EURL- *Salmonella*

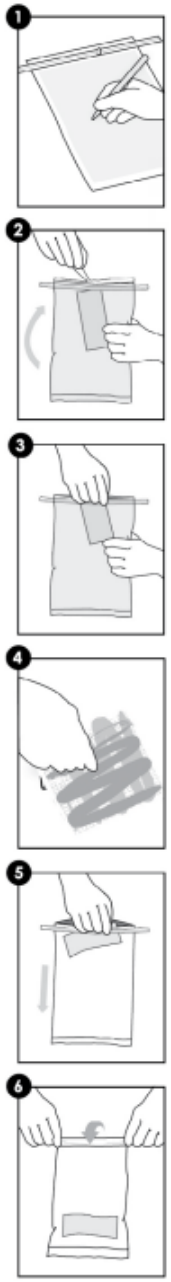
Combined Proficiency test PPS-Food

Detection of *Salmonella* in hygiene swabs (2022)

Irene Pol-Hofstad

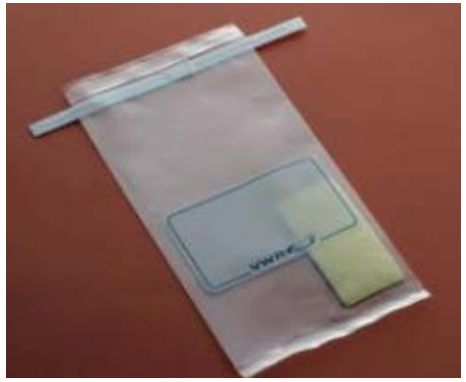
Combined EURL-*Salmonella* Detection study PPS-Food 2022

- No EURL detection study in Food in 2022 due to ILS study ISO/TS 6579-4
- Matrix: hygiene swabs
- Hygiene swab samples are used sample the environment of poultry farms or food production area
- The hygiene swab samples were contaminated at EURL lab
  - high and low concentration of *Salmonella*



From DFA and Hygiena websites

Preparation of samples



- 10 ml Peptone Saline Solution to moisten hygiene swabs
- Leave them to saturate at room temperature
- Add background flora
  - *Citrobacter freundii/Citrobacter youngae and Enterobacter cloacae,*
- Add high and low concentration *Salmonella*
  - *Salmonella* Infantis and *Salmonella* Enteritidis

Pre-test: Stability of samples

- Simulate transport and storage conditions

- Pretest:
  - incubation contaminated hygiene swab samples at 5 °C and 10 °C for up to 3 weeks

- Test for:
  - *Salmonella:* EN-ISO 6579:1-2017
  - *Enterobacteriaceae:* EN-ISO 21528-2
  - Aerobic count: EN-ISO 4833-1:2013

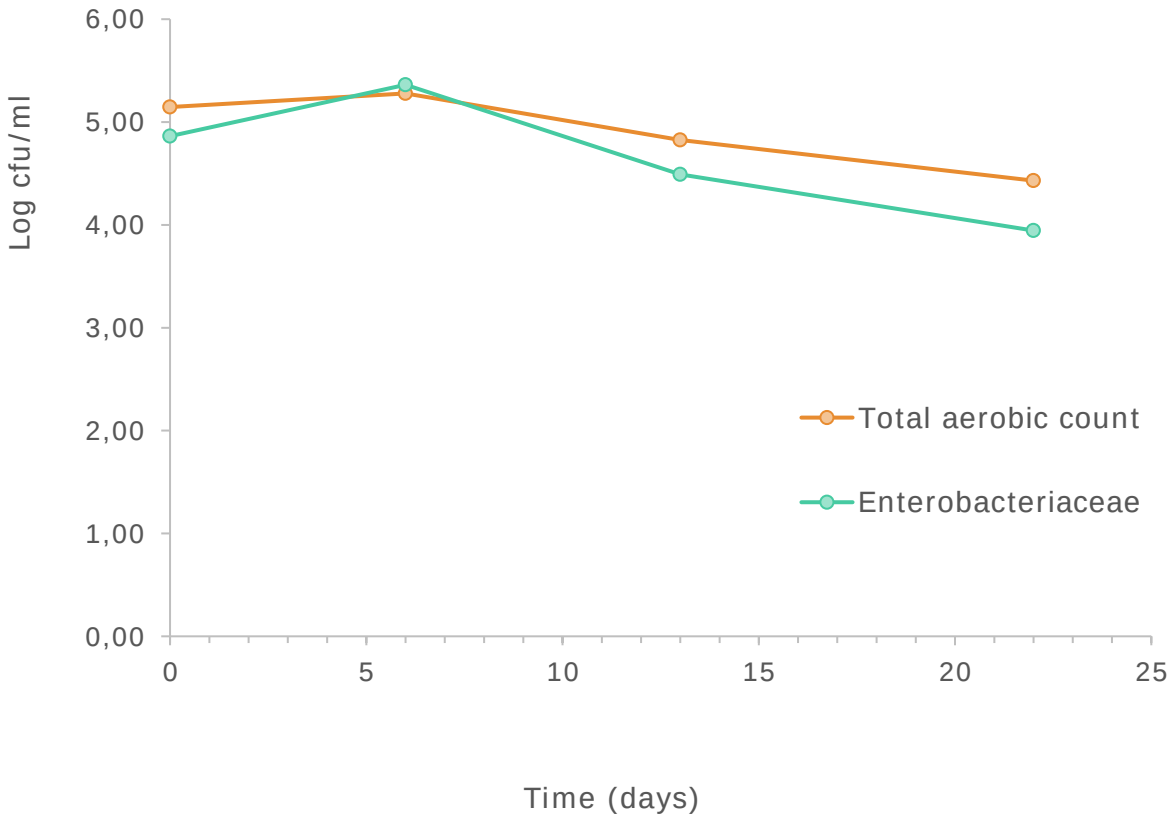
Pre-test: contamination

- Background flora:
  - combination *E. cloacae* and *C. freundii/C. youngae*

- *Salmonella*:
  - combination of *Salmonella Infantis* and *Salmonella Enteritidis*

Conclusion Pre-tests: background flora

– Both aerobic count and *Enterobacteriaceae* are stable



Combination of two strains *Salmonella*

- Three combination:
  - a. *S.* Infantis < *S.* Enteritidis
    - 7 cfu + 17 cfu
  - b. *S.* Infantis = *S.* Enteritidis
    - 14 cfu + 14 cfu
  - c. *S.* Infantis > *S.* Enteritidis
    - 17 cfu + 7 cfu
- Selected 12 colonies to test for serogroup O7 or O9

