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

Verification of methods following EN ISO 16140-3; Practice

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Method validation

Salmonella

NEN-EN-ISO 6579-1:2017

INTERNATIONAL STANDARD

ISO 6579-1

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2017-02

Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 1:
Detection of *Salmonella* spp.

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche, le dénombrement et le sérotypage des Salmonella *—*

Partie 1: Recherche des Salmonella *spp.*

1 Scope

This document specifies a horizontal method for the detection of *Salmonella*. It is applicable to the following:

— products intended for human consumption and the feeding of animals;

— environmental samples in the area of food production and food handling;

— samples from the primary production stage such as animal faeces, dust, and swabs.

With this horizontal method, most of the *Salmonella* serovars are intended to be detected. For the detection of some specific serovars, additional culture steps may be needed. For *Salmonella* Typhi and *Salmonella* Paratyphi, the procedure is described in Annex D.

The selective enrichment medium modified semi-solid Rappaport-Vassiliadis (MSRV) agar is intended for the detection of motile *Salmonella* and is not appropriate for the detection of non-motile *Salmonella* strains.

ISO 16140-3 Verification requirements for a qualitative (detection) method

Method	Performance characteristics	Implementation Verification	(Food) item verification
Qualitative	Estimated LOD_{50} ($eLOD_{50}$)	✓	✓

- **$eLOD_{50}$**: 3 available protocols to determine the $eLOD_{50}$

Implementation Verification

- **Demonstrate competence of the user laboratory to perform the method**

  (obtain expected results on a (food) item used in the validation study)

- **The user laboratory shall:**
  1. review **validation data** for the method,
  2. select 1 (food) item tested during the validation study that belongs within the **scope of laboratory application** of the user laboratory, and
  3. use this **1 (food) item** and the **sample size** used in the validation study to perform implementation verification.

C.1 Performance characteristics RVS broth and MKTTn broth

International interlaboratory studies were organized in 2000 in the frame of the European project SMT CT 96 2098 (see References [8] and [15]). These studies involved 11 laboratories in nine countries in Europe and 10 laboratories in USA and were carried out on fresh cheese curd, dried egg powder, raw poultry meat, and a reference material. The food samples were each tested at low and high levels of contamination, plus a negative control.

The method submitted to the interlaboratory studies was that of ISO 6579:2002 (see Reference [1]), including selective enrichment in RVS broth and MKTTn broth. The procedure for detection of *Salmonella* in food samples as described in ISO 6579:2002 is comparable to the procedure as described in this document.

Table C.3 — Results of data analysis obtained with raw poultry meat samples

Parameter	Trial I raw poultry meat (blank)	Trial I raw poultry meat (low level contamination)[a]	Trial I raw poultry meat (high level contamination)[a]	Trial II raw poultry meat (low level contamination)[a]	Trial II raw poultry meat (high level contamination)[a]
Number of participating collaborators	25	25	25	13	13
Number of samples per collaborator	5	5	5	6	6
Number of collaborators retained after evaluation of the data	20	20	20	13	13
Number of samples retained after evaluation of the data	100	99	100	78	78
Test portion size, in g	25	25	25	25	25
Specificity, in %	100	—	—	nd	nd
Sensitivity, in %	—	98	100	nd	nd
LOD_{50} (95 % confidence interval), in cfu/test portion	—	nd	nd	2,2 (1,5 to 3,2)	

nd = not determined.

[a] Poultry meat samples were artificially contaminated with *Salmonella* Typimurium in Trial I and were naturally contaminated with *Salmonella* spp. in Trial II.

MPN results of the contaminated samples were:

	MPN/25 g
Trial I low level	3,7 (1 to 9,5)
Trial I high level	5,8 (1 to 25)
Trial II low level	0,2 (0,04 to 0,9)
Trial II high level	1,0 (2,2 to 4,5)

C.3 Performance characteristics of MSRV agar for detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage

The precision data of MSRV agar for detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage were calculated from three different interlaboratory studies organized by the EURL-*Salmonella*, RIVM, The Netherlands. This concerned studies organized in 2008[16], 2012[17] and 2013[21]. The samples tested in the three studies were, respectively, chicken faeces, pig faeces, and boot socks. The samples were each tested at two different levels of contamination, plus a negative control. All studies were funded by the European Commission and the latter study was also performed as part of the CEN mandate M381.

