Using genetic markers for detection and subtyping of the emerging *Salmonella enterica* subspecies *enterica* serotype Muenchen

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ABSTRACT Non-typhoidal *Salmonella* (**NTS**) poses a global threat to public health. Poultry, one of the main reservoirs of NTS, is usually not clinically affected by most NTS, yet the economic losses to the poultry industry due to control and mitigation efforts, and due to negative publicity can be tremendous.

NTS strains are routinely characterized into serotypes in a time-consuming, labor-intensive multistep process that requires skilled personnel. Moreover, the discriminatory power of serotyping is limited compared to other subtyping methods. Whole-genome sequence data enable the identification of genetic variation within serotypes. However, sequencing is often limited by available resources, and analyzing and interpreting the genetic data may be time-consuming. Source tracing during epidemiological outbreak investigations requires rapid and efficient characterization of strains to control pathogen spread. Here we designed a multiplex polymerase chain reaction (**PCR**) assay for the detection of genetic variants of *Salmonella* Muenchen, a serotype that has emerged in Israel in the last 3 yr in both clinical human cases and different hosts. Test sensitivity of 99.21% and specificity of 94 to 100% were determined using in-silico PCR with a dataset of 18,282 NTS assemblies from 37 NTS serotypes. Similarly, test sensitivity of 100% and specificity of 96.2 to 100% were determined in-vitro with 120 NTS isolates of 52 serotypes. Moreover, the test enabled differentiation between the common sequence types of serotype Muenchen using both approaches. As opposed to traditional serotyping and other subtyping methods, the designed test allows for rapid and cost-efficient detection of the emerging *S.* Muenchen serotype and its variants in a single step. Future development of similar assays for other dominant serotypes may help reduce the time and cost required for detection and initial characterization of dominant NTS strains. Overall, these tests will be beneficial to both public health and for reducing of the economic losses to the poultry industry due to NTS infections.

Key words: *Salmonella*, genetic characterization, public health, zoonosis, poultry

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INTRODUCTION

Foodborne illness is one of the 10 major global threats to human health (World Health Organization (WHO), 2015). Among the bacterial pathogens, non-typhoidal *Salmonella* (**NTS**) strains are a major cause of this illness (Kirk et al., 2015). In addition to the economic burden of human illness (Kirk et al., 2015), major economic losses may occur to the poultry industry, such as was demonstrated following the former United Kingdom Health Minister Edwina Currie statement that the majority of the United Kingdom eggs were infected with *Salmonella* in 1988 (Knowles et al., 2007).

Salmonella strains are traditionally subtyped to serotypes based on immunological variability of somatic (O) and flagellar (H1 and H2) antigens according to the White–Kauffmann–Le Minor scheme (Brenner et al.,

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2000; Grimont and Weill, 2007). Serotype identification is traditionally conducted using slide agglutination tests with multiple antisera in a multistep process which may also include, in diphasic serotypes, a phase-inversion step (Danan et al., 2009). In a review by Tang et al. (2019), a comparison of *Salmonella* characterization methods was conducted; traditional serotyping was found to be time-consuming (lasting 2−17 d for serotype identification from a single colony) and labor-intensive, and to require skilled personnel to interpret the test results. Alternative approaches included identifying serotypes based on detection and characterization of the genes encoding the somatic (Herrera-Leon et al., 2007) and flagellar (Echeita et al., 2002; Herrera-Leon et al., 2004) antigens. Another approach was based on the detection of a number of genetic markers associated with specific serotypes (Jean-Gilles Beaubrun et al., 2012). However, similar to traditional serotyping, such approaches require multiple steps. Moreover, given the low discriminatory power of traditional methods (Tang et al., 2019), genetic variations within NTS serotypes (Cheng et al., 2019; Elnekave et al., 2020b) are not detected.

Salmonella enterica subspecies *enterica* strains are mainly involved in infections of homeothermic animals (Brenner et al., 2000). Thousands of serotypes were described to date (Cheng et al., 2019), of which certain serotypes often remain dominant over time. However, emergence of specific variants of these serotypes may lead to their periodic increase, as was described with *Salmonella* serotype Typhimurium definitive type (**DT**) 104 (Mather et al., 2013), a *Salmonella* serotype Infantis clone harboring the pESI plasmid (Aviv et al., 2014), and the globally expanding *Salmonella* serotype 4,[5],12:i:- sequence type (**ST**) 34 (Elnekave et al., 2018; Elnekave et al., 2020a).

In Israel, serotypes Muenchen (6,8:d:1,2) and Virginia (8:d:1,2) are emerging following the acquisition of a mega-plasmid named pESI, and similar genomes were also detected in clinical isolates in South Africa, United Kingdom, and the United States of America (Cohen et al., 2022). These serotypes, which were previously identified as two different serotypes based on random expression of the minor antigen O:6, are currently regarded as a single serotype (Mikoleit et al., 2012) that will be referred to as *S.* Muenchen hereafter. The incidence of clinical cases caused by this serotype in Israel has increased dramatically and reached 40% (1055/2671) of all clinical *Salmonella* isolates on 2022 (Cohen et al., 2022). Similarly, an increase in the prevalence of *S.* Muenchen has been found in isolates collected from 'animal and poultry' sources: from 3.8% (63/1672) in 2017 to 15.5% (165/616) in 2019, and from 'food industry and ready-to-eat products' (of various sources): from 4.9% (12/245) in 2017 to 26.8% (127/473) in 2019 (Israel Ministry of Health − Jerusalam Central Laboratories, 2017; Israel Ministry of Health − Jerusalam Central Laboratories, 2019). Despite the rapid increase of this emerging pathogen and the risk to public health, strain characterization has been conducted mainly by serotyping due to limited resources and availability of other fast and reliable methods for identification of the serotype and its genetic variants, which may play a role in its emergence as was demonstrated with many other emerging serotypes, such as *S.* 4,[5],12:i:- ST34 (Elnekave et al., 2018) and *S.* Kentucky ST198 (Le Hello et al., 2011). Under such conditions, there is limited ability to conduct a timely outbreak investigation and detect its potential sources to protect the public.

