Journal of Microbiological Methods 244 (2026) 107471

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A comparative study of *invA* and *ttrC*-based real-time PCR assays for the genus-specific molecular identification of *Salmonella enterica*

Jaweria Riaz [a,*], Maira Napoleoni [b], Giuditta Fiorella Schiavano [c], Jennie Fischer [d], Maria Borowiak [d], Giorgio Brandi [a], Giulia Amagliani [a]

[a] *Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy*
[b] *Regional Reference Center for Enteric Pathogens Marche, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy*
[c] *Department of Humanities, University of Urbino Carlo Bo, Urbino, Italy*
[d] *Department Biological Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany*

ARTICLE INFO

Keywords:
Salmonella enterica
invA gene
ttrC gene
Real-time PCR assays
Specificity
Inclusivity
Exclusivity

ABSTRACT

Aims: The reliability of the *invA* gene, a gold-standard target for PCR-based diagnosis of *Salmonella*, has been questioned due to false-negative results reported by some authors. Thus, evaluation of the inclusivity features of *invA* and *ttrC*-based real-time PCR assays and comparison of the specificity performance of both targets for the genus-specific molecular identification of *Salmonella enterica* has been carried out on a wide strain panel.

Methods and results: Genus-specific identification using two real-time PCR-based assays targeting *invA* and *ttrC* gene fragments was performed on *Salmonella* strains ($n = 421$) of 50 different serovars isolated in Central Italy between 2002 and 2023 from: food and veterinary samples during poultry chain surveillance, human clinical samples, and surface water samples collected from rivers. A 99% inclusivity was recorded for both the *ttrC*-based and the *invA*-based real-time PCR assays, with perfect agreement (Cohen's Kappa = 0.991) with the ISO/TR 6579–3:2014 and with each other.

Conclusions: Our findings suggest that both the *ttrC*-based and *invA*-based PCR assays are equally reliable for the genus-specific identification of a wide *Salmonella enterica* strain panel of epidemiological relevance and are in complete agreement. However, considering the genomic variability in some *Salmonella* serovars, a dual-target PCR approach is recommended to potentially improve diagnostic accuracy.

1. Introduction

Salmonella spp. is a genus of the *Enterobacteriaceae* family that shows prominent variations in serological reactions, genetic makeup, and virulence. The genus is classified into two species: *Salmonella enterica* and *Salmonella bongori*, and more than 2600 serovars. *S. enterica* is divided into six different subspecies, named (or assigned Roman numerals) as follows: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI), although new taxonomic groups have been recently proposed (Chattaway et al., 2021; Pearce et al., 2021). Whereas *S. bongori* has no subspecies, *S. enterica* subspecies *enterica* (*S.*) accounts for the vast majority of human infections, which might also correlate with its widespread distribution in domesticated animals. However, *S. bongori* and serovars of higher *S. enterica* subspecies other than I and II are commonly associated with cold-blooded animals.

Salmonella spp. is a major cause of gastrointestinal infections in humans and animals. The transmission of this foodborne pathogen occurs mainly over the farm-to-fork continuum, and poultry products serve as the leading contributor. Also, in the European Union (EU), salmonellosis has been reported as the second most common cause of zoonosis after campylobacteriosis in humans (EFSA and ECDC, 2024). Invasive non-typhoidal *Salmonella* (NTS) disease has been proven to be a major public health threat (Balasubramanian et al., 2019; EFSA and ECDC, 2024; Wang et al., 2024; White et al., 2022). A worldwide estimate of the gastroenteritis disease burden caused by NTS reported more than 93.8 million cases and 230,000 annual deaths (Majowicz et al., 2010). Considering the epidemiological data, rapid and reliable surveillance of *Salmonella* presence in food is of paramount importance for consumers' protection in several countries, including the EU (Regulation (EC) No 2160/2003, 2003). The PCR-based molecular detection (conventional or real-time) of pathogens in food matrices is considered a fast and accurate

---

* Corresponding author at: Department of Biomolecular Sciences, University of Urbino Carlo Bo, via S. Chiara, 27, 61029 Urbino, Italy.
E-mail address: j.riaz@campus.uniurb.it (J. Riaz).

https://doi.org/10.1016/j.mimet.2026.107471
Received 15 January 2026; Received in revised form 5 March 2026; Accepted 16 March 2026

approach, as a preliminary screening of enrichment cultures, and technological advancement has made it the best choice in molecular diagnosis (Bell et al., 2016). Till now, DNA-based detection of *S. enterica* in food and environmental samples has been carried out using many targets, i.e., unique *agfA* sequences (Siddiky et al., 2022), *viaB* (*S. typhi*) (Kariuki et al., 2022), *hilA* (Guo et al., 2000; Veni et al., 2025), *sdiA* (Halatsi et al., 2006), *ttrC* (Carloni et al., 2018), *fimW* gene (Wen et al., 2020), *ompC* (Tăbăran et al., 2024), and *stn* (Liu et al., 2024), to mention only a few, all considered sensitive and specific.

Pathogenic *Salmonella* strains isolated from poultry harbor various genes associated with *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) (Shaji et al., 2023). SPI-1 enables bacterial penetration of non-phagocytic host cells, while SPI-2 is important for survival within macrophages and for the establishment of systemic infection (Pico-Rodríguez et al., 2024). The *invA* gene encodes an inner membrane protein essential for epithelial cell invasion and presumably induces internalization (Hensel, 2004; Pardo-Roa et al., 2019). The *invA* 139–141 primer set (284 bp), identified as the most selective for *Salmonella* detection (Rahn et al., 1992), showed 99.6% inclusivity and 100% exclusivity using 242 target and 122 non-target strains (Malorny et al., 2003a). Although bioinformatic analysis confirmed the *invA* gene as an important target for *Salmonella* spp. identification due to its wide distribution in different serovars linked to its major role in invasion and pathogenesis (Mkangara et al., 2020; Shanmugasamy et al., 2011), its reliability has been questioned by some authors, as a few false negatives (Buehler et al., 2019; Kreitlow et al., 2021; B. T. Mohammed, 2024; Nde et al., 2008; Turki et al., 2012; Liu et al., 2018; Yanestria et al., 2019) and false-positives (Arnold et al., 2004; Nde et al., 2008; Resendiz-Nava et al., 2019) were being reported. Discrepant *invA*-based PCR results reported by different authors might be attributed to the use of different primer sets, *S. enterica* subsp. IV was not detected by Kreitlow et al. (2021) using LAMP primers, whereas it was successfully detected by Malorny et al. (2003b) using the Rahn et al. (1992) primer set. Moreover, some serotypes of *S. enterica* subsp. *enterica* may lack the *invA* gene sequence, thereby preventing its detection by PCR targeting this gene (Ginocchio et al., 1997; Turki et al., 2012).

In contrast, recent studies highlighted the very high inclusivity and exclusivity of *ttr*RSBCA-based primers (Kreitlow et al., 2021), while Malorny et al. (2004) described first the *ttr*-based *Salmonella* spp. specific detection. The *ttr* locus, positioned near SPI-2, forms the genetic basis of tetrathionate respiration (Hensel et al., 1999). This locus, carrying five genes (*ttrA*, *ttrB*, and *ttrC* encoding the structural protein for tetrathionate reductase, and *ttrR* and *ttrS* encoding a two-component regulator system), is considered significant in the life cycle of the pathogen and is therefore genetically stable in all *Salmonella* spp. (Malorny et al., 2004).