The method submitted to the interlaboratory studies was that of ISO 6579:2002/Amd 1:2007[2] for the detection of *Salmonella* in samples from the primary production stage including selective enrichment on MSRV agar. This method has been incorporated in this document.

Table C.7 — Results of data analysis obtained with chicken faeces samples

Parameter	Chicken faeces +				
	Blank	STM5[a]	STM44[a]	SE7[a]	SE91[a]
Number of participating collaborators	32	32	32	32	32
Number of samples per collaborator	5	5	5	5	5
Number of collaborators retained after evaluation of the data	19	19	19	19	19
Number of samples retained after evaluation of the data	95	95	95	95	95
Test portion size, in g	10	10	10	10	10
Specificity, in %	100	—	—	—	—
Sensitivity per serovar and level, in %	—	96,8	100	67,4	100
LOD_{50} per serovar (95 % confidence interval), in cfu/test portion	—	1,0 (0,7 to 1,4)		4,3 (3,3 to 5,6)	
LOD_{50} overall (95 % confidence interval), in cfu/test portion	—	2,5 (2,1 to 3,0)			

[a] Chicken faeces samples were artificially contaminated with reference materials with the following strains and levels:

Salmonella Typhimurium (STM) at a level of 5 cfu/test portion and a level of 44 cfu/test portion;

Salmonella Enteritidis (SE) at a level of 7 cfu/test portion and a level of 91 cfu/test portion.

(Food) item Verification

- Demonstrate the competence of the user laboratory to perform the validated method with **(food) items that are tested in the user laboratory**.

- **The user laboratory shall:**
  - select **1 challenging (food) item from each (food) category listed within the scope of validation**, that is also a (food) category that is tested within the **scope of laboratory application** of the user laboratory, and
  - use this **1 (food) item and the sample size** used in the validation study to perform the (food) item verification.

Categories: Broad range of foods and other.

Table A. 1: Classification of sample types & suggested target combinations for validation studies

Raw milk & dairy products	Heat processed milk & dairy products	Raw meat & ready-to-cook meat products (except poultry)	Ready-to-eat, ready-to-reheat meat products	Raw poultry & ready-to-cook poultry products	Ready-to-eat, ready-to-reheat meat poultry products
Eggs & egg products (derivatives)	Raw & ready-to-cook fish & seafoods (unprocessed)	Ready-to-eat, ready-to-reheat fishery products	Fresh produce & fruits	Processed fruits & vegetables	Dried cereals, fruits, nuts, seeds and vegetables
Infant formula & infant cereals	Chocolate, bakery products & confectionary	Multi-component foods or meal components	Environmental samples (food or feed production)	Pet food & animal feed	Primary production samples

User laboratory: RIVM-Z&O Laboratory

- Detection of *Salmonella* according to ISO 6579-1
- "a broad range of foods" plus the 3 "other" categories
  - 1 x Implementation verification
  - 8 x (Food) item verification

Verification	Category	Item	Inoculum strain	Strain source
Implementation verification	Raw poultry & ready-to-cook poultry products	Chicken fillet parts	Infantis	Broiler
(Food) item verification	Raw milk & dairy products	Raw milk	Hadar	Human
(Food) item verification	Raw meat & ready-to-cook meat products (except poultry)	Minced meat (beef/pork)	STMmono	Pig meat
(Food) item verification	Eggs & egg products (derivatives)	Whole liquid egg	Enteritidis	Egg
(Food) item verification	Raw & ready-to-cook fish & seafoods (unprocessed)	Mussels	Agama	Shellfish
(Food) item verification	Fresh produce & fruits	Cantaloupe	Virchow	Human
(Food) item verification	Environmental samples (food or feed production	Spent irrigation water		Environmental
(Food) item verification	Pet food & animal feed	Chicken feed	Senftenberg	Cocoa powder
(Food) item verification	Primary production samples (PPS)	Chicken faeces: boot socks	Typhimurium	Chicken: boot swabs

Verification Qualitative methods

- Estimated LOD_{50} ($eLOD_{50}$) determination

Table 3 —Protocols to determine $eLOD_{50}$ and number of replicates needed per inoculation level

Protocol	Inoculation level of the test portion					
	9 × LOD_{50} /test portion	3 × LOD_{50} /test portion	1 × LOD_{50} /test portion	3 to 5 cfu /test portion	Blank	Total number of replicates
1	1	4	4	-	1	10
2	-	3	5	-	1	9
3	-	-	-	7	1	8

NOTE The abbreviation of colony forming units is cfu.

