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Evaluation of Roka Atlas *Salmonella* method for the detection of *Salmonella* in egg products in comparison with culture method, real-time PCR and isothermal amplification assays☆

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ABSTRACT

With the increasing focus on the food safety, rapid methods for the detection of *Salmonella* are crucial for both food industry and regulatory agencies. Recently, many molecular methodologies with diverse technologies have been introduced. Roka Atlas® *Salmonella* Assay (SEN) is a molecular method that uses ribosomal RNA as target for detection, which is theoretically more sensitive than PCR or isothermal amplification methods that target the DNA sequences of single genes. In this study, SEN assay was compared with four PCR- and isothermal amplification-based assays and a culture method, such as the MicroSEQ® *Salmonella* spp. Detection kit (MicroSEQ), 3M™ Molecular Detection Assay (MDA) *Salmonella*, ANSR™ *Salmonella* Assay (ANSR), and Pro-AmpRT™ SALM spp. Kit (Pro-AmpRT). Food samples were prepared and analyzed according to the current U. S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) *Salmonella* culture method. A total of 155 bacterial isolates (121 for *Salmonella* inclusivity and 34 for *Salmeonlla* exclusivity) and 200 egg product samples inoculated at a level of 1–5 CFU/25 g were analyzed. The study also estimated the limit of detection of these molecular methods, and illustrated their advantages and disadvantages. For exclusivity, all 34 non-*Salmonella* isolates were negative by all 5 molecular methods studied. For inclusivity, all 121 *Salmonella* isolates were positive by MDA, ANSR, and Pro-AmpRT methods. However, the SEN and MicroSEQ results were negative for 9 samples inoculated with *Salmonella bongori*. The detection limit of the 5 molecular methods ranged from 1.76 to 3.76 log CFU/mL pre-enrichment culture, with the SEN assay being the most sensitive (1.76 – 2.64 log CFU/mL). The results indicated that the SEN assay was as effective and sensitive in detecting *Salmonella enterica* in egg products as was the FDA BAM culture method and the 4 other isothermal amplification and PCR methods evaluated in the study.

1. Introduction

Salmonella causes more than one million human illnesses annually in the United States, which results in a huge economic cost (CDC, 2014; Food Safety News, 2018a). *Salmonella* outbreaks are usually associated with the consumption of contaminated eggs, poultry, meat, milk, fruits, and vegetables; additionally, some pet animals (e.g. turtle, dog, cats) can be vectors for *Salmonella* (WHO, 2018). Over 2500 *Salmonella* serotypes have been identified: among them, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most frequently isolated serotypes causing human illness worldwide (Lee, Runyon, Herrman, Phillips, & Hsieh, 2015).

Eggs and egg products are considered one of the major sources of salmonellosis, partly due to the widespread consumption throughout the world (EFSA & ECDC, 2014; Foley et al., 2011; Howard, O'Bryan, Crandall, & Ricke, 2012). European Food Safety Authority (EFSA) reported a *Salmonella* outbreak associated with polish eggs from 2016 to 2017. The outbreak had 536 confirmed illnesses, in 16 European Union

☆ Mention of trade names or commercial products in the paper is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the U. S. Food and Drug Administration.

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(EU)/European Economic Area (EEA) countries. And 196 of the 536 cases were identified with *Salmonella* Enteritidis by whole genome sequence (WGS) (ESFA, 2017). In 2016, CDC reported that a multistate (Missouri, Illinois and Kansas) outbreak of *Salmonella* Oranienburg associated with shell eggs caused 8 human illnesses (CDC, 2016). In January 2018, *Salmonella* in homemade mayo infected 174 individuals, and 25 of them were hospitalized in Chile; and the investigation of this outbreak is still undergoing (Food Safety News, 2018b).

With increasing focus on food safety, easy, rapid and accurate methods for the detection of *Salmonella* in foods are highly desired by both industry and regulatory agencies. Recently, many molecular methods and commercial kits with diverse technologies for the detection of *Salmonella* in foods have been introduced. Since real-time PCR and other PCR-based technologies provide much faster and easier operation, than culture methods with equivalent accuracy, similar technologies, such as isothermal nucleic acid amplification based methods have been extensively explored in pathogen detection. These methods include loop-mediated isothermal amplification (LAMP), transcription mediated amplification (TMA), signal mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), and helicase-dependent amplification (HDA), etc. (Gill & Ghaemi, 2008; Kuchta et al., 2014; Mori & Notomi, 2009).

