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

Results Interlaboratory Study validation of ISO/TS 6579-4

Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12,i:-) by polymerase chain reaction (PCR)

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EURL-*Salmonella* workshop May 2023

ISO/TS 6579-4

Scope:
Method is applicable for:

- differentiation of the isolate under analysis between monophasic *Salmonella* Typhimurium and the monophasic variant of another *Salmonella* non-Typhimurium serovar that has the same antigenic formula;
- identification of the isolate under analysis being either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium.

- *Salmonella* Typhimurium (1,4,[5],12:i:1,2)
- Monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-)
- Variant of another non-target serovar
  - *S.* Agama (4,12:i:1,6)
  - *S.* Farsta (4,[5],12:i:e,n,x)
  - *S.* Gloucester (1,4,12,27:i:l,w)
  - *S.* Lagos (1,4,[5],12:i:1,5)
  - *S.* Tsevie (1,4,12:i:e,n,z15)
  - *S.* Tumodi (1,4,12:i:z6)

ISO/TS 6579-4

Describes three different PCR methods:

- **A probe-based multiplex real-time PCR** (PCR method 1):
  Primers and probes published by Maurischat et al., 2015.

- **An agarose gel-based multiplex PCR** (PCR method 2):
  Primers published by EFSA, 2010 and Tennant et al., 2010.

- **An agarose gel-based single target PCR** (PCR method 3):
  Primers published by Maurischat et al., 2015; primers internal control published by Gallien, 2003.

Validation of ISO/TS 6579-4

Validation performed in accordance with **ISO/DIS 17468:2022** ('Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method').
For confirmation and typing methods validation study is based upon **EN ISO 16140-6:2019** ('Microbiology of the food chain – Method validation - Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures').

Validation study performed to determine **performance characteristics**; existed of two parts:

- Method(s) evaluation study
- Interlaboratory study

Interlaboratory study (ILS) ISO/TS 6579-4

- Design ILS based upon info of EN ISO 16140-6 (‘Microbiology of the food chain – Method validation - Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures’):

  - Per PCR method at least 10 valid data sets from at least 10 collaborators needed.
  - A total of (at least) 24 different strains shall be tested:
    - 16 target strains for inclusivity and 8 non-target strains for exclusivity.
  - Strains selected from 172 strains tested during method evaluation study.
  - Like for method(s) evaluation study, typing results found with slide agglutination (for *Salmonella*) was considered as ‘reference method’.

Time frame ILS

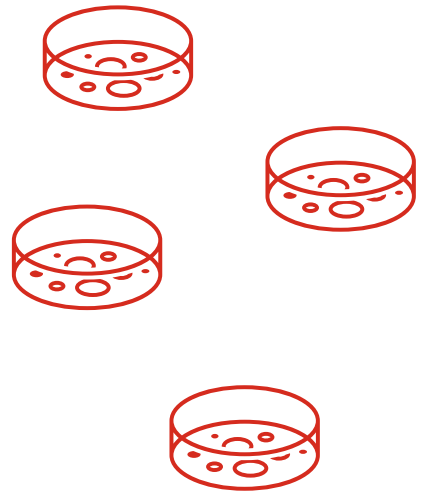
- Feb-March 2022: Call for participants among NRLs-*Salmonella*, members of ISO/TC34/SC9 and ISO/TC34/SC9-WG10.

Number of participants		
PCR method 1	**PCR method 2**	**PCR method 3**
probe-based multiplex real-time PCR	agarose gel-based multiplex PCR	agarose gel-based single target PCR
26	18	13

- Organiser ILS: EURL-*Salmonella.*
- 6 April 2022: informing potential participants on planning ILS.
- 26 April 2022: sending information and documents to participants ILS.
- 16 May – 1 July 2022: Performance ILS.