We used a mixed approach, combining both in-silico and in-vitro analyses, to design a multiplex polymerase chain reaction (**PCR**) assay and evaluate its sensitivity and specificity. The designed multiplex PCR provides a timely and affordable alternative for detection of the *S.* Muenchen serotype and distinguishing between its common STs.

MATERIALS AND METHODS

Data Analyses

Specific genetic markers for the identification of *S.* Muenchen groups were identified and a multiplex PCR assay was developed and validated using a multistep approach that included in-silico and in-vitro analyses. A schematic diagram of the data analyses process described below is included (Figure 1).

1. In-silico analysis

Two sources were used in the in-silico analyses:

a. The National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/; as of March 2021).
b. The dataset and analysis outputs (including the sequence types) of a previously published study (Elnekave et al., 2020b), which included 18,282 publicly available whole-genome sequences (raw reads) that were retrieved from the NCBI database. These genomes belonged to 37 NTS serotypes that were collected from multiple hosts in the United States between 2006 and 2017. The raw reads were all assembled and analyzed using a single pipeline (including multiple quality control cut-offs), which is described elsewhere (Elnekave et al., 2020b).

Identification and validation of target sequences for detection of *S.* Muenchen

We scanned the "gene_presence_absence.csv" output file of a core genome analysis of *Salmonella* isolates of *S.* Muenchen and another 36 NTS serotypes (n = 10 for each serotype), which was generated previously (Elnekave et al., 2020b). This data file includes a list of all genes and which samples it is present in. Using this approach, we were able to detect accessory genes (i.e., genes found in less than 99% of the sequences) that were unique to *S.* Muenchen or to its mainly represented STs (defined based on multi locus sequence typing of 7 genes)

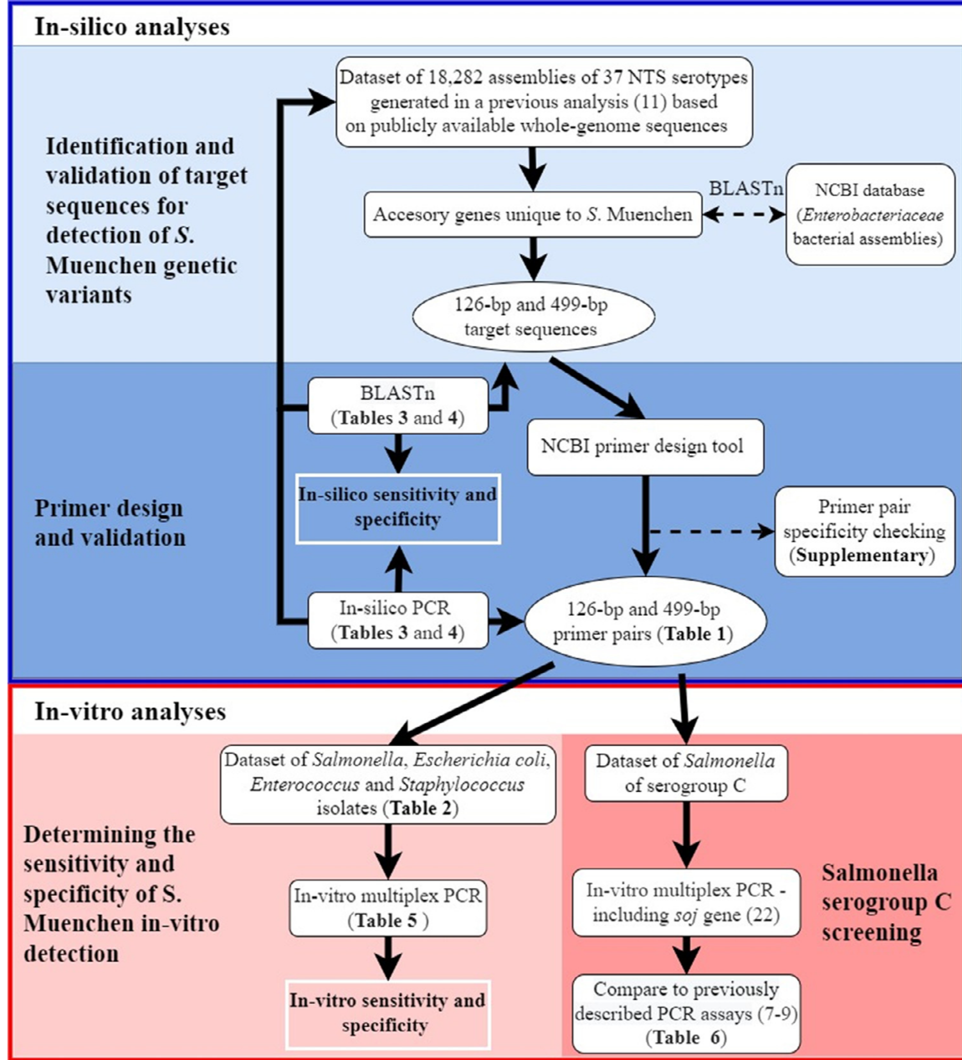

Figure 1. A schematic diagram of the analyses process: in-silico (blue box) and in-vitro (red box) analyses.

in that analysis, that is, ST83 and ST112. The accessory gene names and lengths were then used to seek its sequence in an annotated assembled genome output file ('.gbk' file) that was available through the previous analysis (Elnekave et al., 2020b). Identified gene sequences were uploaded to the nucleotide basic local alignment search tool (BLASTn) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and a search was conducted using the default settings (i.e., word size = 28; Match/Mismatch scores = 1,-2; Gapcosts = 0,2.5; and an e-value = 0.05). BLASTn results were initially scanned and the potential relevance of the target sequence was determined based on its detection in *S.* Muenchen and other *Enterobacteriaceae* bacterial assemblies at NCBI.

