

# Development and Validation of SYBR Green-Based qPCR Technique of Detection and Quantification of *Salmonella enterica*

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## Abstract

*Salmonella enterica* serovars is a leading cause of human gastroenteritis, and the incidence of salmonellosis is constantly increasing, causing millions of infections and many deaths annually. The detection of the pathogen in optimal terms is an essential factor for reducing the impact on the human body. In this work, SYBR Green I-based qPCR method of detection and quantification of *Salmonella enterica* subsp. *enterica*, two pairs of primers were designed using publically available Primer-BLAST software. Primer efficiency was calculated by establishing a standard curve. The specificity, sensitivity, accuracy, and precision of PCR results were tested. Both primer pairs showed an acceptable performance, proving the developed techniques were sensitive, reliable and precise. The validated qPCR technology has a good potential to replace the traditional culture method in microbial diagnosis.

## Keywords

Dilution, DNA, Primer, Real-Time PCR, *Salmonella enterica*

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## 1. Introduction

In recent decades, according to World Health Organization (WHO), the incidence of *Salmonella* infection (salmonellosis) has been increasing worldwide. Contamination of food with *Salmonella* spp. and, as a consequence, the development of salmonellosis is a serious threat that requires great attention to the control of the microbiological purity of food, the development of quick and rapid, accurate methods for its detection.

According to the Bergey's Manual of Systematic Bacteriology, *Salmonella* belongs to the Enterobacteriaceae family, genus—*Salmonella*, which includes 2 species: *Salmonella bongori* and *Salmonella enterica* [1]. *S. enterica* subdivided into six subspecies with about 2500 serovars (serotypes) [2]. These serovars can cause illnesses in human ranging from gastroenteritis to typhoid fever which kills around 500,000 people worldwide per year [3].

The source of salmonellosis, most often, are eggs (up to 90% of cases of salmonellosis are associated with the consumption of raw or insufficiently cooked eggs), meat and dairy products, and, to a lesser extent, fish and fish products, as well as plant products. *Salmonella* is relatively stable in the external environment and is well preserved in room dust (almost up to three months), in open water (11 to 120 days), in meat and sausages (2 - 6 months), in frozen meat (more than six months), in milk at room temperature for up to 10 days and in the refrigerator for up to 20 days; in butter (52 - 128 days); in eggs (over a year), on eggshells (24 days). At 70°C, salmonella die within 5 - 10 minutes, in the thickness of meat they withstand boiling for several hours. In meat stored in a refrigerator at a low above zero temperature, salmonella not only survive, but are also able to reproduce [4].

Recently, salmonella serotypes have become widespread, characterized by resistance to many modern common antibiotics and disinfectants, as well as increased heat resistance [5].

It has been extensively reported that classical microbiological methods, even though reliable, are lengthy and tedious. In this sense molecular applications, relies on the detection of specific *Salmonella* genes by PCR techniques, such as Real-Time PCR [6] [7], which have greatly allowed the reduction in the time needed to achieve the final result and to help control the spread of disease [8].

For designing a reliable PCR assay, it is necessary to pick a good target gene for PCR amplification. Several target genes of *Salmonella* have been reported, such as *invA* [6] [7] [9], *sdia*, *fliC*, *fliB*, *setB*, *hilA* [10] [11] [12] [13] [14].

The *invA* gene lies at centisome 63 region of the *Salmonella* chromosome and is widely used as a target in PCR assays for *Salmonella* detection [10].

The *invA* gene usually codes for a protein of the inner bacterial membrane, which is responsible for the invasion of the intestinal cells of the host [4] and is promising for primer design since it contains conservative and well characterized sequences. In order to validate the PCR assays of *Salmonella* molecular detection it is necessary to test specificity, sensitivity, accuracy, and precision of results [15].

