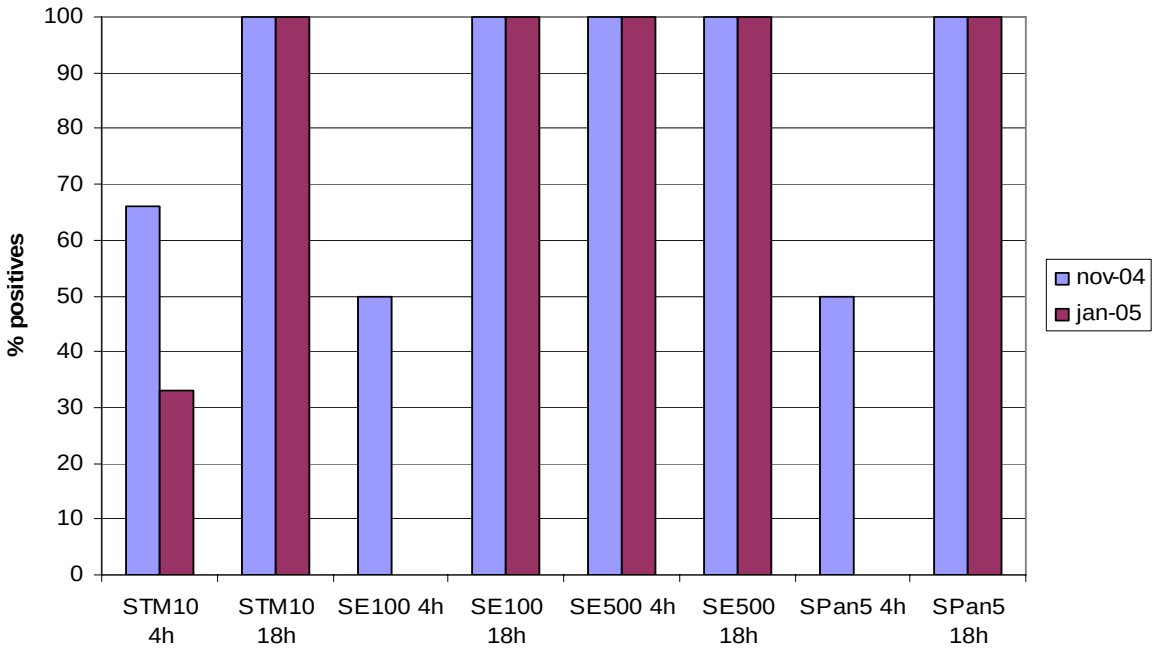

Figure 5 Percentages of positive isolations of control capsules without faeces after 4 h and 18 h of incubation of BPW (tested twice)

The control capsules gave more positive results after 18 h of incubation of BPW than after 4 h of incubation. All samples were found positive for *Salmonella* after 18 h of incubation. The number of positive isolations were variable after 4 h of incubation of BPW.

The percentages of positive isolations of chicken faeces mixed peptone/glycerol 30% artificially contaminated with capsules after 4 h and 18 h of incubation of BPW are shown in Table 13 and Figure 6 (tested three times). The medium combination used was MSRV/XLD.

Table 13 *Percentages of positive isolations of artificially contaminated chicken faeces (capsule + 10 g faeces) mixed with peptone/glycerol 30% after 4 h and 18 h of incubation of BPW (tested three times)*

	Percentages of positives							
Capsules →	**STM10 (n=7)**		**STM 100 (n=4)**		**SE 100 (n=7)**		**SE 500 (n=4)**	
Incubation BPW →	**4h**	**18h**	**4h**	**18h**	**4h**	**18h**	**4h**	**18h**
November 2004	14	86	75	50	28	43	50	100
January 2005	14	28	50	75	14	14	75	0
February 2005	0	71	50	100	0	71	100	0

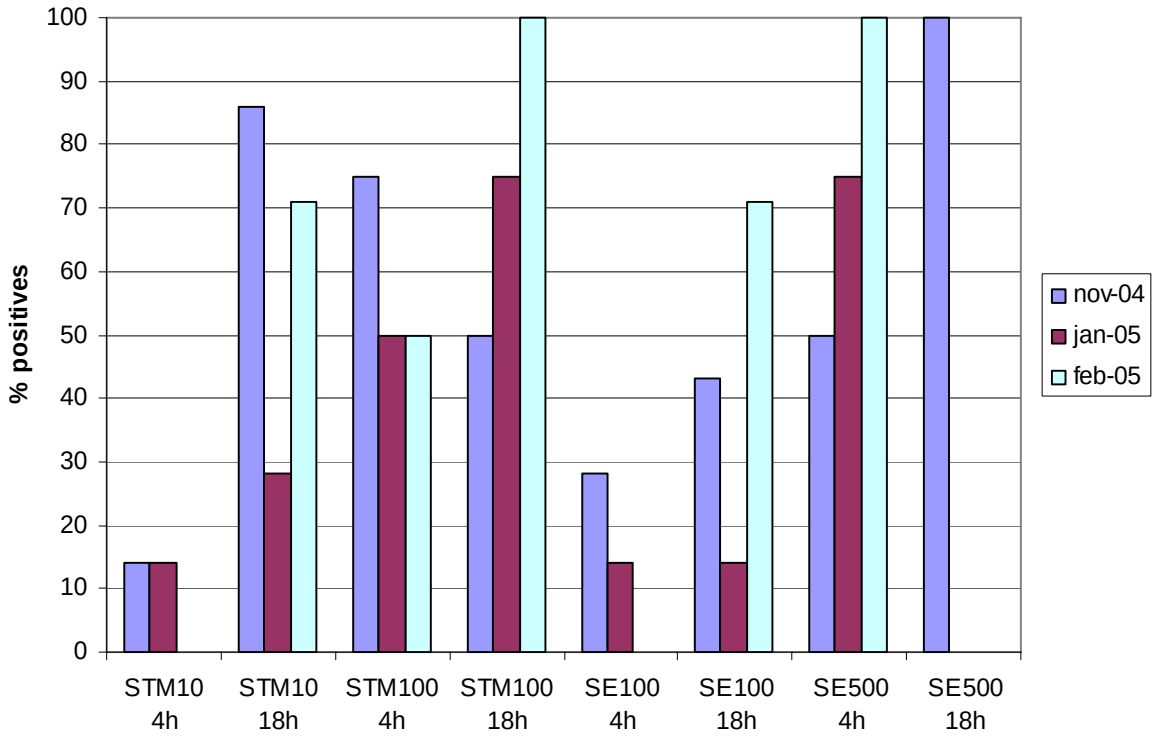

Figure 6 *Percentages of positive isolations of artificially contaminated chicken faeces (capsule + 10 g faeces) mixed with peptone/glycerol 30% after 4 h and 18 h of incubation of BPW (tested three times)*

Salmonella negative chicken faeces mixed with peptone/glycerol 30% and artificially contaminated with *Salmonella* reference materials gave in general more positive results after 18 h of incubation of BPW than after 4 h of incubation, but not all samples were 100% positive.

The percentages of positive isolations of the naturally contaminated chicken faeces mixed with peptone/glycerol 30% and the unmixed pig faeces after 4 h and 18 h of incubation of BPW are shown Table 14 and in Figure 7. MSRV/XLD was used for the chicken faeces and MSRV/BGA was used for the pig faeces.