Being a classical gold standard method, the *invA*-based PCR detection should be highly specific for the *Salmonella* genus since specificity is the main criterion for the diagnostic test. Specificity has been defined as the sum of inclusivity, “the ability to detect the target microorganism from a wide range of other microorganisms”, and exclusivity, “the lack of interference from a relevant range of non-target microorganisms” (ISO 16140-2, 2016; Malorny et al., 2003a). The *invA* gene target has been shown to have limitations in its specificity performance; on the other hand, the *ttrC* gene marker is also as reliable as *invA* for specific detection of genus *Salmonella,* however, it has not been described as a gold standard in literature, but rather a validated alternative. Thus, our work aimed to compare the specificity performance of two real-time PCR assays, using two different target sequences, one from *invA* and the other from the *ttrC* gene, for the genus-specific molecular identification of 421 *Salmonella* strains (50 serovars) isolated from different matrices to obtain a high diversity of strains and serovars as a cross section of strains circulating in Italy, with particular focus on the poultry chain.

2. Materials and methods

2.1. Strain collection and culture conditions

The strain panel used for this study included 421 *Salmonella* strains belonging to the 50 most epidemiologically relevant serovars, including the rare serovars, in Italy, obtained from the Regional Reference Center for Enteric Pathogens (CRRPE), Marche, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, section of Tolentino. It covered a wide time interval of twenty-two years, from 2002 to 2023 (Table 1). Food and veterinary strains (n. 196) have been collected during the *Salmonella* surveillance program for poultry meat, carried out under Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs (European Union, 2005), following the national Poultry monitoring plan (Italian Ministry of Health, 2018, editions from 2008 to 2022) and

Table 1
Salmonella strains used for inclusivity tests. The origin of the strain is reported.

	No. of strains			
Serovars	Food-Vet*	Human	Surface water	Total
S. Abaetetuba	1	0	0	1
S. Abortusovis	1	0	0	1
S. Agama	0	0	1	1
S. Agona	2	0	0	2
S. Anatum	2	0	0	2
S. Blockley	17	0	0	17
S. Bovismorbificans	0	1	0	1
S. Bradford	1	0	0	1
S. Braenderup	1	0	0	1
S. Brandenburg	1	0	0	1
S. Bredeney	11	0	0	11
S. Cerro	2	0	0	2
S. Choleraesuis var. Kunzendorf	1	0	0	1
S. Coeln	3	0	0	3
S. Colorado	1	0	0	1
S. Cubana	1	0	0	1
S. Derby	1	23	17	41
S. Enteritidis	11	19	0	30
S. Fyris	0	1	0	1
S. Give	0	1	0	1
S. Goldcoast	1	0	0	1
S. Hadar	20	0	0	20
S. Haifa	1	0	0	1
S. Heidelberg	1	0	0	1
S. Infantis	64	22	19	105
S. Isangi	1	0	0	1
S. Kapemba	0	0	1	1
S. Kentucky	4	0	0	4
S. Kottbus	1	0	0	1
S. Liverpool	2	0	0	2
S. Livingstone	12	0	0	12
S. London	0	1	0	1
S. Mbandaka	5	0	0	5
S. Montevideo	1	0	0	1
S. Muenchen	1	0	0	1
S. Napoli	0	0	1	1
S. Newport	1	0	0	1
S. Panama	0	1	0	1
S. Poona	1	0	0	1
S. Rissen	1	0	0	1
S. Saintpaul	2	0	0	2
S. Schwarzengrund	1	0	0	1
S. Senftenberg	0	1	0	1
S. Stanley	0	1	0	1
S. Stanleyville	1	0	0	1
S. Strathcona	0	1	0	1
S. Thompson	10	0	0	10
S. Veneziana	2	0	0	2
Monophasic variant of *Salmonella* Typhimurium (MVST)	3	25	27	55
Tot.	196	122	103	421

[*] Food-Vet: strains of food and veterinary origin (see Materials and methods for details).

during self-monitoring controls in food (European Union, 2005) and veterinary sectors (Italian Ministry of Health, 2018, editions from 2008 to 2022). Human strains (n. 122) were isolated from people with gastrointestinal symptoms, some of them hospitalized and were sent to the CRRPE from the Regional hospitals' analysis laboratories participating in Enter-Net surveillance for the Marche region. Moreover, environmental strains (n. 103) isolated by the Regional Agency for Environmental Protection of Marche (ARPAM) in the context of surface water monitoring for drinking water purposes in accordance with the Legislative Decree 152/06, Part III, Annex 2, Section A and sent to CRRPE for serotyping, were included.

In detail, the strains were collected from three different sources: 1) human clinical samples included faeces (n. 115), blood (n. 04), urine samples (n. 02), and wound swabs (n. 01); 2) food and veterinary samples from the poultry chain included fresh chicken meat (n. 56), processed chicken meat (n. 10), meat-based products (n. 04), poultry faeces (n. 23), farm dust (n. 01), boot swabs (n. 90), environmental swabs from food processing environments (n. 02) also from bivalve mollusk (n. 01), fresh pork meat (n. 01), fresh turkey meat (n. 01), ovine organ (n. 01), pepper (n. 01), porcine organ (n. 01), processed beef meat (n. 01) and processed pork meat (n. 03), and 3) surface water samples (n. 103) were collected using sterile containers for microbiological analysis (supplementary material, Table S1).

Strain isolation and identification were accomplished according to the ISO 6579-1:2017/Amd 1:2020 (UNI EN ISO 6579-1:2017/Amd 1, 2020). All *Salmonella* strains, regardless of their origin, were serotyped according to ISO/TR 6579–3:2014 (ISO/TR 6579-3, 2014). Briefly, antigenic formulae and serovars were determined using the White–Kauffmann–Le Minor scheme (Grimont and Weill, 2007) by slide agglutination with commercially available antisera (Statens Serum Institut, Copenhagen, Denmark) against the somatic (O), capsular (Vi), and flagellar (H) antigens.

Since the strain panel comprised a historic collection, *Salmonella* strains were recovered from microbanks (Biolife) that were stored at −80 °C, re-cultured on Xylose Lysine Deoxycholate (XLD) agar (Biolife), with overnight incubation at 37 °C. Sixty-three non-*Salmonella* strains belonging to 18 different bacterial genera were also included in the study to confirm exclusivity (Table 2).

Table 2
Non-*Salmonella* strains used for exclusivity tests.