While most of these PCR and isothermal amplification assays for *Salmonella* detection use DNA as the template, Atlas® *Salmonella* Assay (SEN), on the other hand, was developed based on the combination of target capture of ribosomal RNA (rRNA), TMA, and Hybridization Protection Assay (HPA) technology by Roka Bioscience Inc. (Roka Bioscience, Inc., Warren, NJ). Due to the abundance of rRNA genes that are present in all bacteria, and the low mutation rate and the unique hyper-variable regions for each bacterium, detection of rRNA enables a 10^2–10^3-fold higher target levels with less enrichment time as compared to DNA, (Hogan, 2004; Kohne, 1998; Livezey, Kaplan, Wisniewski, & Becker, 2013). It's reported that the SEN method was 40 times more sensitive than similar DNA based methods with a detection limit of 250 CFU/*Salmonella* per ml (Livezey et al., 2013).

The goal of this study was to evaluate the effectiveness of the SEN assay for detecting *Salmonella* in egg products as compared to the U. S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) culture method, as well as with four DNA-based molecular methods: MicroSEQ® *Salmonella* spp. Detection kit (MicroSEQ), 3M™ Molecular Detection Assay (MDA) *Salmonella*, ANSR™ *Salmonella* Assay (ANSR), and Pro-AmpRT™ SALM spp. kit (Pro-AmpRT). Here, MicroSEQ is a ABI real-time PCR method, ANSR is an isothermal amplification assay, both MDA and Pro-AmpRT are based on the LAMP technology.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

For inclusivity and exclusivity testing, a total of 155 isolates from the collections of the Center for Food Safety and Applied Nutrition (CFSAN), FDA, were used in the study. Details of these isolates were presented in Tables 1 and 2. All isolates were cultured overnight at 35 ± 2 °C in trypticase soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ) and diluted to 4 or 5 log CFU/ml for inclusivity and exclusivity tests.

2.2. Sample preparation

A total of 20 varieties of egg products were used in this study, including 18 egg products ordered online (liquid egg yolk, liquid egg white, eggnog, egg custard, egg custard soft cake, egg rolls cookies, macaroni and cheese, protein plus elbow pasta, extra wide egg noodles, Amish extra wide egg noodles, extra broad egg noodles, egg matzos, whole powdered eggs, egg white powder, egg yolk powder, scrambled eggs with bacon, egg drop Asian soup mix, and egg crystals; http://www.amazon.com/) and 2 egg products purchased at local grocery store (deviled egg halves and egg salad, Maryland). Details of 200 samples analyzed in the study were reported in our previous publication (Hu et al., 2016). In brief, the egg products were inoculated at 1–5 colony-forming units (CFU)/25 g. The inoculated wet egg products were stored at 4 °C for 2–3 days before microbiological analysis, while inoculated dry egg products were stored at room temperature for 2 weeks before analytical assay. Half of the samples were pre-enriched with lactose broth (LB); and the other half were pre-enriched using buffered peptone water (BPW). The rest of the procedure followed FDA BAM, Chapter 5: *Salmonella* (Food and Drug Administration, 2018).

2.3. DNA extraction

InstaGene Matrix kit (Bio-Rad, Hercules, CA) was used for DNA extraction following the manufacturer's protocol. Concisely, an aliquot of 50 μl pre-enriched sample (or 1–3 colonies) was added to a tube containing 200 μl InstaGene matrix, and incubated at 56 °C for 25 min, then 100 °C for 8 min. The tube was centrifuged at 12,000 rpm for 3 min; the sediment was discarded and the supernatant was stored at − 20 °C before use.

2.4. Roka Atlas® Salmonella Detection Assay

Atlas® *Salmonella* Detection Assay (SEN) was carried out using the Roka Bioscience Atlas system (Roka Bioscience, Inc., Warren, NJ) according to the manufacturer's instructions. Briefly, 400 μl of pre-enrichment samples were added into a Roka G1 Modified Sample Transfer Tube (Roka Bioscience). The tubes were placed into the Atlas instrument as directed to run a fully automated protocol. Each assay reagent kit was validated with a set of Atlas *Salmonella* SEN Calibrators provided by the manufacturer. The Atlas instrument generated the first sample results after 3 h and the subsequent sample results at every 5 min thereafter.

2.5. MicroSEQ™ Salmonella spp. Detection kit

When using the MicroSEQ™ *Salmonella* spp. Detection Kit (Life Technologies, Carlsbad, CA), a mixture consisted of 5 μl DNA template and 25 μl distilled water (Invitrogen, Grand Island, NY) was added to each lyophilized *Salmonella* assay bead. The tubes with sample DNA template and *Salmonella* assay beads were loaded and run under the RapidFinder™ Express Software (Applied Biosystems, Inc., Foster City, CA) on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) following the manufacturer's instructions (https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/rapidfinder-express-software.html).