- Enumeration of the inoculum !
  - Plate counts, MPNs
- Acceptability limits

The $eLOD_{50}$, determined according to protocol 1 (see 5.5.1) or protocol 2 (see 5.5.2) shall be compared to the LOD_{50} from the validation study. For implementation verification, use the LOD_{50} value corresponding to the tested (food) item.

For (food) item verification, the $eLOD_{50}$ shall not be > 4 × LOD_{50} observed in the validation study. If no LOD_{50} value corresponds to the tested (food) item, the $eLOD_{50}$ shall not be > 4 cfu/test portion. This acceptability limit is based on the theoretical value of a LOD_{50} of 1 cfu/ test portion.

For protocol 3, there shall be a minimum of 6 positive results out of the 7 replicates tested.

Protocols in ISO 16140-3

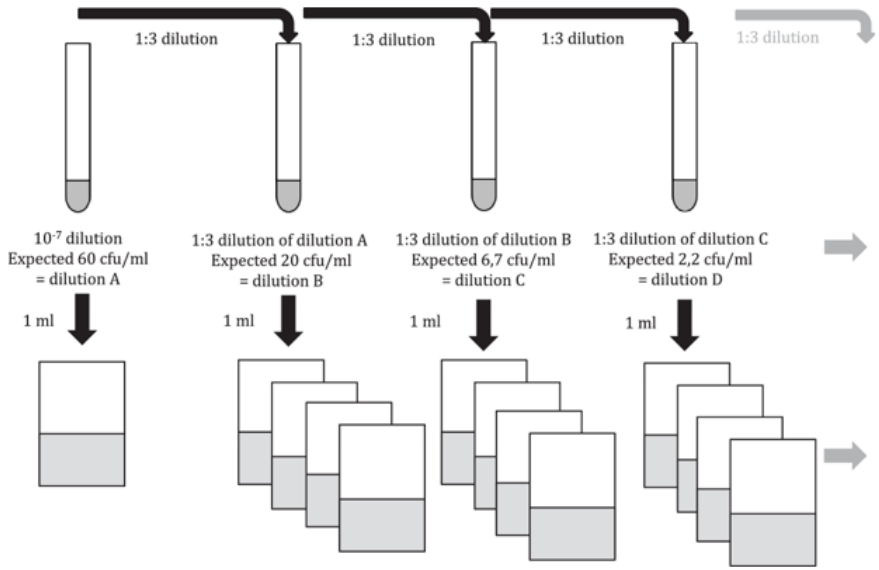

Figure C.3 — Example of the inoculation of the test portions when using protocol 1

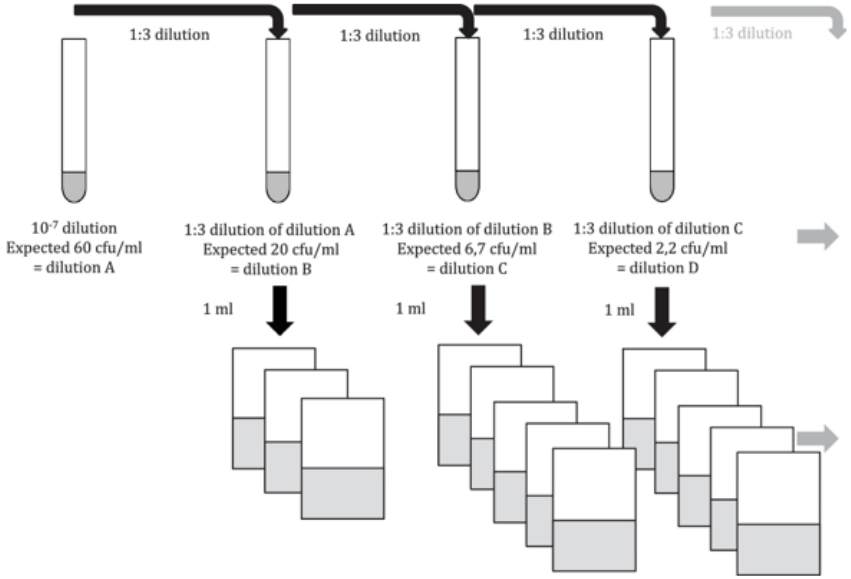

Figure C.4 — Example of the inoculation of the test portions when using protocol 2