## 2. Materials and Methods

### 2.1. Materials

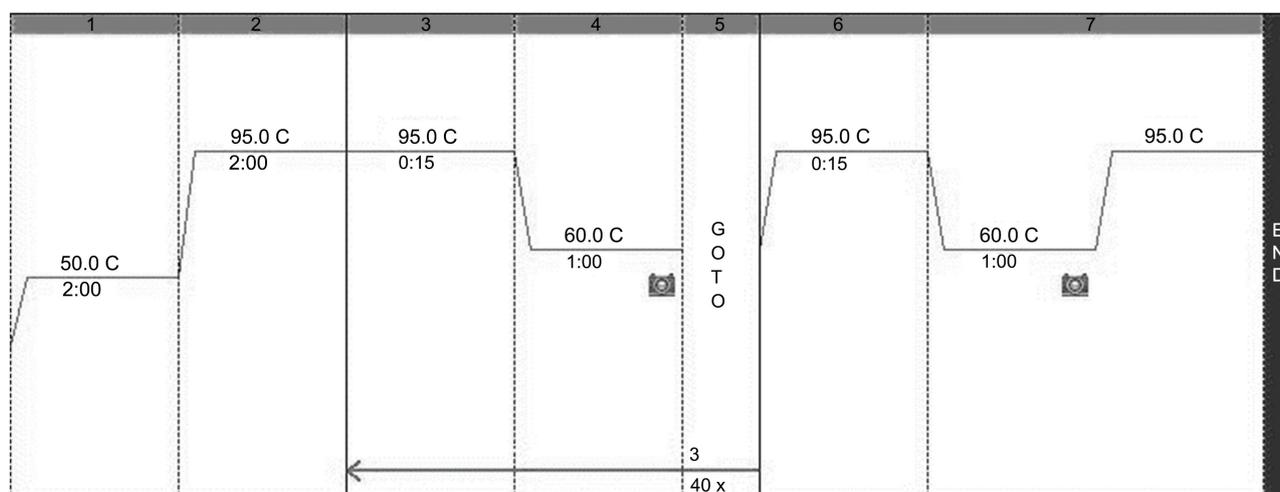
As *Salmonella enterica* DNA standard, microbial DNA standard from *Salmonella enterica*, (Sigmaaldrich MBD0005) was used. For primer specificity check, DNA from liquid cultures of *Escherichia coli*, *Staphylococcus aureus*, *Listeria*

*monocytogenes* was purified using SDS-based method. Briefly, the cells from 1 ml culture were pelleted by centrifugation and re-suspended in 0.6 mL of the Extraction buffer (Tris-HCl 0.2M pH 8.0, NaCl 0.25M, Na<sub>2</sub>EDTA 0.025M, SDS 5% w/v) and heated at 65°C for 1 hour. Then the lysate was centrifuged, supernatant collected to a fresh 1.7 ml tube, followed by chlorophorm extraction and ethanol precipitation. The pellet was washed with 70% ethanol, air dried and dissolved in 100 ul of deionized water; 2 µL of the resulting DNA solution was used per PCR reaction. The qualitative and quantitative verification of the isolated DNA was made by spectrophotometric analyses at Genova Nano Micro-volume spectrophotometer.

## 2.2. Methods

The method used was quantitative real-time PCR (qPCR). qPCR allows precise quantification of specific nucleic acids in a complex solution by fluorescent detection of labeled PCR products. In a real-time PCR, a positive reaction is detected by accumulation of a fluorescent signal. For the experiments, we used SYBR Green I nonspecific dye as the fluorescent agent (Applied biosystems). The quantification cycle (C<sub>q</sub>) represents the number of cycles required for the fluorescent signal to cross the threshold (*i.e.*, to pass the background level). C<sub>q</sub> levels are inversely proportional to the amount of target nucleic acid in the sample. The lower the C<sub>q</sub> level the higher is the concentration of target nucleic acid in the sample. We used 40 cycles of amplification. **Figure 1** showed the cycling conditions used in this work.

qPCR was carried out on a Real-Time PCR Detection Systems CFX96 Touch™ BIORAD. qPCR conditions were as recommended by SybrGreen producer (Applied Biosystems)—initial incubation at 50°C for 2 minutes, initial denaturation at 95°C for 2 minutes, and alternation at 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. For melting curve construction, samples were heated at 95°C for 15 seconds, then incubated at 60°C for 1 minute (1.6°C/s ramp rate),



**Figure 1.** Temperature cycling conditions.

then heated at 95°C for 15 seconds (0.15°C/s ramp rate). Cycling conditions are shown in **Figure 1**. The detection was done at SYBR channel.