Species	Strain ID
Aeromonas spp.	52,845/23
Aeromonas spp.	57,581/23
Aeromonas spp.	34,482/24
Aeromonas spp.	36,027/24
Aeromonas spp.	44,577/24
Aeromonas spp.	47,564/24
Aeromonas spp.	56,140/24
Aeromonas spp.	11,412/25
Aeromonas spp.	18,998/25
Aeromonas spp.	23,528/25
Bacillus cereus	31,028/23
Bacillus cereus	34,398/23
Bacillus cereus	40,427/23
Bacillus cereus	30,622/24
Bacillus cereus	40,730/25
Campylobacter coli	43,465/25
Campylobacter coli	43,702/25
Campylobacter coli	44,198/25
Campylobacter coli	53,628/25
Campylobacter jejuni	2430/26
Campylobacter jejuni	1706
Campylobacter jejuni	2940
Campylobacter jejuni	2997
Campylobacter jejuni	4257
Citrobacter freundii	40,428/23
Citrobacter freundii	46,632/25
Citrobacter freundii	17,983/14
Clostridium difficile	A702
Clostridium difficile	A1024
Clostridium perfringens	A1064
Clostridium perfringens	A1057
Cronobacter spp.	39,849/23
Enterobacter cloacae	ATCC 13047
Enterococcus casseliflavus	V4136
Enterococcus durans	V4150
Enterococcus dysgalactiae	V4181
Enterococcus faecalis	ATCC 29212
Enterococcus faecalis	V4157
Enterococcus faecalis	V4186
Enterococcus faecium	V4194
Escherichia coli	ATCC 25922
Escherichia coli O157:H7	ATCC 35150
Klebsiella pneumoniae	ATCC 10031
Klebsiella pneumoniae	ATCC 13883
Listeria innocua	ATCC 33090
Listeria monocytogenes	ATCC 7644
Mannheimia haemolytica	B2700
Mannheimia haemolytica	B2707
Pasteurella multocida	B2704
Pasteurella multocida	B2695
Pseudomonas aeruginosa	ATCC 10145
Shigella flexneri	ATCC 12022
Staphylococcus aureus	ATCC 6538
Staphylococcus aureus	ATCC 25923
Streptococcus canis	V4163
Streptococcus equisimilis	V4195
Streptococcus suis	V4189
Streptococcus uberis	V4145
Yersinia enterocolitica	15,460/24
Yersinia enterocolitica	19,142/24
Yersinia enterocolitica	25,531/24
Yersinia enterocolitica	39,179/24
Yersinia enterocolitica	50,016/24

2.2. Real-time PCR protocols

Bacterial DNA for real-time PCR assays was obtained by thermal lysis (Russo et al., 2022). Genus-specific qualitative real-time melting curve PCR was carried out in a Real-time thermal cycler Rotor-Gene Q using two different assays: one targeting a sequence of the *invA* gene and the other a sequence of the *ttrC* gene, as described in the following.

2.2.1. *invA*-based real-time PCR assay

The primer set targeting the *invA* gene was based on the protocol described by Rahn et al. (1992). The specific primer pair used to detect *Salmonella* included forward 139 (5′-GTGAAATTATCGCCACGTTCGGGCAA-3′) and reverse 141 (5′-TCATCGCACCGTCAAAGGAAC-3′), which were utilized to get an amplification product of 284 bp (Table 3). The gel-based PCR protocol reported by Arnold et al. (2004) using the same primers was converted to a melting curve real-time PCR protocol by making some specific modifications as follows: The reagents consisted of 1× TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa), 10 μM of both 139 and 141 primers, and 5 μl of DNA template in a final volume of 25 μl. The thermal protocol involved initial denaturation at 95 °C for 10 min, followed by 30 cycles with denaturation at 95 °C for 30 s and annealing at 60 °C for 60 s. Melting curve analysis was performed from 72 to 95 °C, with a 1 °C increase every 5 s. The conversion of the end-point protocol of Arnold et al. (2004) to the real-time PCR assay described above was validated by using *S. enterica* ATCC 13076 DNA as positive control and non-*Salmonella* strains as negative controls, as provided in Table 2. The presence of the 284 bp-specific product was visualized by using 1.5% agarose gel electrophoresis for both assays.

2.2.2. *ttrC*-based real-time PCR assay

The *ttrC* (155 bp)-based genus-specific real-time PCR was performed according to Amagliani et al. (2010), using the same PCR-kit as for the *invA*-based amplification. The reagents included 1× master mix, 10 μM of both *ttr*1 (5′-GCAGGAGGTCTGAATGACG-3′) and *ttr*2 (5′-TTTTCCGCCAGTGAAGATAAC-3′) primers (Table 3) and 5 μl of DNA template, making the total volume up to 25 μl. The thermal conditions

Table 3
Primers used for *invA* and *ttr*C-based real-time PCR assays.

Primer	Sequence 5′-3′ (length, bp)	Position	Gene and GenBank Access. No.	Tm (°C)	Amplicon length (bp)
139	GTGAAATTATCGCCACGTTCGGGCAA (26)	371–396	*invA* M90846.1	76	284
141	TCATCGCACCGTCAAAGGAAC (21)	636–655		71	
*ttr*1	GCAGGAGGTCTGAATGACG (19)	3278–3296	*ttrC* AF282268.1	64	151
*ttr*2	TTTTCCGCCAGTGAAGATAAC (21)	3412–3432		59	

included an initial denaturation of 95 °C for 2 min, followed by 30 cycles of 95 °C for 20 s denaturation and annealing at 63 °C for 60 s. Melting curve analysis was performed from 72 to 95 °C, with a 1 °C increase every 5 s.

2.3. Statistical analysis

Contingency tables were constructed after data collection from clinical, environmental, and food/veterinary strains. The agreement between the results of each *invA* and *ttrC*-based real-time PCR assays and the real strain identity, previously determined using the UNI EN ISO 6579-1:2017/Amd1:2020 (UNI EN ISO 6579-1:2017/Amd 1, 2020), has been assessed by Cohen's Kappa coefficient (Mackinnon, 2000) by using the online tool GraphPad (https://www.graphpad.com/quickcalcs/kappa1/). The value has been interpreted according to Landis and Koch (Landis and Koch, 1977). Inclusivity and exclusivity for both assays were calculated using the following formulae.

Formula (1)

$$\text{Inclusivity} = \frac{TP}{TP + FN} = \frac{400}{400 + 1} = 0.99 \text{ or } 99\%$$

Formula (2)

$$\text{Exclusivity} = \frac{TN}{TN + FP} = \frac{12}{12 + 0} = 1.0 \text{ or } 100\%$$

3. Results

3.1. Genus-specific real-time PCR

The standard PCR previously described by Arnold et al. (2004), once converted into a real-time PCR assay to obtain faster results, was equally able to detect the specific 284 bp target sequence from the *S. enterica* ATCC 13076 used as positive control (not shown). Non-*Salmonella* strains (Table 2) were not amplified. All clinical, food, veterinary, and environmental *Salmonella* strains ($n = 420$) tested positive with both *invA* and *ttrC*-based real-time PCR assays except for one rare serovar, *S.* Abaetetuba (supplementary material, Table S1). Melt analysis of the amplification products revealed peaks ranging from 88.0 °C to 89.2 °C, with an average of 88.35 °C ± 0.23, for the *invA* target, and from 86.5 °C to 89.3 °C, with an average of 87.18 °C ± 0.29 for the *ttrC* target (Fig. 1).

3.2. Agreement analysis and assays inclusivity/exclusivity

Bivariate contingency tables summarize the diagnostic performance of *invA* and *ttrC*-based real-time PCR assays, each separately analyzed in comparison with the reference culture method (ISO/TR 6579–3:2014) (Table 4). A Kappa value of 0.991 with a 95% confidence interval from 0.973 to 1.00 and a Standard error (SE) of 0.009 was observed for both *invA* and *ttrC* gene targets. The observed value indicated, therefore, perfect agreement of each assay with the standard culture method and with each other, according to the same scale of interpretation (Kappa between 0.81 and 1.00) for Kappa statistics according to Landis and Koch (Landis and Koch, 1977). Moreover, 99% inclusivity and 100% exclusivity have been recorded for both targets (Formulas 1 and 2).