Primer design and validation

The NCBI primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used with the default settings to design primers for the selected target sequences. The 'primer pair specificity checking parameters' option was enabled to improve search specificity. The non-redundant nucleotide ('nr') database was selected and the search organism was restricted to *Salmonella* or *Enterobacteriaceae* in 2 separate BLASTs. Primer pairs were selected based on the PCR product length to develop a multiplex PCR for identification of multiple *S.* Muenchen variants (see further on). The PCR primer pair specificity results and the target sequences were further evaluated to estimate their potential relevance for *S.* Muenchen detection (see supplementary information for further details).

While a large database of *Salmonella* and other bacterial sequences is included in NCBI's non-redundant nucleotide database, it does not include all of the assembled genomes in GenBank. Therefore, to further validate the target sequences that were identified in the previous steps, we used a large library of assembled genomes of 37 NTS serotypes (including *S.* Muenchen) that had been created as part of a previous project and stored on a local server at the University of Minnesota (Elnekave et al., 2020b). A local BLASTn was used to identify the target sequences in the different serotypes. The BLASTn was performed using a conservative approach with a 60% coverage threshold and 70% identity to allow for detection any sequences that may resemble the target sequences in *S.* Muenchen and other serotypes.

Table 1. The designed primer pairs for the detection of multiple genetic variants of *Salmonella* Muenchen.

Primer	Sequence (5′-3′)	Amplicon size (bp)	Target gene (amino acids #)	Amplicon sequence reference at NCBI
126-F	AGTTCAGGAGCGTGGTCAAC	126	DNA-binding protein (80)	CP045056.1: 2117876-2118001
126-R	AGACTCGGTCACATGCCATT			
499-F	GAGCTGGTAAAGCAGGGGAA	499	Restriction endonuclease subunit S (577)	CP051416.1: 3997785-3998283
499-R	TTCTAGGCACACGCTTCTGG			

Moreover, as primers may bind and amplify sequences other than the expected targets, we used an in-silico PCR script (https://github.com/egonozer/in_silico_pcr; with the '-m' and '-i' options to allow up to one base mismatch and up to one base insertion or deletion per primer sequence, respectively) to BLAST the 126-bp and 499-bp primer pairs (Table 1) against the assemblies' dataset.

The in-silico sensitivity of the designed multiplex PCR (see further on) was calculated as the percentage of *S.* Muenchen assemblies in which one of the target sequences could be detected or identified by the in-silico-PCR. The in-silico specificity was calculated, for each target sequence separately, as the complementary (to one) of the percentage of non-*S.* Muenchen serotypes in which the target sequence was detected out of all non-*S.* Muenchen *Salmonella* serotypes that were included. In addition, the in-silico specificity was calculated similarly based on the in-silico PCR findings.

2. In-vitro analysis

A set of 2 primer pairs that enabled differentiating between *S.* Muenchen genetic strains were designed in the analysis (Table 1). A multiplex PCR was performed using GoTaq Green Master Mix (Promega, M7121). The reaction was set up in a 25-μL volume according to the supplier's standard application protocol. For optimizing the multiplex PCR results, we first tested the outcomes generated with varying primer pair concentrations (i.e., 0.2 μM, 0.1 μM, or 0.05 μM) and with different concentrations of bacterial DNA (i.e., 0.6 ng/μL, 0.3 ng/μL or 0.15 ng/μL). The amplification program was as follows: initial denaturation for 2 min at 95°C, followed by 44 cycles of amplification: 30 s denaturation at 95°C, 30 s annealing at 55°C, and 40 s elongation at 72° C. A final step of 5 min at 72°C was added for extension finalization. Prior to visualization by 1.5% agarose electrophoresis, the samples were stored at +4°C. Based on the outcomes of the optimization process (supplementary Figure S1), in all analyses the primer pair was added to a final concentration of 0.2 μM, and the bacterial DNA was added to a final concentration of 0.6 ng/μL. *S.* Muenchen isolates of ST126 and ST 83 that were serotyped using slide agglutination and sequenced as part of different analyses (see below) were included as positive controls and H_2O was used as negative control.

Determining the sensitivity and specificity of *S.* Muenchen in-vitro detection

In addition to the in-silico analyses described above, the designed multiplex PCR detection was evaluated in vitro using *Salmonella* isolates collected from multiple hosts and *Escherichia coli, Enterococcus* and *Staphylococcus* isolates collected from poultry (Table 2). The isolate collection included 15 *S.* Muenchen isolates of 3 dominant STs (i.e., ST83, ST112, and ST82), 105 isolates of 53 unique *Salmonella enterica* subspecies *enterica* serotypes, 4 isolates of other *Salmonella enterica* subspecies, and 7 isolates of bacteria other than *Salmonella.* Slide agglutination tests were used to determine the serotypes of the *Salmonella* isolates collected in Israel (n = 117) at the Israeli Ministry of Health central *Salmonella* diagnostic laboratory. Serotyping of 3 isolates collected in the United States was available from a previous study (Elnekave et al., 2019). In-vitro specificity and sensitivity were determined as described above for the in-silico analysis.

Salmonella serogroup C screening

A subset (n = 213) of *Salmonella enterica* subspecies *enterica* isolates belonging to serogroup C that were

Table 2. Sources and hosts of the bacterial isolate collection that was used for in-vitro validation of the designed PCR assay.

