Internal Comparative Evaluation Study of Alternative Enrichment Protocols for Detection of *Salmonella* in Untreated Spices

Tech Note

Introduction

Between 1973 and 2010, there were 14 outbreaks of foodborne illness linked to contaminated spices worldwide. More than 80 percent of the spices in American kitchens come from overseas. In testing shipments of imported spices offered for entry to the U.S. between 2007 and 2010, FDA found that *Salmonella* was prevalent in nearly 7 percent—a rate twice as high as all other imported food regulated by that agency[1]. Detection of *Salmonella* in spices poses several challenges. Many spices contain inhibitory compounds that may provide antibacterial activity against *Salmonella*. These compounds may limit the growth of pathogens under some conditions. These compounds can also bring inhibitory effects to some pathogen detection platforms. Typically, dilutions are used to overcome the effects of inhibitory compounds. In a report from the FDA, they indicate that methods should be developed to focus on ways to negate the effects of inhibitory compounds in spices so method sensitivity can be improved[2].

An internal study was conducted at Bio-Rad's Research and Development center in Marnes la Coquette, France to evaluate alternative enrichment protocols for the detection of *Salmonella* in spices with the iQ-Check® *Salmonella* II real-time PCR kit. The purpose of the study was to increase the recovery of *Salmonella* in artificially spiked, untreated spices while overcoming the high background flora. Alternative protocols were tested and found that adding a selective phase and a re-growing step increased the detection of *Salmonella* in spiked samples. These studies also showed the strong inhibitory characteristic of cinnamon even after a 1:100 dilution. To negate the inhibitory effect of cinnamon, BPW supplemented with an inhibitor neutralizing additive was used and compared to the traditional enrichment method.

Material and Methods

The study was conducted in two phases. Phase one tested 48 spiked samples representing 14 different types of untreated spices and focused on adding a selective supplement to BPW and incorporating a re-growth step. Phase two tested 18 untreated cinnamon samples spiked with *Salmonella* enriched with BPW supplemented with an inhibitor neutralizing additive.

Phase One:

Sample Preparation:

Forty-eight untreated spice samples were collected and aseptically weighed into 25g portions for testing and artificially contaminated with *Salmonella* Abaetetuba ATCC 35640 lyophilized pellets. The lyophilized pellets containing the inoculum culture were dissolved in 10 ml of distilled water and tested for inoculum level. Pellets used contained approximately 695 cfu/pellet and was calculated that 160–200µl was necessary to spike a sample with 10–15 cfu/bag. The *Salmonella* strain was added to the spice samples in BPW within 15 minutes after dissolving the pellet.

iQ-Check Prep, CFX and kits

Background Flora:

For each of the 48 samples, the total flora was enumerated by testing dilutions of the sample in Plate Count Agar. Plates were incubated 3 days at 30°C on Plate Count Agar. Table 1 shows the total background flora and spike levels for each sample.

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Table 1. Total flora and spike level of spice samples