### 3. Results and Discussion

#### 3.1. Primer Design

For detection of *Salmonella enterica* subsp. *enterica*, two pairs of home designed primers were developed. Primers were designed using Primer-BLAST software, available at <https://www.ncbi.nlm.nih.gov/tools/>. During primer design, the default values were used for most parameters except for PCR product size which was set to the value of 70 - 200, Primer melting temperatures (T<sub>m</sub>) was set up to be within 60 for Min, 61 for Opt, 63 for Max; Max GC in primer 3' end was set to the value of 2, Max Self Complementarity and Max Pair Complementarity-5 for the parameter Any. The template used was the nucleic acid sequence LC111465.1 for *invA* gene (invasive antigen A) from GenBank. Designing primers targeting this gene can be a good choice, due to its conservative and well characterized sequences.

**Table 1** showed sequences and characteristics of the primers used in this work. Primer pairs *se21-22* and *se23-24* were used for qPCR analysis.

BLAST analysis of the primers showed that these primers were capable of recognizing a vast number of *Salmonella enterica* serovars, including *Typhi*, *Ad-jame*, *Typhimurium*, *Infantis*, *Worthington*, *Albany*, *Heidelberg*, *Enteritidis*, *Anatum*, *Kentucky*, *Weltevreden* etc.

#### 3.2. Primer Experimental Testing and Validation

##### *Primer specificity check*

Primer specificity is the ability of primers to recognize only target DNA rather than unrelated DNA sequences.

First, the specificity of the primers was checked bioinformatically (*in silico*) to assure that no overlapping with other species is detected by BLAST. Second, the specificity of the primers was checked experimentally (*in vitro*). Purified DNA samples extracted from *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, as well as standard DNA of *Salmonella enterica* were used as a template

**Table 1.** The sequences and characteristics of the primers.

Primer	Sequence (5'->3')	Template strand	Length	T <sub>m</sub>	GC%	Self compl.	Self 3' compl.
se21-	GCTTCCAGTTGGTCCAGCAT	Plus	20	60.61	55	5	3
22	CGGGAGCTTGGCTATGTGTT	Minus	20	60.39	55	4	0
Product length	131						
se23-	CGTGGCATGTCTGAGCACTT	Plus	20	60.95	55	4	3
24	ACGCATGAAGAGGGGGAGAA	Minus	20	60.91	55	4	0
Product length	198						

in PCR with the tested pairs of primers. **Figure 2** showed the results obtained with primer pair *se21-22* (**Figure 2(a)**) and primer pair *se23-24* (**Figure 2(b)**).

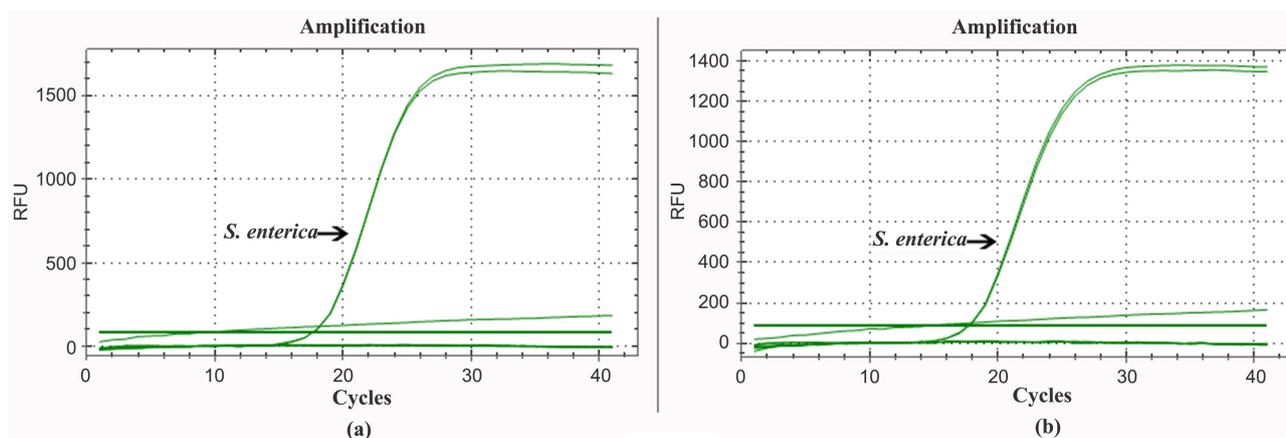
Neither of the primer pairs amplified irrelevant DNA sequences. One can see, that the amplification is observed only in the samples with *Salmonella enterica* standard DNA as a template, while no amplification occurs in the no template control and irrelevant DNA samples.