4. Discussion

The *invA* is considered a gene marker of unique nature for *Salmonella*, after it was first acknowledged by Rahn et al. (1992), and many protocols for the genus-specific molecular detection of this pathogen are based on this gene (Cremonesi et al., 2016; Hoorfar et al., 2000; Huoy et al., 2024; Singh and Mustapha, 2013). In the current study, we used the primer set initially described by Rahn et al. (1992), subsequently validated on 242 target strains (Malorny et al., 2003b), and extensively used by several authors in recent years (Chevez et al., 2024; Nazari Moghadam et al., 2023; Pino et al., 2025). We tested this primer set on a large number of strains from different origins, including epidemiologically important serovars, and compared it with another primer set targeting the genus-specific sequence of *ttrC* (Amagliani et al., 2010). Both the *ttrC* and *invA* primer sets offered 99% inclusivity, with only one strain showing false-negative results for both *ttrC* and *invA* gene targets. Moreover, the exclusivity evaluation (100%) carried out in the present study complemented previous results from Rahn et al. (1992) and Amagliani et al. (2010), which assessed the specificity of the *invA* and *ttrC* primer sets, respectively. Rahn et al. tested the *invA* primer set against 142 non-*Salmonella* strains belonging to 21 genera, while Amagliani et al. evaluated the *ttr*C primers against 10 genera. Our results, combined with these previous findings, suggest that the *invA* and *ttrC* primer sets are reliable targets for detecting *S. enterica*.

However, the reliability of the *invA* gene as a gold standard has been questioned in the past by some authors, using either the same (Arnold et al., 2004; Nde et al., 2008; Resendiz-Nava et al., 2019; Yanestria et al., 2019) or different primer sets (Buehler et al., 2019; Kreitlow et al., 2021; B. T. Mohammed, 2024; Turki et al., 2012), due to some failures of PCR-based protocols (gel-based or real-time). Both false negatives (Yanestria et al., 2019; Nde et al., 2008) and false positives (Arnold et al., 2004; Nde et al., 2008; Resendiz-Nava et al., 2019) were recorded with the 139–141 *invA* primers. Some of them (Resendiz-Nava et al., 2019) suggested the use of *ttrA/C* genes for reliable detection after successful discrimination of *Salmonella* and non-*Salmonella* strains.

The *ttr* locus is required for tetrathionate respiration in anaerobic environments and is likely to be significant within the life cycle of *Salmonella* spp. (Hensel et al., 1999). Malorny et al. (2004) claimed that *ttr* genes in all *Salmonella* strains should be considered genetically stable and could be suggested as a reliable target for *Salmonella* detection, in line with the recent report from Kreitlow et al. (2021).

The *invA*, as an integral part of SPI-1, is involved in encoding the effector protein of a T3SS (Type III Secretion System) that is important for the non-phagocytic invasion of bacteria in epithelial cells (Galán et al., 1992; Hu et al., 2021), although it is not essential for the survival of certain *Salmonella*. Indeed, its sequence is likely to be susceptible to genetic variation or to be even missing; *S.* Kentucky, *S.* Senftenberg, and *S.* Litchfield are known to occasionally lack this sequence (Ginocchio et al., 1997). Additionally, *invA* diversity across *Salmonella* serovars has been initially described by Ginocchio et al. (1997) and more recently by Branchu et al. (2018), while Buehler et al. (2019) listed 362 of 2058 nucleotide sites that were variable among *invA* sequences.

Since PCR-based methods require high levels of homogeneity between primer and target sequences to avoid significant reductions in PCR detection efficiency (Lefever et al., 2013). The *invA* diversity caused by single-nucleotide polymorphisms (SNPs) could be responsible for false-negative results. Especially less common subspecies and serovars

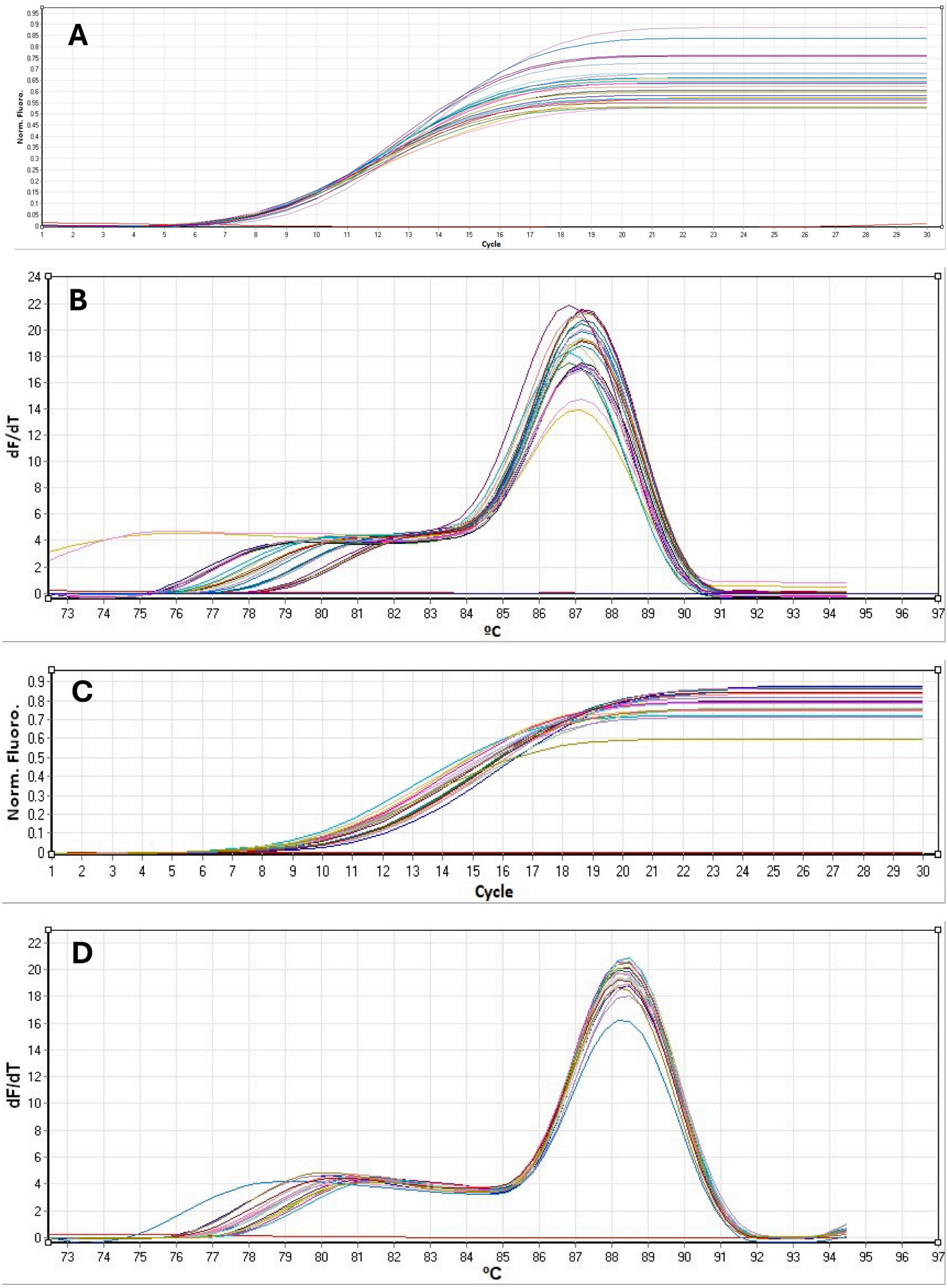

Fig. 1. A-B showing the amplification and melting curves of the *ttrC* gene target, and C-D showing the amplification and melting curves of the *invA* gene target.

