Evaluation of an Alternative Chromogenic Agar Plating Method for Detection and Confirmation of *Salmonella* in Cannabis Flower, Cannabis-Infused Gummies, Cannabis-Infused Chocolate, and Cannabis-Derived Concentrates

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Abstract

The iQ-Check™ *Salmonella* II PCR Detection Kit is commonly used to screen for *Salmonella* in various matrices and has been validated by AOAC International for a broad range of foods. However, it is also possible to screen for contamination on agar plates. Here we evaluated an alternative screening and confirmation method for cannabis flower (10 g), cannabis-infused gummies (25 g), cannabis-infused chocolate (25 g), and cannabis-derived concentrates (5 g) that consisted of a direct streak of primary and secondary enrichment broths onto RAPID'*Salmonella* Agar and compared the results to Standard Method Performance Requirements (SMPR) 2020.002 (AOAC 2020). *Salmonella* typically form magenta colonies on RAPID'*Salmonella* Agar (Figure 1), allowing easy differentiation from competing organisms. Statistical analysis demonstrated no difference between the alternative and SMPR methods.

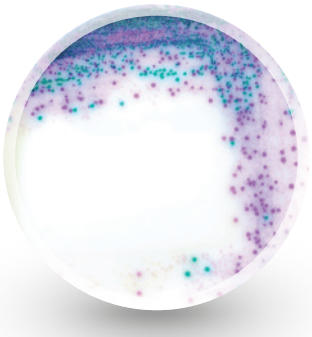

Fig. 1. Visual differences between *Salmonella* and competing organisms. *Salmonella* typically form magenta colonies on RAPID'*Salmonella* Agar. Competing organisms form blue or white colonies or are completely inhibited.

Introduction

Salmonella has been recognized as a primary cause of foodborne illness worldwide. This genus of bacteria is classified into two species: *S. enterica* and *S. bongori*, the former containing the majority of serotypes associated with human disease. *Salmonella* can contaminate a wide range of foods, including poultry, meat, eggs, dairy, fruit, and vegetables, as well as pet food. With the growing legalization of cannabis in the United States, many states have enacted requirements for testing cannabis products for the presence of *Salmonella*. In most cases, particularly in the U.S., *Salmonella* infection is characterized by acute gastroenteritis. Symptoms include diarrhea, fever, abdominal cramps, and vomiting lasting 4 to 7 days in most people.

Methods

The inoculation procedure was followed as outlined in Appendix J of AOAC's Microbiology Validation Guidelines (Latimer 2023). Prior to inoculation, a total aerobic plate count (APC) was performed for each matrix following procedures outlined in the Bacteriological Analytical Manual, Chapter 3: Aerobic Plate Count (U.S. Food and Drug Administration 2021). Matrices were prescreened for natural contamination of *Salmonella* species following culture procedures outlined in SMPR 2020.002. No natural contamination was found in any of the matrices, so they were artificially contaminated, targeting three levels, measured in colony forming units (CFU) per test portion: noninoculated (0), low (0.2–5), and high (5–10).

For cannabis flower, lyophilized *Salmonella* Typhimurium American Type Culture Collection (ATCC) 14028 pellets were crushed and diluted with nonfat dry milk that previously screened negative for *Salmonella* species. A bulk lot of cannabis flower was artificially inoculated with a low level expected to yield fractional positive results (5–15 positive results) and a high level expected to yield all positive results. For all other matrices, a single colony of a *Salmonella* strain was transferred to Buffered Peptone Water Standard, dehydrated (Bio-Rad Laboratories, Inc., catalog #12013259) and incubated

overnight at 37°C. Cannabis-infused gummies were artificially inoculated with *Salmonella* Newport ATCC 6962, cannabis-infused chocolate with *Salmonella* Typhimurium ATCC 14028, and cannabis-derived concentrates with *Salmonella* Heidelberg ATCC 8326. The freshly grown, nonstressed culture was diluted to an appropriate level to achieve fractional positive results for the low level and all positive results for the high level. Matrices were held at ambient temperature (20–25°C) for 2 weeks prior to analysis. A most probable number (MPN) analysis was performed on the day of testing to determine inoculation levels.

