Rapid Detection of *Salmonella* spp. in Water Samples by Real-Time PCR

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Abstract

Regular monitoring of water quality is a key component of water risk management. The speed and accuracy of water tests directly impact the identification, management, and mitigation of disruptions in the water supply. Conventional *Salmonella* detection is usually performed using a culture-based method, which is time-consuming, labor-intensive, and unsuitable for high-throughput analysis. A rapid and sensitive protocol for detecting *Salmonella* spp. in water samples was evaluated using the iQ-Check *Salmonella* II Kit (Bio-Rad Laboratories, Inc., catalog #3578123). The iQ-Check *Salmonella* II Kit allows potential problems to be identified quickly by both low- and high-volume laboratories, thus ensuring the safety of the water supply and minimizing impacts on public health.

Introduction

Salmonella spp. are common bacteria throughout the world. They are usually classified as pathogens, although their virulence and pathogenesis vary widely. The natural hosts of *Salmonella* include humans, agricultural and domestic livestock, and wild animals, including birds. Humans and animals can excrete these bacteria while harboring them asymptomatically, thus making it impossible to eliminate them from the environment. *Salmonella* are largely classified into two main species: *S. enterica* and *S. bongori*, the former comprising most of the serotypes associated with human disease. Salmonellosis is characterized by acute gastroenteritis. Symptoms include diarrhea, fever, stomach pain, and vomiting, lasting 4–7 days in most people. *Salmonella* infections can cause various morbidities and mortality, especially in developing countries. The prevalence is especially high in low-resource regions where poor sanitation and a lack of clean water are common issues.

Since water is a recognized vehicle of infection, the presence and absence of *Salmonella* is regularly monitored in water systems. *Salmonella* can be present in all types of domestic and agricultural wastewater, fresh water (including ground and drinking waters), and sea water. Water can also become contaminated with *Salmonella* after flooding or other natural disasters.

Methods

The objective of this study was to evaluate the capacity of the iQ-Check *Salmonella* II Kit to detect *Salmonella* spp. in water samples.

The study evaluated two methods to prepare water samples before enrichment:

- Method 1: For all samples greater than 10 ml, the water sample was filtered using a 0.40-µm membrane. The membrane was then added to the broth for enrichment following the ISO 19250:2010 guidelines for the detection of *Salmonella* spp. This method is suitable for all types of filterable water and was evaluated on network water and pond/river water.
- Method 2: For samples less than 200 ml, water was added directly into 2x-concentrated broth. This method is suitable for nonfilterable water samples and was evaluated on pond/river water.

After these methods were used to prepare the water samples, bacteria were recovered using two methods: (i) the iQ-Check *Salmonella* II Kit and (ii) by regrowth of bacteria in Rappaport Vassiliadis Soya (RVS) Broth (Bio-Rad, #3555773), and streaking on Xylose Lysine Deoxycholate (XLD) Agar Plates (Bio-Rad, #3541751) and RAPID'*Salmonella* Agar Plates (Bio-Rad, #3563961), as described in ISO 19250:2010.

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Water Samples

Water samples were collected from the water network and from a pond in Marnes-la-Coquette, France. Samples were divided into 100-ml aliquots for water filtration and inoculated with stressed *Salmonella* strains.

Strain Preparation and Stress

Two *Salmonella* strains were used in this study (Table 1).

Table 1. Description of the *Salmonella* strains selected for this study.

Genus and Species	Serovar	Collection	Strain Number
Salmonella enterica subsp. enterica	Paratyphi B	ATCC	8759
Salmonella enterica subsp. enterica	Paratyphi A	ATCC	9150

For all tests, an isolated colony was cultured for 20 hr at 37 ± 1°C in Trypto-Casein-Soy Broth (Bio-Rad, #3553454), then diluted in water supplemented with NaCl and incubated at room temperature to stress the strain prior to adding it to the water sample.

The stress level was measured by plating the stressed culture on selective media (RAPID'*Salmonella* Agar Plates) and nonselective media (Trypto-Casein-Soy Agar, Bio-Rad, #3563884B). The media were incubated for 4 hr at ambient temperature, after which the number of colonies were counted. The results were expressed as the log difference between the counts of the two plates, which indicates the stress level.