***Salmonella* strain + aimed cfu**	**Actual cfu**	
	XLD	**TSA**
SI 7 cfu	2	15
SI 14 cfu	13	16
SI 17 cfu	7	25
SE 7 cfu	13	7
SE 14 cfu	24	19
SE 17 cfu	25	19

Combination of two strains *Salmonella*

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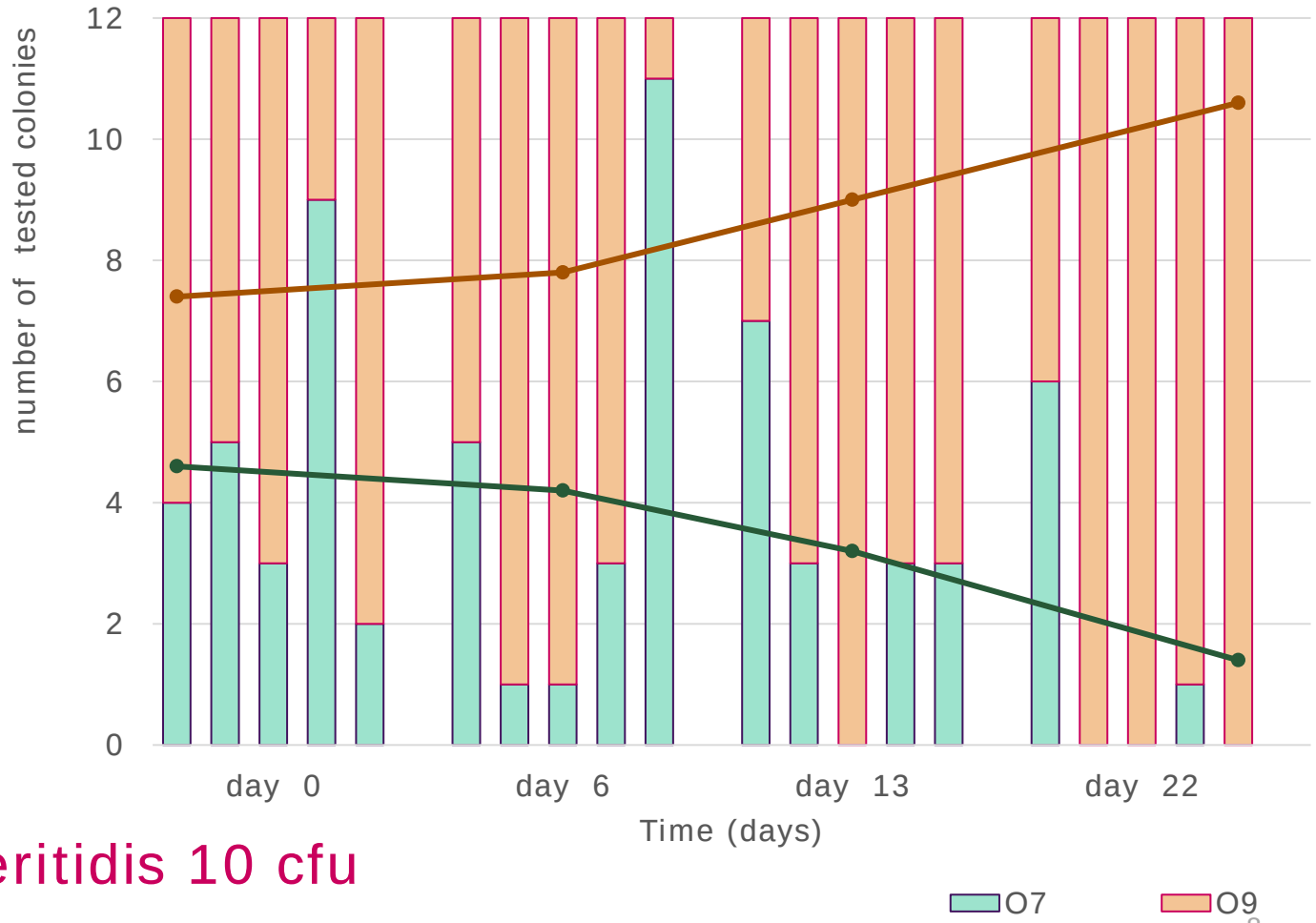


S. Infantis 40 cfu + *S.* Enteritidis 10 cfu

Study design

Hygiene swabs moistened with 10 ml PSS

- 6 x Low level SI 14 cfu + *E. cloacae* and *C. freundii* (10^6 cfu/sample)
- 4 x High level: SI 40 cfu + SE 10 cfu + *E. cloacae and C. freundii* (10^6 cfu/sample)
- 4 x Negative samples (no *Salmonella* added)
  - 2 samples: *E. cloacae* and *C. freundii* (10^6 cfu/sample)
  - 2 samples*: E. cloacae* and *C. youngae* (10^6 cfu/sample)

- Process controls:
  - C1: 1 procedure control (hygiene swab samples with sterile peptone saline solution (PS) only)
  - C2: 1 positive control sample (laboratories' own *Salmonella* control strain)

Participants

In total: 34 NRLs PPS and 34 NRLs Food

- 27 NRLs from 27 EU Member States
- 11 NRLs from EU (potential) candidate Member States or European Free Trade Associations countries (EFTA)
- 1 NRL from a third country