Source	Host	Subspecies *enterica* serotypes (# of isolates)	Others (#)	*S.* Muenchen STs[1] (#)
Egg and Poultry board, Israel	Poultry	28 (45)	*E. coli* (3) *Enterococcus* (3) *Staphylococcus* (1)	NA (7)
Ministry of Health, Israel	Human, environment and unknown	39 (72)	subsp. *arizonae* (1) subsp. *diarizonae* (2) subsp. *salamae* (1)	ST82[2] (3) NA (2)
Veterinary diagnostic laboratories – University of Minnesota, St Paul, USA	Swine	1 (3[3])		ST83 (1) ST112 (1) Unknown (1)

[1]Available information of the sequence types (STs) is indicated for *Salmonella* Muenchen isolates.
[2]Sequence types (STs) were obtained through the Israeli MOH from three isolates that were sequenced as part of a different study. No additional genetic data or genetic analyses of these isolates were conducted as part of this study.
[3]These isolates were sequenced and analyzed (including retrieving the sequence types) as part of a previous study (Elnekave et al., 2019). The sequences NCBI Biosample numbers: SAMN10423937 (ST83), SAMN07420490 (ST112), and SAMN10423910 (unknown ST).

collected from poultry in Israel during 2021 were used to further validate the described multiplex PCR. For these, serogrouping was conducted by slide agglutination at the Egg and Poultry Board *Salmonella* diagnostic laboratory, but without serotype determination. *S.* Muenchen isolates were then identified based on their antigenic formula using a multistage procedure that included three previously described PCR assays (Echeita et al., 2002; Herrera-Leon et al., 2004; Herrera-Leon et al., 2007) (see supplementary information for further details). The results were compared to the multiplex PCR assay described above, which was adapted to include primers for identification of *S.* Infantis based on the presence of the *soj* gene (Ye et al., 2021). For this purpose, the elongation step in the multiplex PCR protocol was extended to 1 min and the *soj* primers were added in a final concentration of 0.4 μM.

RESULTS

A set of 2 primer pairs was designed for direct detection of *S.* Muenchen from different genetic groups using a multiplex PCR (Table 1). The target sequences of 126 bp and 499 bp, which were amplified by these primers, allowed the detection and differentiation of common STs in silico (Table 3) and in vitro (supplementary Figure S2).

The PCR primer pairs and the target sequences detected with these sets of primers were first estimated in silico against a dataset of 18,282 assemblies of 37 NTS serotypes, which was available through a previous study (Elnekave et al., 2020b). The analyses outcomes were similar when using the primer pairs (in-silico PCR) or target sequences BLAST (Tables 3 and 4). Of the 382 *S.* Muenchen strains screened in silico for target sequences, 181 (47.4%) had the 126-bp target sequence, 198 (51.8%) had the 499-bp target sequence and 3 (0.7%) had none (Table 3). The detected sequences were of full length and had at least 99.21% identity to the target sequences. The same results were obtained with the in-silico PCR (Table 3) and full-length primers (with 100% identity) were found in all detected assemblies. Overall, the calculated in-silico sensitivity when using in-silico PCR or target sequences BLAST for both primer sets was 99.21%.

Table 3. In-silico analysis results. The detection of the PCR target sequences in a database of 382 *Salmonella* Muenchen assemblies using a basic local alignment search tool (BLAST).

MLST	Target sequence sizeDetected[1]/Total (%)	
	126-bp	499-bp
ST112[2]	160/162 (98.8)	0/162 (0)
ST1567	2/2 (100)	0/2 (0)
ST1606	12/12 (100)	0/12 (0)
ST2169	1/1 (100)	0/1 (0)
ST2229	2/2 (100)	0/2 (0)
ST2795	0/2 (0)	2/2 (100)
ST2870	2/2 (100)	0/2 (0)
ST2881	0/1 (0)	0/1 (0)
ST3211	1/1 (100)	0/1 (0)
ST83	0/196	196/196 (100)
Unknown ST	1/1 (100)	0/1 (0)

[1]The same results were obtained by in-silico PCR using the 126-bp and 499-bp primers (see text for details).

[2]None of the target sequences were detected in 2/162 of ST112 *Salmonella* Muenchen assemblies.

Of the 36 non-*S.* Muenchen serotypes screened in silico for the target sequences, 8 (22%) had the 126-bp target sequence, 1 (2.7%) had the 499-bp target sequence and 27 (75%) had none (Table 4). However, the full-length 126-bp target sequence (with 100% sequence identity) was only detected in 2 of the *S.* Enteritidis and one *S.* Montevideo assemblies. Moreover, isolates of serotypes Enteritidis, Montevideo, Heidelberg, Javiana, Mbandaka, Anatum, Muenster, and Poona, in which partial lengths (i.e., 72−83% coverage) and sequence identity (i.e., 89.13−100%) of the 126-bp target sequence were detected, were included in the in-vitro analysis (Table 5) and none of these serotypes was detected as *S.* Muenchen by the multiplex PCR assay (below). The full-length 499-bp target sequence was found in all *S.* Norwich assemblies (n = 110; 100% coverage and 98.2% identity). This serotype was not available for in-vitro analysis. In addition, of the 36 non-*S.* Muenchen serotypes screened using the in-silico PCR, only 2 assemblies of *S.* Enteritidis and one *S.* Montevideo were detected by the 126-bp primers. In these, a full length (100% identity) target sequence was also detected (above). The 499-bp primers were not detected in any serotype other than *S.* Muenchen using the in-silico PCR.

Based on the target sequence BLAST detection results, the calculated in-silico specificity, that is, classifying other *Salmonella* subspecies *enterica* serotypes as non-*S.* Muenchen, was 78% and 97.2% for the 126-bp and 499-bp target sequence sizes, respectively. However, using the in-silico PCR results, the calculated in-silico specificity was 94 and 100% for the 126-bp and 499-bp target sequence sizes, respectively.