#### Primer efficiency

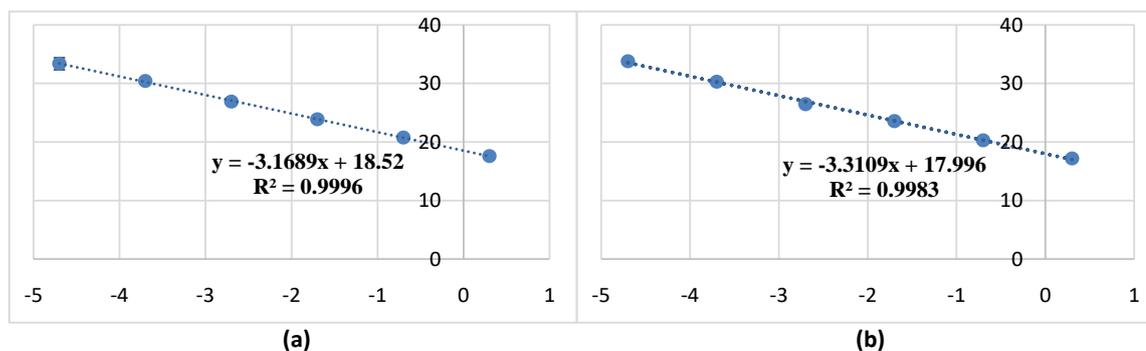
Ideally, if the amplification efficiency is 100%, the amount of newly synthesized PCR amplicon doubles each cycle. The efficiency of the reaction can be estimated from the slope of the standard curve. Practically, primers are suitable for real-time PCR analysis with the efficiencies 90% - 110%.

Primer efficiency was calculated by establishing the standard curve. The ranges of the standard curve of *Salmonella enterica* standard DNA were 2 ng/ul -  $2 \times 10^6$  ng/ul, each standard was tested in triplicate. **Figure 3** showed the standard curves for the primer pair *se21-22* (**Figure 3(a)**) and *se23-24* (**Figure 3(b)**).

Using the standard curve and the online primer efficiency calculator (<https://www.thermofisher.com/ro/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html>), primer efficiency was calculated. For primer pair *se21-22*, primer efficiency was 106.81%. For



**Figure 2.** Primer specificity test *se21-22* (a) and *se23-24* (b). Only *Salmonella enterica* DNA shows the amplification curve.



**Figure 3.** The standard curves for the primer pairs *se21-22* (a) and *se23-24* (b).

primer pair *se23-24*, primer efficiency was 100.46%, which makes both primer pairs suitable for use in qPCR.

### ***LOD and LOQ***

Generally, the LOD (limit of detection) is defined as the lowest amount of analyte, which can be detected with a more than stated percentage of confidence, but not necessarily quantified as an exact value [16]. Another definition describes LOD as the lowest concentration level which can be determined as statistically different from blank. However, in PCR, for the limited concentration of template DNA (analyte), the output of the reaction will be either amplification, or no amplification at all, making it impossible to set a cut-off value after which the sample will be considered positive [17].