Spice	Total flora (cfu/g)	Spike level (cfu/sample)	Spice	Total flora (cfu/g)	Spike level (cfu/sample)
Paprika 1	1.75×10^5	15	*Cinnamon 1*	500	10
Paprika 2	$>3\times10^5$	15	*Cinnamon 2*	500	10
Paprika 3	1.67×10^5	15	*Cinnamon 3*	<1000	9
Black pepper 1	2.48×10^6	9	*Cinnamon 4*	<100	10
Black pepper 2	7.15×10^5	15	*Cinnamon 5*	500	10
Black pepper 3	1.64×10^6	15	*Garlic flakes 1*	3×10^3	6
Basil 1	2.9×10^6	6	*Garlic flakes 2*	1.7×10^4	9
Basil 2	1.4×10^6	9	*Garlic flakes 3*	1150	9
Basil 3	3.2×10^6	10	*Garlic 1*	$>3\times10^4$	9
Marjoram 1	2.4×10^4	9	*Oregano 1*	2×10^4	9
Marjoram 2	8×10^3	10	*Oregano 2*	3.3×10^5	9
Marjoram 3	3.5×10^4	10	*Oregano 3*	1.25×10^6	9
Cumin 1	$>3\times10^4$	6	*Oregano 4*	1.5×10^4	9
Cumin 2	1.06×10^4	15	*Sage 1*	9.1×10^4	9
Cumin 3	1.45×10^3	15	*Sage 2*	$>3\times10^4$	6
Red pepper 1	2.6×10^6	6	*Sage 3*	1.1×10^5	10
Red pepper 2	1.9×10^5	9	*Sage 4*	6.9×10^4	10
Red pepper 3	2.65×10^5	15	*Sage 5*	4.5×10^3	10
Red pepper 4	5.6×10^5	15	*Thyme 1*	$>10^5$	6
Red pepper 5	1.95×10^5	15	*Thyme 2*	10^4	9
White pepper 1	7×10^4	6	*Thyme 3*	$>3\times10^5$	10
White pepper 2	1.95×10^4	15	*Parsley 1*	5×10^3	6
White pepper 3	3.7×10^4	15	*Parsley 2*	200	10
Pepper 1	5×10^3	10	*Parsley 3*	200	10

Sample Protocols:

Reference Method

Each of the 48 samples was prepared according to the dilution ratio described in the FDA BAM Chapter 5 *Salmonella*[3]. Dilutions were carried out in Buffered Peptone Water. Potassium sulfite was added to BPW for garlic flakes. After mixing, the pH values were verified with a pH-meter and adjusted to 6.7–6.8 if needed, using NaOH or HCl. Primary enrichments in BPW were incubated at 35°C for 24 hours then transferred to Rappaport Vassiliadis and Tetrathionate broths. Both broths were incubated for 24 hours at 41.5°C and subsequently streaked on *Salmonella* ChromID (bioMerieux) and RAPID'*Salmonella* Agar (RSLM, Bio-Rad, product #356-3961).

iQ-Check PCR Method

A set of 48 artificially contaminated samples was enriched according to the standard Bio-Rad enrichment protocol of BPW incubated at 35°C for 24 hours. A second set of 48 samples was enriched in an alternative protocol of BPW supplemented with the RAPID'*Salmonella* selective capsule (Bio-Rad product #356-4710 or 10× product #356-4709) and incubated at 41.5°C for 20 hours. A re-grow step was used by transferring 1 ml of this alternative enrichment to 9 ml of BPW and incubated at 37°C for 4 hours. Samples were tested on the iQ-Check *Salmonella* PCR kit and subsequently streaked to RSLM agar.

DNA Preparation and PCR

DNA extractions were performed according to the Bio-Rad iQ-Check Easy protocol. With the exception of 8 samples, the iQ-Check Prep Automated System was used. The PCR reactions were carried out in a CFX Deep Well using the iQ-Check *Salmonella* II kit (Bio-Rad product #357-8123).

Phase Two:

Sample Preparation:

Eighteen untreated cinnamon samples were prepared similarly to the Phase One testing using *Salmonella* Abaetetuba ATCC 35640. The *Salmonella* strain was added to the cinnamon samples in BPW within 15 minutes after dissolving of the pellet. Samples were enriched according to the dilution ratio described in the FDA BAM Chapter 5 *Salmonella*.

Background Flora:

For each sample, the total flora was enumerated by testing dilutions of the sample in Plate Count Agar. Plates were incubated 3 days at 30°C. Table 2 shows the total background flora and spike levels for each sample.

Table 2. Total flora and spike level of cinnamon samples

Cinnamon sample	Total flora	Spike level (cfu/sample)
1	$<10^3$	9
2	1.65×10^3	9
3	1.05×10^3	9
4	300	14
5	<100	14
6	<100	14
7	5.5×10^3	14
8	$^1.95\times10^3$	14
9	800	14
10	1×10^3	14
11	7×10^3	11
12	100	11
13	300	11
14	1.11×10^4	11
15	1.16×10^4	11
16	4.6×10^3	11
17	2×10^3	11
18	100	11

Sample Protocol:

iQ-Check PCR Method

Each of the 18 samples was enriched in BPW and BPW supplemented with an inhibitor neutralizing additive and incubated at 35°C for 24 hours. Samples were tested on the iQ-Check *Salmonella* PCR kit and subsequently streaked to RSLM agar.