Strains tested in ILS

- 16 monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-)
  - Including 1 mSTm showing inconsistent results between PCR methods
- 2 (biphasic) *Salmonella* Typhimurium (1,4,[5],12:i:1,2)
  - Including 1 positive control
- 6 strains of other *Salmonella* serovars:
  - *S.* Agama (4,12:i:1,6);
  - *S.* Farsta (4,[5],12:i:e,n,x);
  - *S.* Gloucester (1,4,12,27:i:l,w);
  - *S.* Lagos (1,4,[5],12:i:1,5);
  - *S.* Coeln (1,4,[5],12:y:1,2);
  - *S.* Augustenborg (6,7,14:i:1,2).
- 2 strains of other *Enterobacteriaceae*:
  - *Escherichia coli*
  - *Enterobacter cloacae*



ILS - general

- For this interlaboratory study, monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) was considered as the only target strain.
- All participants received 26 strains in agar vials
- Requested primers, probes, IAC and PCR mix were shipped with dry-ice
  - Others use own primers, probes etc.
- Use of different brands for PCR mix and use of different (real-time) thermal cyclers

ILS – multiplex real-time PCR (PCR 1 - Annex B)

- 26 participants, from 18 different countries.
- Data from 6 participants excluded from calculations for technical reasons like:
  - Deviations from the protocol (e.g. no IAC used);
  - Non-valid results;
  - Temperature abuse of sent reagents.

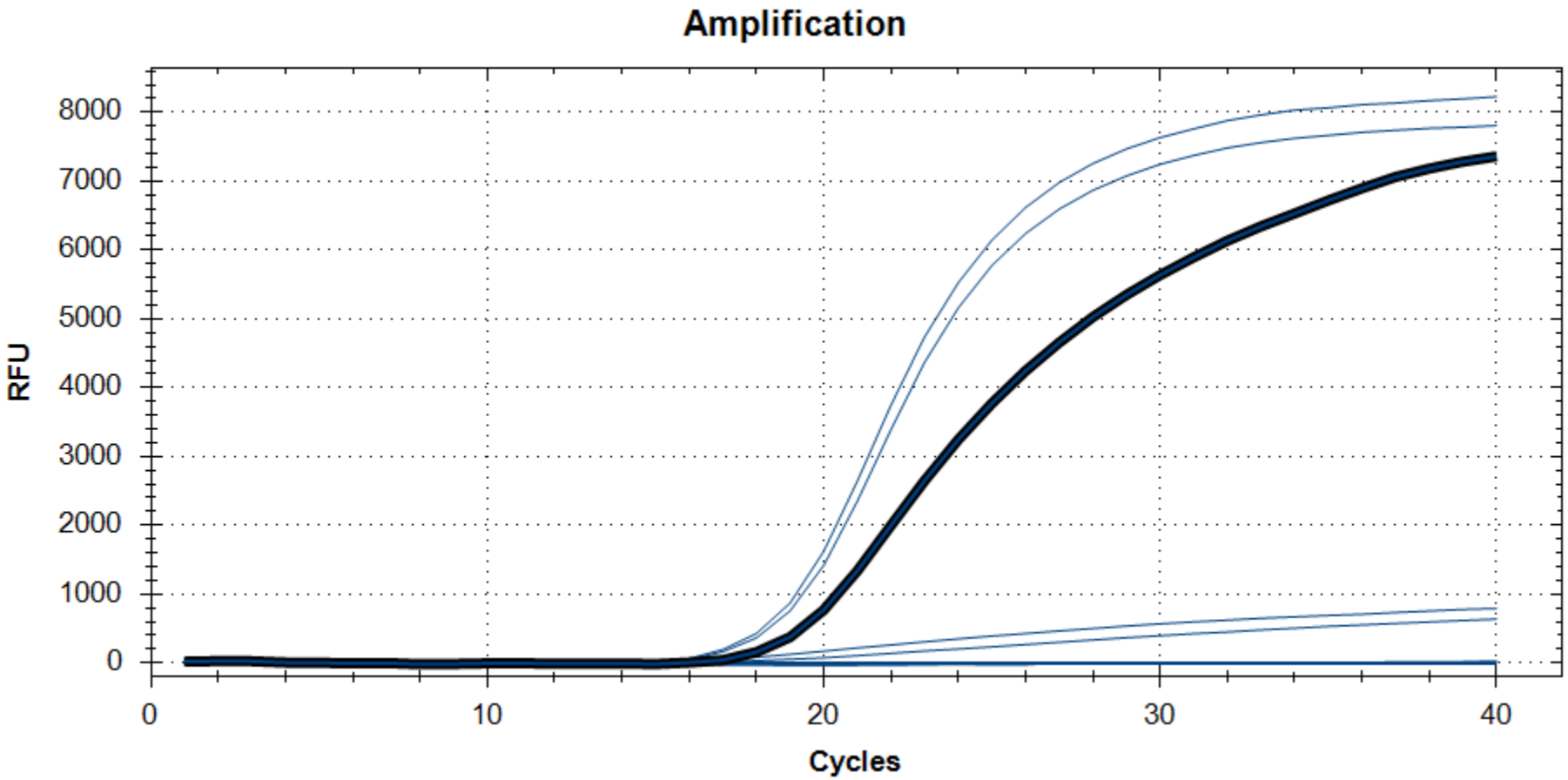
ILS – multiplex real-time PCR (PCR 1 - Annex B)