2.6. 3M™ Molecular Detection Assay Salmonella

The 3M™ Molecular Detection Assay *Salmonella* (3M Food Safety, St. Paul, MN) was performed on the 3M™ Molecular Detection System (MDS, 3M Food Safety). Pre-enriched sample (20 μl) was transferred into a lysis tube and mixed well. The lysis tubes were incubated at 100 ± 1 °C for 15min, and then placed on a chill block for 10min and set on the bench (room temperature) for 5min. Afterwards, 20 μl of lysed sample was transferred to a reagent tube and loaded into a 3M Molecular Detection Speed Loader Tray (3M Food Safety). Matrix control, reagent control, and negative control were added following the manufacturer's instrument. Results were recorded after a 75min run.

2.7. ANSR™ Salmonella Assay

The ANSR™ *Salmonella* Assay (Neogen, Lansing, MI) was performed

Table 1
Salmonella inclusivity and test results.

Isolate ID	Serotype/Sero-formula	SEN[a]	MicroSEQ[b]	MDA[c]	ANSR[d]	Pro-AmpRT[e]
4000 H	Agona	+	+	+	+	+
1988 H	Alachua	+	+	+	+	+
CNM-3685/03	Brandenburg	+	+	+	+	+
3029 H	Brisbane	+	+	+	+	+
1158 H	Cerro	+	+	+	+	+
1061 H	Cubana	+	+	+	+	+
02–0062	Enteritidis	+	+	+	+	+
13–2	Enteritidis	+	+	+	+	+
17905	Enteritidis	+	+	+	+	+
18579	Enteritidis	+	+	+	+	+
18580	Enteritidis	+	+	+	+	+
22689	Enteritidis	+	+	+	+	+
SE10	Enteritidis	+	+	+	+	+
SE12	Enteritidis	+	+	+	+	+
SE22	Enteritidis	+	+	+	+	+
SE26	Enteritidis	+	+	+	+	+
CDC_07ST000857	Enteritidis	+	+	+	+	+
CDC_08-0253	Enteritidis	+	+	+	+	+
CDC_08-0254	Enteritidis	+	+	+	+	+
CDC_2010K_1441	Enteritidis	+	+	+	+	+
CDC_2010K_1543	Enteritidis	+	+	+	+	+
1941 H	Fresno	+	+	+	+	+
768 H	Gera	+	+	+	+	+
1108447–08	Give	+	+	+	+	+
CNM-3578/03	Hadar	+	+	+	+	+
02–0105	Heidelberg	+	+	+	+	+
579082–8	Heidelberg	+	+	+	+	+
607310–1	Heidelberg	+	+	+	+	+
607309–34	Heidelberg	+	+	+	+	+
3170 H	Inverness	+	+	+	+	+
2080 H	Javiana	+	+	+	+	+
1070 H	Johannesburg	+	+	+	+	+
2069 H	Michigan	+	+	+	+	+
1 H	Montevideo	+	+	+	+	+
1501 H	Muenchen	+	+	+	+	+
1250 H	Muenster	+	+	+	+	+
02–0061	Newport	+	+	+	+	+
CM 01	Poona	+	+	+	+	+
1465 H	Rubislaw	+	+	+	+	+
CM 02	Saintpaul	+	+	+	+	+
2105 H	Saphra	+	+	+	+	+
1097 H	Senftenberg	+	+	+	+	+
94–0708	*S. bongori*	−	−	+	+	+
95–0123	*S. bongori*	−	−	+	+	+
96–0233	*S. bongori*	−	−	+	+	+
CNM-256	*S. bongori*	−	−	+	+	+
CNM-262	*S. bongori*	−	−	+	+	+
95–0321	*S. bongori*	−	−	+	+	+
ATCC 43975	*S. bongori*	−	−	+	+	+
SarC (11) RKS 3041	*S. bongori*	−	−	+	+	+
SarC (12) RKS 3044	*S. bongori*	−	−	+	+	+
3030 H	Tornow	+	+	+	+	+
02–0115	Typhimurium	+	+	+	+	+
K0507	Typhimurium	+	+	+	+	+
H8289	Typhimurium	+	+	+	+	+
H8290	Typhimurium	+	+	+	+	+
H8292	Typhimurium	+	+	+	+	+
H8293	Typhimurium	+	+	+	+	+
H8294	Typhimurium	+	+	+	+	+
2009K0191	Typhimurium	+	+	+	+	+
2009K0208	Typhimurium	+	+	+	+	+
2009K0224	Typhimurium	+	+	+	+	+
2009K0226	Typhimurium	+	+	+	+	+
2009K0230	Typhimurium	+	+	+	+	+
2009K0234	Typhimurium	+	+	+	+	+
2009K0350	Typhimurium	+	+	+	+	+
AM01797	Typhimurium/DT104	+	+	+	+	+
AM03380	Typhimurium/DT104	+	+	+	+	+
AM03759	Typhimurium/DT104	+	+	+	+	+
AM04695	Typhimurium/DT104b	+	+	+	+	+
2433	Typhi	+	+	+	+	+
2308 H	Urbana	+	+	+	+	+
885 H	Vietnam	+	+	+	+	+
CNM-3663/03	Virchow	+	+	+	+	+