Table 4
Contingency tables for real-time PCR results in comparison with strain identity assessed through the ISO/TR 6579–3:2014. *invA* targeting assay (A); *ttrC* targeting assay (B).

A	Positive (*invA* PCR)	Negative (*invA* PCR)
Positive (Culture)	420 (True positives)	1 (False Negatives)
Negative (Culture)	0 (False positives)	63 (True Negatives)
B	**Positive (*ttrC* PCR)**	**Negative (*ttrC* PCR)**
Positive (Culture)	420 (True positives)	1 (False Negatives)
Negative (Culture)	0 (False positives)	63 (True Negatives)

might carry *invA* polymorphisms, as explained by Buehler et al. for *S. enterica* subsp. *salamae* (Buehler et al., 2019). The *ttrC* and *invA* false-negative result was reported in this study for a rare serovar, *S.* Abaetetuba, obtained from fresh pork meat. The *ttrC*-negative result could be attributed to a single-nucleotide deletion observed in the binding site of the forward primer sequence in the whole genome sequence of a reference genome (*S.* Abaetetuba str. ATCC 35640; Accession number: NZ_CP007532.1; Bioproject: PRJNA170546) on the NCBI database. If our strain also possesses the same deletion, it could have prevented efficient primer annealing, leading to a negative PCR result. Likewise, the false-negative *invA* result suggests the presence of strain-specific polymorphisms that were not observed in the reference genome. Although we did not perform WGS, our hypothesis for amplification failure for a single serovar could also be supported by a recent study, which documented several non-synonymous mutations through WGS, including both targets (*ttrC* and *invA*) used for detecting *Salmonella* (Hu et al., 2021).

Nevertheless, according to the WHO (https://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal, lastly accessed October 2025), all *Salmonella* serovars can cause disease in humans and may be present in a wide range of hosts and environments. Therefore, detection methods should also account for uncommon serovars occurring not only in food matrices but also in primary production, food-related environments, and surface waters.

Although the present study did not include all known *Salmonella* serovars, which are estimated to be more than 2600, it should be considered that the serovars investigated here are the most prevalent and epidemiologically relevant in the food and veterinary field, as indicated by EFSA and ECDC in their last report (EFSA and ECDC, 2024). Indeed, while *S.* Enteritidis and *S.* Typhimurium prevalence decreased thanks to vaccinations and other control programs in the primary production and in the poultry chain (Commission Regulation (EC), 2006), *S.* Infantis is now ranked among the top four serovars and the most isolated in broiler production (EFSA and ECDC, 2024), and it's gaining increasing concern, especially if considering multidrug-resistant strains possessing antimicrobial resistance genes and the pESI-like plasmid (Russo et al., 2024). The rare serovars like *S.* Breanderup in melons (Moore et al., 2024), *S.* Umbilo in rocket (Rosner et al., 2024), and diverse serovars in sesame (Anyogu et al., 2024) often cause outbreaks and are important to take into consideration for PCR detection. However, strains more often associated with cold-blooded animals and known as opportunistic pathogens with low prevalence in human infections might also be considered, since they can induce extraintestinal infections, causing a broad spectrum of serious diseases (Lamas et al., 2018).

To overcome such issues, reported in the literature, a different primer set could be advisable. In line with this, the revised instructions of the US FDA, Bacteriological Analytical Manual (Wallace et al., 2024), reporting the two FDA-developed molecular methods for rapid screening of pre-enrichment cultures through loop-mediated isothermal amplification (LAMP) and real-time PCR (qPCR) (www.fda.gov/media/107724/download, lastly accessed December 2025), include *invA* primers other than those of Rahn et al. (1992).

In recent years, also thanks to the increasing application of WGS analysis, several characteristic elements, such as integrases, transposases and insertion sequence (*IS*) elements that contribute to mobilization as well as instability of pathogenicity islands have been identified in *S. enterica* (Branchu et al., 2018). Such variability could affect the reliability of a PCR assay using primers targeting genes within the above-mentioned pathogenicity islands. Indeed, mutations or indels can occur within primer and probe target regions, and depending on the location of these mutations, PCR sensitivity can be diminished, in some cases resulting in false-negative results. Attention should be paid to uncommon serovars or emerging variants, as stated by Rockett et al. (2020), who documented nucleotide diversity in primer and probe sequences occurring within the 52 serovars investigated.

Although our individual PCR assays showed high inclusivity, we recommend using a dual-target PCR approach, taking into consideration the genetic variability described by others. This will further minimize the risk of false-negative results, especially in the case of rare serovars or emerging genetic lineages and improve specificity and reliability. Given the increasing reliance on PCR systems for *Salmonella* detection, it is essential to regularly monitor genomic diversity in PCR target regions, particularly among prevalent and emerging serovars of public health significance. This can be achieved by leveraging the growing number of NGS sequence data (Rockett et al., 2020) and continuing to isolate and sequence *Salmonella* strains to track genetic variability among new emerging lineages (Allard et al., 2013; Mohammed and Dubie, 2022).

5. Conclusion

The comparison of *invA* and *ttrC-based* real-time PCR assays showed 99% inclusivity across a diverse strain set of *S. enterica* serovars, including the most epidemiologically prevalent and also the rare ones. Hence, the concerns raised in the literature on the *invA* reliability using a similar or different primer set of Rahn et al. (1992) were largely unconfirmed in our dataset, with only one *S.* Abaetetuba strain testing negative, his gold standard (*invA*) was successfully validated for routine surveillance of *Salmonella* serovars in Italy. Concerning the rare serovars and ongoing genetic diversity (SNPs), a two-target PCR approach can guarantee comprehensive detection. Standard methods of microbial detection should regularly be re-evaluated over time, and continuous isolation and characterization of *Salmonella* strains are important for ensuring the ongoing effectiveness and feasibility of these methods. The selection and monitoring of reliable amplification targets and primer sets for food pathogen detection in various sample types, in line with the One Health approach, is of paramount importance for protecting consumers' safety and public health.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2026.107471.

CRediT authorship contribution statement

Jaweria Riaz: Writing – original draft, Methodology, Investigation. **Maira Napoleoni:** Writing – review & editing, Resources. **Giuditta Fiorella Schiavano:** Supervision. **Jennie Fischer:** Writing – review & editing. **Maria Borowiak:** Investigation, Formal analysis. **Giorgio Brandi:** Supervision. **Giulia Amagliani:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Funding

Funding from the University of Urbino, Dipartimento di Scienze Biomolecolari (Funding for Food Safety Research Projects, Delibera n. 30/2020 of 05th February 2020) was received for this study.

Declaration of competing interest

The authors declare no competing interests regarding the publication of this research article.

Acknowledgements

The authors would like to thank the Regional Reference Center for Enteric Pathogens (CRRPE) for the Marche Region of the IZSUM, the Regional Agency for Environmental Protection of Marche (ARPAM) and Regional Hospitals' Laboratories Participating in Enter-Net Surveillance for Marche Region.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. References used for primer design can be found in NCBI GenBank: M90846.1 (for invA) and AF282268.1 (for the ttr region).

References

Allard, M.W., Luo, Y., Strain, E., Pettengill, J., Timme, R., Wang, C., Li, C., Keys, C.E., Zheng, J., Stones, R., Wilson, M.R., Musser, S.M., Brown, E.W., 2013. On the evolutionary history, population genetics and diversity among isolates of *SalmonellaEnteritidis* PFGE pattern JEGX01.0004. PLoS One 8 (1), e55254. https://doi.org/10.1371/journal.pone.0055254.