ISO 16140-3 Testing Conditions

To verify the capacity of the iQ-Check *Salmonella* II Kit method to accurately detect the presence of *Salmonella* in water samples, the estimated level of detection at 50% (eLOD50) protocol (Table 2) was used with one uninoculated sample and seven inoculated samples for each type of water. A target amount of 3–5 CFU per test was used to spike each bag.

Two independent strains, one for each matrix, were spiked at the targeted amount (3–5 CFU) after stressing the bacteria. The level of spike and stress was recorded to ensure acceptability within the ISO 16140-3 requirements. A maximum target of 5 CFU and minimum stress level of 0.5 log was expected. Count levels below 3 CFU per bag were accepted if the number of positive results was above the acceptance criteria, as allowed by ISO 16140-3 guidelines.

Preparation and Enrichment of Water Samples for the Detection of *Salmonella* spp.

Salmonella cells can be present in low numbers and injured in the aqueous environment, so a pre-enrichment step is required to resuscitate the bacteria. All enrichments were performed using Buffered Peptone Water Plus (BPW Plus) in either liquid (Bio-Rad, #3554179) or powder (Bio-Rad, #3564684) forms using the sample and enrichment conditions described in Table 3. For water filtration, a 0.4-µm pore size membrane was used (Millipore Sigma, #HTTP04700).

Table 3. Sample preparation and enrichment conditions.

Water Sample	Sample Size	Method of Preparation	Media	Incubation Time and Temperature
Network water	100 ml	Method 1: Filtered water and membrane added to the media	50 ml BPW Plus	16 hr at 37 ± 1°C
Pond/river water	100 ml	Method 1: Filtered water and membrane added to the media	50 ml BPW Plus	16 hr at 37 ± 1°C
Pond/river water	100 ml	Method 2: Water added directly to the media	100 ml BPW Plus 2x-concentrated*	16 hr at 37 ± 1°C

* BPW Plus 2x-concentrated was prepared by adding twice the amount of BPW Plus powder into water compared to the normal BPW Plus formula. BPW, Buffered Peptone Water.

The following detection methods all utilized samples from the same enrichment bag.

PCR Method

A 100-µl aliquot was sampled from the enrichment bag and treated with iQ-Check Free DNA Removal Solution (FDRS, Bio-Rad, #3594970) to remove potential free DNA in the enriched sample. While the use of FDRS is not required, removing free DNA allowed for the eLOD to be verified under the most stringent conditions. Samples were extracted using the Easy I protocol described in the iQ-Check *Salmonella* II Kit. At the end of the extraction, 5 µl was used to perform the analysis using the iQ-Check *Salmonella* II PCR Kit. Amplification was conducted using the FAST thermal protocol on the CFX96 Touch Deep Well System (Bio-Rad, #3600037). Analysis was performed using CFX Manager Software Industrial Diagnostic Edition version 3.1 (Bio-Rad, #3593893).

Table 2. The eLOD50 strategy and acceptance criteria per the ISO 16140-3:2021 guidelines.

	Inoculation Level of the Test Portion					
Protocol	High Level, 9 × eLOD50/ Test Portion	Intermediate Level, 3 × eLOD50/Test Portion	Low Level, 9 × eLOD50/ Test Portion	3–5 CFU/Test Portion	Blanks	Total Number of Replicates
1	1	4	4	–	1	10
2	–	3	5	–	1	9
3	–	–	–	7	1	8
Method	**Performance Characteristics**			**Acceptibility Limits**		
Qualitative	eLOD50			Protocols 1 and 2: eLOD50 ≤ 4 × LOD50 Protocol 3: ≥6 out of 7 positive results		

* CFU, colony forming units; eLOD50, estimated level of detection at 50%; LOD50, level of detection.

Direct Chromogenic Media Testing

Without shaking the bag at the end of the enrichment, 10-µl inoculating loops were used to streak the sample onto RAPID'*Salmonella* plates. Plates were incubated for 24 hr at 37 ± 1°C. Characteristic single colonies were assessed by PCR for confirmation.

Adapted ISO 19250:2010 Method

The secondary enrichment was performed by adding 0.1 ml of the primary BPW Plus enrichment into 10 ml of RVS broth. After incubation for 24 hr at 41.5 ± 1°C, 10 µl of the sample was streaked onto both RAPID'*Salmonella* and XLD agar plates and incubated for 24 hr at 37 ± 1°C. Characteristic colonies were assessed by PCR for confirmation.