(*continued on next page*)

Table 1 (*continued*)

Isolate ID	Serotype/Sero-formula	SEN[a]	MicroSEQ[b]	MDA[c]	ANSR[d]	Pro-AmpRT[e]
CNM-1029/02	4,5,12:b:	+	+	+	+	+
00–0163	II 58:l,z13,z28:z6	+	+	+	+	+
00–0324	II 47:d:z39	+	+	+	+	+
01–0227	II 48:d:z6	+	+	+	+	+
01–0249	II 50:b:z6	+	+	+	+	+
CNM-169	II 53:lz28:z39	+	+	+	+	+
CNM-176	II 39:lz28:enx	+	+	+	+	+
CNM-4290/02	II 13,22:z29:enx	+	+	+	+	+
CNM-466/03	II 4,12:b:	+	+	+	+	+
CNM-5936/02	II 18:z4,z23:	+	+	+	+	+
01–0089	IIIa 41:z4,z23:	+	+	+	+	+
01–0204	IIIa 40:z4,z23:	+	+	+	+	+
01–0324	IIIa 48:g,z51:	+	+	+	+	+
02–0111	IIIa 21:g,z51:	+	+	+	+	+
CNM-247	IIIa 51:gz51:	+	+	+	+	+
CNM-259	IIIa 62:g,z51:	+	+	+	+	+
CNM-3527/02	IIIa 48:z4,z23,z32:	+	+	+	+	+
CNM-7302/02	IIIa 48:z4,z23:	+	+	+	+	+
01–0170	IIIb 60:r:e,n,x,z15	+	+	+	+	+
01–0221	IIIb 48:i:z	+	+	+	+	+
01–0248	IIIb 61:k:1,5, (7)	+	+	+	+	+
02–0188	IIIb 61:l,v:1,5,7	+	+	+	+	+
CNM-3511/02	IIIb 48: z10: e,n,x,z15	+	+	+	+	+
CNM-4190/02	IIIb 38:z10:z53	+	+	+	+	+
CNM-750/02	IIIb 60:r:z	+	+	+	+	+
CNM-834/02	IIIb 50:i:z	+	+	+	+	+
01–0133	IV 50:g,z51:	+	−	+	+	+
01–0147	IV 48:g,z51:	+	−	+	+	+
01–0149	IV 44:z4,z23:	+	−	+	+	+
01–0276	IV 45:g,z51:	+	−	+	+	+
01–0551	IV 16:z4,z32:	+	+	+	+	+
CNM-1904/03	IV 11:z4,z23:	+	+	+	+	+
CNM-4708/03	IV 6,7:z36:	+	−	+	+	+
ST-16	IV 16:z4,z32:	+	+	+	+	+
ST-21	IV 40:g,z51:	+	+	+	+	+
ST-22	IV 40:z4,z24:	+	+	+	+	+
CDC_07-0708	I 4, [5],12:i:	+	+	+	+	+
CDC_08-0061	I 4, [5],12:i:	+	+	+	+	+
CDC_08-0134	I 4, [5],12:i:	+	+	+	+	+
CDC_07-835	I 4, [5],12:i:	+	+	+	+	+
CDC_07-934	I 4, [5],12:i:	+	+	+	+	+
CDC_07-922	I 4, [5],12:i:	+	+	+	+	+
1121	VI 6,14,25:z10:1, (2),7	+	+	+	+	+
1415	VI 11:b:1,7	+	+	+	+	+
1937	VI 6,7:z41:1,7	+	+	+	+	+
2229	VI 11:a:1,5	+	+	+	+	+
811	VI 6,14,25:a:e,n,x	+	+	+	+	+

[a] SEN, Atlas® *Salmonella* Assay.
[b] MicroSEQ, MicroSEQ® *Salmonella* spp. Detection kit.
[c] MDA, 3M™ Molecular Detection Assay *Salmonella*.
[d] ANSR, ANSR™ *Salmonella* Assay.
[e] Pro-AmpRT, Pro-AmpRT™ SALM spp. kit.

according to the manufacturer's instructions: pre-enriched sample (50 μl) was transferred into a cluster tube containing 450 μl mixed lysis reagent solution, cluster tubes with samples and lysis reagents were places in a heat block at 37 °C for 10min, then at 80 °C for 20 min. After the completion of the lysis incubation, 50 μl of lysed sample was added into a reaction tube. Amplification was conducted on the ANSR Pathogen Detection System (PDS, Neogen). The ANSR reader provided positive, negative, or invalid (need to be retested) results in 10min.