Table 14 *Percentages of positive isolations of naturally contaminated faeces (of chickens and pigs) unmixed and mixed with peptone/glycerol 30% after 4 h and 18 h of incubation of BPW*

	Percentage positives	
	November-2004	January-2005
Poultry faeces mixed 4h BPW	100	90
Poultry faeces mixed 18h BPW	5	10
Pig faeces 4h BPW unmixed	---	45
Pig faeces 18h BPW unmixed	---	100

For pig faeces the highest number of positives was considered as 100%

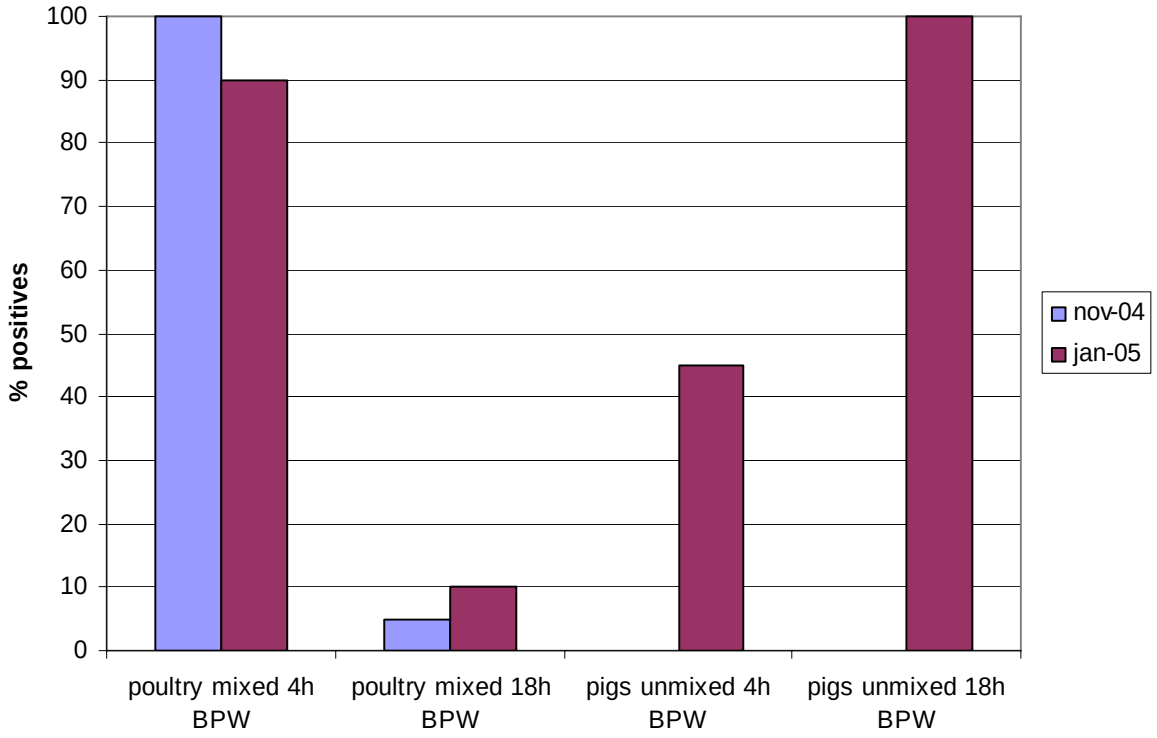

Figure 7 *Percentages of positive isolations of naturally contaminated faeces (of chickens and pigs) unmixed and mixed with peptone/glycerol 30% after 4 h and 18 h of incubation of BPW*

The percentages of positive isolations of the artificially contaminated chicken faeces not mixed with a preservation medium after 4 h and 18 h of incubation of BPW are shown in Table 15 and Figure 8. The medium combination used was MSRV/XLD.

Table 15 *Percentages of positive isolations of artificially contaminated chicken faeces not mixed (capsule + 10 g faeces) after 4 h and 18 h of incubation of BPW*

	Percentages of positives							
Capsules →	**STM10 (n=7)**		**STM 100 (n=4)**		**SE 100 (n=7)**		**SE 500 (n=4)**	
Incubation BPW →	**4h**	**18h**	**4h**	**18h**	**4h**	**18h**	**4h**	**18h**
February 2005	0	100	50	100	0	100	0	100
March 2005	0	100	0	100	0	100	0	100

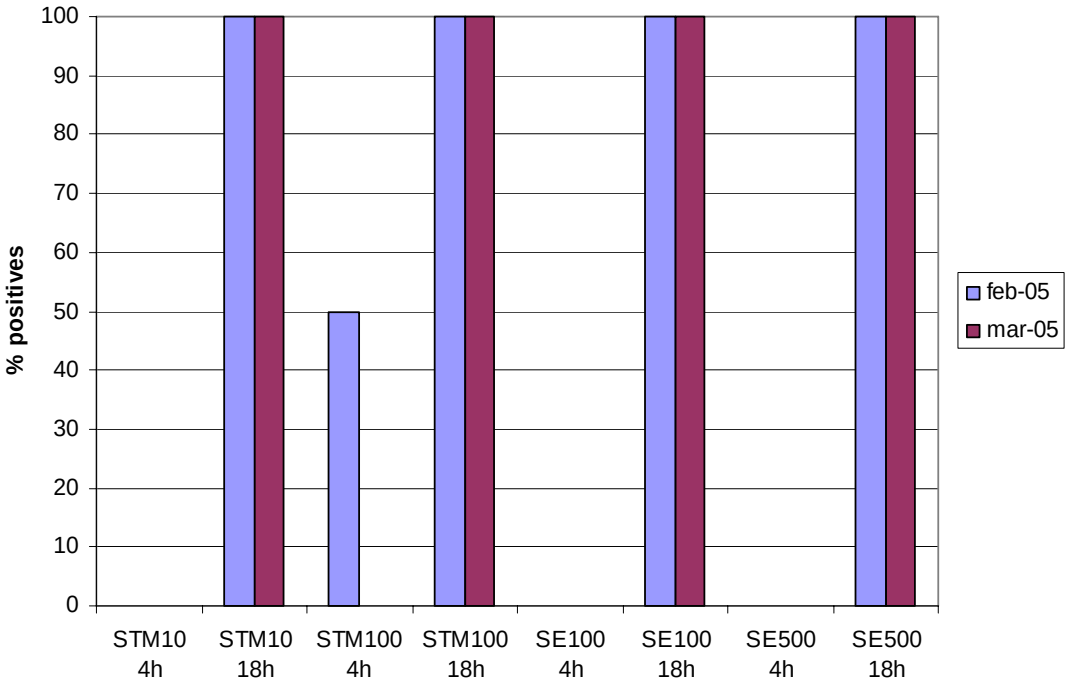

Figure 8 *Percentages of positive isolations of artificially contaminated chicken faeces not mixed (capsule + 10 g faeces) after 4 h and 18 h of incubation of BPW*

Salmonella positive (naturally contaminated) chicken faeces, mixed with peptone/glycerol 30%, showed opposite results to artificially contaminated faeces mixed with peptone/glycerol 30%. Mixed naturally contaminated faeces showed more positive isolations after 4 h of incubation in BPW while mixed artificially contaminated faeces generally showed more positives after 18 h of incubation in BPW. If the faeces was not mixed with peptone/glycerol, originating from chicken or from pigs, 100% positive results were found after 18 h of incubation of BPW. After 4 h of incubation of BPW only a few of these samples were found positive.

3.7 Influence of glycerol on the detection of *Salmonella*

The percentages of positive isolations of artificially contaminated chicken faeces, mixed 1:1 with TSB/glycerol or peptone/glycerol solutions and not mixed faeces, after 4 h and 18 h of incubation of BPW are shown in Table 16 and Figures 9 and 10.

Table 16 Percentages of positive isolations of artificially contaminated chicken faeces (capsule + 10 g faeces) mixed and not mixed with a preservation medium after 4 h and 18 h of incubation of BPW (medium combination MSRV/XLD)

	Percentages of positives							
Capsules →	**STM10 (n=7)**		**STM 100 (n=4)**		**SE 100 (n=7)**		**SE 500 (n=4)**	
Incubation BPW →	**4 h**	**18 h**	**4 h**	**18h**	**4 h**	**18 h**	**4 h**	**18 h**
Faeces mixed with TSB/glycerol 30%	100	14	75	75	14	14	75	75
Faeces mixed with TSB/glycerol 15%	71	86	75	100	14	57	75	100
Faeces mixed with Peptone/glycerol 30%	57	28	75	25	14	14	25	50
Faeces mixed with Peptone/glycerol 15%	57	86	100	100	14	71	50	100
Not mixed faeces	0	100	25	100	0	100	0	100

The isolation of *Salmonella* from the artificially contaminated chicken faeces mixed with a 30% glycerol solution showed in general more positive isolations after 4 h of incubation of BPW than after 18 h of incubation of BPW. However, the faeces mixed with a 15% glycerol solution showed more positive isolations after 18 h of incubation of BPW than after 4 h of incubation of BPW. All unmixed faeces samples artificially contaminated with capsules were tested positive for *Salmonella* after 18 h of incubation of BPW. A high concentration of glycerol in the faeces resulted in less positive isolations after 18 h of incubation of BPW when compared to a low concentration of glycerol or in case of absence of glycerol in the faeces. In Table 17 these results are shown in the sensitivity rates.