Results

A summary of the spike and stress levels obtained for each strain is shown in Table 4. All spike conditions fell inside the acceptable range of the ISO 16140-3 guidelines in terms of spike level (3–5 CFU per bag) and stress level (above 0.5 log).

For method 1, both water samples assessed with the iQ-Check *Salmonella* PCR Kit were negative for *Salmonella* spp. in the unspiked sample, and a minimum six out of seven of the spiked samples were positive for *Salmonella* spp. The ISO method produced a negative result for the unspiked sample and all spiked samples were positive, confirming PCR results. While one spiked network water sample was negative according to the iQ-Check *Salmonella* PCR Kit results, the stress level of the bacteria was particularly high (close to the limit of 3 CFU), and the test results remain in the acceptable range to pass ISO 16140-3 eLOD criteria (≥6 out of 7). Detailed PCR results are presented in Tables 5 and 6, and Figures 1 and 2.

Table 4. Inoculation and stress levels obtained for each matrix and method evaluated.

Method and Water Type	Genus and Species	Serovar	Spike Level, CFU/100 ml Water	Stress Level, log
Method 1 / Network water	*Salmonella enterica* subsp. *enterica*	Paratyphi B	3.5	0.95
Method 1 / Pond/river water	*Salmonella enterica* subsp. *enterica*	Paratyphi A	3.2	0.59
Method 2 / Pond/river water	*Salmonella enterica* subsp. *enterica*	Paratyphi B	3.4	0.79

CFU, colony forming units.

Table 5. Results of the PCR, direct inoculation, and ISO method for network water testing with method 1.

			50 ml of BPW Plus, 16 hr at 37°C		
Water Sample	Spike, CFU	Rep	iQ-Check *Salmonella* II Kit with Easy I Extraction with FDRS	10-µl Direct Streak on RAPID'*Salmonella* Plates	ISO 19250:2010 Protocol with Regrowth in RVS Broth and Streak on XLD and RAPID'*Salmonella* Plates
100 ml of network water filtered through a 0.4-µm membrane	0	Negative control	Negative	Negative	Negative
	3.5	1	Negative	Negative	Positive
	3.5	2	Positive	Negative	Positive
	3.5	3	Positive	Negative	Positive
	3.5	4	Positive	Positive	Positive
	3.5	5	Positive	Positive	Positive
	3.5	6	Positive	Positive	Positive
	3.5	7	Positive	Positive	Positive

CFU, colony forming units; BPW, Buffered Peptone Water; FDRS, iQ-Check Free DNA Removal Solution; RVS, Rappaport Vassiliadis Soya; XLD, Xylose Lysine Deoxycholate.

Table 6. Results of the PCR, direct inoculation, and ISO method for pond/river water testing with method 1.

			50 ml of BPW Plus, 16 hr at 37°C		
Water sample	Spike, CFU	Rep	iQ-Check *Salmonella* II Kit with Easy I Extraction with FDRS	10-µl Direct Streak on RAPID'*Salmonella* Plates	ISO 19250:2010 Protocol with Regrowth in RVS Broth and Streak on XLD and RAPID'*Salmonella* plates
100 ml of pond/river water filtered through a 0.4-µm membrane	0	Negative control	Negative	Negative	Negative
	3.2	1	Positive	Positive	Positive
	3.2	2	Positive	Negative	Positive
	3.2	3	Positive	Positive	Positive
	3.2	4	Positive	Positive	Positive
	3.2	5	Positive	Positive	Positive
	3.2	6	Positive	Positive	Positive
	3.2	7	Positive	Positive	Positive

CFU, colony forming units; BPW, Buffered Peptone Water; FDRS, iQ-Check Free DNA Removal Solution; RVS, Rappaport Vassiliadis Soya; XLD, Xylose Lysine Deoxycholate.