2.8. Pro-AmpRT™ SALM spp. kit

The Pro-AmpRT™ SALM spp. kit (OptiGene, Horsham, UK) was performed on the Genie III system (OptiGene), according to the manufacturer's instructions. Firstly, 5.0 μl primer mix was added to a 15 μl isothermal master mix immediately before use; then 5.0 μl DNA template was added to this amplification mix. The amplification program was run under an isothermal temperature at 66 °C for 30min, and then annealing from 98 °C to 80 °C with a ramping at 0.05 °C per sec.

2.9. Sensitivity test

In the attempt to compare the sensitivity of all the commercial detection kits described above, ten-fold serial dilutions (10^0 - 10^{-8}) of 3 different pre-enriched samples (3 egg samples) were prepared using distilled water, then analyzed by the real-time PCR, Atlas instrument, 3M MDS, ANSR PDS, and Genie III system, respectively.

2.10. Statistical analysis

Duncan's multiple range test was conducted to compare the differences ($P < 0.05$) of detection limits of different molecular methods. Fisher's exact test was performed to determine significant differences of effectiveness ($P < 0.05$) of different detection methods. SAS 9.3_M 1. (SAS Institute, Cary, NC) was used for all statistical analysis.

Table 2
Salmonella exclusivity and test results.

Isolate ID	Bacteria	SEN[a]	MicroSEQ[b]	MDA[c]	ANSR[d]	Pro-AmpRT[e]
ATCC 55055	*Bacillus cereus*	–	–	–	–	–
6A16	*Bacillus cereus*	–	–	–	–	–
3A16	*Bacillus subtilis*	–	–	–	–	–
ATCC 19268	*Bacillus thuringiensis*	–	–	–	–	–
3476	*Bacillus amyloliquifaciens*	–	–	–	–	–
1391	*Brenneria nigrifluens*	–	–	–	–	–
47N	*Citrobacter Freundii*	–	–	–	–	–
E604	*Cronobacter sakazaki*	–	–	–	–	–
E265	*Cronobacter malonaticus*	–	–	–	–	–
E464	*Cronobacter dublinensis*	–	–	–	–	–
8645	*Erwinia mallotivora*	–	–	–	–	–
ATCC 43888	*E. coli* O157:H7	–	–	–	–	–
EC P1334	*E. coli* O91:H21	–	–	–	–	–
EC CL-15	*E. coli* O113:K75:H21	–	–	–	–	–
NES 14	*Klebsiella pneumoniae*	–	–	–	–	–
ATCC 51780 (1/2b)	*Listeria monocytogenes*	–	–	–	–	–
ATCC 15313 (4b)	*Listeria monocytogenes*	–	–	–	–	–
ATCC 19120	*Listeria grayi*	–	–	–	–	–
ATCC 25401	*Listeria grayi(murrayi)*	–	–	–	–	–
ATCC 35897	*Listeria welshimeri*	–	–	–	–	–
ATCC 19119	*Listeria ivanovii*	–	–	–	–	–
ATCC 35967	*Listeria seeligeri*	–	–	–	–	–
N/A	*Listeria innocua*	–	–	–	–	–
66N	*Proteus vulgaris*	–	–	–	–	–
ATCC 27853	*Pseudomonas aeruginosa*	–	–	–	–	–
SHI0168	*Shigella sonnei*	–	–	–	–	–
670	*Shigella flexneri*	–	–	–	–	–
973	*Shigella dysenteriae*	–	–	–	–	–
970	*Shigella boydii*	–	–	–	–	–
ATCC 25923	*Staphylococcus aureus*	–	–	–	–	–
11629	*Vibrio cholerae*	–	–	–	–	–
7708	*Vibrio metschnikovii*	–	–	–	–	–
17802	*Vibrio parahaemolyticus*	–	–	–	–	–
29306	*Vibrio vulnificus*	–	–	–	–	–

[a] SEN, Atlas® *Salmonella* Assay.
[b] MicroSEQ, MicroSEQ® *Salmonella* spp. Detection kit.
[c] MDA, 3M™ Molecular Detection Assay *Salmonella*.
[d] ANSR, ANSR™ *Salmonella* Assay.
[e] Pro-AmpRT, Pro-AmpRT™ SALM spp. kit.

3. Results

3.1. Inclusivity and exclusivity test

For inclusivity, MDA, ANSR, and Pro-AmpRT assays detected all 121 *Salmonella* isolates (Table 1). SEN assays detected the 112 *Salmonella enterica* isolates, but failed to detect the 9 *Salmonella bongori* isolates. MicroSEQ failed to detect 5 of 10 *Salmonella* IV group isolates, as well as all of the 9 *Salmonella bongori* isolates (Table 1). For exclusivity, all 34 non-*Salmonella* isolates were negative by all five molecular reagent kits studied: SEN, MicroSEQ, MDA, ANSR, Pro-AmpRT (Table 2).