- 5 participants seemed to have difficulties with interpretation of real-time PCR results / threshold setting:
  - Observation by participants for some samples: lower RFU and higher C_t. Mostly *fliA-IS200* target for other serovar samples
  - Raw data requested

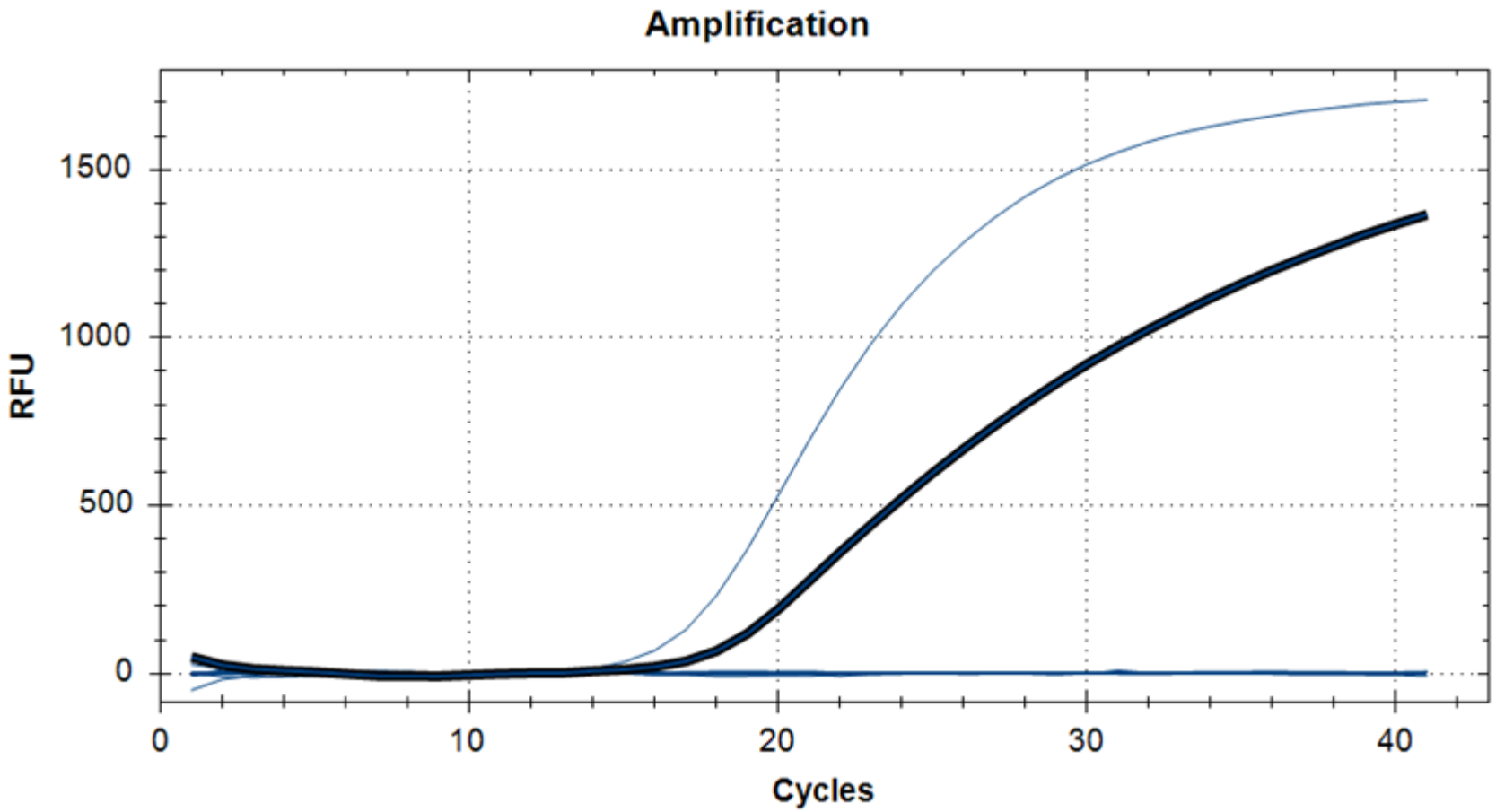
› PCR 1 - Example

Target	fliA-IS200	fljB-hin	hin-iroB	IAC	
Fluorescent	FAM	YY	ROX	Cy5	
Samples	Ct value	Ct value	Ct value	Ct value	
22ILS-01	18,63	-	-	21,72	1,4,[5],12:i:-
22ILS-02	19,1	-	-	22,1	1,4,[5],12:i:-
22ILS-03	**28,63**	16,94	-	21,38	1,4,[5],12:i:-
22ILS-04	19,03	-	-	22,12	1,4,[5],12:i:-
22ILS-05	**27,86**	17,66	**-**	22,02	1,4,[5],12:i:-
22ILS-06	17,93	-	-	22,02	1,4,[5],12:i:-
22ILS-07	18,39	-	18,81	23,04	1,4,[5],12:i:-
22ILS-08	17,44	-	-	22,09	1,4,[5],12:i:-
22ILS-09	**32,49**	17,53	**-**	21,6	1,4,[5],12:i:-
22ILS-10	18,24	-	-	21,85	1,4,[5],12:i:-
22ILS-11	**31,46**	18,99	**-**	21,78	1,4,[5],12:i:-
22ILS-12	19,57	-	-	22,06	1,4,[5],12:i:-
22ILS-13	19,14	-	-	22,14	1,4,[5],12:i:-
22ILS-14	19,04	-	-	22,14	1,4,[5],12:i:-
22ILS-15	17,65	18,02	18,12	24,37	1,4,[5],12:i:1,2
22ILS-16	**34,64**	19,07	19,31	22,65	1,4,[5],12:i:1,2
22ILS-17	18,4	-	18,68	22,4	1,4,[5],12:i:-
22ILS-18	18,91	-	-	21,6	1,4,[5],12:i:-
22ILS-19	-	-	-	22,31	Other serovars
22ILS-20	19,51	-	19,96	22,25	1,4,[5],12:i:-
22ILS-21	18,61	19,03	-	21,65	1,4,[5],12:i:-