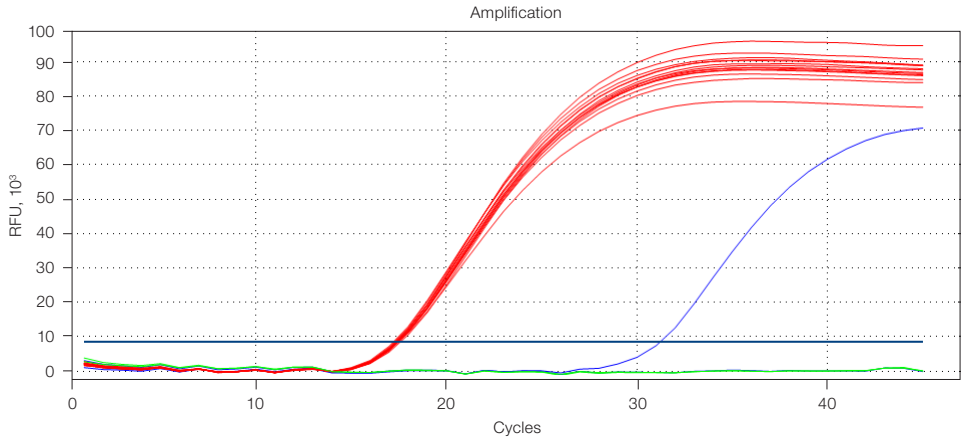


Fig. 1. PCR curves obtained for unspiked (green) and spiked (red) samples. Positive and negative controls are shown in blue. RFU, relative fluorescence units.

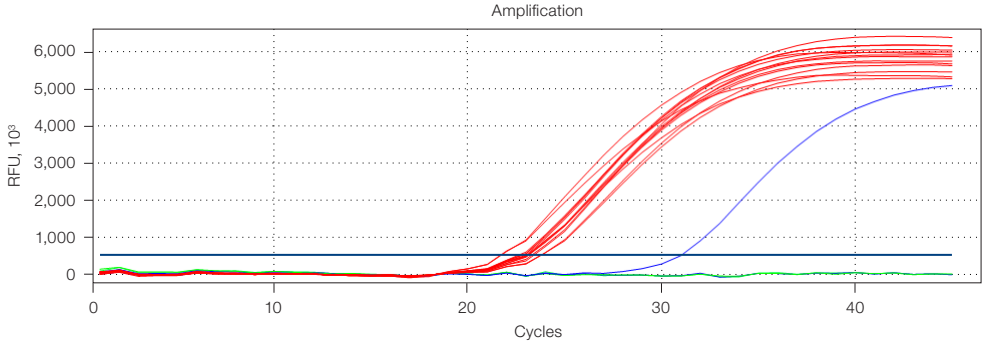


Fig. 2. PCR curves obtained for unspiked (green), spiked (red) samples. Positive and negative controls are shown in blue. RFU, relative fluorescence units.

During these tests, direct inoculation on RAPID'*Salmonella* after the first enrichment showed inferior performance with a significant difference compared to the ISO method in both water samples. While low levels of background flora were observed in the network water, pond/river water showed a high amount of interfering flora, making the reading difficult without RVS regrowth (Figure 3). According to those results, regrowth of the primary enrichment in RVS broth (similar to the ISO 19250:2010 method) is recommended for all water types.

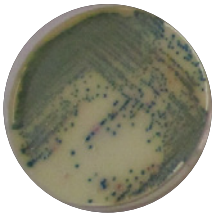
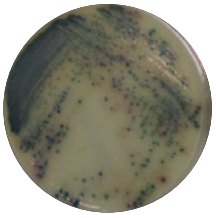
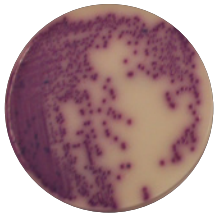
	Unspiked Sample		Spiked Sample	
Sample	Direct Streak	After RVS Broth Secondary Enrichment	Direct Streak	After RVS Broth Secondary Enrichment
Pond/river water				

Fig. 3. View of the RAPID'*Salmonella* chromogenic on unspiked sample and spiked sample with or without the regrowth in RVS broth. RVS, Rappaport Vassiliadis Soya.

For method 2, pond/river water samples evaluated with the iQ-Check *Salmonella* II Kit produced a negative result for the unspiked sample and seven positives out of seven for the spiked samples. The ISO method produced negative results for the unspiked sample and seven positives out of seven, confirming PCR results. These results are in the acceptable range to pass ISO 16140-3 eLOD criteria, as described in Table 2. Table 7 and Figure 4 detail the PCR results.

Table 7. Results of the PCR, direct inoculation, and ISO method for pond/river water testing with method 2.