Amagliani, G., Omiccioli, E., Brandi, G., Bruce, I.J., Magnani, M., 2010. A multiplex magnetic capture hybridisation and multiplex real-time PCR protocol for pathogen detection in seafood. Food Microbiol. 27 (5), 580–585. https://doi.org/10.1016/j.fm.2010.01.007.

Anyogu, A., Somorin, Y.M., Oladipo, A.O., Raheem, S., 2024. Food safety issues associated with sesame seed value chains: current status and future perspectives. Heliyon 10 (16), e36347. https://doi.org/10.1016/j.heliyon.2024.e36347.

Arnold, T., Scholz, H.C., Marg, H., Rösler, U., Hensel, A., 2004. Impact of invA-PCR and culture detection methods on occurrence and survival of Salmonella in the flesh, internal organs and lymphoid tissues of experimentally infected pigs. J. Veterin. Med. Ser. B: Infect. Diseas. Veterin. Publ. Health 51 (10), 459–463. https://doi.org/10.1111/j.1439-0450.2004.00808.x.

Balasubramanian, R., Im, J., Lee, J.-S., Jeon, H.J., Mogeni, O.D., Kim, J.H., Rakotozandrindrainy, R., Baker, S., Marks, F., 2019. The global burden and epidemiology of invasive non-typhoidal *Salmonella* infections. Hum. Vaccin. Immunother. 15 (6), 1421–1426. https://doi.org/10.1080/21645515.2018.1504717.

Bell, R.L., Jarvis, K.G., Ottesen, A.R., McFarland, M.A., Brown, E.W., 2016. Recent and emerging innovations in *Salmonella* detection: a food and environmental perspective. Microb. Biotechnol. 9 (3), 279–292. https://doi.org/10.1111/1751-7915.12359.

Branchu, P., Bawn, M., Kingsley, R.A., 2018. Genome variation and molecular epidemiology of *Salmonella enterica* Serovar Typhimurium Pathovariants. Infect. Immun. 86 (8). https://doi.org/10.1128/IAI.00079-18.

Buehler, A.J., Wiedmann, M., Kassaify, Z., Cheng, R.A., 2019. Evaluation of inva diversity among salmonella species suggests why some commercially available rapid detection kits may fail to detect multiple salmonella subspecies and species. J. Food Prot. 82 (4). https://doi.org/10.4315/0362-028X.JFP-18-525.

Carloni, E., Rotundo, L., Brandi, G., Amagliani, G., 2018. Rapid and simultaneous detection of Salmonella spp., *Escherichia coli* O157, and *Listeria monocytogenes* by magnetic capture hybridization and multiplex real-time PCR. Folia Microbiol. 63 (6), 735–742. https://doi.org/10.1007/s12223-018-0617-0.

Chattaway, M.A., Langridge, G.C., Wain, J., 2021. Salmonella nomenclature in the genomic era: a time for change. Sci. Rep. 11 (1), 7494. https://doi.org/10.1038/s41598-021-86243-w.

Chevez, Z.R., Dunn, L.L., da Silva, A.L.B.R., Rodrigues, C., 2024. Prevalence of STEC virulence markers and Salmonella as a function of abiotic factors in agricultural water in the southeastern United States. Front. Microbiol. 15. https://doi.org/10.3389/fmicb.2024.1320168.

Commission Regulation (EC), 2006. No 1177/2006 of 1 August 2006 implementing Regulation (EC) No 2160/2003 of the European Parliament and of the Council as Regards Requirements for the Use of Specific Control Methods in the Framework of the National Programmes for the Control of Salmonella in Poultry, 212 C.F.R.

Cremonesi, P., Cortimiglia, C., Picozzi, C., Minozzi, G., Malvisi, M., Luini, M., Castiglioni, B., 2016. Development of a droplet digital polymerase chain reaction for rapid and simultaneous identification of common foodborne pathogens in soft cheese. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01725.

EFSA, ECDC, 2024. The European Union One Health 2023 zoonoses report. EFSA J. 22 (12). https://doi.org/10.2903/j.efsa.2024.9106.

European Union, 2005. Commission regulation (EC) no 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union 50, 1–26. Retrieved from. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32005R2073.

Galán, J.E., Ginocchio, C., Costeas, P., 1992. Molecular and functional characterization of the Salmonella invasion gene invA: homology of InvA to members of a new protein family. J. Bacteriol. 174 (13), 4338–4349. https://doi.org/10.1128/jb.174.13.4338-4349.1992.

Ginocchio, C.C., Rahn, K., Clarke, R.C., Galán, J.E., 1997. Naturally occurring deletions in the centisome 63 pathogenicity island of environmental isolates of Salmonella spp. Infect. Immun. 65 (4), 1267–1272. https://doi.org/10.1128/iai.65.4.1267-1272.1997.

Grimont, P.A., Weill, F.-X., 2007. Antigenic Formulae of the Salmonella Serovars, 9. WHO Collaborating Centre for Reference and Research on Salmonella, pp. 1–166. Retrieved from https://www.pasteur.fr/sites/default/files/veng_0.pdf.

Guo, X., Chen, J., Beuchat, L.R., Brackett, R.E., 2000. PCR detection of *Salmonella enterica* serotype Montevideo in and on raw tomatoes using primers derived from *hilA*. Appl. Environ. Microbiol. 66 (12), 5248–5252. https://doi.org/10.1128/AEM.66.12.5248-5252.2000.

Halatsi, K., Oikonomou, I., Lambiri, M., Mandilara, G., Vatopoulos, A., Kyriacou, A., 2006. PCR detection of *Salmonella* spp. using primers targeting the quorum sensing gene *sdiA*. FEMS Microbiol. Lett. 259 (2), 201–207. https://doi.org/10.1111/j.1574-6968.2006.00266.x.

Hensel, M., 2004. Evolution of pathogenicity islands of *Salmonella enterica*. Int. J. Med. Microbiol. 294 (2–3), 95–102. https://doi.org/10.1016/j.ijmm.2004.06.025.

Hensel, M., Hinsley, A.P., Nikolaus, T., Sawers, G., Berks, B.C., 1999. The genetic basis of tetrathionate respiration in *Salmonella typhimurium*. Mol. Microbiol. 32 (2), 275–287. https://doi.org/10.1046/j.1365-2958.1999.01345.x.

Hoorfar, J., Ahrens, P., Rådström, P., 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. 38 (9), 3429–3435. https://doi.org/10.1128/JCM.38.9.3429-3435.2000.

Hu, L., Cao, G., Brown, E.W., Allard, M.W., Ma, L.M., Zhang, G., 2021. Whole genome sequencing and protein structure analyses of target genes for the detection of Salmonella. Sci. Rep. 11 (1), 20887. https://doi.org/10.1038/s41598-021-00224-7.

Huoy, L., Vuth, S., Hoeng, S., Chheang, C., Yi, P., San, C., Chhim, P., Thorn, S., Ouch, B., Put, D., Aong, L., Phan, K., Nasirzadeh, L., Tieng, S., Bongcam-Rudloff, E., Sternberg-Lewerin, S., Boqvist, S., 2024. Prevalence of Salmonella spp. in meat, seafood, and leafy green vegetables from local markets and vegetable farms in Phnom Penh, Cambodia. Food Microbiol. 124, 104614. https://doi.org/10.1016/j.fm.2024.104614.