22ILS-22	**30,42**	18,71	**-**	21,67	1,4,[5],12:i:-
22ILS-23	19,8	-	-	22,07	1,4,[5],12:i:-
22ILS-24	**18,41**	**-**	**-**	**21,75**	1,4,[5],12:i:-
22ILS-25	18,8	-	19,28	22,09	1,4,[5],12:i:-
Pos. cont.	19,57	20,07	20,11	22,13	1,4,[5],12:i:1,2

- PCR 1 Example: *fliA-IS200* target



› PCR 1 Example: *fliA-IS200* target



ILS – multiplex real-time PCR (PCR 1 - Annex B)

- Additional info added to clause B.3 for better guidance for the interpretation of the results

"The quantification cycle (Cq) value, or cycle threshold (Ct) value, is dependent on the thermal cycler model and analysis software used for the assay and shall be determined for the users' own thermal cycler. **A positive sample generates a typical amplification curve, with at least the exponential phase (see ISO 22119:2011).**

The positive (process) control (e.g. an isolate of biphasic *Salmonella* Typhimurium; see B.2.3.2.3) can help with the interpretation of the amplification curve and for setting the threshold to determine the C_t value of the target sequence of the isolate under analysis. Additionally, if an isolate is positive for different target sequences (see Table B.4), it is expected that the C_t value of the different targets, excluding IAC, is in the same order of magnitude. Further information and guidance for interpretation of real-time PCR results can be found in ISO 22174."