Following the in-silico approach, an in-vitro multiplex PCR assay was designed using the two primer sets (Table 1). The 126-bp or the 499-bp amplicons were found in various STs of *S.* Muenchen, including its Rough:d:1,2 variants and test sensitivity for *S.* Muenchen (and its variants) detection was 100% (Table 5). The 126-bp and 499-bp target sequences were detected in none and two (3.8%; *S.* Manhattan and *S.* Blockley) of the 53 unique *Salmonella enterica* subspecies *enterica* serotypes tested, respectively (Table 5). The in-vitro specificity of the test, that is, classifying other *Salmonella* subspecies *enterica* serotypes as non-*S.* Muenchen, was 100% and 96.2% for the 126-bp and 499-bp target sequences, respectively. The PCR amplicons were not detected in isolates of other *Salmonella enterica* subspecies or bacterial species, except for 2 out of 3 *E. coli* isolates in which the 126-bp target sequence was detected (Table 5). The latter agreed with the primer blast specificity results (see supplementary information). Furthermore, the antigenic formula was determined for a subset of *Salmonella* subgroup C isolates (n = 213) using previously published PCR assays (see above). The identification of *S.* Muenchen with the newly developed multiplex

Table 4. In-silico analysis results. The detection of the PCR target sequences in a database of 18,282 non-typhoid *Salmonella* assemblies of 37 serotypes using basic local alignment search tool (BLAST).

Serogroup	Serotype	Target sequence size Detected/Total (%) 126 bp	Target sequence size Detected/Total (%) 499 bp	Tested in vitro
B	4,[5],12:i:-	0/1015	0/1015	-
	Agona	0/300	0/300	+
	Copenhagen	0/587	0/587	+
	Derby	0/407	0/407	+
	Heidelberg	1/930 (0.1)[2]	0/930	+
	Reading	0/224	0/224	+
	Saintpaul	0/541	0/541	+
	Schwarzengrund	0/457	0/457	+
	Typhimurium	0/1926	0/1926	+
C_1	Bareilly	0/128	0/128	+
	Braenderup	0/345	0/345	+
	Hartford	0/110	0/110	-
	Infantis	0/964	0/964	+
	Mbandaka	1/304 (0.3)[2]	0/304	+
	Montevideo	2/594 (0.3)[5]	0/594	+
	Norwich	0/110	110/110 (100)[6]	-
	Ohio	0/113	0/113	+
	Oranienburg	0/172	0/172	+
	Tennessee	0/96	0/96	+
	Thompson	0/317	0/317	-
C_2	Hadar	0/381	0/381	+
	Kentucky	0/1635	0/1635	+
	Muenchen	181/382 (47.4)[1]	198/382 (51.8)[1]	+
	Newport	0/1159	0/1159	+
D_1	Berta	0/179	0/179	+
	Dublin	0/297	0/297	+
	Enteritidis	41/2113 (1.9)[3]	0/2113	+
	Javiana	1/558 (0.2)[2]	0/558	+
E_1	Anatum	646/646 (100)[4]	0/646	+
	London	0/109	0/109	-
	Meleagridis	0/94	0/94	+
	Muenster	135/135 (100)[2]	0/135	+
	Uganda	0/101	0/101	+
E_4	Senftenberg	0/302	0/302	+
G	Poona	126/154 (81.8)[2]	0/154	+
K	Cerro	0/250	0/250	+
R	Johannesburg	0/147	0/147	+

[1]Detection varied by ST. Please see Table 3 for further details.
[2]A sequence of 92-bp was detected (identity: 89.1−92.3%).
[3]The detected length varied: 98-bp (n = 38; identity = 83.7%), 105-bp (n = 1; identity = 100%), full length (n = 2; identity = 100%). The latter were also detected by in-silico PCR using the 126-bp primers (see text for details), and were defined as outliers of *S.* Enteritidis in a previous analysis (Elnekave et al., 2020b).
[4]Partial detection: 91 bp (n = 470; identity = 92.3%) and 92bp (n = 176; identity = 92.4%).
[5]The detected length varied: 92-bp (n = 1; identity = 88%) and full length (n = 1; identity = 100%). The latter was also detected by in-silico PCR using the 126-bp primers (see text for details).
[6]Full length was detected.

Table 5. In-vitro analysis results. The amplicon sizes detected using the designed primers in a collection of non-typhoid *Salmonella enterica* subspecies *enterica* of 9 serogroups, and *Salmonella enterica* of other subspecies, *Escherichia Coli*, *Enterococcus* and *Staphylococcus* isolates.

		Amplicon size (positive/tested)				Amplicon size (positive/tested)	
Serogroup (# tested)	Serotype	126 bp	499 bp	Serogroup (# tested)	Serotype	126 bp	499 bp
B (24)	Agona	0/2	0/2	C_2 (31)	Altona	0/1	0/1
	Bredeney	0/2	0/2		Blockley	0/3	1/3
	Copenhagen	0/2	0/2		Emek	0/2	0/2
	Derby	0/2	0/2		Hadar	0/2	0/2
	Hato	0/2	0/2		Kentucky	0/3	0/3
	Heidelberg	0/2	0/2		Manhattan	0/2	2/2
	Java	0/1	0/1		Muenchen	14/15[1]	1/15[2]
	monophasic	0/2	0/2		Newport	0/2	0/2
	Reading	0/2	0/2		Rough:d:1,2	1/1	0/1
	Saintpaul	0/2	0/2	D_1 (11)	9,12:lv:-	0/1	0/1
	Schwarzengrund	0/2	0/2		Berta	0/2	0/2
	Typhimurium	0/3	0/3		Dublin	0/2	0/2
C_1 (32)	Afula	0/1	0/1		Enteritidis	0/4	0/4
	Bareilly	0/2	0/2		Javiana	0/2	0/2
	Braenderup	0/2	0/2	E_1 (12)	Anatum	0/2	0/2
	Concord	0/3	0/3		Give	0/2	0/2
	Edinburg	0/1	0/1		Meleagridis	0/2	0/2
	Infantis	0/6	0/6		Muenster	0/2	0/2
	Livingstone	0/1	0/1		Orion	0/2	0/2
	Mbandaka	0/2	0/2		Uganda	0/2	0/2
	Montevideo	0/2	0/2	E_4 (4)	Liverpool	0/2	0/2
	Ohio	0/2	0/2		Senftenberg	0/2	0/2
	Oranienburg	0/2	0/2	G (2)	Poona	0/2	0/2
	Rissen	0/2	0/2	K (2)	Cerro	0/2	0/2
	Rough:r:1,5	0/1	0/1	R (2)	Johannesburg	0/2	0/2
	Tennessee	0/1	0/1				
	Virchow	0/4	0/4				
Additional							
S. enterica subsp. arizonae		0/1	0/1	*Escherichia coli*[3]		2/3	0/3
S. enterica subsp. diarizonae serotype 65:z10:e,n,x,z15 strain		0/2	0/2	*Enterococcus* species[3]		0/3	0/3
S. enterica subsp. salamae		0/1	0/1	*Staphylococcus* species[3]		0/1	0/1

[1]Including ST82 (n = 3), ST112 (n = 1), unknown ST (n = 1) and non-available ST (n = 9).
[2]Including ST83 (n = 1).
[3]Poultry origin.