ISO 16140-2, 2016. Microbiology of Food and Animal Feed—Method Validation—Part 2: Protocol for the Validation of Alternative (Proprietary) Methods against a Reference Method. International Organization for Standardization, Geneva, Switzerland.

ISO/TR 6579-3, 2014. Microbiology of the Food Chain—Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella—Part 3: Guidelines for Serotyping of Salmonella spp. International Organization for Standardization, Geneva, Switzerland.

Italian Ministry of Health, 2018. National Control Plan for Salmonellosis in Poultry 2019–2021 (Piano Nazionale di Controllo delle Salmonellosi negli Avicoli 2019–2021). Retrieved from. https://www.salute.gov.it/imgs/C_17_pubblicazioni_2849_allegato.pdf.

Kariuki, F., Getanda, P., Nyachieo, A., Juma, G., Kinyanjui, P., Kamau, J., 2022. Evaluation of the detection of staA, viaB and sopE genes in Salmonella spp. using the polymerase chain reaction (PCR). Arch. Microbiol. 204 (1), 25. https://doi.org/10.1007/s00203-021-02654-3.

Kreitlow, A., Becker, A., Schotte, U., Malorny, B., Plötz, M., Abdulmawjood, A., 2021. Evaluation of different target genes for the detection of *Salmonella* sp. by loop-mediated isothermal amplification. Lett. Appl. Microbiol. 72 (4), 420–426. https://doi.org/10.1111/lam.13409.

Lamas, A., Miranda, J.M., Regal, P., Vázquez, B., Franco, C.M., Cepeda, A., 2018. A comprehensive review of non-enterica subspecies of *Salmonella enterica*. Microbiol. Res. 206, 60–73. https://doi.org/10.1016/j.micres.2017.09.010.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33 (1), 159. https://doi.org/10.2307/2529310.

Lefever, S., Pattyn, F., Hellemans, J., Vandesompele, J., 2013. Single-nucleotide polymorphisms and other mismatches reduce performance of quantitative PCR assays. Clin. Chem. 59 (10), 1470–1480. https://doi.org/10.1373/clinchem.2013.203653.

Liu, Y., Singh, P., Mustapha, A., 2018. Multiplex high resolution melt-curve real-time PCR assay for reliable detection of Salmonella. Food Control 91, 225–230. https://doi.org/10.1016/j.foodcont.2018.03.043.

Liu, B., Meng, C., Han, S., Li, Q., Miao, X., Wang, Z., Xu, C., Kang, X., Jiao, X., Pan, Z., 2024. Development of a 1-step multiplex PCR assay for the detection of *S. enteritidis*, *S. pullorum*, *S. typhimurium*, and *S. infantis* associated with poultry production. Poult. Sci. 103 (9), 104043. https://doi.org/10.1016/j.psj.2024.104043.

Mackinnon, A., 2000. A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. Comput. Biol. Med. 30 (3), 127–134. https://doi.org/10.1016/S0010-4825(00)00006-8.

Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., Jones, T.F., Fazil, A., Hoekstra, R.M., 2010. The global burden of Nontyphoidal *Salmonella* gastroenteritis. Clin. Infect. Dis. 50 (6), 882–889. https://doi.org/10.1086/650733.

Malorny, B., Hoorfar, J., Bunge, C., Helmuth, R., 2003a. Multicenter validation of the analytical accuracy of salmonella PCR: towards an international standard. Appl. Environ. Microbiol. 69 (1). https://doi.org/10.1128/AEM.69.1.290-296.2003.

Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., Bunge, C., Dorn, C., Helmuth, R., 2003b. Interlaboratory diagnostic accuracy of a Salmonella specific PCR-based method. Int. J. Food Microbiol. 89 (2–3), 241–249. https://doi.org/10.1016/S0168-1605(03)00154-5.

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., Helmuth, R., 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl. Environ. Microbiol. 70 (12), 7046–7052. https://doi.org/10.1128/AEM.70.12.7046-7052.2004.

Mkangara, M., Mbega, E.R., Chacha, M., 2020. Molecular identification of *Salmonella typhimurium* from village chickens based on inva and spvC genes. Vet. World 13 (4). https://doi.org/10.14202/vetworld.2020.764-767.

Mohammed, B.T., 2024. Identification and bioinformatic analysis of invA gene of Salmonella in free range chicken. Braz. J. Biol. 84. https://doi.org/10.1590/1519-6984.263363.

Mohammed, Y., Dubie, T., 2022. Isolation, identification and antimicrobial susceptibility profile of *Salmonella* isolated from poultry farms in Addis Ababa, Ethiopia. Vet. Med. Sci. 8 (3), 1166–1173. https://doi.org/10.1002/vms3.762.

Moore, H.L., Aabye, M., Hoban, A., Rosner, B., Lefevre, S.K., Litrup, E., Müller, L., Ethelberg, S., Simon, S., Balasegaram, S., Larkin, L., Jernberg, C., Takkinen, J., 2024. Multinational collaboration in solving a European Salmonella Braenderup outbreak linked to imported melons, 2021. Euro Surveill 29 (1). https://doi.org/10.2807/1560-7917.ES.2024.29.1.2300273.

Nazari Moghadam, M., Rahimi, E., Shakerian, A., Momtaz, H., 2023. Prevalence of *Salmonella typhimurium* and *Salmonella enteritidis* isolated from poultry meat: virulence and antimicrobial-resistant genes. BMC Microbiol. 23 (1), 168. https://doi.org/10.1186/s12866-023-02908-8.

Nde, C.W., Fakhr, M.K., Doetkott, C., Logue, C.M., 2008. An evaluation of conventional culture, invA PCR, and the real-time PCR iQ-check kit as detection tools for Salmonella in naturally contaminated premarket and retail Turkey. J. Food Prot. 71 (2), 386–391. https://doi.org/10.4315/0362-028X-71.2.386.

Pardo-Roa, C., Salazar, G.A., Noguera, L.P., Salazar-Echegarai, F.J., Vallejos, O.P., Suazo, I.D., Schultz, B.M., Coronado-Arrázola, I., Kalergis, A.M., Bueno, S.M., 2019. Pathogenicity island excision during an infection by *Salmonella enterica* serovar Enteritidis is required for crossing the intestinal epithelial barrier in mice to cause systemic infection. PLoS Pathog. 15 (12), e1008152. https://doi.org/10.1371/journal.ppat.1008152.

Pearce, M.E., Langridge, G.C., Lauer, A.C., Grant, K., Maiden, M.C.J., Chattaway, M.A., 2021. An evaluation of the species and subspecies of the genus Salmonella with whole genome sequence data: proposal of type strains and epithets for novel *S. enterica* subspecies VII, VIII, IX, X and XI. Genomics 113 (5), 3152–3162. https://doi.org/10.1016/j.ygeno.2021.07.003.

Pico-Rodríguez, J.T., Martínez-Jarquín, H., Gómez-Chávez, J. de J., Juárez-Ramírez, M., Martínez-Chavarría, L.C., 2024. Effect of *Salmonella* pathogenicity island 1 and 2 (SPI-1 and SPI-2) deletion on intestinal colonization and systemic dissemination in chickens. Vet. Res. Commun. 48 (1), 49–60. https://doi.org/10.1007/s11259-023-10185-z.