ILS – multiplex real-time PCR (PCR 1 - Annex B)

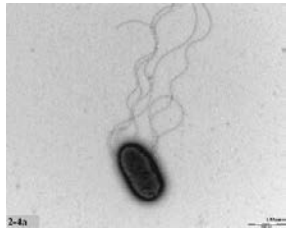
- Additional info added to clause B.3 for better guidance for the interpretation of the results
  - Re-interpretation of results

- 5 participants agreed with re-interpretation real-time PCR results / threshold settings.
- Before re-interpretation 2 inclusivity deviations and 25 exclusivity deviations.
- After re-interpretation 1 inclusivity deviation and 1 exclusivity deviation.

ILS – gel-based multiplex PCR (PCR 2 - Annex C)

- 18 participants, from 14 different countries.
- Data from 1 participant excluded from calculations for technical reasons (temperature abuse of sent reagents).

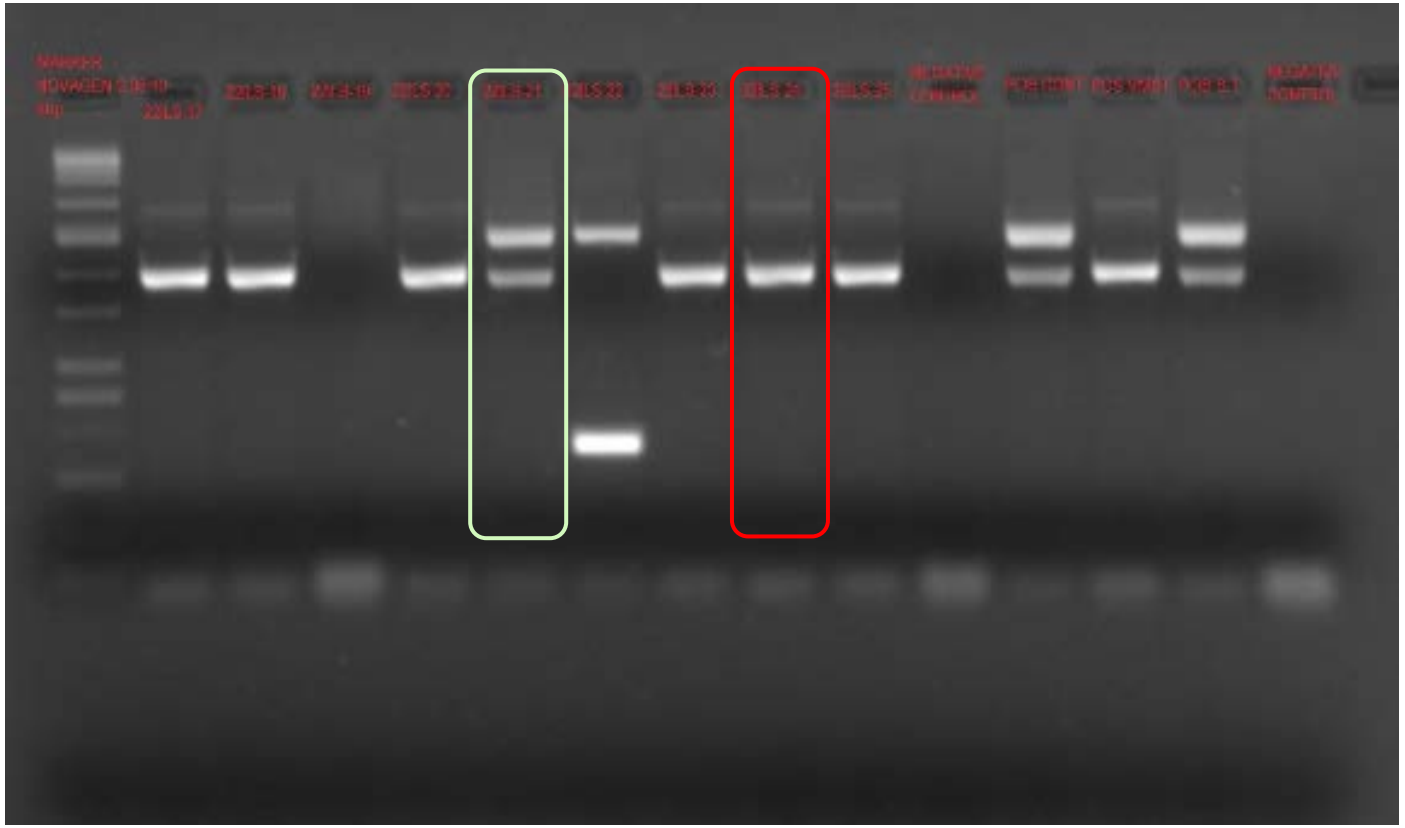
- Inconsistent monophasic *S.* Typhimurium was identified as (biphasic) *S.* Typhimurium by all participants, as in de method comparison study (inclusivity deviations).
- One participant identified sample 24 wrongly as monophasic *S.* Typhimurium (exclusivity deviation).