Table 6. In-vitro screening of *Salmonella enterica* subspecies *enterica* belonging to serogroup C. Comparison of the detection of *S.* Muenchen isolates using the designed single multiplex PCR assay and the identification based on the antigenic formula using previously described PCR assays.

		Multiplex PCR amplicon size		
Serotype	6,8:d:1,2[1]	126 bp	499 bp	*S.* Infantis (*soj* gene[2])
Muenchen	176/176 (100)	176/176 (100)	0/176 (0)	0/176 (0)
Infantis	0/14 (0)	0/14 (0)	0/14 (0)	14/14 (100)
Other	0/23 (0)	0/23 (0)	0/23 (0)	0/23 (0)

[1]Based on detection of three antigenic markers previously published (see supplementary for further details).
[2]As described by Ye et al. (Ye et al., 2021).

PCR assay of these isolates was in full agreement with its antigenic formula (Table 6).

DISCUSSION

Genomic variation within *Salmonella* serotypes is not fully represented using the traditional serotyping methods (Worley et al., 2018; Elnekave et al., 2020b). The importance of rapid and efficient identification of serotypes and their variants has been demonstrated over the years with the emergence of multiple genomic variants of different *Salmonella* serotypes (Le Hello et al., 2011; Elnekave et al., 2018; Garcia-Soto et al., 2020). Timely detection of the potential sources as part of an epidemiological outbreak investigation is essential for implementing the appropriate control measures and protecting public health.

Among the available subtyping methods, whole-genome sequence data provide the best discrimination between and within serotypes (Cheng et al., 2019). In addition, whole-genome sequences enable identifying important structures, such as virulence factors and antimicrobial resistance genes (Cheng et al., 2019). The decreasing cost of sequencing has led to increased use of whole-genome sequence data by public health laboratories the world over (Ashton et al., 2016; Brown et al., 2019). Despite its usefulness and the reduced price, sequencing of all *Salmonella* isolates collected by public health laboratories may be impossible due to limited resources and the need for timely diagnoses. For example, the Ministry of Health laboratories in Israel received about 4,000 NTS isolates collected from various hosts during 2019 (Israel Ministry of Health – Jerusalam Central Laboratories, 2019). However, available resources support the sequencing of only a few hundred NTS isolates every year. Moreover, the sequencing process may be time-consuming and require additional expertise for analysis and interpretation of the results (Tang et al., 2019). Therefore, in addition to the sequencing efforts, there is a need to develop a low-cost, fast, and reliable method for the detection of certain serotypes and their variants, such as targeted multiplex PCR assays. Multiplex PCR assays have been developed for the detection of some of the common serotypes such as *S.* Enteritidis and *S.* Typhimurium (Soumet et al., 1999; Liu et al., 2012), but they only enable serotype identification, and cannot distinguish between genetic variants.

Here we designed a multiplex PCR assay with 2 highly sensitive and specific primer sets that allowed not only detection of the emerging *S.* Muenchen serotype but also identification and characterization of 2 main ST groups. One group (126-bp band) included ST112 and ST82, and the less common ST1567, ST1606, ST2169, ST2229, ST2870, and ST3211. The other group (499-bp band) included ST83 and the less common ST2795. In addition, the target sequences (126 bp and 499 bp) were not detected in the less common *S.* Muenchen ST2881 in the in-silico analysis, or in ST84 using BLAST against assemblies (ERR271989 and ERR2719893) that were downloaded from EnteroBase (https://enterobase.warwick.ac.uk/; data not shown).

In-silico specificity was determined based on the detection of nucleotide fragments that covered at least 60% and had at least 90% identity to the designed target sequences in a collection of 18,282 assemblies of 37 NTS serotypes (Elnekave et al., 2020b). Using these criteria, an 22% false detection rate for the 126-bp target sequence was determined. However, the full-length 126-bp target sequence was only found twice in *S.* Enteritidis assemblies of a rare ST and in one *S.* Montevideo assembly. Moreover, these *S.* Enteritidis and *S.* Montevideo assemblies were the only non-*S.* Muenchen assemblies detected using the in-silico PCR. Based on these findings only 5.5% false detection is determined for the developed Multiplex PCR. This is also supported by the higher specificity determined based on the in-vitro analyses of multiple NTS serotypes, including those partially detected in silico. For the 499-bp target sequence, a false detection varying between 0 and 2.8% was determined in silico based on the in-silico PCR and BLAST of the target sequence, respectively. This high specificity was also supported in vitro.

Approximately 1,600 of the 2,600 *Salmonella* serotypes that have been described belong to subspecies *enterica* (Cheng et al., 2019). Many of these serotypes may be involved in human infections; nevertheless, almost 70% of the clinical cases are caused by the 20 most prevalent serotypes (Centers for Disease Control and Prevention (CDC), 2016). The database used for the in-silico analyses in this study was based on 37 serotypes with public health significance that were defined

as these including metadata for at least 100 isolates, of which 1 or more were retrieved from humans (Elnekave et al., 2020b). Therefore, the in-silico specificity found in this study is likely to provide a good estimate of its specificity when used in the field. Moreover, the complementary in-silico approach taken in this study which included 'primer pair specificity testing' was not only restricted to the 37 serotypes and had similar findings (see supplementary information).