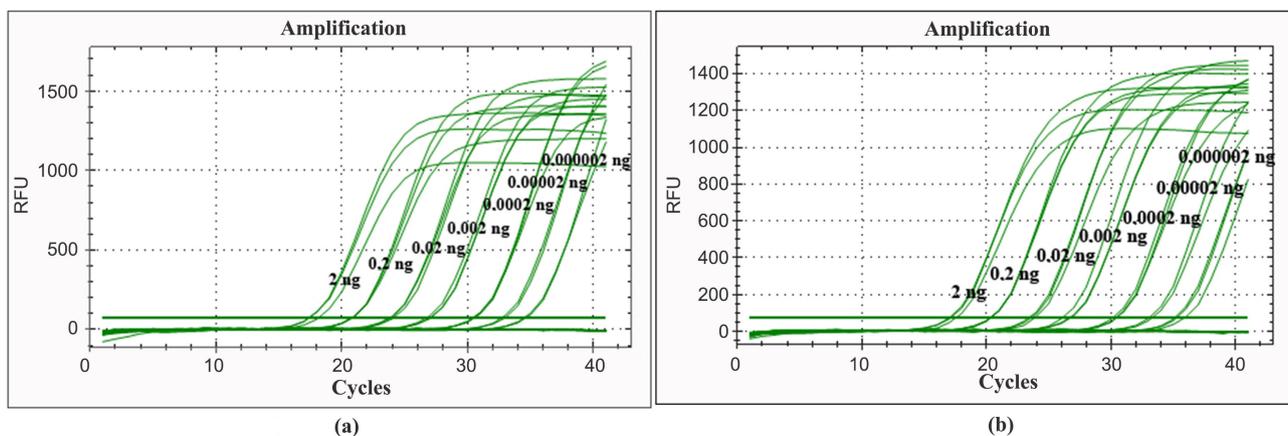
Since the definitions reported were described for chemical methods and are not perfectly suited for PCR, other approaches have been proposed. It is generally accepted to consider the LOD as the minimum concentration of nucleic acid or number of cells, which always gives a positive PCR result in over 95% of replicas tested [18].

In this case, the LOD was determined by establishing the standard curve and analyzing which concentration gives a positive PCR signal in over 95% of the replicas. The ranges of the standard curve of *Salmonella enterica* standard DNA were 2 ng/μl - 2 × 10<sup>6</sup> ng/μl, each standard was tested in triplicate. The amount of 2 × 10<sup>-5</sup> ng/μl was reliably detectable throughout the assay with both primer pairs (Figure 4).

The LOQ (limit of quantification) was defined as the smallest amount of analyte, which can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation [16]. For qPCR, the LOQ is determined as the lowest concentration of analyte, which gives a variability (CV, coefficient of variation) below 25%. In this assay, LOQ was equal to LOD, and was 2 × 10<sup>-5</sup> ng/μl for both primer pairs.

### ***Precision***

Precision is defined as the degree of agreement of measurements under specified conditions. Intra-assay precision was determined from experiment results in an



**Figure 4.** Determination of detection limit for primer pair *se21-22* (a) and *se23-24* (b).

individual run. The standard deviation (SD) and coefficient of variation (CV) of each standard were from 0.05 to 1.03 cycles and 2.5% to 3.1%. For primer pair *se21-22* and 0.02 - 1.13 cycles and 0.1% to 3.35% for primer pair *se23-24*. **Table 2** and **Table 3** showed the results of intra-assay precision tests for both primer pairs.

Inter-assay precision was determined from experimental results from three different runs on three different days. The SD and CV were 0.23 - 0.8 and 1.34 to 2.9%. For primer pair *se21-22* and 0.27 - 0.97 cycles and 1.53% to 3.15% for primer pair *se23-24*. **Table 4** and **Table 5** showed the results of inter-assay precision tests for both primer pairs.

**Table 2.** Intra-assay precision (repeatability) test for primer pair *se21-22*.

Standard, ng/μl	Mean Ct (n = 3)	STDEV	Coefficient variation
2	17.59719888	0.438542	2.492112398
0.2	20.7553349	0.053635	0.258415242
0.02	23.88458118	0.186543	0.781017251
0.002	26.89655218	0.208508	0.775220683
0.0002	30.4138247	0.070077	0.230410339
0.00002	33.38192428	1.038433	3.110765388