3.2. Detection limits of different molecular assays

Pre-enriched samples with levels of *Salmonella* ranging from 7.38 to 9.64 log CFU/ml were serial diluted to determine the detection limits of the different molecular assays. The results showed that detection limits of the 5 molecular assays ranged from 1.76 to 3.76 log CFU/ml pre-enrichment for individual sample; and the SEN assay had a range of 1.76 - 2.64 log (data not shown). The SEN assay had the lowest average detection limit (1.92 ± 1.10 log CFU/ml) among the 5 molecular assays studied, which was significantly lower than those for MicroSEQ and ANSR assays with values at 3.25 ± 0.51 and 3.42 ± 0.99 log CFU/ml, respectively. Detection limits of MDA (2.75 ± 1.18 log CFU/ml) and Pro-AmpRT (2.75 ± 0.57 log CFU/ml) had no significant difference ($P > 0.05$) with those of SEN, MicroSEQ and ANSR assays (Table 3).

3.3. Effectiveness of different molecular assays for the detection of *Salmonella* in egg products

Results comparing the culture method and the molecular assays for the detection of *Salmonella* in egg products are presented in Table 4. All 40 uninoculated samples were negative for *Salmonella* by all 6 detection assays. Both the SEN assay and culture method detected 127 positive samples out of a total of 160 samples. The MircoSEQ, MDA, and Pro-AmpRT assays detected 125 positive samples; and ANSR assay identified 124 positives. Statistically, there were no significant differences among molecular assays studied and the FDA BAM culture method for the detection of *Salmonella* in egg products ($P > 0.05$).

4. Discussion

The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* contains majority of the 2579 identified serotypes, and 99% of reported human *Salmonella* isolates in the U.S. belongs to this subspecies (CDC, 2011; WHO, 2018). The results from our inclusivity study revealed that MicroSEQ and SEN assays could not detect *Salmonella bongori*, while MDA, ANSR, and Pro-AmpRT were capable of detecting *Salmonella bongori*. For SEN assay, our data confirmed with a previous report (Kwong et al., 2013) that SEN assay was unable to detect *Salmonella bongori*. For MicroSEQ assay, previous validation test on *Salmonella* detection did not include *Salmonella bongori* (Balachandran et al., 2011); our result revealed the discrepancy between the two *Salmonella* species in response to the

Table 3
Detection limits for *Salmonella* of different assays studied.

	SEN[a]	Pro-AmpRT[b]	MDA[c]	MicroSEQ[d]	ANSR[e]
Detection limit (log CFU/ml pre-enrichment)	1.92 ± 1.10B[f]	2.75 ± 0.57AB	2.75 ± 1.18AB	3.25 ± 0.51A	3.42 ± 0.99A

[a] SEN, Atlas® *Salmonella* Assay.
[b] Pro-AmpRT, Pro-AmpRT™ SALM spp. kit.
[c] MDA, 3M™ Molecular Detection Assay *Salmonella*.
[d] MicroSEQ, MicroSEQ® *Salmonella* spp. Detection kit.
[e] ANSR, ANSR™ *Salmonella* Assay.
[f] Means followed with the same letter are not significantly different.

Table 4
Effectiveness of different assays for the detection of *Salmonella* in egg products.

	# of positive/# of total samples tested					
	Culture	SEN[a]	MicroSEQ[b]	MDA[c]	ANSR[d]	Pro-AmpRT[e]
Egg products	127/160	127/160	125/160	125/160	124/160	125/160
Fisher's exact test [f]		1.0000	0.8914	0.8914	0.7859	0.8914
Uninoculated	0/40	0/40	0/40	0/40	0/40	0/40

[a] SEN, Atlas® *Salmonella* Assay.
[b] MicroSEQ, MicroSEQ® *Salmonella* spp. Detection kit.
[c] MDA, 3M™ Molecular Detection Assay *Salmonella.* And the MDA and ANSR detect results were from our previous publication (Hu et al., 2016).
[d] ANSR, ANSR™ *Salmonella* Assay.
[e] Pro-AmpRT, Pro-AmpRT™ SALM spp. kit.
[f] Fisher's exact test was conducted using SAS 9.3_M 1. And the Fisher's exact test calculated by the comparison of each method with culture method.

MicroSEQ assay. For ANSR assay, other reports also showed that it could effectively detect *Salmonella bongori* (6 serotypes: Brookfield, Malawi, Maregrosso, Balboa, Bongor, Camdeni), while two reports indicated that it could not detect one serotype from *Salmonella enterica* (*Salmonella* ser. Weslaco) (Caballero et al., 2015; Mozola et al., 2013).