DNA Preparation and PCR

For the 18 samples, DNA extractions were performed according to the Bio-Rad iQ-Check Easy protocol. The PCR reactions were carried out in a CFX Deep Well using the iQ-Check *Salmonella* II kit.

Results:

Phase One

With the exception of cinnamon and parsley, for all the spice samples tested the total flora was over 103 cfu/g (Table 1). The spike levels were between 6 and 15 cfu of S. Abaetetuba ATCC 35640 per sample. In most cases, the ratio between the targeted bacteria and the background flora was less than 1/100. Among 48 samples tested, 45 were detected by the BAM reference method (Table 3). The three negative samples were cinnamon.

There was no difference in the final results obtained with the ChromID or the RAPID'*Salmonella*. However, RAPID'*Salmonella* plates were more often scored higher meaning it was easier to pick presumptive colonies.

Table 3. Comparative results between standard enrichment protocol, new enrichment protocol and BAM

	Enrichment BPW 24 hours 35°C			Enrichment BPW + RAPID'Salmonella Capsule 20 hours 41.5°C / Re-growth BPW 4 hours 37°C			
Spice	No Dilution	1:10 Dilution	RSLM	No Dilution	1:10 Dilution	RSLM	BAM
Paprika 1	POS	POS	+	POS	POS	+	+
Paprika 2	POS	POS	+	POS	POS	+	+
Paprika 3	INH	POS	+	POS	POS	+	+
Black pepper 1*	POS	POS	+	POS	POS	+	+
Black pepper 2	POS	POS	+	POS	POS	+	+
Black pepper 3	POS	POS	+	POS	POS	+	+
Basil 1	INH	NEG	–	POS	POS	–	+
Basil 2*	INH	POS	–	POS	POS	+	+
Basil 3	INH	POS	–	POS	POS	–	+
Marjoram 1*	INH	POS	+	POS	POS	+	+
Marjoram 2	INH	POS	+	POS	POS	+	+
Marjoram 3	NEG	POS	+	POS	POS	+	+
Cumin 1	POS	POS	+	POS	POS	+	+
Cumin 2	POS	POS	+	POS	POS	+	+
Cumin 3	POS	POS	+	POS	POS	+	+
Red pepper 1	POS	POS	+	POS	POS	+	+
Red pepper 2*	POS	POS	+	POS	POS	+	+
Red pepper 3	POS	POS	+	POS	POS	+	+
Red pepper 4	POS	POS	+	POS	POS	+	+
Red pepper 5	POS	POS	+	POS	POS	+	+
White pepper 1	POS	POS	+	POS	POS	+	+
White pepper 2	POS	POS	+	POS	POS	+	+
White pepper 3	POS	POS	+	POS	POS	+	+
Pepper 1	POS	POS	+	POS	POS	+	+
Cinnamon 1	NEG	NEG	–	NEG	NEG	–	–
Cinnamon 2	POS	POS	–	NEG	NEG	–	+
Cinnamon 3*	POS	POS	+	POS	POS	+	+
Cinnamon 4	NEG	NEG	–	NEG	NEG	–	–
Cinnamon 5	NEG	NEG	–	NEG	NEG	–	–
Garlic flakes 1	POS	POS	+	POS	POS	+	+
Garlic flakes 2*	POS	POS	+	POS	POS	+	+
Garlic flakes 3	POS	POS	+	POS	POS	+	+
Garlic 1	POS	POS	+	POS	POS	+	+
Oregano 1	POS	POS	+	POS	POS	+	+
Oregano 2	POS	POS	+	POS	POS	+	+
Oregano 3	POS	POS	–	POS	POS	+	+
Oregano 4	POS	POS	+	POS	POS	+	+
Sage 1*	INH	POS	+	POS	POS	+	+
Sage 2	INH	INH	+	POS	POS	+	+
Sage 3	INH	INH	+	POS	POS	+	+
Sage 4	INH	NEG	–	POS	POS	+	+
Sage 5	INH	INH	+	POS	POS	+	+
Thyme 1	INH	POS	–	POS	POS	–	+
Thyme 2*	INH	POS	+	POS	POS	+	+
Thyme 3	INH	POS	+	POS	POS	+	+