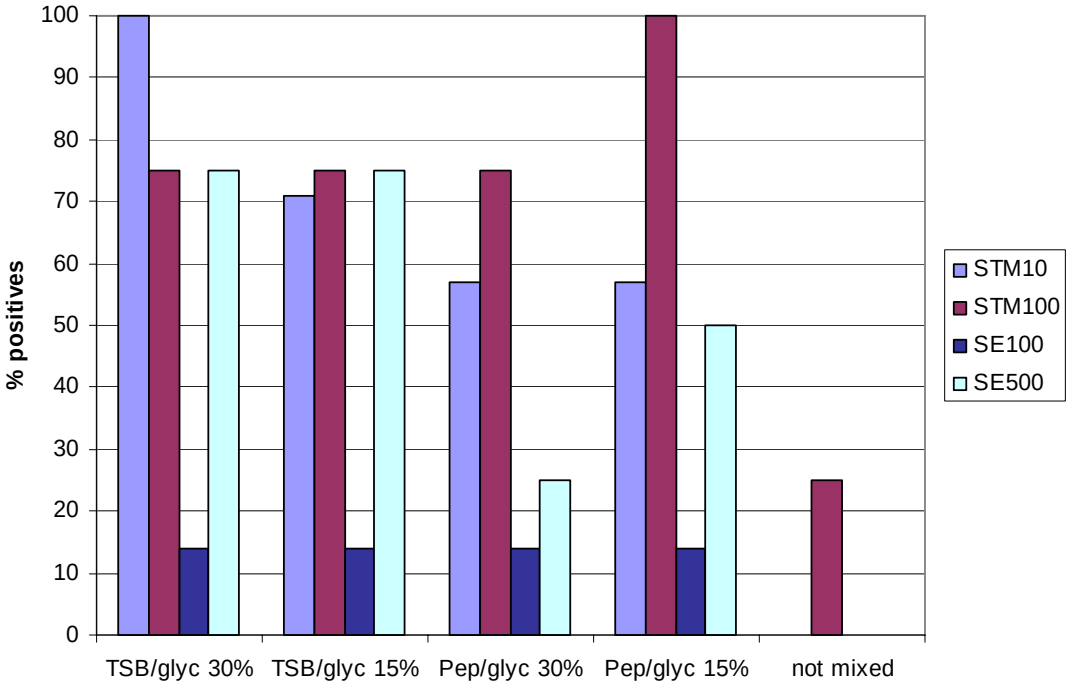

Figure 9 *Percentages of positive isolations of artificially contaminated chicken faeces mixed and not mixed (capsule + 10 g faeces) after 4 h of incubation of BPW (medium combination MSRV/XLD)*

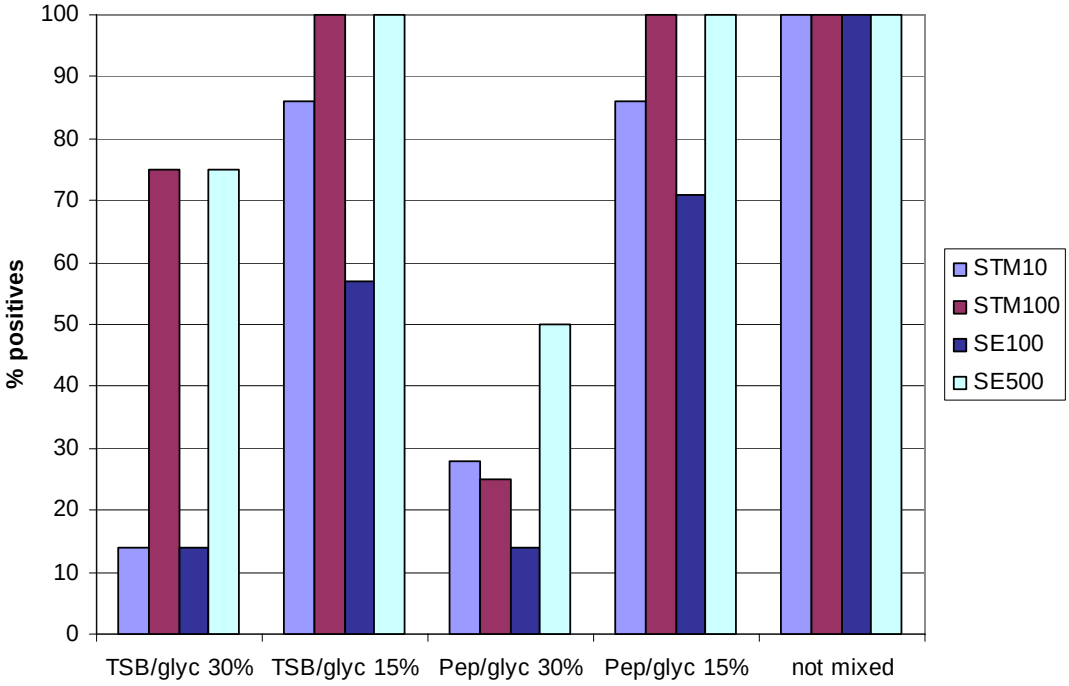

Figure 10 *Percentages of positive isolations of artificially contaminated chicken faeces mixed and not mixed (capsule + 10 g faeces) after 18 h of incubation of BPW (medium combination MSRV/XLD)*

Table 17 Sensitivity rates after 4 h and 18 h of incubation of BPW for chicken faeces, unmixed and mixed with different preservation media and artificially contaminated with capsules (n=22)

		4 h BPW	**18 h BPW**
Unmixed faeces	number of samples	22	22
	positive samples	1	22
	Sensitivity in %	***4.6***	***100.0***
Faeces 1:1 mixed with TSB/glycerol 30%	number of samples	22	22
	positive samples	14	8
	Sensitivity in %	***63.6***	***36.4***
Faeces 1:1 mixed with TSB/glycerol 15%	number of samples	22	22
	positive samples	12	18
	Sensitivity in %	***54.6***	***81.8***
Faeces 1:1 mixed with peptone/glycerol 30%	number of samples	22	22
	positive samples	9	6
	Sensitivity in %	***40.9***	***27.3***
Faeces 1:1 mixed with Peptone/glycerol 15%	number of samples	22	22
	positive samples	11	19
	Sensitivity in %	***50.0***	***86.4***

The sensitivity was calculated according to the following formula:

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

3.8 Comparison between skim milk and peptone/glycerol as preservation media for storage of faeces samples

The percentages of positive isolations of capsules in combination with unmixed chicken faeces, faeces 1:1 mixed with peptone glycerol 15% and faeces 1:1 mixed with double strength skim milk after 4 h and 18 h of incubation of BPW are shown in Table 18 and Figures 11 and 12.