**Table 3.** Intra-assay precision (repeatability) test for primer pair *se23-24*.

Standard, ng/μl	Mean Ct (n = 3)	STDEV	Coefficient variation
2	17.20860213	0.270594865	1.572439543
0.2	20.29236189	0.024829105	0.122356902
0.02	23.58279697	0.254664027	1.079872025
0.002	26.47513677	0.351875914	1.329080629
0.0002	30.30124086	0.157424314	0.519530916
0.00002	33.80120295	1.13293754	3.351766923

**Table 4.** Inter-assay precision (intermediate precision) test for primer pair *se21-22*.

Pair <i>se21-22</i>	Mean Ct			STDEV	CV, %
	Standard, ng/μl	Day 1	Day2		
2	17.67344	17.5972	17.38514	0.149387	0.8511151
0.2	21.17401	20.75533	20.86401	0.217252	1.0379384
0.02	24.08814	23.88458	24.42158	0.271105	1.1234537
0.002	27.60883	26.89655	28.02483	0.570585	2.0740953
0.0002	30.78307	30.41382	31.76068	0.69595	2.2460243
0.00002	34.17652	33.38192	35.15967	0.890538	2.6009194

**Table 5.** Inter-assay precision (intermediate precision) test for primer pair *se23-se24*.

Pair <i>se23-24</i>	Mean Ct				
	Standard, ng/μl	Day 1	Day 2	Day3	STDEV
2	17.67044	17.2086	17.19989	0.269193	1.5506851
0.2	21.11432	20.29236	20.73872	0.411485	1.9864003
0.02	24.12672	23.5828	24.28787	0.369449	1.5394268
0.002	27.51393	26.47514	28.8854	0.73097	2.6783822
0.0002	30.53868	30.30124	32.09845	0.97632	3.1515081
0.0002	34.23234	33.8012	34.8602	0.532535	1.5526751

### Accuracy

Accuracy is defined as a degree of conformity of a value generated by a procedure to the assumed or accepted true value [19]. In other words, it shows how close are the experimental values to the reference values.

For accuracy measurement, DNA of *Salmonella enterica* of three different concentrations (0.04, 0.01 and 0.025 ng) was used as a template in real-time PCR. The real-time PCR experiments were carried out by 9 replicates on different days.

**Table 6** showed the percentage recovery for pair of primers *se21-22*, and **Table 7** showed the percentage recovery for pair of primers *se23-24*. The average recovery was 81.8% - 111.4% for *se21-22* and 63.4% - 99.8% for pair of primers *se23-24*.

### Linearity

The linearity of this method for both primer pairs was evaluated by calculating coefficients of determination ( $R^2$ ). Three standard curves were created by plotting mean  $C_T$  values against the DNA concentration. Data from three independent experiments were used for building the standard curves. **Figure 5** showed the standard curves of three independent experiments for the primer pair *se21-22*. The  $R^2$  of the standard curves exceeded 0.99, which meets the accepted criterion the  $R^2 > 0.98$ .

**Figure 6** showed the standard curves of three independent experiments for the primer pair *se23-24*. The  $R^2$  of the standard curves exceeded 0.99, which met the accepted criterion the  $R^2 > 0.98$ .

At present, conventional culture-based methods are considered to be gold standard for detecting food-borne pathogenic bacteria. However, these methods have a number of disadvantages. They are time-consuming and labor intensive. Besides, it is difficult to use them for quantification of the pathogenic bacteria. Thus, there is a need for rapid, sensitive and specific methods suitable for high throughput analysis and capable of quantifying the disease-causing bacteria.

