

VALIDATION OF BACGENE® SALMONELLA ASSAY FOR THE DETECTION OF SALMONELLA IN SPICE MATRICES

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ABSTRACT

Spices are known to have antimicrobial and PCR inhibitory components. Consequently, many PCR platforms have limited or no validations with spice matrices. In order to validate the performance of the BACGene *Salmonella* spp. real-time PCR assay, seven spice matrices were chosen based on their inhibitory and antimicrobial properties and inoculated in accordance with AOAC appendix J sensitivity studies and enriched following the FDA BAM sample preparation guidelines. These spices were inoculated with either *Salmonella enterica* ser. Typhimurium or *Salmonella enterica* ser. Abaetetuba and incubated according to the FDA Bacteriological Analytical Manual's (BAM) protocols. Following incubation, an aliquot of the primary enrichment was transferred to brain heart infusion broth (BHI) to further dilute out PCR inhibitors, and reincubated for three hours to allow for regrowth. All enrichments were also culturally confirmed using FDA BAM Chapter 5 as the reference method. For all spice matrices tested, BACGene *Salmonella* spp. PCR results showed 100% agreement with the reference method. Nine additional spices underwent matrix verification following the FDA's "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds" and demonstrated PCR detection in all inoculated replicates. The results demonstrate that BACGene *Salmonella* can reliably perform and detect *Salmonella* in spice matrices that have been enriched according to the FDA BAM primary enrichment methods with a BHI regrow step.

INTRODUCTION

Spices and seasonings are key ingredients in many segments of the food supply chain, and present unique food safety risks due to the fact that they are frequently added to ready-to-eat foods without any subsequent lethality processing steps. Spices and seasonings have been associated with *Salmonella* outbreaks¹ and led to several recalls², highlighting the importance of a robust pathogen testing program for spice and seasoning manufacturers.

These matrices present unique challenges for pathogen testing due to the antimicrobial nature of many spices³. In order to adequately grow and detect *Salmonella*, these antimicrobial compounds must either be neutralized or diluted out during pre-enrichment and prior to detection⁴. Broadly, the US Food and Drug Administration's Bacterial Analytical Manual (FDA BAM) Chapter 5 identifies five different enrichment protocols based on a spice's microbial inhibitory capacity⁵. These categories and their recommended enrichment protocols are shown below in Table 1.

In addition to inhibiting microbial growth, many spices also contain compounds that may inhibit the PCR detection assay. These compounds generally work in one (or more) of three major ways: inhibition of DNA polymerases, binding magnesium, or denaturing DNA⁶. Examples include proteinases, which can degrade *Taq* polymerase, calcium or other ions which can compete with magnesium chloride, a needed cofactor for *Taq* polymerase, or the presence of high magnesium chloride concentrations. Other compounds that may inhibit PCR include certain phenolic groups⁷, hemes⁸, polyphenolic compounds, complex polysaccharides, and fats or oils⁹. Any compounds which can drastically alter the composition of the PCR reaction chemistry can be inhibitory as well, including matrices with a very high salt concentration and/or basic or acidic matrices.

MATERIALS AND METHODS

Matrices and dilutions

Seven spices were chosen for validation from the categories listed in Table 1. For this study, black pepper, garlic powder, mustard, cinnamon, oregano, cloves, and dried basil were tested. Thirty samples of each matrix were weighed out and diluted with prewarmed enrichment media as specified in Table 1. Samples were allowed to sit for 60 minutes, then the pH was measured and adjusted if necessary to 6.8 +/- 0.2.

An additional nine spice matrices were chosen for matrix verification studies¹¹. Anise seeds, fennel seeds, red pepper, and white pepper were verified according to the non-inhibitory spice method. Cilantro leaves, ground coriander, rubbed sage, and thyme leaves were verified according to the leafy spice method. Onion powder was tested according to the onion and garlic method. Samples were allowed to sit for 60 minutes, then the pH was measured and adjusted if necessary to 6.8 +/- 0.2.

Table 1: Different enrichment requirements for various spices as defined by FDA BAM Chapter 5

SPICE GROUP	TEST SAMPLE SIZE	ENRICHMENT BROTH	SAMPLE:BROTH RATIO
Non-Inhibitory Spices: Black Pepper, White Pepper, Celery Flake, Chili Powder, Cumin, Parsley, Sesame, Rosemary, Thyme	25g	Tryptic Soy Broth	1:9
Onion and Garlic	25g	Tryptic Soy Broth +0.5% K ₂ SO ₃	1:9
Moderately Inhibitory Spices: Allspice, Cinnamon, Oregano	37.5g	Tryptic Soy Broth	1:99
Moderately Inhibitory Spices requiring neutralization: Mustard	37.5g	Tryptic Soy Broth +0.5% K ₂ SO ₃	1:99
Significantly Inhibitory Spices: Cloves	3.75g	Tryptic Soy Broth	1:999
Leafy Spices: Basil	25g	Tryptic Soy Broth	1:19

Bacterial inoculation

Samples were inoculated with *Salmonella* following pH adjustment. Black pepper, oregano, cinnamon, mustard and dried basil were inoculated with *Salmonella enterica* ser. Typhimurium, and garlic powder and cloves were inoculated with *Salmonella enterica* ser. Abaetetuba. Organisms were purchased as lyophilized BioBalls (Biomérieux) and rehydrated according to manufacturer's instructions. For each matrix, inoculation levels and number of replicates were chosen based on AOAC Official Methods of Analysis Appendix J matrix studies protocol¹⁰. Five samples were spiked at a high level (8-12 cfu per sample), twenty samples were spiked at a fractional level (0.8-1.2 cfu per sample), defined as a level where approximately half of the samples would be statistically expected to receive a very low inoculum (1-2 cfu), while the remaining samples would be expected to receive no organism. Additionally, five samples were left uninoculated as negative controls. All enrichments were incubated at 37°C for 22-24 hours.