Photo: Maskot bildbyrå; Europa.eu; nl.depositphotos.com

Transport

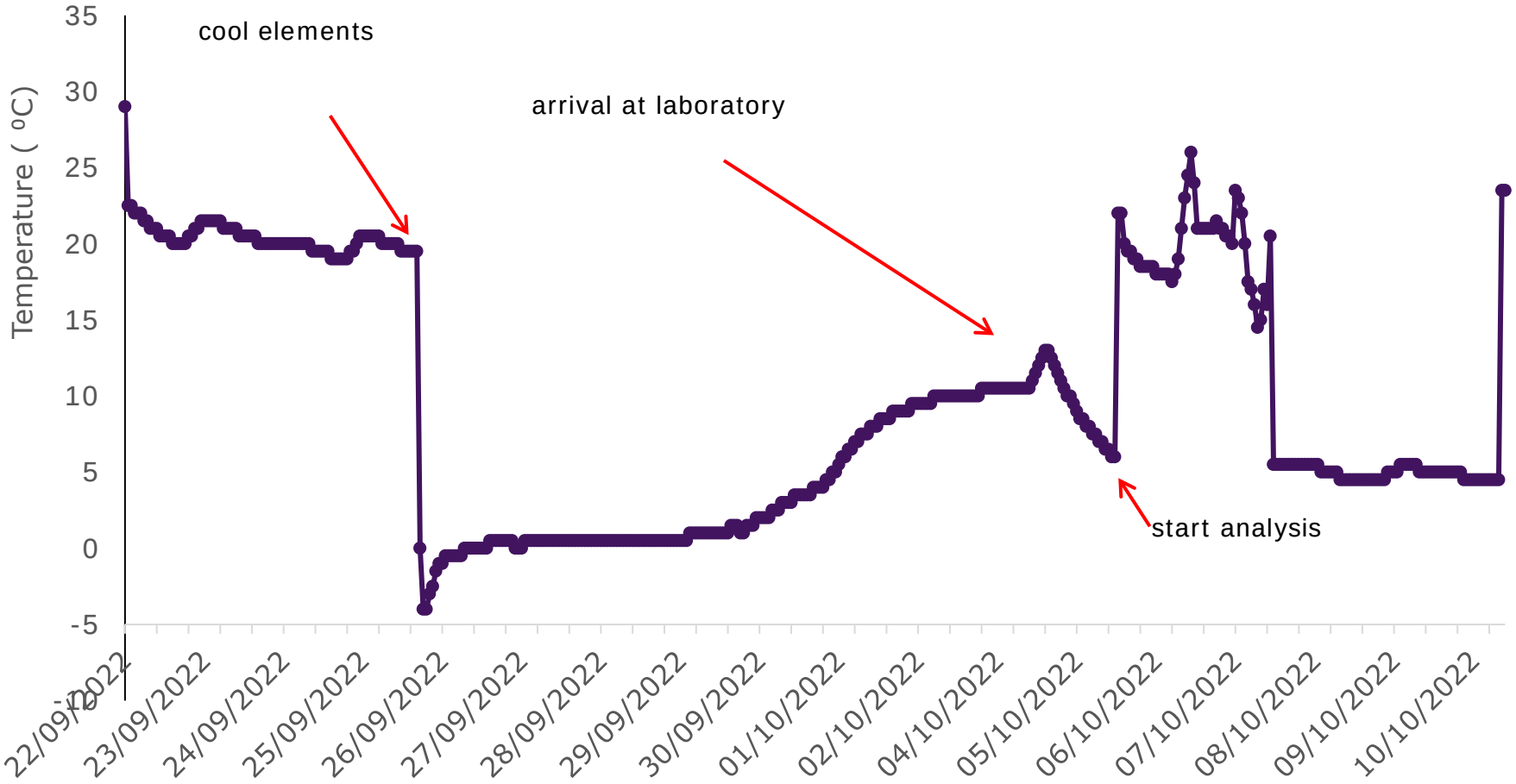
Transport time

- 51 parcels: 1 day
- 7 parcels: 2 days
- 5 parcels: 3 days
- 4 parcels: 4 days
- Lab code 23: 8 days

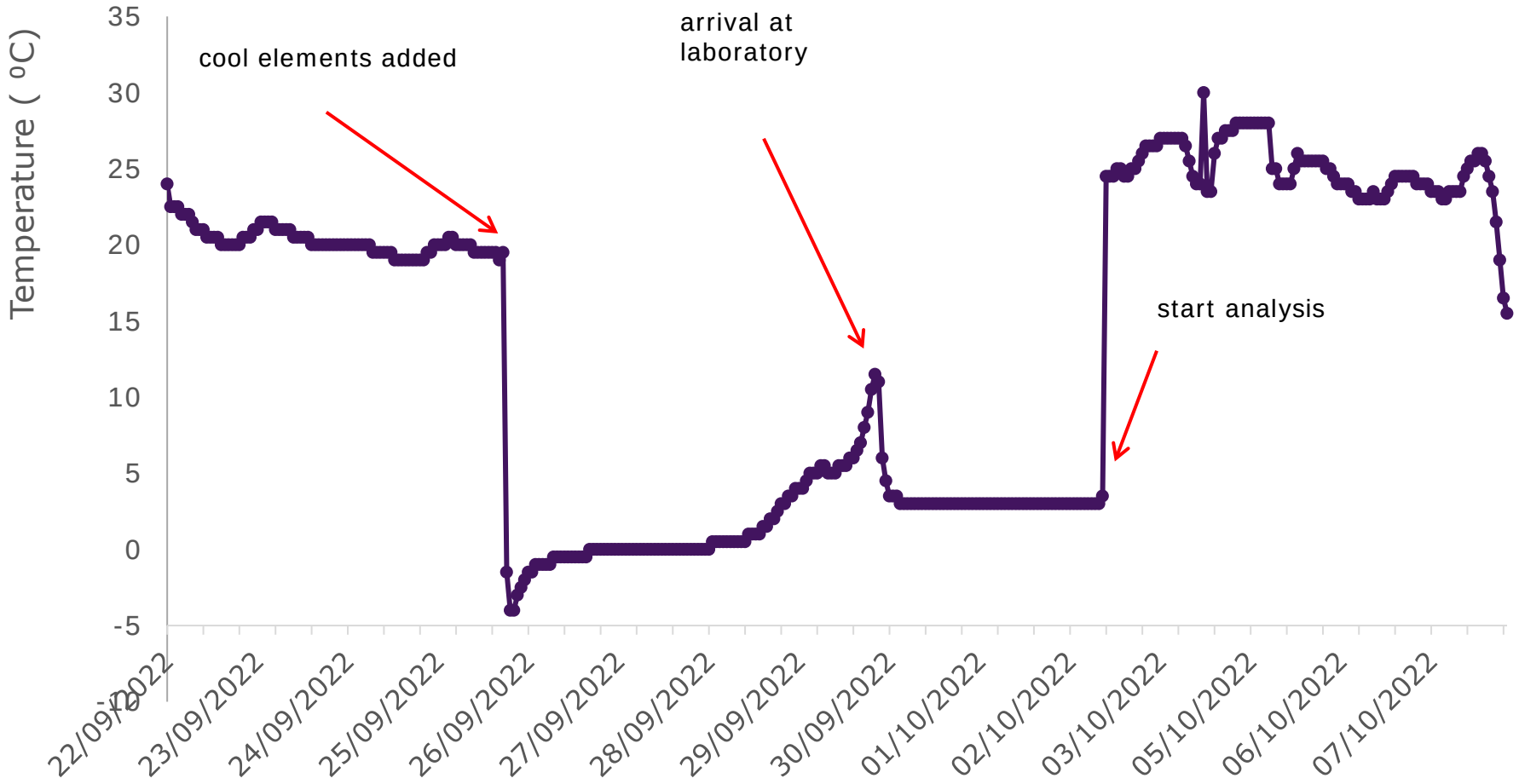
Temperature during transport and storage:

- temperature probe between samples
- transport temperature: - 3 °C till + 4 °C
- storage temperature: mostly 0 °C – 9 °C

Temperature profile parcel lab code 23



Temperature profile parcel lab code 21



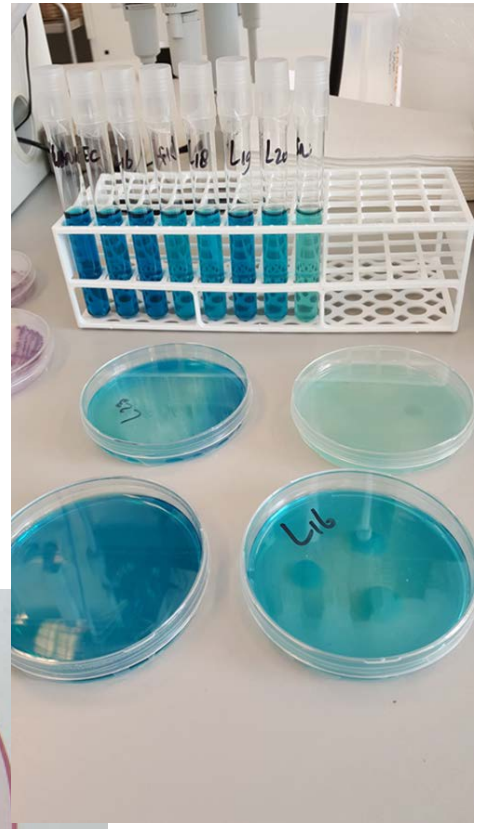
Method EURL *Salmonella* PT PPS-Food

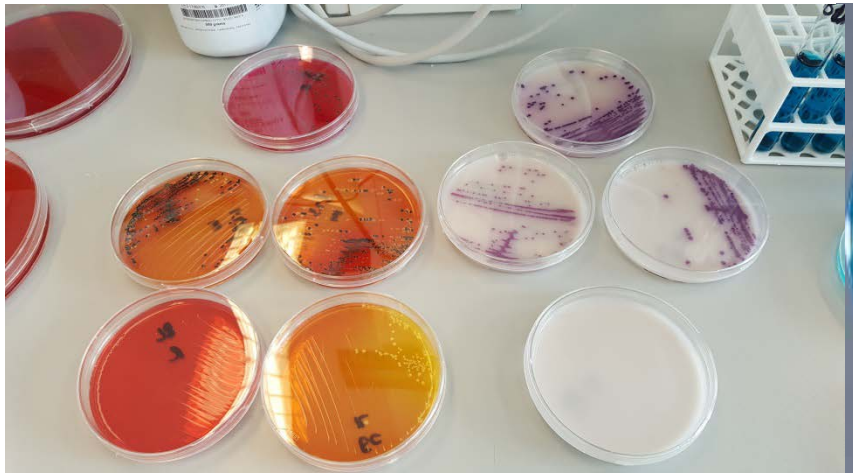
Prescribed: ISO 6579-1:2017

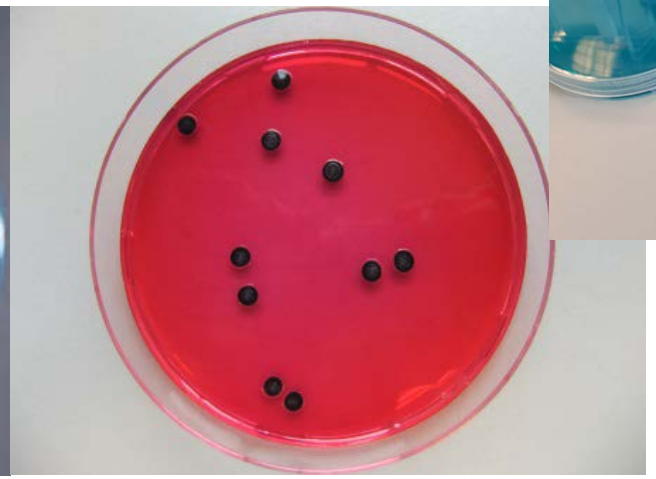
PPS samples: - Selective enrichment on MSRV

Food samples: - Selective enrichment on MKTTn and RVS or MSRV

- Isolation medium XLD & 2nd agar of choice







PT samples: Artificial contamination

Date of testing	Sample	Low level SI cfu / sample	High level SI + SE cfu / sample
21 Sept 2022	Inoculum of diluted culture	8	30 + 8
3 Oct 2022	Inoculated hygiene swabs, stored 5°C MPN (95% confidence limit)	2,2 (0,9-5,5)	17,4 (6,5-45)
21 sept 2022	Inoculum of mixture	***E. cloacae* + *C. freundii* (cfu / ml)** $8,0 \times 10^5$	***E. cloacae* + *C. youngae* (cfu / ml)** $9,7 \times 10^5$