Table 18 *Percentages of positive isolations of artificially contaminated chicken faeces (capsule + 10 g faeces) mixed and unmixed after 4 h and after 18 h of incubation of BPW (medium combination MSRV/XLD)*

	Percentages of positives							
Capsules →	**STM10 (n=7)**		**STM 100 (n=4)**		**SE 100 (n=7)**		**SE 500 (n=4)**	
Incubation BPW →	**4 h**	**18 h**	**4 h**	**18h**	**4 h**	**18 h**	**4 h**	**18 h**
Faeces mixed with peptone/glycerol 15%	43	28	100	25	0	28	75	75
Faeces mixed with double strength skim milk	14	71	50	100	0	57	50	100
Not mixed faeces	14	86	100	100	0	100	75	100

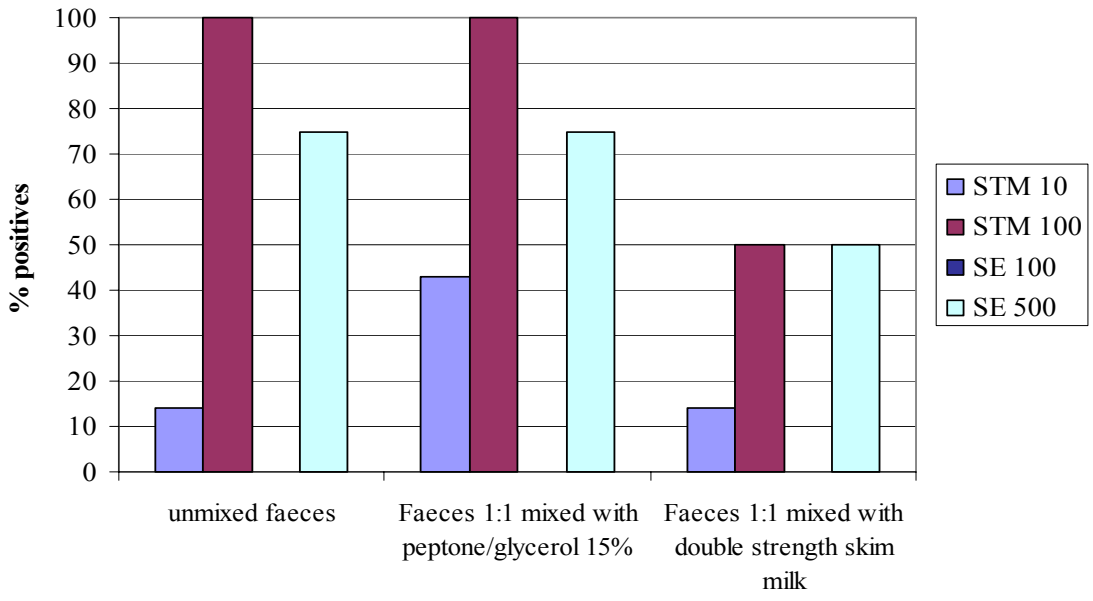

Figure 11 *Percentages of positive isolations of mixed and unmixed chicken faeces artificially contaminated with capsules after 4 h of incubation of BPW (medium combination MSRV/XLD)*

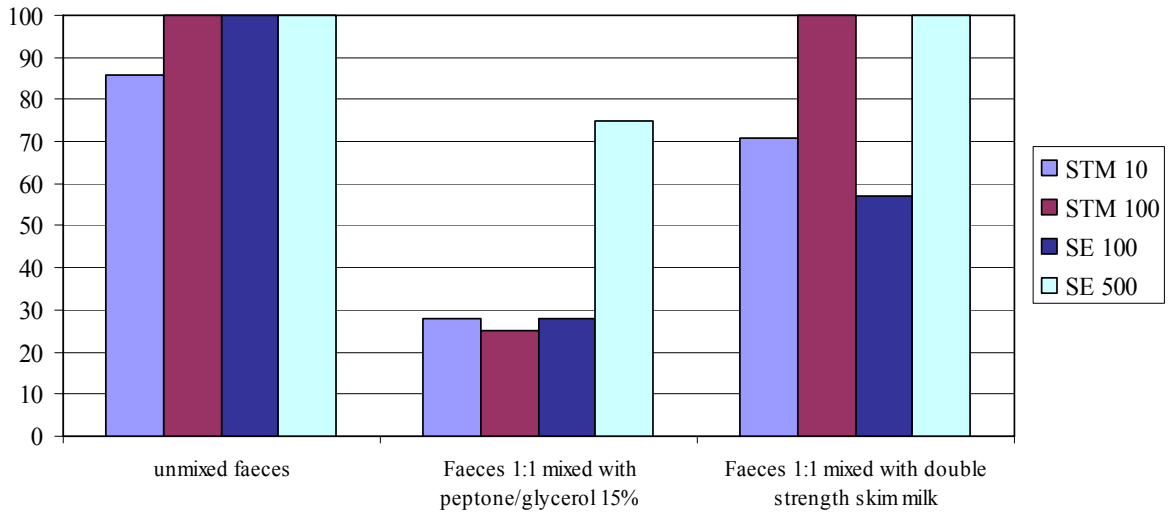

Figure 12 *Percentages of positive isolations of mixed and unmixed chicken faeces artificially contaminated with capsules after 18 h of incubation of BPW (medium combination MSRV/XLD)*

The artificially contaminated faeces samples mixed with the peptone/glycerol solution showed, in contrast with the unmixed faeces and the faeces mixed with double strength skim milk, more positive isolations after 4 h of incubation of BPW than after 18 h of incubation of BPW. The faeces mixed with double strength skim milk gave in general more positive isolations than faeces mixed with peptone/glycerol after 18 h of incubation of BPW. The unmixed faeces showed the highest percentages of positive isolations after 18 h of incubation of BPW. The sensitivity rates for all artificially contaminated faeces samples (n=22) are given in Table 19. The highest sensitivity rate was found for the unmixed artificially contaminated chicken faeces after 18 h of incubation of BPW.

Table 19 *Sensitivity rates after 4 h and 18 h of incubation of BPW for chicken faeces, unmixed and mixed with different preservation media and artificially contaminated with capsules (n=22)*

		4 h BPW	**18 h BPW**
Unmixed faeces	number of samples	22	22
	positive isolations	8	21
	Sensitivity in %	***36.4***	***95.5***
Faeces 1:1 mixed with peptone/glycerol 15%	number of samples	22	22
	positive samples	10	8
	Sensitivity in %	***45.5***	***36.6***
Faeces 1:1 mixed with double strength skim milk	number of samples	22	22
	positive samples	5	17
	Sensitivity in %	***22.7***	***77.3***

For calculation of the sensitivity rate see Table 17.

The log_{10} values of the total number of aerobic bacteria (cfp/gram) of the mixed and unmixed negative chicken faeces stored at different temperatures during 77 days are given it Table 20 and Figure 13.

Table 20 *Log_{10} values of the total aerobic count of mixed and unmixed negative faeces stored at different temperatures for 11 weeks*

	Unmixed faeces	Faeces 1:1 mixed with peptone/glycerol 15%		Faeces 1:1 mixed with double strength skim milk	
Day	stored at (5 ± 3) °C	stored at (5 ± 3) °C	stored at (-20 ± 5) °C	stored at (5 ± 3) °C	stored at (-20 ± 5) °C
0	9.53	9.41	9.41	9.36	9.36
7	9.36	9.38	9.30	9.53	9.44
14	9.51	9.38	9.45	9.56	9.45
28	9.04	8.14	9.05	9.28	9.06
56	8.87	6.21	9.03	9.04	9.08
77	8.63	n.c.	9.02	8.76	9.01