In the detection limit study, results indicated that the SEN assay had the lowest detection limit, with an average detection limit of 1.92 log CFU/ml pre-enriched samples. The other four molecular assays had detection limits ranging from 2.75 to 3.42 log CFU/g (data not shown). In addition, for inoculated egg products, the SEN assay detected 127 positive of 160 egg products samples, which 100% matched the results of culture method. All other methods (MicroSEQ, MDA, ANSR, Pro-AmpRT) missed 2 or 3 samples compared to the culture method and SEN assay. We analyzed the 3 culture positive samples missed by these methods, the *Salmonella* populations in these samples were below their detection limits. Statistically, all 6 methods are equally effective for the detection of *Salmonella* in pre-enriched samples (Table 4).

The cause of the difference in the results may be due to the different targets and/or technology used by the SEN, MicroSEQ, MDA, ANSR, Pro-AmpRT assays. The five different molecular assays adopted different principles for the detection of *Salmonella* (Table 5). Among them, SEN, MDA, ANSR, and Pro-AmpRT assays are isothermal amplification based detection kits, but the mechanisms for the detection are different. The SEN assay was designed to use rRNA as detection target, based on the combination of target capture, TMA, and hybridization protection assay (HPA). MDA and Pro-AmpRT assay utilize LAMP technology while targeting DNA, while ANSR assay is based on nicking enzyme amplification reaction (NEAR™) technology (Van Ness, Van Ness, & Galas, 2003). MicroSEQ kit, on the other hand, is based on TaqMan® real-time PCR technology to amplify a unique microorganism-specific DNA target sequence and a TaqMan® probe for detecting the amplified *Salmonella* sequence. Each of the five methods has some unique features; and we tried to compare the pros and cons of each of them for appropriate usages (Table 5 and Fig. 1).

Since PCR-based methods can detect a single copy of genomic DNA, each bacterial contains 10^2 to 10^4 copies/cell of rRNA, the rRNA detecting SEN assay has an advantage over PCR-based methods (Livezey et al., 2013; H.; Yang et al., 2012). Furthermore, the larger sample size (400 μl) used for detection by SEN assay versus 20 or 50 μl used by other molecular kits also contributed to the higher sensitivity of SEN assay. It was reported that the SEN assay was 40 times more sensitive than DNA-based methods (Livezey et al., 2013). Another advantage of

Table 5
Comparison of the characteristics of different assays and instrument used in the study.

Instrument and company	Technology (target)	Time per run (tests per run)	Portable
Roka Atlas™ System (Roka Bioscience, Inc.)	TMA (rRNA)	3–5h (1–120 testes) (process 300 tests in 8 h, or 500 tests in 12 h in one operator)[a]	No
7500 Fast System (Applied Biosystems, Inc.); RapidFinder Express Software	TaqMan probe based real-time PCR (DNA)	40min (96 tests)	No
3M™ Molecular Detection System (3M Food Safety, Inc.)	LAMP (DNA)	60 min (96 tests)	Yes
ANSR® Pathogen Detection System (Neogen Ltd.)	Isothermal amplification (DNA)	10min (16 tests)	Yes
Genie III (OptiGene Co.)	LAMP (DNA)	~38min (8 tests)	Yes

[a] The first 5 result will be generated after 3h, subsequent 5 results will come in every 5min thereafter.

this method is its fully automated instrument, which greatly reduces the risk of false negatives, and offers a walk-away freedom for the operators. In a report of the evaluation of SEN assay, the results indicated that it was a rapid and accurate tool for detecting *Salmonella enterica* in 12 kinds of foods and 3 surfaces (fresh ground chicken, deli cooked turkey, raw cod, raw cookie dough, fresh ground beef, nonorganic creamy peanut butter, string cheese, milk chocolate, cocoa powder, pasteurized dried whole egg, stainless steel, plastic, sealed concrete, and nacho cheese seasoning). The SEN assay also showed higher sensitivity than the reference method (U.S. Department of Agriculture-Food Safety and Inspection Service/Microbiology Laboratory Guidebook, USDA-FSIS/MLG) for detecting *Salmonella* from pasteurized dried whole eggs (Kwong et al., 2013). Chaney et al. (2017) found a strong positive correlation between *Salmonella* enumeration result and SEN assay signal-to-cutoff values. In addition, the SEN assay was demonstrated as a very helpful method to test *Salmonella* from freeze-dried probiotic culture (Shannon, Greve, Legan, & Solutions, 2017).