Pino, M., Mujica, K., Mora-Uribe, P., Garcias-Papayani, H., Paillavil, B., Avendaño, C., Flores-Crisosto, D., Norambuena, R., Rojas-Martínez, V., Aguilera, M., Muñoz, N.C., Cifuentes, P., Pieringer, H., Ulloa, S., 2025. Research note: reduction of Salmonella load in Brazilian commercial chicken farms using INSPEKTOR®: a bacteriophage-based product. Poult. Sci. 104 (1), 104544. https://doi.org/10.1016/j.psj.2024.104544.

Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galán, J.E., Ginocchio, C., Curtiss, R., Gyles, C.L., 1992. Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of Salmonella. Mol. Cell. Probes 6 (4). https://doi.org/10.1016/0890-8508(92)90002-F.

Regulation (EC) No 2160/2003, 2003. Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the Control of Salmonella and Other Specified Food-borne Zoonotic Agents, Vol. L 325.

Resendiz-Nava, C., Esquivel-Hernandez, Y., Alcaraz-Gonzalez, A., Castaneda-Serrano, P., Nava, G.M., 2019. PCR assays based on invA gene amplification are not reliable for salmonella detection. Jundishapur J. Microbiol. 12 (2). https://doi.org/10.5812/jjm.68764.

Rockett, R.J., Arnott, A., Wang, Q., Howard, P., Sintchenko, V., 2020. Genomic surveillance enables suitability assessment of *Salmonella* gene targets used for culture-independent diagnostic testing. J. Clin. Microbiol. 58 (9). https://doi.org/10.1128/JCM.00038-20.

Rosner, B.M., Simon, S., Nielsen, S., Köberl-Jelovcan, S., Gymoese, P., Werber, D., Meinen, A., Pietsch, M., Flieger, A., Fischer, J., Lamparter, M.C., Küffel, F., Költringer, F., Kornschober, C., Müller, L., Falkenhorst, G., Maritschnik, S., 2024. Multinational investigation of a Salmonella Umbilo outbreak reveals rocket salad and baby spinach as the likely infection vehicles, Europe, 2024. Eurosurveillance 29 (46). https://doi.org/10.2807/1560-7917.ES.2024.29.46.2400728.

Russo, I., Bencardino, D., Napoleoni, M., Andreoni, F., Schiavano, G.F., Baldelli, G., Brandi, G., Amagliani, G., 2022. Prevalence, antibiotic-resistance, and replicon-typing of *Salmonella* strains among serovars mainly isolated from food chain in Marche region, Italy. Antibiotics 11 (6), 725. https://doi.org/10.3390/antibiotics11060725.

Russo, I., Fischer, J., Uelze, L., Napoleoni, M., Schiavano, G.F., Andreoni, F., Brandi, G., Amagliani, G., 2024. From farm to fork: spread of a multidrug resistant Salmonella Infantis clone encoding blaCTX-M-1 on pESI-like plasmids in Central Italy. Int. J. Food Microbiol. 410, 110490. https://doi.org/10.1016/j.ijfoodmicro.2023.110490.

Shaji, S., Selvaraj, R.K., Shanmugasundaram, R., 2023. Salmonella infection in poultry: a review on the pathogen and control strategies. Microorganisms 11 (11), 2814. https://doi.org/10.3390/microorganisms11112814.

Shanmugasamy, M., Velayutham, T., Rajeswar, J., 2011. Inv a gene specific PCR for detection of Salmonella from broilers. Vet. World 562. https://doi.org/10.5455/vetworld.2011.562-564.

Siddiky, N.A., Sarker, S., Khan, S.R., Rahman, T., Kafi, A., Samad, M.A., 2022. Virulence and antimicrobial resistance profile of non-typhoidal *Salmonella enterica* serovars recovered from poultry processing environments at wet markets in Dhaka, Bangladesh. PLoS One 17 (2), e0254465. https://doi.org/10.1371/journal.pone.0254465.

Singh, P., Mustapha, A., 2013. Multiplex TaqMan® detection of pathogenic and multi-drug resistant Salmonella. Int. J. Food Microbiol. 166 (2), 213–218. https://doi.org/10.1016/j.ijfoodmicro.2013.07.023.

Tăbăran, A., Dan, S.D., Colobaţiu, L.M., Mihaiu, M., Condor, S., Mărgăoan, R., Crişan-Reget, O.L., 2024. Evaluation of multidrug resistance of Salmonella isolated from pork meat obtained from traditional slaughter systems in Romania. Microorganisms 12 (11), 2196. https://doi.org/10.3390/microorganisms12112196.

Turki, Y., Ouzari, H., Mehri, I., Ben Aissa, R., Hassen, A., 2012. Biofilm formation, virulence gene and multi-drug resistance in Salmonella Kentucky isolated in Tunisia. Food Res. Int. 45 (2), 940–946. https://doi.org/10.1016/j.foodres.2011.05.031.

UNI EN ISO 6579-1:2017/Amd 1, 2020. Microbiology of the Food Chain — Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella — Part 1: Detection of Salmonella spp. Amendment 1: Broader Range of Incubation Temperatures, Amendment to the Status of Annex D, and Correction of the Composition of MSRV and SC. International Organization for Standardization, Geneva, Switzerland.

Veni, K., Stephen, J., Lekshmi, M., Nayak, B.B., Kumar, S.H., 2025. A polymerase chain reaction (PCR) method to detect emerging multidrug-resistant *Salmonella* Infantis harboring the pESI plasmid in seafood. J. AOAC Int. 108 (1), 56–61. https://doi.org/10.1093/jaoacint/qsae081.

Wallace, H., Andrews, H.W., Jacobson, Andrew, Ge, Beilei, Zhang, Guodong, Hammack, Thomas, Food and Drug Administration, 2024. Bacteriological Analytical Manual (BAM) Chapter 5: Salmonella. (May 2024 Edition. ed.). https://www.fda.gov/media/178914/download?attachment.

Wang, Z., Huang, C., Liu, Y., Chen, J., Yin, R., Jia, C., Kang, X., Zhou, X., Liao, S., Jin, X., Feng, M., Jiang, Z., Song, Y., Zhou, H., Yao, Y., Teng, L., Wang, B., Li, Y., Yue, M., 2024. Salmonellosis outbreak archive in China: data collection and assembly. Sci. Data 11 (1), 244. https://doi.org/10.1038/s41597-024-03085-7.

Wen, J., Gou, H., Zhan, Z., Gao, Y., Chen, Z., Bai, J., Wang, S., Chen, K., Lin, Q., Liao, M., Zhang, J., 2020. A rapid novel visualized loop-mediated isothermal amplification method for Salmonella detection targeting at fimW gene. Poult. Sci. 99 (7), 3637–3642. https://doi.org/10.1016/j.psj.2020.03.045.

White, A.E., Tillman, A.R., Hedberg, C., Bruce, B.B., Batz, M., Seys, S.A., Dewey-Mattia, D., Bazaco, M.C., Walter, E.S., 2022. Foodborne illness outbreaks reported to National Surveillance, United States, 2009–2018. Emerg. Infect. Dis. 28 (6). https://doi.org/10.3201/eid2806.211555.

Yanestria, S.M., Rahmaniar, R.P., Wibisono, F.J., Effendi, M.H., 2019. Detection of *invA* gene of *Salmonella* from milkfish (*Chanos chanos*) at Sidoarjo wet fish market, Indonesia, using polymerase chain reaction technique. Vet. World 12 (1). https://doi.org/10.14202/vetworld.2019.170-175.