n.c.: not countable because of overgrowth by fungi

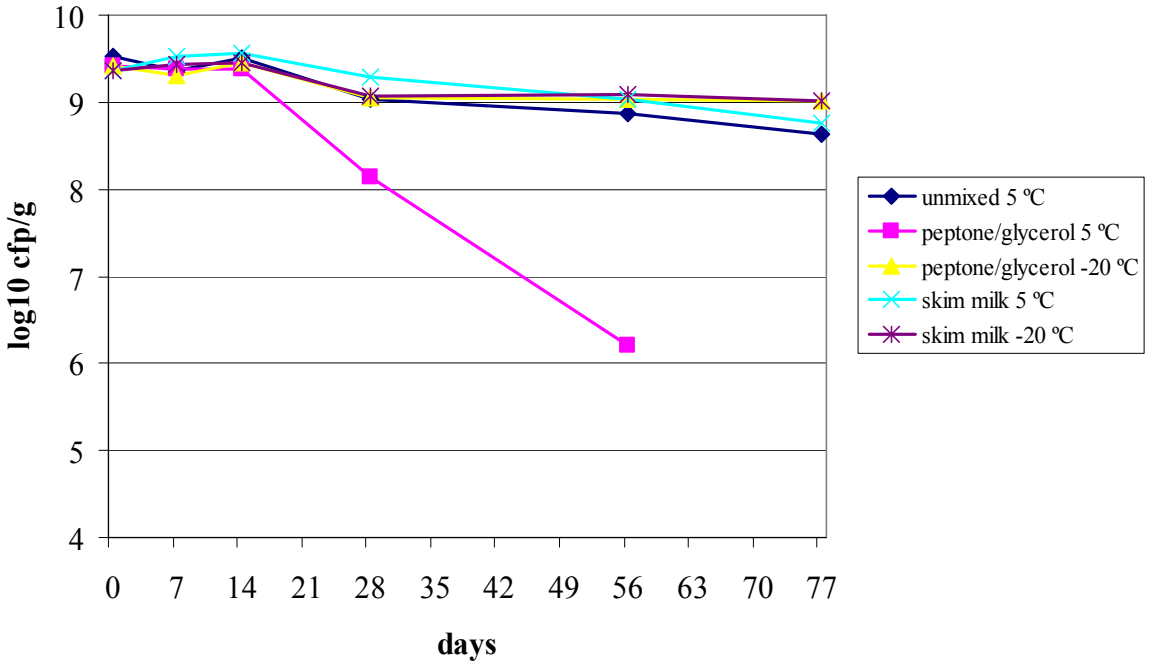

Figure 13 *Log_{10} values of the total aerobic counts of the mixed and unmixed negative faeces stored at different temperatures for 11 weeks*

The total number of aerobic bacteria of mixed and unmixed faeces samples stored at (5 ± 3) ºC and of mixed faeces stored at (-20 ± 5) ºC were stable for at least 11 weeks. The total number of aerobic bacteria of the faeces mixed with peptone/glycerol solution stored at

(5 ± 3) ºC was only stable for 2 weeks. After 11 weeks of storage at (5 ± 3) ºC of faeces mixed with peptone/glycerol solution, the faeces contained a lot of fungi which made it impossible to determine the total number of aerobic bacteria.
The log_{10} values of the total number of Enterobacteriaceae (cfp/gram) of the mixed and unmixed negative chicken faeces stored at different temperatures during 77 days are given it Table 21 and Figure 14.

Table 21 *Log_{10} values of the number of Enterobacteriaceae of the mixed and unmixed negative faeces stored at different temperatures for 11 weeks*

	Unmixed faeces	Faeces 1:1 mixed with peptone/glycerol 15%		Faeces 1:1 mixed with double strength skim milk	
Day	stored at (5 ± 3) °C	stored at (5 ± 3) °C	stored at (-20 ± 5) °C	stored at (5 ± 3) °C	stored at (-20 ± 5) °C
0	7.04	7.04	7.04	7.02	7.02
7	6.50	7.04	7.20	7.18	6.87
14	6.19	6.89	6.69	7.24	6.31
28	4.36	4.02	6.87	6.89	6.24
56	0	0	6.13	6.00	6.06
77	0	0	6.28	0	6.88

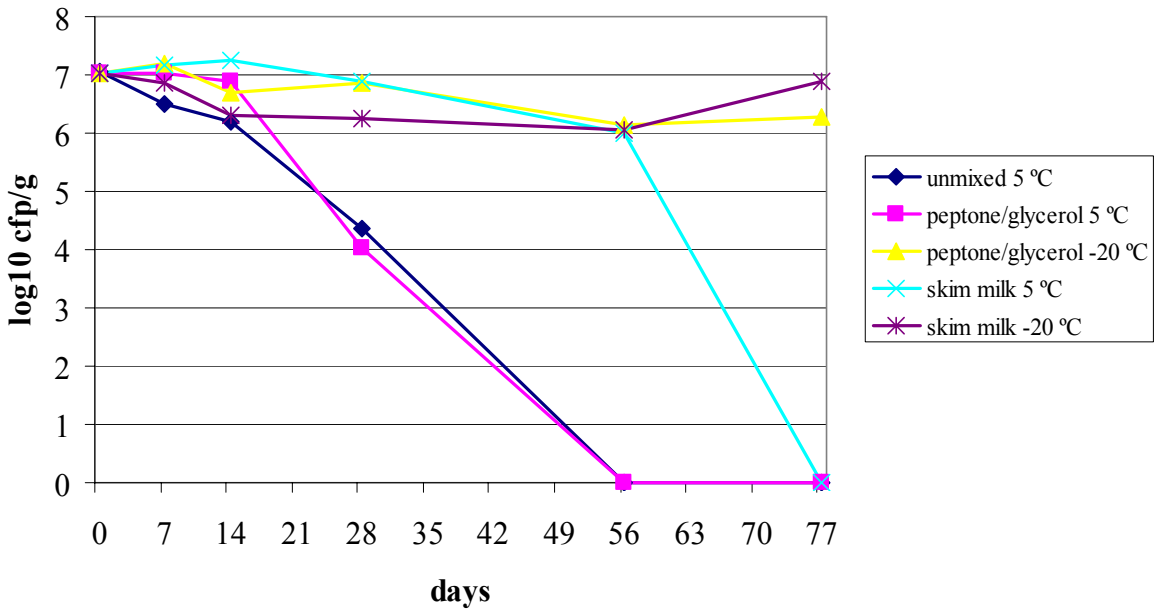

Figure 14 *Log_{10} values of the number of Enterobacteriaceae of the mixed and unmixed negative faeces stored at different temperatures for 11 weeks*

The number of Enterobacteriaceae of the unmixed chicken faeces and the chicken faeces mixed with peptone/glycerol 15% stored at (5 ± 3) ºC was stable for 2 weeks. The number of Enterobacteriaceae of faeces mixed with double strength skim milk stored at (5 ± 3) ºC was stable for 8 weeks. The mixed faeces stored at (-20 ± 5) ºC showed a stable number of Enterobacteriaceae for at least 11 weeks.

3.9 Influence of glycerol on the growth of *Salmonella*

The growth curves of *Salmonella* Typhimurium and of *Salmonella* Enteritidis in BPW, containing 0.75% glycerol, 1.5% glycerol or no glycerol, incubated at (37 ± 1) ^{o}C for 24 h are given in Tables 22, 23 and Figures 15 and 16. The BPW was initially inoculated with one capsule STM100 or one capsule SE 100

Table 22 *Number of S. Typhimurium grown in BPW, containing different concentrations of glycerol, incubated at (37 ± 1) ^{o}C for 24 h. Initial inoculum in BPW was one capsule STM100*

Time (hours)	BPW without glycerol (cfp/ml)	0.75% glycerol in BPW (cfp/ml)	1.5% glycerol in BPW (cfp/ml)
0	1.0E+00	1.0E+00	1.0E+00
2	1.0E+00	1.5E+00	1.0E+00
4	7.5E+01	4.0E+01	6.5E+01
6	2.5E+03	1.8E+03	2.6E+03
8	8.2E+04	7.4E+04	6.5E+04
24	9.7E+08	4.2E+07	1.3E+07