- Protocol 3: direct use of reference material (3-5 cfu/ml), 7 samples

Elaborated workflow *at RIVM-Z&O*

- Combination of Protocol 1, Protocol 2, Protocol 3 (*for research reasons only*)
- Aiming at a concentration for Dilution A at 27 cfu/ml
- Enumeration of overnight culture (ON) of inoculation strain
- ON dilutions kept in fridge and used to prepare Dilution A
- Actual inoculation dilutions B, C, D, prepared from Dilution A by 1:3 steps
- Both plate counts and MPN (*for research reasons only*) for enumeration of 1 ml dilutions (A, B, C, D)
- Testing inoculated samples (P1, P2, P3) for presence of *Salmonella*

	Matrix:	X=voer, Z=mest, E=heelei, F=mosselen					
	Entmateriaal:	Stam S6	Stam S8	Stam S10	Stam S11	Analist(en): Wendy	
		MSRV				MKTTn	
		24 uur	48 uur	XLD	BSA	XLD	BSA
NR:	Monster omschrijving	Verdacht (V) / Niet-Verdacht (NV) / Geen groei (-)					
X20	Blanco voer	NV	NV			NV	NV
X21	Enten met 1 ml Dil. B	V		V	V	V	V
X22	Enten met 1 ml Dil. C	V		V	V	V	V
X23	Enten met 1 ml Dil. C	V		V	V	V	V
X24	Enten met 1 ml Dil. C	V		V	V	V	V
X25	Enten met 1 ml Dil. C	V		V	V	V	V
X26	Enten met 1 ml Dil. D	V		V	V	V	V
X27	Enten met 1 ml Dil. D	V		V	V	V	V
X28	Enten met 1 ml Dil. D	V		V	V	V	V
X29	Enten met 1 ml Dil. D	NV	NV			NV	NV
X30	Enten met 1 ml Dil. D	V		V	V	V	V
X31	Enten met 1 ml Dil. D	V		V	V	V	V
X32	Enten met 1 ml Dil. D	V		V	V	V	V

Enumeration of inocula

Code	Omschrijving	# ml in onderzoek	BPW (+/- troebel)			Groei op TSA (+/-)		
			1	2	3	1	2	3
S 6	Dilution D	1 ml	+	-	+		-	
S 6	Dilution E	1 ml	-	-	-	-	-	-
S 6	Dilution F	1 ml	-	-	-	-	-	-
S 8	Dilution D	1 ml	+	+	+			
S 8	Dilution E	1 ml	+	+	+			
S 8	Dilution F	1 ml	+	-	-		-	-
S 10	Dilution D	1 ml	+	+	-			-
S 10	Dilution E	1 ml	+	-	-		-	-
S 10	Dilution F	1 ml	-	-	-	-	-	-
S 11	Dilution D	1 ml	+	-	-		-	-
S 11	Dilution E	1 ml	+	-	-		-	-
S 11	Dilution F	1 ml	-	-	-	-	-	-

MONSTER NR	PRODUCT	aantal kolonies per plaat (14 cm, 1 ml)					
		Dil. A	Dil. A	Dil. B	Dil. B	Dil. C	Dil. C
		Stam afhankelijk	Stam afhankelijk	is 20 ml Dil. A plus 40 ml PFZ	is 20 ml Dil. A plus 40 ml PFZ	is 20 ml Dil. B plus 40 ml PFZ	is 20 ml Dil. B plus 40 ml PFZ
Dil A is 2,3 ml -6 plus 57,7 ml PFZ (verwachting 27 cfu/ml)	Ent S6 op NA	36	37	8	10	3	6
Dil A is 2,5 ml -6 plus 57,5 ml PFZ (verwachting 27 cfu/ml)	Ent S8 op NA	47	31	12	12	6	5
Dil A is 2,1 ml -6 plus 57,9 ml PFZ (verwachting 27 cfu/ml)	Ent S10 op NA	37	35	15	10	1	2
Dil A is 1,4 ml -6 plus 58,6 ml PFZ (verwachting 27 cfu/ml)	Ent S11 op NA	31	27	10	13	5	3