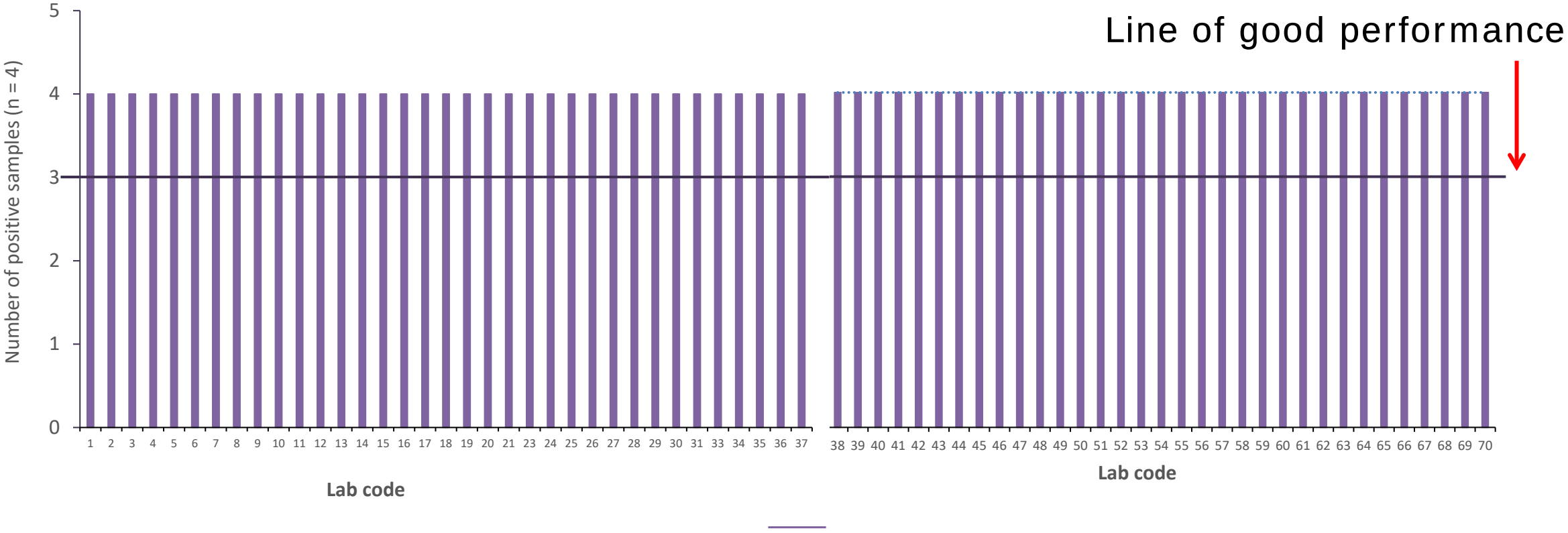
Deviations from ISO

Lab code	BPW		RVS		MKTTn			MSRV		
	Incubation time	T (ºC)	pH	T (ºC)	T (ºC)	pH	Novobiocin	T (ºC)	pH	Novobiocin
EN ISO 6579-1	16–20 h	37	5,0-5,4	41,5	37	7,0–8,2	40 mg/l	41,5	5,1–5,4	10 mg/l
2	19,5	37	0	41,5	37	?	40 mg/L			
13	24	37	5,1	41,5	37	7,9	4 mg/L			
19	19 h 45	37			37	0	40 mg/L	41,5	5,28	10 mg/L
27	20	37	5,43	41,5	37	8,02	10 mg/L	41,5	5,42	10 mg/L
28	20	37	5,43	41,5	37	8,02	10 mg/L	41,5	5,42	10 mg/L
31	20	37	7,2	41,5	37	8,2	20 mg/L	41,5	5,2	20 mg/L
36	20	37	5,31	41,5	37	7,42	39,02 mg/L			
51	20	36	5,2	41,5	36	6,6	40mg/l			
52	20	37	5,36	37						
56	20	37						41,5	5,6	10 mg/L
62	18 h 55	37			41,7	8	40 mg/L	41,7	5,4	10 mg/L

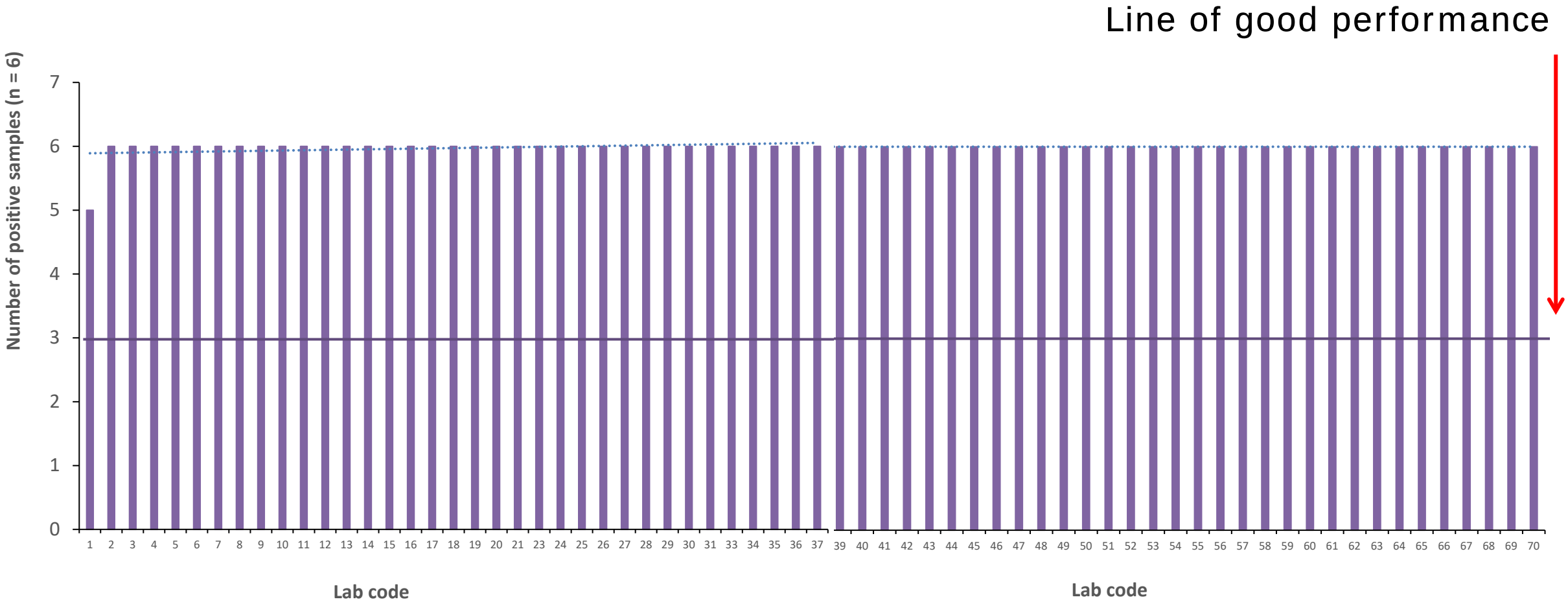
Samples: specificity, sensitivity, accuracy