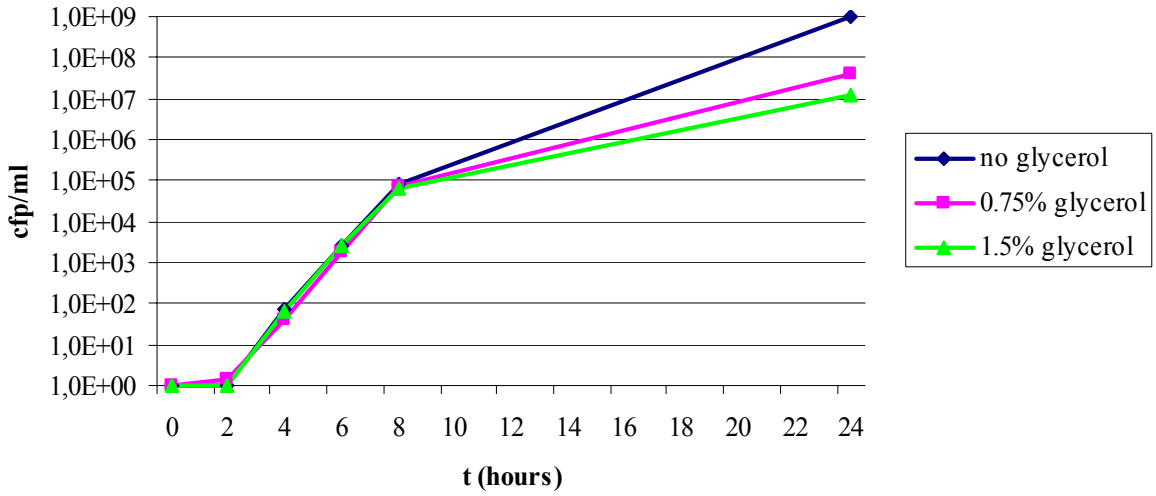

Figure 15 *Number of S. Typhimurium during 24 h of incubation in BPW containing different concentrations of glycerol, and incubated at (37 ± 1) ^{o}C. Initial inoculum in BPW was one capsule STM100*

Until 8 h of incubation of *S.* Typhimurium in BPW at (37 ± 1) °C, no differences were found between the three different BPW solutions in the number of *S.* Typhimurium. After 24 h of incubation, the final concentration of *S.* Typhimurium was 2 $\log_{10}$ lower in BPW containing 1.5% glycerol than in BPW without glycerol. In BPW containing 0.75% glycerol the concentration of *Salmonella* was 1 $\log_{10}$ (cfp/ml) lower when compared to BPW without glycerol.

Table 23 *Number of S. Enteritidis grown in BPW containing different concentrations of glycerol, incubated at (37 ± 1) °C for 24 h. Initial inoculum in BPW was one capsule SE100*

	SE100		
Time (hours)	**BPW without glycerol (cfp/ml)**	**0.75% glycerol in BPW (cfp/ml)**	**1.5% glycerol in BPW (cfp/ml)**
0	1.0E+00	1.0E+00	1.0E+00
2	1.0E+00	1.0E+00	1.0E+00
4	1.0E+00	1.5E+01	1.0E+00
6	1.8E+02	2.0E+02	1.1E+02
8	5.7E+03	5.3E+03	2.3E+03
24	1.0E+09	1.0E+08	1.9E+08

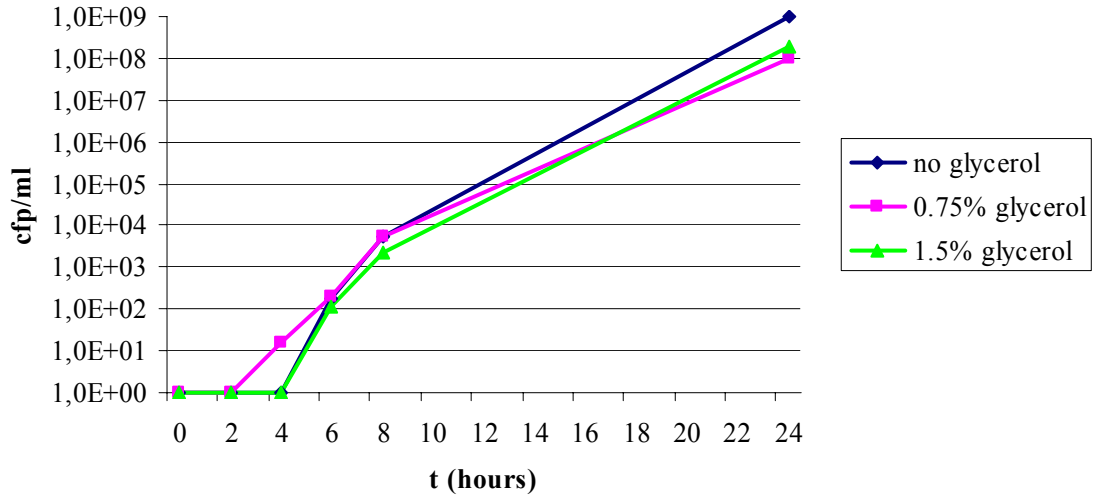

Figure 16 *Number of S. Enteritidis during 24 h of incubation in BPW containing different concentrations glycerol and incubated at (37 ± 1) °C. Initial inoculum in BPW was one capsule SE100*

S. Enteritidis grew slower than *S.* Typhimurium during the first 8 h of incubation in BPW at (37 ± 1) °C. Until 8 h of incubation, no differences were found between the three different BPW solutions in the number of *S.* Enteritidis. After 24 h of incubation, the final concentration of *S.* Enteritidis was 1 $\log_{10}$ lower in BPW containing glycerol than in BPW without glycerol.

4. Discussion and conclusions

ISO 6579 is primarily intended for the isolation of *Salmonella* spp. from food and feeding stuffs and is not always suitable for the detection of *Salmonella* spp. from other matrices. For the isolation of *Salmonella* from animal faeces and environmental samples, many studies have shown that MSRV is an appropriate medium for selective enrichment. Therefore a new annex of ISO 6579 was drafted, describing this medium.
This new Annex D of ISO 6579 describes the detection of *Salmonella* spp. in:
- animal faeces (like poultry, pigs, cattle);
- environmental samples in the area of the primary production stage (like dust).

MSRV is intended for the detection of motile Salmonellae and is less appropriate for the detection of non-motile Salmonellae. If non-motiles are expected it is advised to use beside MSRV also a liquid selective enrichment broth like Rappaport-Vassiliadis.

The addition of novobiocin to the MSRV facilitates the detection of *Salmonella* by inhibiting the competitive background flora. On the other hand, novobiocin can negatively influence the motility of micro-organisms (Soutourina et al., 2001). This was clearly shown, as for all tested samples (faeces of chicken, cattle and pigs and dust) the migration of *Salmonella* on MSRV containing a low concentration of novobiocin (0.01 g/L) was larger than on MSRV containing a higher concentration of novobiocin (0.02 g/L). Furthermore, more pig faeces samples were found positive for *Salmonella* when using MSRV containing 0.01 g/L novobiocin instead of MSRV containing 0.02 g/L novobiocin.

The best storage temperature for *Salmonella* positive chicken faeces mixed with peptone/glycerol 30% was (-20 ± 5) ^{o}C. The number of *Salmonella* and the number of aerobic bacteria remained stable for at least 14 days. A higher storage temperature revealed a decrease in the number of *Salmonella*. The number of aerobic bacteria remained stable for at least 14 days when stored at different temperatures (in the range of -20 ºC up to +20 ºC), independent whether the faeces was mixed or not.

No significant correlation was found between the dilution factor in BPW and the influence of the background flora on the detection of *Salmonella*. For pig and chicken faeces not mixed with peptone/glycerol and for control capsules no differences were found between a 1/10 dilution and a 1/100 dilution in BPW. Variable results were found when the chicken faeces was mixed with peptone/glycerol 30%. After 4 h of incubation of BPW, more *Salmonella* positive samples were found in the 1/10 dilution in BPW when compared to the 1/100 dilution in BPW. If the same BPW was incubated for 18 h, more *Salmonella* positive samples were found in the 1/100 dilution in BPW.