			100 ml of BPW Plus, 16 hr at 37°C		
Water sample	**Spike, CFU**	**Rep**	**iQ-Check *Salmonella* II Kit with Easy I Extraction with FDRS**	**10-µl Direct Streak on RAPID'*Salmonella* Plates**	**ISO 19250:2010 Protocol with Regrowth in RVS Broth and Streak on XLD and RAPID'*Salmonella* Plates**
100 ml of pond/river water filtered through a 0.4-µm membrane	0	Negative control	Negative	Negative	Negative
	3.4	1	Positive	Negative	Positive
	3.4	2	Positive	Positive	Positive
	3.4	3	Positive	Positive	Positive
	3.4	4	Positive	Positive	Positive
	3.4	5	Positive	Positive	Positive
	3.4	6	Positive	Positive	Positive
	3.4	7	Positive	Positive	Positive

CFU, colony forming units; BPW, buffered peptone water; FDRS, iQ-Check Free DNA Removal Solution; RVS, Rappaport Vassiliadis Soya; XLD, Xylose Lysine Deoxycholate.

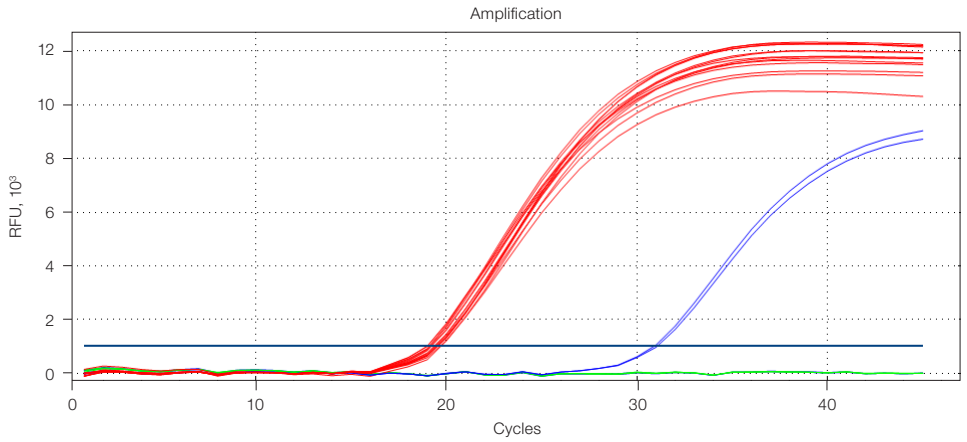


Fig. 4. PCR curves obtained for unspiked (green) and spiked (red) samples. Positive and negative controls are shown in blue. RFU, relative fluorescence units.

Conclusion

Two methods for the detection of *Salmonella* spp. in water samples were evaluated and verified according to the ISO 16140-3:2021 eLOD50 method. They allow for the use of initial screening using real-time PCR with the iQ-Check *Salmonella* II Kit and the possibility to confirm the results with a regrowth in RVS and the use of XLD and RAPID'*Salmonella* Chromogenic Media. The methods are described in Table 8.

The iQ-Check *Salmonella* II PCR Kit provides a useful tool for routine water quality monitoring and for rapid screening during outbreaks. This kit could help identify potential threats posed by *Salmonella* contamination and allow for an immediate response to a potential public health issue.

Table 8. Summary of verified methods.

Matrix	**Sample Preparation and Enrichment**	**Screening Test**	**Confirmation Test**
All filterable water types with volume >10 ml	Filtration through a 0.4-µm membrane and incubation in 50 ml BPW Plus at 37°C for 16 hr	iQ-Check *Salmonella* II PCR Kit (Easy I extraction) with or without FDRS treatment	Regrowth in RVS broth and streak on XLD and RAPID'*Salmonella* Plates
Nonfilterable sample type with volume <200 ml	Addition of same volume of BPW Plus 2x directly to the sample and incubation at 37°C for 16 hr		

BPW, Buffered Peptone Water; FDRS, iQ-Check Free DNA Removal Solution; RVS, Rappaport Vassiliadis Soya; XLD, Xylose Lysine Deoxycholate.

In laboratory settings with routine water samples and sampling volumes, confirmation of these results is recommended.

Further Reading

Kurtz JR et al. (2017). *Salmonella* infection: Interplay between the bacteria and host immune system. Immunol Lett 190, 42–50.

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International Standardization Organization (2010). ISO 19250:2010 Water quality — Detection of *Salmonella* spp.

International Standardization Organization (2021). ISO 16140-3:2021 Microbiology of the food chain.

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