Control samples		All labs n = 68	EU NRLs only	EU NRLs PPS n = 28	EU NRLs Food n =28
Procedure control n=1	No. of samples	68	56	28	28
	No. of negative samples	68	56	28	28
	Specificity in %	**100%**	**100%**	**100%**	**100%**
Positive control (own *Salmonella*) n=1	No. of samples	68	56	28	28
	No. of positive samples	68	56	28	28
	Sensitivity in %	**100%**	**100%**	**100%**	**100%**
All control samples n=2	No. of samples	136	112	56	56
	No. of correct samples	136	112	56	56
	Accuracy in %	**100%**	**100%**	**100%**	**100%**

Results: High concentration SI + SE



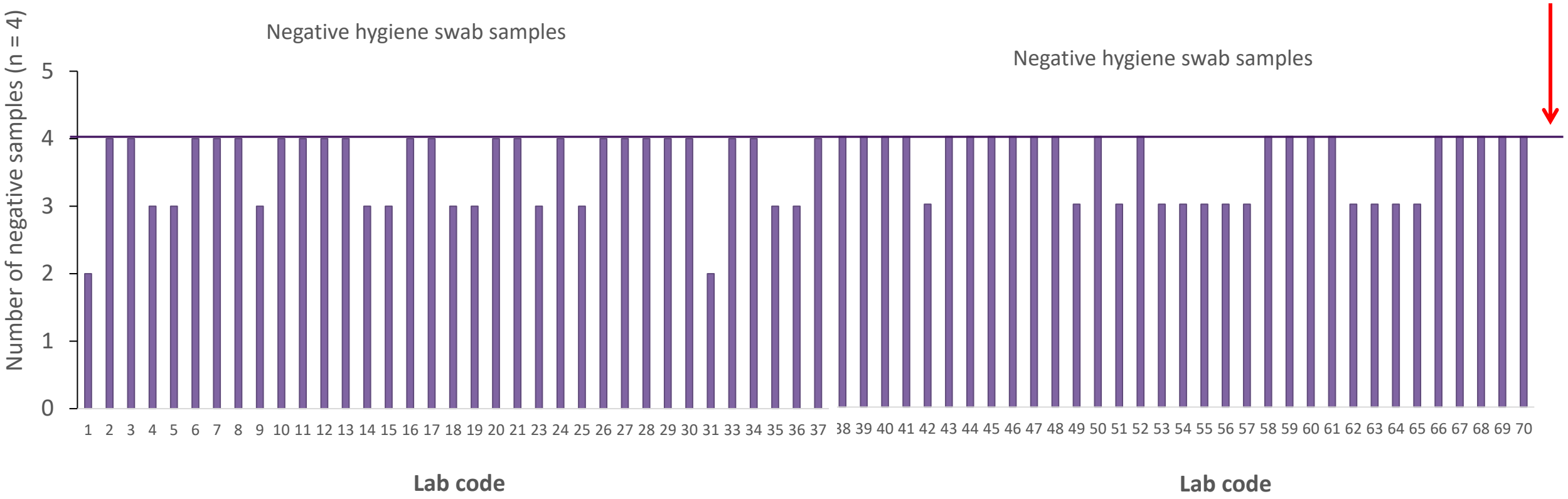
Results: Low concentration SI



- 4 labs reported serotype for positive samples
- Questions concerning different *Salmonella* types in different samples or in same sample using different methods or being both NRL Food and NRL PPS

Results: Negative samples

Normally: Line of good performance



Overall results

	Number of positive samples		
	Negative n=4	SI low n=6	SI + SE high n=4
Criteria good performance	0	≥3	≥3
All other NRLs (n = 43)	0	6	4
Lab code (4, 5, 9, 14, 15, 18, 19, 23, 25, 35, 36, 42, 49, 51, 53, 54, 55, 56, 57, 62, 63, 64, 65 (n = 23; 12 NRLs PPS + 11 NRLs Food)	1	6	4
Lab code 31 (NRL PPS)	2	6	4
Lab code 1 (NRL PPS)	2	5	4

Negative samples: investigation

- In total n = 272 negative samples, 245 correct negative (89%)
- n = 27 samples positive (11%)
- 25 labs 1 or 2 samples positive
- Positives found in B1, B8, B9 and B14 serie
- 1 false positive reporting

Samples	Contamination	# labs Correct	# labs False	Lab codes
B1	Negative (EC/CF)	57	11	4, 5, 18, 19, 23, 31, 51, 56, 57, 64, 65,
B2	Low SI	68		
B3	High SI/SE	68		
B4	Low SI	68		
B5	Low SI	68		
B6	Low SI	68		
B7	Low SI	68		
B8	Negative (EC/CF)	64	4	25, 31, 49, 54
B9	Negative (EC/CY)	63	5	1, 35, 53, 62, 63
B10	High SI/SE	68		
B11	High SI/SE	68		
B12	High SI/SE	68		
B13	Low SI	67	1	1
B14	Negative (EC/CY)	61	7	1, 9, 14, 15, 36, 42, 55
C1	Negative	68		
C2	Positive	68		

In detail investigation:

- Test spare negatives samples
- Test our stocks
- Questions labs involved

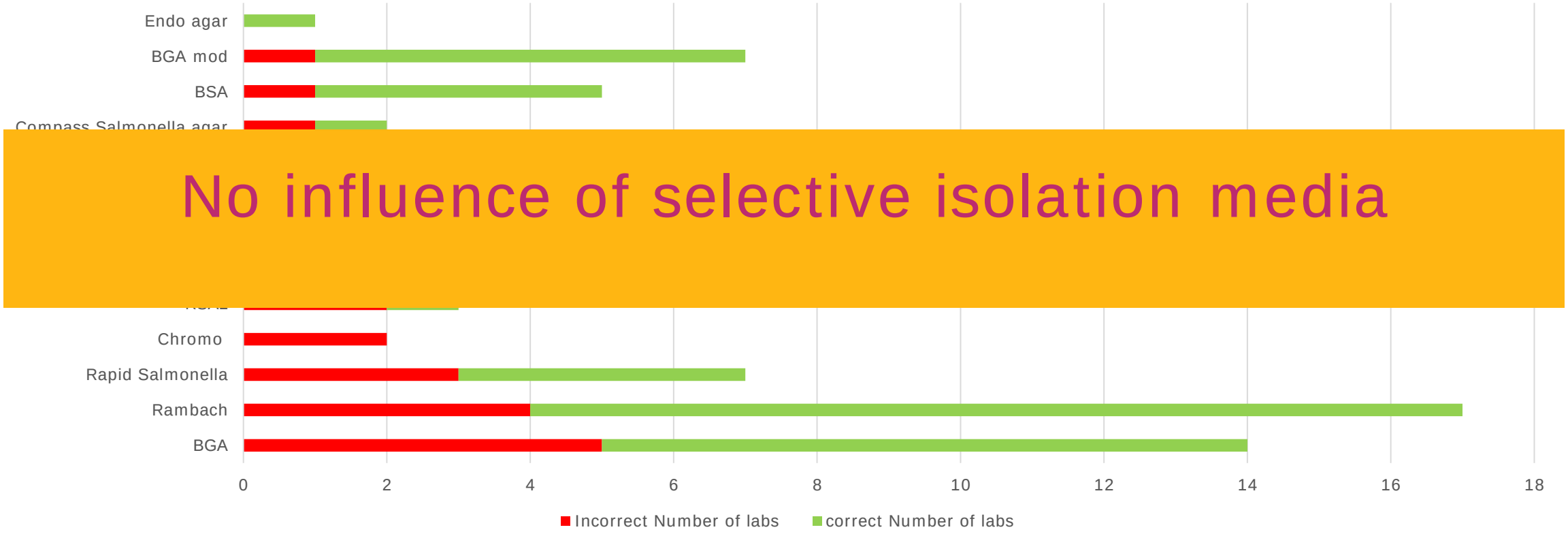
RESULTS

- 7 spare sets: n = 28 neg samples: all negative
- Stocks: *E. cloacae, C. freundii, C. youngae, S. Infantis, S. Enteritidis: all pure*

Information labs

- Influence selective enrichment media
- Influence isolation media
- Influence confirmation methods
- Pos control strains
- Serotyping
- WGS

Influence of selective isolation media



No influence of selective isolation media

Information labs

- Influence selective enrichment media
- Influence isolation media
- Influence confirmation methods
- Pos control strains
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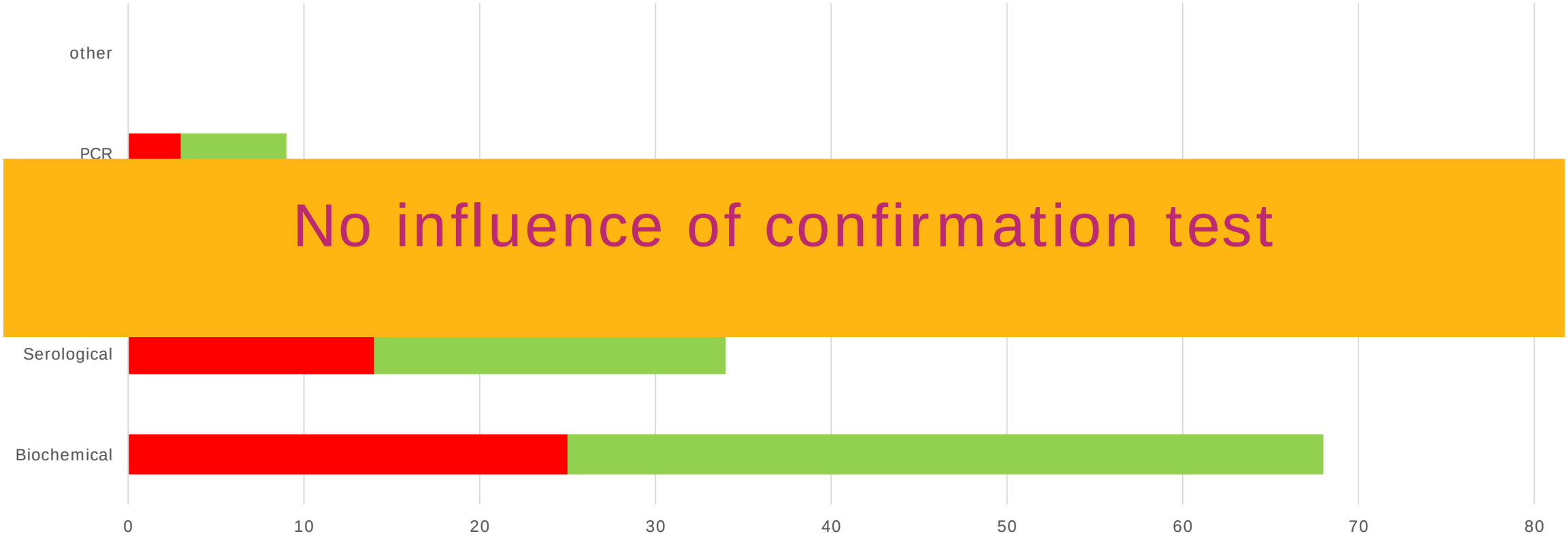
- WGS

Influence of type of confirmation test



No influence of confirmation test

Information labs

- Influence selective enrichment media
- Influence isolation media
- Influence confirmation methods
- Pos control strains
- Serotyping
- WGS