› PCR 2 - Example

Sample 21
Expectation: 1,4,[5],12:i:-

Sample 24
Expectation: *Enterobacter cloacae*



fljB 1389 bp
fliA-fliB 1000 bp

fliA-fliB 250 bp

Table C.4 – Interpretation of PCR results (expected fragment sizes in bp)

Target sequence	1,4,[5],12:i:-	1,4,[5],12:i:1,2	Other serovars	Other 2nd H-phase monophasic serovars
fliA-fliB	1 000	1 000	250	250
fljB	-	1 389	1 389	-

ILS – gel-based single target PCR (PCR 3 - Annex D)

- 13 participants, from 11 different countries.
- Data from 1 participant excluded from calculations for technical reasons (temperature abuse of sent reagents).
- 10 inclusivity deviations:
  - from three participants.
  - caused by less intense bands for target *fljB-hin*, considered as positive.
  - results were reported as STm instead of mSTm.
- 1 exclusivity deviation by one participant.
- 20 results indicated as missing values because one of the target sequences was negative in combination with a negative IAC:
  - 1 participant with 12 missing values;
  - 1 participant with 6 missing values;
  - 1 participant with 2 missing values.

- Example - *fljB-hin* target

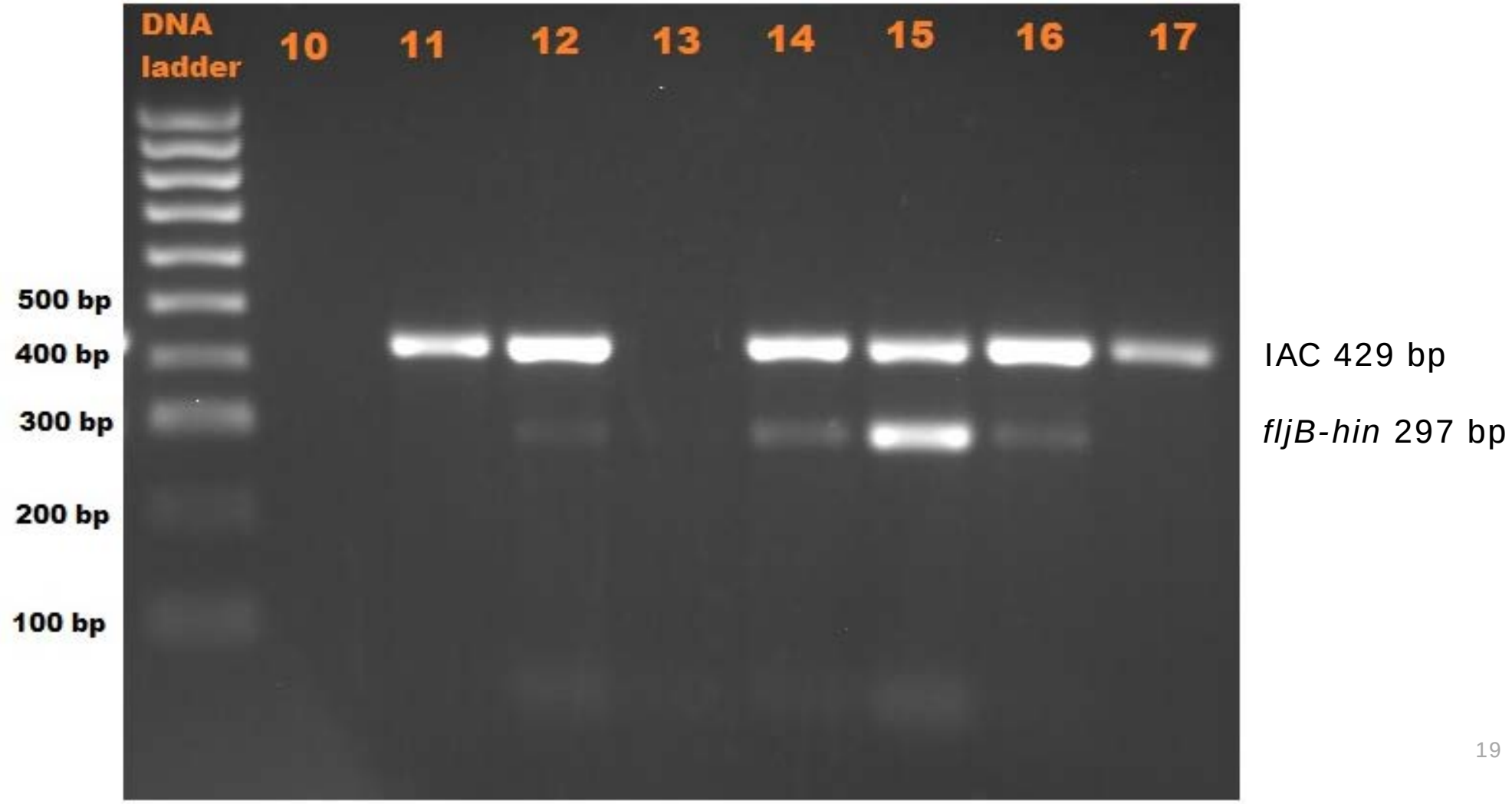


Table E.3 Details of the interlaboratory study of the three PCR protocols.

Details of the interlaboratory study				
Method	**Number of participating collaborators**	**Number of samples per collaborator**	**Number of collaborators retained after evaluation of data**	**Number of samples retained after evaluation of the data**