MPN table
in ISO 16140-3

0,62 [0,15 -2,6] cfu/test portion

CFU/ml Dilution D

1,35 cfu/test portion

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5 **Qualitative methods - Technical protocol for verification; calculation and lookup tool for comparing results with results of the verification**

Estimated LOD50 (eLOD50) determination

5.5 **Evaluation of results (protocol 1)**

LOD_{50} (cfu/portion): 2 — Published validation data of the method. If no validation data is available, assume an LOD_{50} of 1 cfu/portion

1 **Table 4 - Estimation of $eLOD_{50}$ based on the number of positive results per level of contamination** 10 11 12

	High inoculation level *targeted 9 x LOD_{50} / test portion* *= targeted 18 cfu/test portion*	Intermediate inoculation level *targeted 4 x LOD_{50} / test* *= targeted 8 cfu/test portion*	Low inoculation level *targeted 1 x LOD_{50} / test portion* *= targeted 2 cfu/test portion*	Blank level *= targeted 0 cfu/test portion*	$eLOD_{50}$ *cfu/test portion*	Lookup result
1440	1/1	4/4	4/4	0/1	< 1,0 x LIL [a]	not
1430	1/1	4/4	3/4	0/1	= 0,5 x LIL	0,5
1420	1/1	4/4	2/4	0/1	= 0,7 x LIL	0,7
1410	1/1	4/4	1/4	0/1	= 1,0 x LIL	1
1400	1/1	4/4	0/4	0/1	= 1,5 x LIL	1,5
1340	1/1	3/4	4/4	0/1	= 0,7 x LIL	0,7
1330	1/1	3/4	3/4	0/1	= 1,0 x LIL	1
1320	1/1	3/4	2/4	0/1	= 1,3 x LIL	1,3
1310	1/1	3/4	1/4	0/1	= 1,7 x LIL	1,7
1300	1/1	3/4	0/4	0/1	= 2,3 x LIL	2,3
1240	1/1	2/4	4/4	0/1	= 1,1 x LIL	1,1
1230	1/1	2/4	3/4	0/1	= 1,5 x LIL	1,5
1220	1/1	2/4	2/4	0/1	= 1,9 x LIL	1,9
1210	1/1	2/4	1/4	0/1	= 2,6 x LIL	2,6
1200	1/1	2/4	0/4	0/1	= 3,7 x LIL	3,7
1140	1/1	1/4	4/4	0/1	Unreliable MPN result	not
1130	1/1	1/4	3/4	0/1	= 2,1 x LIL	2,1
1120	1/1	1/4	2/4	0/1	= 2,8 x LIL	2,8
1110	1/1	1/4	1/4	0/1	= 4,0 x LIL	4
1100	1/1	1/4	0/4	0/1	= 6,3 x LIL	6,3
1040	1/1	0/4	4/4	0/1	Unreliable MPN result	not
1030	1/1	0/4	3/4	0/1	= 3,0 x LIL	3
1020	1/1	0/4	2/4	0/1	= 4,3 x LIL	4,3
1010	1/1	0/4	1/4	0/1	= 6,7 x LIL	6,7
1000	1/1	0/4	0/4	0/1	= 14,0 x LIL	14

NOTE The MPN calculator used can be found at: http://standards.iso.org/iso/7218.

[a] LIL = Low inoculation level.

[b] Unreliable MPN result: MPN combination is very unlikely to occur. Experiment shall be repeated

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5 Qualitative methods - Technical protocol for verification; calculation and lookup tool for comparing results with results of the verification

Estimated LOD50 (eLOD50) determination

	Implementation verification	(Food) item verification				
	(Food) item 1	(Food) item 2	(Food) item 3	(Food) item 4	(Food) item 5	(Food) item 6
Actual low inoculum level (LIL; cfu/test portion	1,0	1,5	0,1	10,0	1,5	2,5

Results per Inoculum level *(frequency of positive duplicates per (food) item test portion and per inoculum level: enter or select 0, 1 or 2 in each cell)*