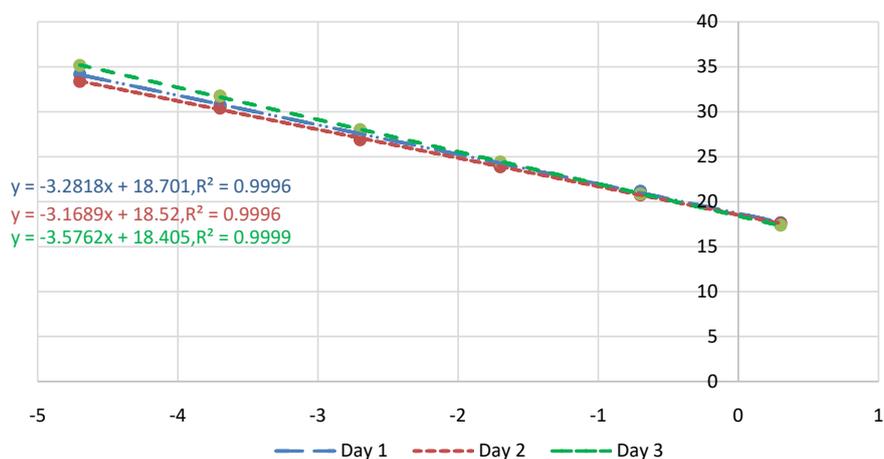
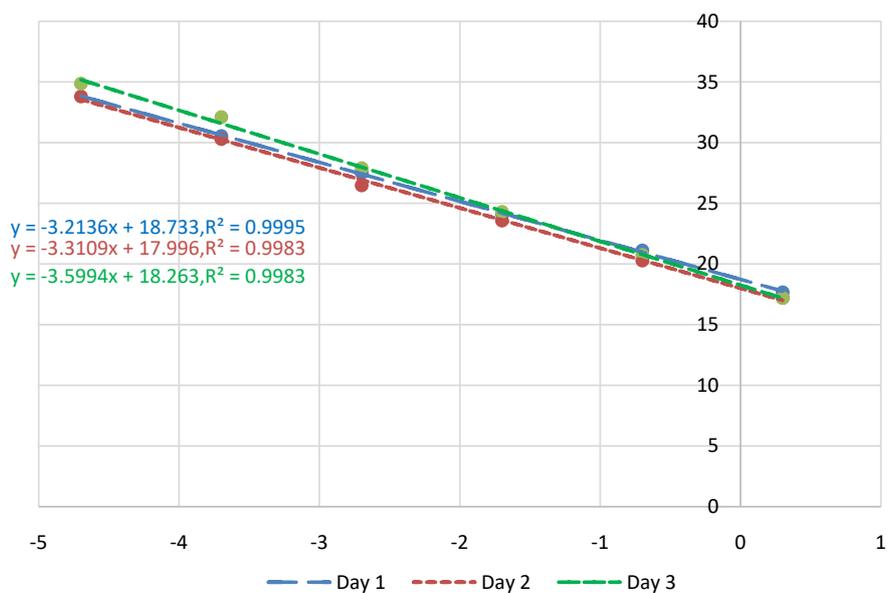
In this paper we describe development and validation of SYBR Green-based qPCR technique of detection and quantification of *Salmonella enterica*. This technique allows for a high throughput processing of samples, produces results within hours, is inexpensive and available.

**Table 6.** Accuracy test for primer pair *se21-se22*.

Pair <i>se21-22</i>	concentration, ng/ul	measured, ng/ul	average recovery, %	CV, %
	0.02	0.022279742	111.4	0.929436
	0.005	0.00491326	98.3	1.4093
	0.00125	0.001022459	81.8	3.995766

**Table 7.** Accuracy test for primer pair *se23-24*.

Pair <i>se23-24</i>	concentration, ng/ul	measured, ng/ul	average recovery, %	CV, %
	0.02	0.019951833	99.8	0.999474
	0.005	0.004585367	91.7	1.493163
	0.00125	0.000792106	63.4	2.492201

**Figure 5.** The standard curves of three independent experiments for the primer pair *se21-22*.**Figure 6.** The standard curves of three independent experiments for the primer pair *se23-24*.

qPCR-based techniques are a simple and rapid alternative to traditional culturing methods of pathogen detection in food samples. They are becoming increasingly popular due to the fast progress in sequencing techniques, and increasing number of genomic sequences of various pathogens available in open access databases. These advances make it possible to design primers to virtually any organism. Moreover, qPCR-based methods can be designed not only for detection of a pathogen and its quantification, but also for serovar typing.

#### 4. Conclusion

In this work, we developed and validated a sensitive, reliable and precise technique of detection and quantification of *Salmonella enterica* based on SYBR Green qPCR method. qPCR technology has a good potential of replacing tradition culture method in microbial diagnostics, especially when fast and sensitive or high throughput diagnostics is required.

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#### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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