Multiplex real-time PCR (Annex B)	26	25	20	500
Gel-based multiplex PCR (Annex C)	18	25	17	425
Gel-based single target PCR (Annex D)	13	25	12	280[a]

[a]20 missing values

Table E.4 Inclusivity and exclusivity results of the interlaboratory study of the three PCR protocols; considering monophasic *Salmonella* Typhimurium as target strain and (biphasic) *Salmonella* Typhimurium, other *Salmonella* serovars and other *Enterobacteriaceae* as non-target strains

Method	Performance characteristic	Number of different strains	Total number of results	Inclusivity agreement	Inclusivity deviation[a]	Exclusivity agreement	Exclusivity deviation[a]
Multiplex real-time PCR (Annex B)	Inclusivity	16	320	319	1	NA	NA
	Exclusivity	9	180	NA	NA	179	1
Gel-based multiplex PCR (Annex C)	Inclusivity	16	272	255	17	NA	NA
	Exclusivity	9	153	NA	NA	152	1
Gel-based single target PCR (Annex D)	Inclusivity	16	177[b]	167	10	NA	NA
	Exclusivity	9	103[c]	NA	NA	102	1

[a]: More information about the inclusivity deviations and exclusivity deviations is given in the last three paragraphs in clause E.2
[b] 15 missing values
[c] 5 missing values

Next

- Publication of ISO/TS 6579-4 in 2024

- Report of the method comparison study and the interlaboratory study validation of ISO/TS 6579-4

Thank you for listening!

Special thanks to all ILS participants, NRL-*Salmonella* Germany (BfR), Kirsten Mooijman, Angela van Hoek and Wendy van Overbeek