Spice	Enrichment BPW 24 hours 35°C			Enrichment BPW + RAPID'Salmonella Capsule 20 hours 41.5°C / Re-growth BPW 4 hours 37°C			BAM
	No Dilution	1:10 Dilution	RSLM	No Dilution	1:10 Dilution	RSLM	
Parsley 1	POS	POS	+	POS	POS	+	+
Parsley 2	POS	POS	+	POS	POS	+	+
Parsley 3	POS	POS	+	POS	POS	+	+
Total positive results	30	40	38	44	44	41	45
Total negative results	4	5	10	4	4	7	3
Total inhibition	14	3	N/A	0	0	N/A	N/A
Total spices	48	48	48	48	48	48	48

*: DNA extraction performed manually

The 48 samples enriched in BPW supplemented with the RAPID'*Salmonella* Capsule with a 4-hour re-growth step were tested on the iQ-Check PCR kit undiluted and with a 1:10 dilution. Forty-four samples were positive on both undiluted and diluted PCR samples. The four samples that were negative were cinnamon and no undiluted sample showed PCR inhibition (Table 3).

Table 3 also shows the RAPID'*Salmonella* plates streaked after the 4-hour re-growth step. Forty-one sample exhibited positive growth. Of the seven samples that were scored as negative, five had no growth; the four cinnamon samples that were also negative on PCR and a Thyme sample that was positive on PCR. The other two samples had a high concentration of non-typical *Salmonella* growth.

Phase Two

All 18 untreated cinnamon samples were positive on the iQ-Check *Salmonella* II kit when enriched in BPW supplemented with an inhibitor neutralizing additive and incubated at 35°C for 24 hours (Table 4). Additionally, for all the samples tested, we observed a perfect correlation between the iQ-Check *Salmonella* II results and the cultures on RAPID'*Salmonella* agar. iQ-Check *Salmonella* II assays gave non ambiguous positive results after enrichment in BPW supplemented with neutralizing additive. Furthermore, no PCR inhibition was recorded. Table 4 also shows the results of the same 18 samples enriched in BPW only.

Table 4. iQ-Check and RSLM Results for Untreated Cinnamon Samples

Cinnamon sample	Enrichment BPW 24 hours 35°C		Enrichment BPW + inhibitor neutralizing additive 24 hours 35°C	
	RSLM	iQ-Check	RSLM	iQ-Check
1	–	NEG	+	POS
2	–	NEG	+	POS
3	–	NEG	+	POS
4	+	POS	+	POS
5	+	POS	+	POS
6	+	POS	+	POS
7	+	POS	+	POS
8	+	POS	+	POS
9	–	NEG	+	POS
10	–	NEG	+	POS
11	–	NEG	+	POS
12	–	NEG	+	POS
13	–	NEG	+	POS
14	+	POS	+	POS
15	+	POS	+	POS
16	+	POS	+	POS
17	–	NEG	+	POS
18	–	NEG	+	POS
Total positive results	8	8	18	18
Total negative results	10	10	0	0
Total samples	18	18	18	18

The improvement of the growth of *Salmonella* in Buffered Peptone Water with an inhibitor neutralizing additive is clearly illustrated with cinnamon sample 3 as seen on Image 1 and 2 below. This sample is positive after a single enrichment in BPW but only 2 colonies were detected on the RAPID'*Salmonella* agar. For the same sample, a dense culture of *Salmonella* was observed on the plate streaking from the enrichment of BPW plus the inhibitor neutralizing additive.