Each 10 g test portion of cannabis flower was added to 90 ml Buffered Peptone Water (BPW), homogenized, and incubated for 18–22 hr at 37 ± 1°C. Each 25 g test portion of cannabis-infused gummies and cannabis-infused chocolates was added to 225 ml BPW (prewarmed, 37°C), homogenized, and incubated for 18–22 hr at 37 ± 1°C. Each 5 g test portion of cannabis-derived concentrate was added to 45 ml BPW (prewarmed, 37°C), homogenized, and incubated for 18–22 hr at 37 ± 1°C. Samples were streaked to RAPID'*Salmonella* Agar (Bio-Rad, #3563963) and incubated for 24 ± 2 hr at 37 ± 1°C.

In addition, samples were subcultured to Rappaport-Vassiliadis (RV) and tetrathionate (TT) broths for confirmation according to SMPR 2020.002. Each tube was streaked to xylose lysine desoxycholate (XLD) and RAPID'*Salmonella* Agar plates. Typical magenta colonies on RAPID'*Salmonella* Agar were identified as *Salmonella* and were easily selected for further confirmation.

Typical growth on either XLD or RAPID'*Salmonella* Agar was transferred to triple sugar iron (TSI) and lysine iron agar (LIA) slants. Test portions with typical results for *Salmonella* were streaked to tryptic soy agar (TSA) and confirmed using polyvalent O and polyvalent H serology tests. Final identification was performed using Analytical Profile Index (API) 20E. Figure 2 describes the study design.

Probability of detection (POD) statistical analysis was performed using the AOAC Binary Workbook with Unpaired Reference, v5-2 (AOAC 2013; Wehling et al. 2011). MPN analysis values were obtained using the Least Cost Formulations MPN Calculator (Least Cost Formulations Ltd 2016).

Results

POD analysis results are presented in Table 1.

Cannabis Flower

The APC result for the cannabis flower was 1.9 x 10^4 CFU/g. MPN analysis resulted in 0.59 CFU/test portion for the low inoculation level and 3.26 CFU/test portion for the high inoculation level. Of the five replicates analyzed at the noninoculated level, zero presumptive positive results were observed. For the 20 samples observed at the low inoculation level, seven presumptive positive results were observed. Of the five replicates analyzed at the high inoculation level, five presumptive positive results were observed. No discrepancies were seen between the presumptive and confirmed results, with confirmation results for all samples matching the presumptive results. All RAPID'*Salmonella* plate confirmation results matched SMPR 2020.002 confirmation results.