Serotype Manhattan (6,8:d:1,5), which was detected in silico as part of the primer BLAST results (see supplementary information) was also detected in vitro. Therefore, identification of the 499-bp amplicon in the multiplex PCR will require an additional verification step, such as detection of the H2:1,5 antigen using the PCR method described by Echeita et al. (2002). Using a PCR for detection of the H2:1,5 antigen will also allow for identification of the Blockley serotype (6,8:k:1,5), which one of its 3 tested isolates was detected by the 499-bp amplicon in vitro. A similar approach (but with the H2:1,6 antigen) may be taken to distinguish from serotype Norwich (6,7:e, h:1,6), which was detected in silico by the 126-bp target (BLAST against the NTS database) but was not included in the in-vitro assay. However, it is important to note that serotype Norwich was not detected by the in-silico PCR using the 126-bp primers. In addition, has a result of the identification in silico of the 126-bp target, in *E. coli* (see supplementary information), we included a limited number of bacterial species and strains other than *Salmonella* that may be found in poultry, and detected the 126-bp amplicon in *E. coli* also in vitro. Given this possible misclassification, a step for species differentiation is required. However, such a step is already routinely conducted using selective growth media (i.e., modified semi-solid Rappaport−Vassiliadis [**MSRV**], xylose lysine deoxycholate [**XLD**], and Brilliant green agar) in the *Salmonella* diagnostic laboratories, which follow International Standard Organization (**ISO**) protocols, such as ISO-6579-1 "Microbiology of the food chain − horizontal method for the detection, enumeration, and serotyping of *Salmonella*".

Overall, the multiplex PCR assay presented here allowed rapid and reliable identification of the emerging *S.* Muenchen serotype. In contrast to traditional identification, this method involves fewer steps, is less time-consuming, does not require subjective interpretation of the results and allows to further characterize the serotype strains into specific genetic variants. The latter may be of great importance for detecting and controlling of emerging genetic strains within serotypes. Moreover, given the low number of NTS serotypes involved in most clinical cases every year, the development of additional similar assays should be encouraged. These assays could either replace or decrease the use of traditional serotyping (i.e., requiring only serogroup identification) for most *Salmonella* isolates and allow for better and faster identification of serotype strains in the field. This, in turn, will allow timely detection of emerging strains and potential outbreak cases, thereby protecting public health and reducing the economic losses to the poultry industry.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102181.

REFERENCES

Ashton, P. M., S. Nair, T. M. Peters, J. A. Bale, D. G. Powell, A. Painset, R. Tewolde, U. Schaefer, C. Jenkins, T. J. Dallman, E. M. de Pinna, K. A. Grant, and G. Salmonella. 2016. Whole Genome Sequencing Implementation. 2016. Identification of Salmonella for public health surveillance using whole genome sequencing. Peer J. 4:e1752.

Aviv, G., K. Tsyba, N. Steck, M. Salmon-Divon, A. Cornelius, G. Rahav, G. A. Grassl, and O. Gal-Mor. 2014. A unique megaplasmid contributes to stress tolerance and pathogenicity of an emergent Salmonella enterica serovar Infantis strain. Environ. Microbiol. 16:977–994.

Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. Salmonella nomenclature. J. Clin. Microbiol. 38:2465–2467.

Brown, E., U. Dessai, S. McGarry, and P. Gerner-Smidt. 2019. Use of whole-genome sequencing for food safety and public health in the United States. Foodborne Pathog. Dis. 16:441–450.

Centers for Disease Control and Prevention (CDC). 2016. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): human isolates surveillance report for 2014 (Final Report) Department of Health and Human Services, CDC, Atlanta, GA.

Cheng, R. A., C. R. Eade, and M. Wiedmann. 2019. Embracing diversity: differences in virulence mechanisms, disease severity, and host adaptations contribute to the success of nontyphoidal salmonella as a foodborne pathogen. Front. Microbiol. 10:1368.

Cohen, E., O. Kriger, S. Amit, M. Davidovich, G. Rahav, and O. Gal-Mor. 2022. The emergence of a multidrug resistant Salmonella Muenchen in Israel is associated with horizontal acquisition of the epidemic pESI plasmid. Clin. Microbiol. Infect, doi:10.1016/j.cmi.2022.05.029.

Danan, C., S. Fremy, F. Moury, M. Bohnert, and A. Brisabois. 2009. Determining the serotype of isolated Salmonella strains in the veterinary sector using the rapid slide agglutination test. J. Ref. 2:13–18.

Echeita, M. A., S. Herrera, J. Garaizar, and M. A. Usera. 2002. Multiplex PCR-based detection and identification of the most common Salmonella second-phase flagellar antigens. Res. Microbiol. 153:107–113.

Elnekave, E., S. Hong, A. E. Mather, D. Boxrud, A. J. Taylor, V. Lappi, T. J. Johnson, F. Vannucci, P. Davies, C. Hedberg, A. Perez, and J. Alvarez. 2018. Salmonella enterica Serotype 4,[5],12:i:- in Swine in the United States midwest: an emerging multidrug-resistant clade. Clin. Infect. Dis. 66:877–885.

Elnekave, E., S. L. Hong, S. Lim, D. Boxrud, A. Rovira, A. E. Mather, A. Perez, and J. Alvarez. 2020a. Transmission of multidrug-resistant Salmonella enterica subspecies enterica 4,[5],12:i:- sequence

type 34 between Europe and the United States. Emerg. Infect. Dis. 26:3034–3038.

Elnekave, E., S. L. Hong, S. Lim, S. S. Hayer, D. Boxrud, A. J. Taylor, V. Lappi, N. Noyes, T. J. Johnson, A. Rovira, P. Davies, A. Perez, and J. Alvarez. 2019. Circulation of plasmids harboring resistance genes to quinolones and/or extended-spectrum cephalosporins in multiple Salmonella enterica serotypes from swine in the United States. Antimicrob. Agents Chemother. 63:e02602-18.