Inoculum level of the test portions	(Food) item 1	(Food) item 2	(Food) item 3	(Food) item 4	(Food) item 5	(Food) item 6
High inoculum	1	1	0	1	1	1
Intermediate	4	4	0	4	3	0
Low inoculum	4	2	1	4	1	0
Blank (uninoculated)	0	0	0	1	0	0
	1440	1420	10	1441	1310	1000
$eLOD_{50}$ (cfu/test portion)	< 1,0	= 0,7 x LIL	Unreliable MPN result	Unreliable MPN result	= 1,7 x LIL	= 14,0 x LIL
$eLOD_{50}$ (cfu/test portion) or repeat test	**< 1,0**	**1,05**	**Repeat test**	**Repeat test**	**2,55**	**35,00**

5.6 **Acceptance criteria (protocol 1)**

The $eLOD_{50}$ values in tab 'Qualitative test' shall not be > 4 x LOD_{50} observed in the validation study

If the LOD_{50} is not known (e.g. for reference methods with no published data), $eLOD_{50}$ shall be ≤ 4 cfu/test portion

Acceptable $eLOD_{50}$ (cfu/portion) **8** = 4 * LOD_{50}

Acceptable result (≤ 8 cfu/portion)	**Accepted**	**Accepted**	**Not accepted**	**Not accepted**	**Accepted**	**Not accepted**

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Combined results, per Protocol

Food item: Animal feed

Protocol 1

Dilution A	Dilution B	Dilution C	Dilution D	Blank
Strain S6	(1:3 of A)	(1:3 of B)	(1:3 of C)	
na	1/1	4/4	3/4	0/1

Protocol 2

Dilution A	Dilution B	Dilution C	Dilution D	Blank
Strain S6	(1:3 of A)	(1:3 of B)	(1:3 of C)	
na	na	3/3	4/5	0/1

Protocol 3

Dilution A	Dilution B	Dilution C	Dilution D	Blank
Strain S6	(1:3 of A)	(1:3 of B)	(1:3 of C)	
na	na	na	6/7	0/1

Evaluation of the results, per Protocol

Food item: Animal feed **Inoculation strain S6**

	Results	Table 6/Table 8 ISO 16140-3	Low Inoculation Level (LIL) (cfu/test portion)	$eLOD_{50}$ (cfu/test portion)	Published validation data or assume 1 cfu/test portion LOD_{50} (cfu/test portion)	$4 \times LOD_{50}$ (cfu/test portion)	Qualitative Verification Acceptability Limit $e\ LOD_{50} \leq 4 \times LOD_{50}$
P1	1-4-3-0	= 0,5 x LIL	1,4	0,7	1	4	Accepted
	1-4-3-0	= 0,5 x LIL	0,62 [0,15 - 2,6]	0,31	1	4	Accepted
P2	3-4-0	= 0,4 x LIL	1,4	0,56	1	4	Accepted
	3-4-0	= 0,4 x LIL	0,62 [0,15 - 2,6]	0,25	1	4	Accepted
P3	6-0	na	1,4 (< [3-5])	na	na	minimum 6/7	Accepted
	6-0	na	0,62 (< [3-5])	na	na	minimum 6/7	Accepted

User laboratory: RIVM-Z&O Laboratory

- Detection of *Salmonella* according to ISO 6579-1
- "a broad range of foods" plus the 3 "other" categories
  - 1 x Implementation verification
  - 8 x (Food) item verification

P1:

Verification	Category	Item	Inoculum strain	Strain source
Implementation verification	Raw poultry & ready-to-cook poultry products	Chicken fillet parts	Infantis	Broiler
(Food) item verification	Raw milk & dairy products	Raw milk	Hadar	Human
(Food) item verification	Raw meat & ready-to-cook meat products (except poultry)	Minced meat (beef/pork)	STMmono	Pig meat
(Food) item verification	Eggs & egg products (derivatives)	Whole liquid egg	Enteritidis	Egg
(Food) item verification	Raw & ready-to-cook fish & seafoods (unprocessed)	Mussels	Agama	Shellfish
(Food) item verification	Fresh produce & fruits	Cantaloupe	Virchow	Human
(Food) item verification	Environmental samples (food or feed production	Spent irrigation water		Environmental
(Food) item verification	Pet food & animal feed	Chicken feed	Senftenberg	Cocoa powder
(Food) item verification	Primary production samples (PPS)	Chicken faeces: boot socks	Typhimurium	Chicken: boot swabs

Thank you for your attention !

Any questions ?