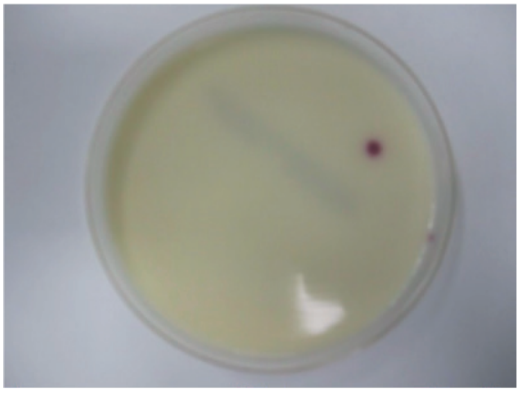

Image 1: Salmonella culture from Cinnamon Sample 3 after enrichment in BPW Only

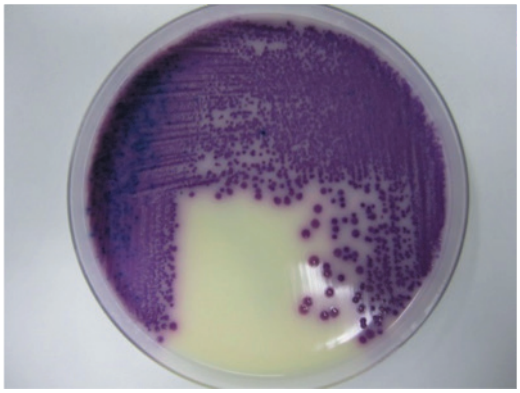

Image 2: Salmonella culture from Cinnamon Sample 3 after enrichment in BPW + inhibitor neutralizing additive

Conclusion:

Phase One

Forty-eight untreated spice samples were tested by the BAM reference method and the iQ-Check *Salmonella* II PCR method using an alternative enrichment protocol.

The results obtained with an enrichment step in BPW supplemented with the RAPID'*Salmonella* selective capsule and subsequent re-growing phase in BPW were far superior than using a traditional enrichment in BPW only. No PCR inhibition was observed using this alternative protocol. The addition of RAPID'*Salmonella* agar to the confirmation steps helped to isolate *Salmonella* by making the colonies easy to see.

Cinnamon is a strongly inhibitory spice. Negative results with the PCR test are correlated by the results of the RAPID'*Salmonella* plates on which no growth was observed. For this matrix, a specific protocol was investigated in Phase Two of this study.

Phase Two

Two enrichment protocols for the detection of *Salmonella* in untreated cinnamon were tested with 18 spiked samples. The protocol including enrichment in BPW with an inhibitor neutralizing additive was able to detect all the samples while less than half of them were positive with an enrichment in BPW that wasn't supplemented. Adding a neutralizing additive to BPW to neutralize the inhibitory effect of cinnamon and improve the growth of *Salmonella* was clearly demonstrated.

Discussion:

As the FDA continues to place an emphasis on the microbial quality of spices and mitigating risk to human health, the detection of *Salmonella* in spices is in the spotlight. The difficulty in detecting *Salmonella* in spices due to the inhibitory compounds and high background flora can be decreased with improved enrichment steps without affecting the sensitivity that comes with dilutions. By combining an improved enrichment with the sensitivity and specificity of the iQ-Check *Salmonella* II real-time PCR kit, time-to-results can be reduced as compared to cultural methods and an increased confidence in results are obtained from using a rapid pathogen detection method.

References:

1. Food and Drug Administration (FDA). 2013. Draft Risk Profile: Pathogens and Filth in Spices [Online]. Available: http://www.fda.gov/downloads/Food/FoodScienceResearch/RiskSafetyAssessment/UCM367337.pdf. Accessed December 16, 2014.

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3. Andrews, W.H., Hammack, T and Jacobson, A. (May 2014) FDA Bacteriological Analytical Manual, Chapter 5 *Salmonella* [Online]. Available: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm. Accessed December 16, 2014.

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