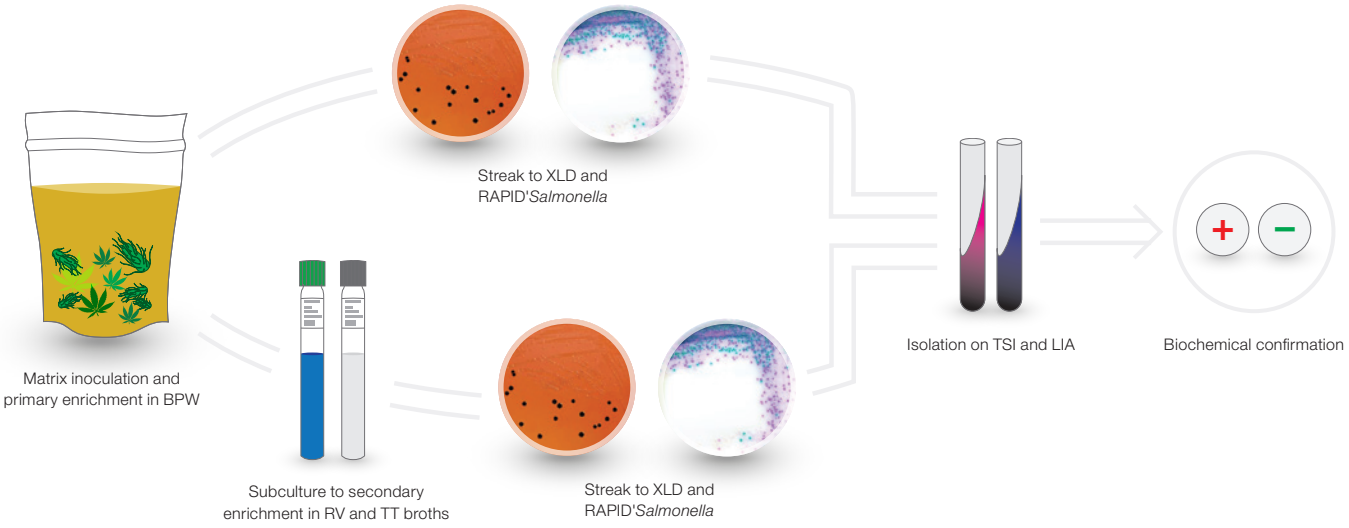

Fig. 2. Study design. The workflow consisted first of inoculating and enriching the sample in BPW. Subsequent streaking on plates and subculturing of the sample, then isolation on TSI and LIA, led to confirmation of *Salmonella*. BPW, buffered peptone water; LIA, lysine iron agar; RV, Rappaport-Vassiliadis; TSI, triple sugar iron; TT, tetrathionate; XLD, xylose lysine desoxycholate.

Table 1. POD results. RAPID'*Salmonella* presumptive vs. confirmed results (paired) – POD results.

			Presumptive			Confirmed				
Matrix and Inoculation	MPN/Test Portion	N	x	POD_{cp}	95% CI	x	POD_{cc}	95% CI	$dPOD_{cp}$	95% CI*
Cannabis flower, 10 g (*Salmonella* Typhimurium ATCC 14028)	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(–0.47, 0.47)
	0.59 (0.31, 1.01)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	(–0.13, 0.13)
	3.26 (1.33, 7.99)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(–0.47, 0.47)
Cannabis infused chocolate, 25 g (*Salmonella* Typhimurium ATCC 14028)	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(–0.47, 0.47)
	0.65 (0.32, 1.15)	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0.00	(–0.13, 0.13)
	4.07 (2.15, 234)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(–0.47, 0.47)
Cannabis infused gummies, 25 g (*Salmonella* Newport ATCC 6962)	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(–0.47, 0.47)
	0.83 (0.44, 1.44)	20	10	0.50	0.30, 0.70	10	0.50	0.30, 0.70	0.00	(–0.13, 0.13)
	4.65 (3.37, 234)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(–0.47, 0.47)
Cannabis derived concentrate, 5 g (*Salmonella* Heidelberg ATCC 8326)	NA	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	(–0.47, 0.47)
	0.80 (0.42, 1.35)	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0.00	(–0.13, 0.13)
	6.74 (3.18, 187)	5	5	1.00	0.57, 1.00	5	1.00	0.57, 1.00	0.00	(–0.47, 0.47)

* If the confidence interval of a dPOD does not contain zero, then 95% CI is the statistically significant difference at the 5% level.
$dPOD_{CP}$, difference between the candidate method–presumptive result and candidate method–confirmed result POD values; MPN, most probable number based on the POD of culture confirmation of test portions using the Least Cost Formulations MPN Calculator, with 95% confidence interval; N, number of test portions; NA, not applicable; POD, probability of detection; POD_{CC}, candidate method–confirmed positive outcomes divided by the total number of trials; POD_{CP}, candidate method–presumptive positive outcomes divided by the total number of trials; x, number of positive test portions; 95% CI, 95% confidence interval.