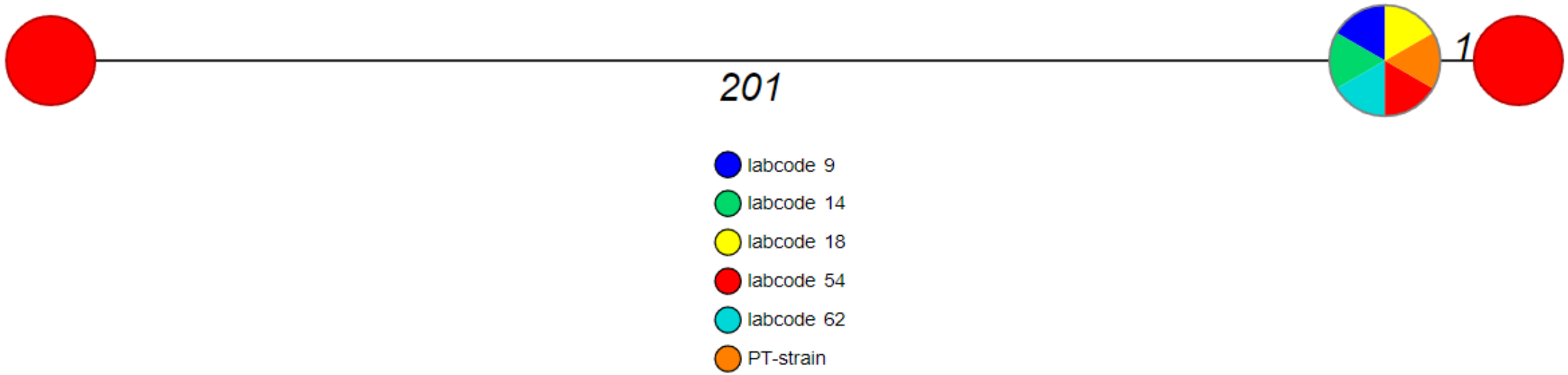
Serotyping results

PT sample			Lab code																
			1	4/5	12	14	18/19	35	36	42	48	51	53	54	55	56/57	60/61	62/63	2e method 62
B1	N	N (EC/CF)		O9 SE			SE					SE				SE			
B2	L	SI	SI	O7	SI	SI				SI			O7				SI	O7	O7 SI
B3	H	SI + SE	SE	SI+SE	SE	SE				SE			O7				SI	O9	O7 SI
B4	L	SI	SE	O7	SI+SE	SI				SI			O7				SI	O7	O7 SI
B5	L	SI	SI	O7	SI	SE				SI			O7				SI	O9	O7 SI
B6	L	SI	SI	O7	SI	SE				SI			O7				SI	O7	O7 SI
B7	L	SI	SI	O7	SI	SI				SI			O7				SI	O7	O7 SI
B8	N	N (EC/CF)									SE			SE					
B9	N	N (EC/CY)	SE					SE					SE					O9	
B10	H	SI + SE	SE	O7	SI SE	SE				SE			O7				SI	O9	O7 SI
B11	H	SI + SE	SE	O7+O9	SE	SE				SI+SE			O9				SI	O9	O7 SI
B12	H	SI + SE	SE	O7	SE	SE				SI+SE			O7				SI	SE	O7 SI
B13	L	SI		O7 SI	SI	SI				SI			O7				SI	SI	O7 SI
B14	N	N (EC/CY)	SI			SE			SE	SE					SE				

correct	1 serovar i.s.o. 2 serovars	Unexpected serovar	wrong

- Serotype all positive results or only when ordered
- 5 colonies are selected and type with sera or usually max 1 colony, depending on morphology. 6 colonies selected.
- 1 colony from each media
- In PT only 1 colony serotyped since it is expected that 1 serovar is used
- Different serovars found in 3 samples reason to check the rest
- Normally 1 colony tested and content with 1 serovar per sample

Minimum spanning tree (MST): cgMLST analyses of the strains from the negative samples



Strains found in the negative samples (B1, B8, B9 and B14) are identical to the SE strain used to contaminate the negative samples

Explanation:

contaminated background flora

E. cloacae strain is used in all samples

- Large number of labs finding *Salmonella* in negative samples: cross contamination is unlikely
- 10% negative samples scored positive (90% correct negative)
- Positives reported in all 4 series of negative samples
- 14 labs serotyped their isolated strain as *S*. Enteritidis
- WGS data showed that “false” positive strain identical to PT strain

New Perfomance criteria

Contamination level	% positive	# positive samples / total # samples
Hygiene swab samples		
***S.* Infantis + *S.* Enteritidis high-level**	Min. 80 %	Min. 3/4
***S.* Infantis low-level**	Min. 50 %	Min. 3/6
Negative (no *Salmonella* added)	Not evaluated*	Not evaluated*
Control samples		
Procedure control (BPW only)	0 %	0 /1
Positive control with *Salmonella*	100 %	1 /1

* 100% *Salmonella*-free matrix cannot be guaranteed, so that an incidental positive result with a *Salmonella* strain different from the inoculation strain is still considered as acceptable.

Samples: specificity, sensitivity, accuracy

Hygiene swab samples		All participants n=68	EU NRLs only n=56	EU NRLs PPS n=28	EU NRLs Food n = 28
Negative samples n=4	No. of samples	272	224	112	112
	No. of negative samples	245	204	101	103
	Specificity in %	**90,1%**	**91,1%**	**90,2%**	**92,0%**
Low level SI n=6	No. of samples	408	336	168	168
	No. of positive samples	407	336	168	168
	Sensitivity in %	**99,8%**	**100%**	**100%**	**100%**
High level SI + SE n=4	No. of samples	272	224	112	112
	No. of positive samples	272	224	112	112
	Sensitivity in %	**100%**	**100%**	**100%**	**100%**
All hygiene swab samples with *Salmonella* n=10	No. of samples	680	560	280	280
	No. of positive samples	679	560	280	280
	Sensitivity in %	**99,9%**	**100%**	**100%**	**100%**
All hygiene swab samples (pos. and neg.) n=14	No. of samples	952	784	392	392
	No. of correct samples	924	764	381	383
	Accuracy in %	**97,1%**	**97,4%**	**97,2%**	**97,7%**

Alternative methods

- 20 labs performed a second method

- PCR method, VIDAS
- Mostly accredited
- 12 labs used in routinely

- Results similar to bacteriological method

Conclusions combined PT-PPS- Food 2022

- Control samples:
  - Excellent performance: 100% correct scores

- *Contaminated hygiene swab* samples:
  - High level SI + SE: 100% correct score
  - Low level SI: 1 lab scored 1 sample negative
  - Negative samples: 25 labs scored 1 or 2 samples positive
  - Most probable explanation: background flora (*E. cloacae*) contaminated

Timetable PT PPS 2023

EURL-*Salmonella* Proficiency Test Primary Production Stage 2023
Detection of *Salmonella* in chicken faeces

Week	Date	Subject
27-35		E-mailing the link to the registration form for the Proficiency Test. Please **register by 31 August 2023** at the latest.
39		E-mailing the link for the result form to the participants. E-mailing the protocol and instructions for the result form to the NRLs. Preparation of media by the NRLs.
39	Monday 25 September 2023	Shipment of the parcels to the participants as Biological Substance Category B (UN 3373).
40	**Monday 2 October 2023**	**Start performance of the Proficiency Test.**
44	1 November 2023 at the latest	Deadline for completing the result form: **1 November 2023** (23:59h CET) After this deadline the result form will be closed.
	December 2023	Interim summary report

Thank you all for your participation in this study

EURL Salmonella

Co-funded by the European Union