Elnekave, E., S. L. Hong, S. Lim, T. J. Johnson, A. Perez, and J. Alvarez. 2020b. Comparing serotyping with whole-genome sequencing for subtyping of non-typhoidal Salmonella enterica: a large-scale analysis of 37 serotypes with a public health impact in the USA. Microb. Genom. 6.

Garcia-Soto, S., M. Y. Abdel-Glil, H. Tomaso, J. Linde, and U. Methner. 2020. Emergence of multidrug-resistant Salmonella enterica subspecies enterica serovar infantis of multilocus sequence type 2283 in German broiler farms. Front. Microbiol. 11:1741.

Grimont, P. A., and F.-X. Weill. 2007. Antigenic formulae of the Salmonella serovars. WHO Collab. Centre Ref. Res. Salmonella 9:1–166.

Herrera-Leon, S., J. R. McQuiston, M. A. Usera, P. I. Fields, J. Garaizar, and M. A. Echeita. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of Salmonella spp. J. Clin. Microbiol. 42:2581–2586.

Herrera-Leon, S., R. Ramiro, M. Arroyo, R. Diez, M. A. Usera, and M. A. Echeita. 2007. Blind comparison of traditional serotyping with three multiplex PCRs for the identification of Salmonella serotypes. Res. Microbiol. 158:122–127.

Israel Ministry of Health – Jerusalam Central Laboratories. 2017. Jerusalem central laboratories - 2017 annual report (Hebrew). Accessed Oct. 2022. https://www.health.gov.il/PublicationsFiles/LAB_JER2017.pdf.

Israel Ministry of Health – Jerusalam Central Laboratories. 2019. Jerusalem central laboratories - 2019 annual report (Hebrew). Accessed Oct. 2022. https://www.health.gov.il/PublicationsFiles/LAB_JER2019.pdf.

Jean-Gilles Beaubrun, J., C. M. Cheng, K. S. Chen, L. Ewing, H. Wang, M. C. Agpaoa, M. C. Huang, E. Dickey, J. M. Du, D. M. Williams-Hill, B. Hamilton, S. A. Micallef, R. E. Rosenberg Goldstein, A. George, S. W. Joseph, A. R. Sapkota, A. P. Jacobson, B. D. Tall, M. H. Kothary, K. Dudley, and D. E. Hanes. 2012. The evaluation of a PCR-based method for identification of Salmonella enterica serotypes from environmental samples and various food matrices. Food. Microbiol. 31:199–209.

Kirk, M. D., S. M. Pires, R. E. Black, M. Caipo, J. A. Crump, B. Devleesschauwer, D. Dopfer, A. Fazil, C. L. Fischer-Walker, T. Hald, A. J. Hall, K. H. Keddy, R. J. Lake, C. F. Lanata, P. R. Torgerson, A. H. Havelaar, and F. J. Angulo. 2015. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med. 12:e1001921.

Knowles, T., R. Moody, and M. G. McEachern. 2007. European food scares and their impact on EU food policy. Br. Food J 109:43–67.

Le Hello, S., R. S. Hendriksen, B. Doublet, I. Fisher, E. M. Nielsen, J. M. Whichard, B. Bouchrif, K. Fashae, S. A. Granier, N. Jourdan-Da Silva, A. Cloeckaert, E. J. Threlfall, F. J. Angulo, F. M. Aarestrup, J. Wain, and F. X. Weill. 2011. International spread of an epidemic population of Salmonella enterica serotype Kentucky ST198 resistant to ciprofloxacin. J. Infect. Dis. 204:675–684.

Liu, B., X. Zhou, L. Zhang, W. Liu, X. Dan, C. Shi, and X. Shi. 2012. Development of a novel multiplex PCR assay for the identification of Salmonella enterica Typhimurium and Enteritidis. Food Control 27:87–93.

Mather, A. E., S. W. Reid, D. J. Maskell, J. Parkhill, M. C. Fookes, S. R. Harris, D. J. Brown, J. E. Coia, M. R. Mulvey, M. W. Gilmour, L. Petrovska, E. de Pinna, M. Kuroda, M. Akiba, H. Izumiya, T. R. Connor, M. A. Suchard, P. Lemey, D. J. Mellor, D. T. Haydon, and N. R. Thomson. 2013. Distinguishable epidemics of multidrug-resistant Salmonella Typhimurium DT104 in different hosts. Science 341:1514–1517.

Mikoleit, M., M. S. Van Duyne, J. Halpin, B. McGlinchey, and P. I. Fields. 2012. Variable expression of O:61 in Salmonella group C2. J. Clin. Microbiol. 50:4098–4099.

Soumet, C., G. Ermel, V. Rose, N. Rose, P. Drouin, G. Salvat, and P. Colin. 1999. Identification by a multiplex PCR-based assay of Salmonella typhimurium and Salmonella enteritidis strains from environmental swabs of poultry houses. Lett. Appl. Microbiol. 29:1–6.

Tang, S., R. H. Orsi, H. Luo, C. Ge, G. Zhang, R. C. Baker, A. Stevenson, and M. Wiedmann. 2019. Assessment and comparison of molecular subtyping and characterization methods for Salmonella. Front. Microbiol. 10:1591.

World Health Organization (WHO). 2015. Global action plan on antimicrobial resistance. 2015. Accessed Oct. 2, 2022. http://apps.who.int/iris/bitstream/handle/10665/193736/9789241509763_eng.pdf?sequence=1.

Worley, J., J. Meng, M. W. Allard, E. W. Brown, and R. E. Timme. 2018. Salmonella enterica phylogeny based on whole-genome sequencing reveals two new clades and novel patterns of horizontally acquired genetic elements. mBio 9:e02303-18.

Ye, Q., Y. Shang, M. Chen, R. Pang, F. Li, X. Xiang, B. Zhou, C. Wang, S. Zhang, and J. Zhang. 2021. Mining and evaluating novel serovar-specific Salmonella C1 serogroup genes by polymerase chain reaction analysis. LWT 141:110821.