At the low inoculation level, the study data indicate with 95% confidence that the true difference between the RAPID'*Salmonella* method presumptive and confirmed results could be as small as –0.13 or as large as 0.13, with a best estimate of 0.00. At the high inoculation level, the true difference could be as small as –0.47 or as large as 0.47, with a best estimate of 0.00.

Cannabis-Infused Chocolate

The APC result for the cannabis-infused chocolate was 60 CFU/g. MPN analysis resulted in 0.65 CFU/test portion for the low inoculation level and 4.07 CFU/test portion for the high inoculation level. Of the five replicates analyzed at the noninoculated level, zero presumptive positive results were observed. For the 20 samples observed at the low inoculation level, eight presumptive positive results were observed. Of the five replicates analyzed at the high inoculation level, five presumptive positive results were observed. No discrepancies were seen between the presumptive and confirmed results, with confirmation results for all samples matching the presumptive results. All RAPID'*Salmonella* plate confirmation results matched SMPR 2020.002 confirmation results.

At the low inoculation level, the study data indicate with 95% confidence that the true difference between the RAPID'*Salmonella* method presumptive and confirmed results could be as small as –0.13 or as large as 0.13, with a best estimate of 0.00. At the high inoculation level, the true difference could be as small as –0.47 or as large as 0.47, with a best estimate of 0.00.

Cannabis-Infused Gummies

The APC result for the cannabis-infused gummies was <1 CFU/g. MPN analysis resulted in 0.83 CFU/test portion for the low inoculation level and 4.65 CFU/test portion for the high inoculation level. Of the five replicates analyzed at the noninoculated level, zero presumptive positive results were observed. For the 20 samples observed at the low inoculation level, ten presumptive positive results were observed. Of the five replicates analyzed at the high inoculation level, five presumptive positive results were observed. No discrepancies were seen between the presumptive and confirmed results, with confirmation results for all samples matching the presumptive results. All RAPID'*Salmonella* plate confirmation results matched SMPR 2020.002 confirmation results.

At the low inoculation level, the study data indicate with 95% confidence that the true difference between the RAPID'*Salmonella* method presumptive and confirmed results could be as small as –0.13 or as large as 0.13, with a best estimate of 0.00. At the high inoculation level, the true difference could be as small as –0.47 or as large as 0.47, with a best estimate of 0.00.

Cannabis-Derived Concentrate

The APC result for the cannabis-derived concentrate was <1 CFU/g. MPN analysis resulted in 0.80 CFU/test portion for the low inoculation level and 6.74 CFU/test portion for the high inoculation level. Of the five replicates analyzed at the noninoculated level, zero presumptive positive results were observed. For the 20 samples observed at the low inoculation level, nine presumptive positive results were observed. Of the five replicates analyzed at the high inoculation level, five presumptive positive results were observed. No discrepancies were seen between the RAPID'*Salmonella* presumptive and confirmed results, with confirmation results for all samples matching the presumptive results. All RAPID'*Salmonella* plate confirmation results matched SMPR 2020.002 confirmation results.

At the low inoculation level, the study data indicate with 95% confidence that the true difference between the RAPID'*Salmonella* method presumptive and confirmed results could be as small as –0.13 or as large as 0.13, with a best estimate of 0.00. At the high inoculation level, the true difference could be as small as –0.47 or as large as 0.47, with a best estimate of 0.00.

Discussion

A chromogenic media was evaluated as part of the iQ-Check *Salmonella* II AOAC validation extension for various cannabis matrices. The RAPID'*Salmonella* method successfully detected target *Salmonella* species in cannabis flower (10 g), cannabis-infused gummies (25 g), cannabis-infused chocolate (25 g), and cannabis-derived concentrate (5 g). POD analysis proved that the study data did not demonstrate a statistically detectable difference from zero between the RAPID'*Salmonella* method–presumptive and reference method–confirmed results. The use of chromogenic media has the advantage of simplifying the selection of typical colonies for detection and/or confirmation of target organisms. This increases the overall ease of use of the method.

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