The two tested incubation times of BPW (4 h and 18 h) showed variable results. For control capsules, artificially contaminated chicken faeces and for naturally contaminated pig faeces

(both latter not mixed with a preservation medium), more positive results were found after 18 h of incubation of BPW when compared to 4 h of incubation. However, when the faeces was mixed with peptone/glycerol different results were found. Naturally contaminated chicken faeces mixed with peptone/glycerol 30% showed much more positive results after 4 h of incubation of BPW than after 18 h of incubation of BPW. However, artificially contaminated chicken faeces mixed with peptone/glycerol 30% showed in general more positive results after 18 h of incubation of BPW. The variable results found with faeces mixed with peptone/glycerol as well as the information as described by Chun et al (1972), resulted in the decision to further test the influence of glycerol on the detection of *Salmonella*. Chicken faeces mixed (1:1) with a preservation medium containing 30% ($^{v}/_{v}$) glycerol showed less positive isolations after 18 h of incubation of BPW when compared to faeces mixed (1:1) with preservation media containing 15% ($^{v}/_{v}$) glycerol or when compared to unmixed faeces. All unmixed faeces samples were found positive after 18 h of incubation of BPW. These results indicated that glycerol may have a negative influence on the detection of *Salmonella*. The reason for adding glycerol to the faeces was to stabilise the micro-organisms at low temperatures. This works well as was shown in the stability studies. Skim milk, which contains no glycerol, was tried as alternative preservation medium. When the faeces were mixed with double strength skim milk and stored at (-20 ± 5) ^{o}C, the total number of aerobic bacteria and the number of Enterobacteriaceae remained stable for at least 11 weeks. These results are similar to those found with faeces mixed with peptone/glycerol. When stored at (5 ± 3) ºC, the number of Enterobacteriaceae was stable for 9 weeks. When these mixed faeces were artificially contaminated with *Salmonella* reference materials, 77% of the samples were found positive for *Salmonella* after 18 h of incubation of BPW. Whereas 100% positives would have been expected. With unmixed faeces, artificially contaminated with *Salmonella* reference materials, 95% of the samples were found positive after 18 h of incubation of BPW, showing that also skim milk seems to influence the detection of *Salmonella*. It was therefore decided to no longer mix the faeces with a preservation medium. Even in unmixed faeces, the total number of aerobic bacteria was stable for at least 11 weeks and the number of Enterobacteriaceae was still stable for two weeks. These results indicate that for interlaboratory comparison studies it is possible to use unmixed faeces which can be stored for a few weeks at 5 ^{o}C.

The experiments to determine the effect of glycerol on the growth of *Salmonella*, showed again that glycerol had a negative influence on the growth of *Salmonella*. The negative influence of glycerol on the growth of *Salmonella* could explain the variable results which were found in the experiments where faeces mixed with a glycerol solution were used. The highest number of positive *Salmonella* isolations was found when unmixed faeces were used, incubated for 18 h in BPW and further analysed on MSRV containing 0.01 g/L novobiocin.

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Annex 1. Calculation of T2

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. Hence $T_2/(I-1)$ is expected to be equal to one. For the variation between capsules of one batch, the Poisson distribution is the theoretical smallest possible variation which could be achieved. However overdispersion is expected and $T_2/(I-1)$ will mostly be larger than 1 (Heisterkamp et al., 1993). The general accepted variation for a batch will therefore be $T_2/(I-1) \leq 2$.

Annex 2. Draft Annex D of ISO 6579 (Sept '06)

Annex D
(normative)

Detection of *Salmonella* spp. in animal faeces and in samples of the primary production stage

D.1 Introduction

ISO 6579 is primarily intended for the isolation of *Salmonella* spp. from food and feeding stuffs and is not always suitable for the detection of *Salmonella* spp. from other matrices.

This annex is applicable to the detection of *Salmonella* spp. in:

- animal faeces (like poultry, pigs, cattle);
- environmental samples in the area of the primary production stage (like dust).

The method in this annex is based upon ISO 6579, with a different selective enrichment. Therefore, where possible reference will be made to the full text of ISO 6579.

The selective enrichment medium as described in this annex (being Modified Semi-solid Rappaport Vassiliadis: MSRV) is intended for the detection of motile Salmonellae and is not appropriate for the detection of non-motile Salmonellae.

NOTE The non-motile *Salmonella* serovars *Salmonella* Gallinarum and *Salmonella* Pullorum do not seem to survive long in environmental samples and will therefore rarely be detected in faecal or environmental (like dust) samples (regardless of the method). The number of other non-motile *Salmonella* serovars in faecal samples seems to be generally low. For example, in a study of Voogt et al (2001) in which circa 1 000 faecal samples of poultry layer flocks and circa 900 faecal samples of broiler flocks were analysed, less than 1% of the total number of samples were positive in a selective broth and at the same time negative on MSRV (and likely to be non-motile). Similar results were found in a Dutch study with circa 3 200 faecal samples of pigs (non-published data). On the other hand, in the case of the Voogt study, up to almost 40% of positive samples would not have been detected (i.e. false negatives) if only a selective broth (in this case Rappaport Vassiliadis) had been used instead of a semi-solid medium.

D.2 Normative references

See Ch. 2 of ISO 6579

Additional:

ENV ISO 11133-1: 2000, Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media – Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory

ISO/TS 11133-2: 2003, Microbiology of food and animal feeding stuffs – Guidelines on preparation and production of culture media – Part 2: Practical guidelines on performance testing of culture media.

D.3 Terms and definitions

See Ch. 3 of ISO 6579.

D.4 Principle

D.4.1 General

The detection of *Salmonella* in animal faeces and in samples of the primary production stage necessitates four stages, as described in Ch. 4 of ISO 6579.

D.4.2 Pre-enrichment in non-selective liquid medium

Buffered Peptone Water (BPW) is inoculated at ambient temperature with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h.

D.4.3 Enrichment on selective semi-solid medium

Modified Semi-solid Rappaport Vassiliadis (MSRV) agar plates are inoculated with the culture obtained in D.4.2.

The MSRV is incubated at 41.5 °C ± 1 °C for 24 h ± 3 h. If a plate is negative after 24 h it is incubated for a further 24 h ± 3 h.

D.4.4 Selective plating and identification

From the culture obtained in D.4.3, two selective solid media are inoculated:

- Xylose Lysine Deoxycholate (XLD) agar;
- Any other solid selective medium complementary to XLD agar (see 4.4 of ISO 6579).

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h ± 3 h.
The second selective medium is incubated in accordance with the manufacturer's instructions.

D.4.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated-out as described in D.4.4, and their identity is confirmed by means of appropriate biochemical and serological tests.

D.5 Culture media, reagents and sera

D.5.1 General

For current laboratory practice, see ISO 7218

All media and reagents needed for this annex are described in Annex B of ISO 6579, except for Modified Semi-solid Rappaport Vassiliadis (MSRV) medium, which is described in D.5.2. Alternatively, dehydrated complete media or diluents may be used. Follow, in that respect, the manufacturer's instructions.

NOTE The composition of MSRV as described by De Smedt et al. (1986), contained 20 mg/L novobiocin. However, from a scientific point of view, 10 mg/L novobiocin is preferred. In studies performed at the CRL-*Salmonella,* more *Salmonella* positive results were found in pig faeces samples when tested with MSRV containing 10 mg/L than with MSRV containing 20 mg/L novobiocin (Veenman et al., 2006). Furthermore, when testing different animal faeces (pigs, chicken, cattle) and naturally contaminated dust, the migration zones on MSRV containing 10 mg/L novobiocin were (much) larger than on MSRV containing 20 mg/L novobiocin (Veenman *et al.,* 2006). Influence of novobiocin on bacterial motility is earlier described by Soutourina et al. (2001).

For the preparation of the selective plating agar media (see B.4, XLD-agar) standard size Petri dishes can be used (90 mm or 100 mm) instead of large size Petri dishes (140 mm).

D.5.2 Modified Semi-solid Rappaport Vassiliadis medium (MSRV)

D.5.2.1 Base medium

Composition

Enzymatic digest of casein	4.6	g
Acid hydrolysate of casein	4.6	g
Sodium chloride (NaCl)	7.3	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5	g
Magnesium chloride anhydrous ($MgCl_2$)	10.9	g
Malachite green oxalate	0.04	g
Agar	2.7	g
Water	1 000	ml

Preparation

Suspend the ingredients into the water.
Heat to boiling with agitation. **Do not autoclave**.
Do not hold the medium at high temperatures longer than necessary.
Cool the medium to 47-50 °C.