For the spices that underwent verification, seven replicates of each spice were inoculated as described above with less than 30 cfu of either *Salmonella enterica* ser. Typhimurium or *Salmonella enterica* ser. Abaetetuba. All enrichments were incubated at 37°C for 22-24 hours.

BACGene PCR

Enriched samples were pulled from incubation, and 10µL of each enrichment was transferred to 500µL of BHI broth. These samples were reincubated for an additional 3 hours at 37°C. Cell lysates were prepared as described in the BACGene *Salmonella* spp. user guide and tested by PCR using BACGene *Salmonella* spp. reagents (Gold Standard Diagnostics). Results were analyzed by FastFinder software (Ugentec) and recorded as presumptive or negative.

Cultural detection

Primary enrichments were all tested according to FDA BAM Chapter 5 for the presence of *Salmonella*. Briefly, 0.1mL of the enrichment was transferred to 10mL Rappaport-Vassiliadis (RV) broth and incubated at 42°C for 24 hours. In parallel, 1mL of the enrichment was transferred to 10mL of tetrathionate (TT) broth and incubated at 35°C for 24 hours. Following incubation, both the RV and TT secondary enrichments were subcultured onto Hektoen Enteric (HE) agar, Xylose Lysine Desoxycholate (XLD) agar, and RAPID' *Salmonella* agar (Bio-Rad). All plates were incubated at 35°C for 24 hours. Typical *Salmonella* colonies were isolated and confirmed. Following confirmations, results were recorded as detected or not detected.

RESULTS

For all spices that were validated in this study, all of the uninoculated controls had no *Salmonella* detected by either BACGene *Salmonella* spp. PCR or by cultural methods. Conversely, all samples spiked with 8-12 cfu of *Salmonella* were detected by both BACGene *Salmonella* spp. PCR and cultural methods. Samples spiked with 0.8-1.2 cfu of *Salmonella* yielded fractionally-positive results for each of the matrices tested, and in these samples, there was 100% agreement between PCR and culture (Table 2).

Table 2: Summary of results

SPICE	SAMPLE SIZE	ENRICHMENT MEDIUM (VOLUME)	HIGH INOCULATED PCR DETECTED	FRACTIONAL INOCULATED PCR DETECTED	UNINOCULATED PCR DETECTED	AGREEMENT % (PCR & CULTURE)
Basil	25g	TSB (475 mL)	5/5	13/20	0/5	100%
Black Pepper	25g	TSB (225 mL)	5/5	11/20	0/5	100%
Cinnamon	37.5g	TSB (3,376 mL)	5/5	10/20	0/5	100%
Clove	3.75g	TSB (3,370 mL)	5/5	12/20	0/5	100%
Garlic	25g	TSB+K ₂ SO ₃ (225 mL)	5/5	10/20	0/5	100%
Mustard	37.5g	TSB+K ₂ SO ₃ (3,376 mL)	5/5	11/20	0/5	100%
Oregano	37.5g	TSB (3,376 mL)	5/5	12/20	0/5	100%

For the nine spice matrices that underwent matrix verifications, all 7 inoculated samples produced presumptive positive results by BACGene *Salmonella* spp. PCR, demonstrating that the method worked on all of these matrices with no indication of growth inhibition or PCR inhibition.

Table 3: Summary of verification results

SPICE	SAMPLE SIZE	ENRICHMENT MEDIUM (VOLUME)	INOCULATED PCR DETECTED
Anise Seeds	25g	TSB (225 mL)	7/7
Red Pepper	25g	TSB (225 mL)	7/7
Fennel Seeds	25g	TSB (225 mL)	7/7
White Pepper	25g	TSB (225 mL)	7/7
Cilantro Leaves	25g	TSB (475 mL)	7/7
Ground Coriander	25g	TSB (475 mL)	7/7
Rubbed Sage	25g	TSB (475 mL)	7/7
Thyme Leaves	25g	TSB (475 mL)	7/7
Onion Powder	25g	TSB+K ₂ SO ₃ (225 mL)	7/7

CONCLUSIONS AND SIGNIFICANCE

This validation and verification data demonstrates the effectiveness of BACGene *Salmonella* spp. across a wide variety of spice matrices. Use of the FDA BAM suggested primary enrichment protocols, combined with a three hour regrow in BHI broth, allowed equivalent *Salmonella* detection compared to cultural methods, with no inhibition on the PCR assay. Importantly, in previous work it was observed that some—but not all—of these matrices were capable of inhibiting the PCR reaction in the absence of a BHI regrow (data not shown). By including the BHI regrow step in all spice matrices, the risk of PCR inhibition was completely eliminated, and this allows for a uniform protocol to be adopted for all spices and seasoning blends.

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