D.5.2.2Novobiocin solution

Composition

Novobiocin sodium salt	0.05	g
Water	10	ml

Preparation

Dissolve the novobiocin sodium salt in the water.
Sterilize by filtration through a filter with a pore size of 0.22 μm.
The solution can be stored for up to 4 weeks at 5 °C ± 3 °C or in small portions (e.g. of 2 ml) at –20 °C for up to one year.

D.5.2.3Complete medium

Composition

Base medium (D.5.2.1)	1 000	ml
Novobiocin solution (D.5.2.2)	2	ml

Preparation

Aseptically add 2 ml of the novobiocin solution (D.5.2.2) to 1000 ml of base medium (D.5.2.1) at 47-50 °C. Mix carefully.
The final pH should be 5.2 (5.1 – 5.4) at 20-25 °C.
Pour carefully into plates up to a final volume of 15-20 ml in Petri dishes with a diameter of 90 mm.
Allow the medium to solidify before moving and handle with care.
Store the plates, **with surface upwards**, for up to 2 weeks at 5 °C ± 3 °C in the dark.
Do not invert the plates, as the semi-solid agar is too sloppy to do so.
Any plates in which the semi-solid agar has liquefied or fragmented should not be used.
Immediately before use, and only if necessary, dry the surface of the agar plates carefully, for example by placing them with the lids off and the agar surface **upwards** in a Laminar Air Flow cabinet. Mind not to overdry the medium.

D.6 Apparatus and glassware

See Ch. 6 of ISO 6579.

Additional:

Sterile loops of 1 µl

D.7 Sampling

See Ch. 7 of ISO 6579.

D.8 Preparation of test sample

See Ch. 8 of ISO 6579.

Generally an amount of sample is added to a quantity of BPW to yield a 1/10 dilution (e.g. 25 g of sample added to 225 ml of BPW). However, for some type of samples it may be necessary to use another ratio.

D.9 Procedure

D.9.1 Non-selective pre-enrichment

Pre-warm the BPW to room temperature before use.
Mix samples well by the most suitable means for the sample type.
Weigh the sample and add it to the appropriate quantity of BPW (see D.8). Incubate the jars at 37 °C ± 1 °C for 18 h ± 2 h.

D.9.2 Selective enrichment

Allow the MSRV plates to equilibrate at room temperature if they were stored at a lower temperature.
Inoculate the MSRV plates with 3 drops of incubated BPW culture. The 3 drops should total 0.1 ml and be placed separately and equally spaced on the surface of the medium.

NOTE When taking a subculture from BPW, it is very important not to disturb particulate samples. Therefore, containers should be moved carefully, and not mixed, shaken or swirled. Aim to extract an inoculum from the largest volume of free fluid nearest the interface between container and surface of culture, but it is advisable to go deeper if there are particulates floating on the surface.

Incubate the inoculated MSRV plates at 41.5 °C ± 1 °C for 24 h ± 3 h.
Do not invert the plates.

Positive plates will show a grey-white, turbid zone extending out from the inoculated drop. The turbid zone is characterized by a white halo with a clearly defined edge.

If the plates are negative after 24 h, reincubate for a further 24 h ± 3 h.

D.9.3 Selective plating

Allow the Xylose Lysine Deoxycholate agar (XLD) plates and the second selective plating medium (see 5.2.4.2 of ISO 6579) to equilibrate at room temperature if they were stored at a lower temperature. If necessary dry the surface of the plates before use.
Observe the MSRV plate (if necessary on a clear white surface or light box). Determine where the furthest point of spread of opaque growth from the inoculation points is and dip a loop of 1 µl just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV are extracted. Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating medium using a new sterile loop.

NOTE 1 By plating-out little material from MSRV (using a 1 µl loop), well isolated colonies can be obtained by using only one standard size Petri dish (90-100 mm) with selective plating agar. The use of large dishes (140 mm) will therefore not be necessary.

Incubate the XLD plates inverted at 37 °C ± 1 °C for 24 h ± 3 h.
Incubate the second selective plating medium in accordance with the manufacturer's instructions.

Return negative MSRV plates to the 41.5 °C incubator and incubate for a further 24 h ± 3 h. Repeat the selective plating procedure after 48 h of incubation of MSRV.

Typical colonies of *Salmonella* grown on XLD-agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE 2 *Salmonella* H_2S negative variants (e.g. *Salmonella* Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening (also see 9.4.4 of ISO 6579).

Check the second selective plating medium after the appropriate incubation time for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

D.9.4 Confirmation

For confirmation of the typical colonies, isolated on the selective plating media, follow the instructions as given in Ch. 9.5 of ISO 6579. In 9.5.2. of ISO 6579 it is prescribed to streak isolated colonies from the selective plating media onto nutrient agar before performing the biochemical confirmation. However, this extra cultural step is not necessary if well-isolated colonies (of a pure culture) are available on the selective plating media. If this is the case perform the biochemical confirmation directly on a typical (suspect), well-isolated colony of each selective plating medium.

D.10 Expression of results

See Ch. 10 of ISO 6579.

D.11 Test report

See Ch. 11 of ISO 6579.

D.12 Quality assurance

See Ch. 12 of ISO 6579.

For the performance testing of media, the information as described in ENV ISO 1133-1 and in ISO/TS 11133-2 is followed. However, in these ISO documents, procedures are given for selective broths as well as for selective agar media for the detection of *Salmonella,* but not for semi-solid media like MSRV. The procedure given below can be used for testing the performance of MSRV and is based upon the procedure and test strains as described for selective (enrichment) media for the detection of *Salmonella* (like MKTTn and RVS, see B.2 and B.3 of ISO 6579) in ISO/TS 11133-2.

The procedure given below has been extracted from ISO/TS 11133-2, 5.4.2.1, but with an adapted concentration of the test strains. The references given are references to the chapters of ISO/TS 11133-2. The procedure, test strains and criteria are summarised in Table 1.

- Inoculation of target micro-organisms: Inoculate MSRV for each test organism with *ca.* 10^4 cfu/ 0,1 ml (for preparation of the inoculum see 5.2.1);
- Inoculation of non-target micro-organisms: Inoculate MSRV for each test organism with $10^5 – 10^6$ cfu/ 0.1 ml (for preparation of the inoculum see 5.2.1);
- Inoculation of target and non-target micro-organisms as a mixed culture: Inoculate MSRV with a mixed culture containing *ca.* 10^4 cfu/ 0,1 ml of target micro-organisms and $10^5 – 10^6$ cfu/ 0,1 ml of non-target micro-organisms (for preparation of the inoculums see 5.2.1).

Incubate the MSRV plates at 41.5 °C ± 1 °C and assess the plates after 24 h ± 3 h and after 48 h ± 6 h.

Table 1 Performance testing of MSRV

Function	Control strains	Final concentration in the inoculum of 0.1 ml	Incubation of MSRV	Criteria
Specificity	*S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076	10^4 cfu	41.5 °C ± 1 °C, 2x 24 h ± 3 h	Grey-white, turbid zone extending out from the inoculated drop. After 48 h, the turbid zones of the 3 drops will be (almost) fully migrated over the plate
Selectivity	*E. coli* ATCC 25922 or ATCC 8739 *E. faecalis* ATCC 29212 or ATCC 19433	10^5 – 10^6 cfu	41.5 °C ± 1 °C, 2x 24 h ± 3 h	Possible growth at the place of the inoculated drop without a turbid zone
Productivity	*S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076 + *E. coli* ATCC 25922 or ATCC 8739 + *P. aeruginosa* ATCC 27853	10^4 cfu 10^5 – 10^6 cfu 10^5 – 10^6 cfu	41.5 °C ± 1 °C, 2x 24 h ± 3 h	Grey-white, turbid zone extending out from the inoculated drop. After 48 h, the turbid zones of the 3 drops will be (almost) fully migrated over the plate Possible extra: subculture with 1 µl loop just inside the border of the opaque growth and spread onto XLD. Incubate at 37 °C ± 1 °C for 24 h ± 3 h. Criteria: growth of characteristic colonies in majority

Remark: In general *S.* Typhimurium will show faster growth and larger migration zones than *S.* Enteritidis.