As an LAMP based detection kit, MDA assay utilizes *Bst* polymerase to amplify the specific target gene (*inv*A gene) of *Salmonella*, which generates bioluminescent lights that can reach a limit of detection of 1–5 CFU/sample (3M Food Safety, 2018). In a report of *Salmonella* detection from 675 samples (ready to eat meat & poultry, fish & seafood, dairy, produce, eggs), the MDA method was able to detect *Salmonella* with 99.7% test efficacy, 0% false negative rate, and 1% false positive rate (3M Food Safety, 2012). In another study on raw duck wing, raw mung bean sprouts, and processed fishballs with the inoculation level of 0–1 log CFU/25 g, the MDA method was demonstrated to have 100% sensitivity and specificity in *Salmonella* detection, except for one false positive and one negative in duck wing samples compared to the International Organization for Standardization method (Lim, Zheng, Miks-Krajnik, Turner, & Yuk, 2015). The MDA assay also successfully detected *Salmonella* in several other food matrices, such as raw ground beef, wet dog food, dry nuts, fruits, wastewater, and river water samples (Bird et al., 2013; Loff, Mare, de Kwaadsteniet, & Khan, 2014; M. H. Yang & Peter, 2013).

Similar to the MDA method, Pro-AmpRT assay uses LAMP technology to detect *Salmonella* on Genie III system, but the Pro-AmpRT assay uses an isothermal master mix that contains a novel DNA polymerase (LF polymerase) isolated from Geobacillus sp. SSD, GspSSD. The LF polymerase tested higher speed in the fluorescent LAMP reaction as compared to *Bst* DNA polymerase (OptiGene, 2018). The Genie III system (dual fluorescence channels) and its former version Genie II System (single fluorescence channel) were designed for LAMP based methods to detect *Hedyotis diffusa*, Cyprinid herpesvirus 2, *Campylobacter jejuni*, etc. (Dong, Cho, Hahn, & Cho, 2014; He et al., 2013; Li et al., 2013). Recently, LAMP technology was reported to be equivalent or better than PCR for the detection of *Salmonella* in foods with the advantage of higher specificity and sensitivity, less cell damage, and less susceptibility to the inhibition from media or food matrix (Enosawa et al., 2003; Fakruddin, 2011; Fortes, David, Koeritzer, & Wiedmann, 2013; Kokkinos, Ziros, Bellou, & Vantarakis, 2014; Shao, Zhu, Jin, & Chen, 2011; Techathuvanan & D'Souza, 2012; Tomita, Mori, Kanda, & Notomi, 2008; Ye et al., 2011; Zhang, Brown, & González-Escalona, 2011). Our paper is the first study on evaluation of Pro-AmpRT assay for *Salmonella* detection in egg products, and it was found to be a reliable and accurate method for the detection of *Salmonella*.

ANSR assay utilizes a unique amplification reaction technology (patent-pending) to amplify DNA. The resultant molecular beacons fluorescence can be detected by the ANSR reader, with a detection limit of 1 CFU/analytical unit or 4 log CFU/mL post-enrichment (Neogen, 2018). In the current study, detection limit of ANSR assay was 2.85–3.76 log CFU/ml, depending on the food matrices. In a study on the detection of *Salmonella* from chicken carcass rinse, raw ground turkey, raw ground beef, hot dogs, oat cereal, and environmental surfaces, ANSR method was found to have 95% sensitivity and 98% specificity, with no statistically significant differences between ANSR and the USDA/FSIS or FDA BAM reference culture method in the number of positive samples detected (Mozola et al., 2014). Comparable results in the detection of *Salmonella* by ANSR kit was also found in a variety of food matrix, such as ground turkey, ground beef, pet food, ice cream, soy flour, almonds, peanut butter, spinach, black pepper, raw frozen shrimp, cocoa powder, dried pasteurized egg, and pasteurized liquid egg (Caballero et al., 2014; Caballero et al., 2015; Foti, Zhang, Biswas, Mozola, & Rice, 2014).

The real-time PCR based MicroSEQ assay can provide a detection limit of 1–3 CFU/25 g food per the kit description (Applied Biosystems, 2018). The RapidFinder™ Express Software run on the 7500 Fast Real-Time PCR System to provide an automated analysis with the intuitional result of positive/negative/invalid, instead of C_T value. In a comparison study of MicroSEQ and reference method for the detection of *Salmonella* in foods, the MicroSEQ kit was proven to be successful in the detection of *Salmonella* in raw ground beef, raw chicken, raw shrimp, Brie cheese, shell eggs, cantaloupe, chocolate, black pepper, dry infant formula, and dry pet food, with a contamination level of 0.2–10 CFU/25 g, but it might cause false-positive results when the inoculation level was at 0.2–2 CFU/25 g in pet food (Balachandran et al., 2011; Balachandran et al., 2012; Cloke et al., 2016). In another study, comparable result was found in the *Salmonella* detection of diced tomatoes, chocolate, and deli ham test by using the MicroSEQ assay linked with the Pathatrix® 10-Pooling *Salmonella* spp. Kit (Wall, Conrad, Latham, & Liu, 2014). Although MicroSEQ assay was found as the most sensitive method with the provided lower C_T values in a comparison study of real-time PCR based methods, it was demonstrated to have less effective performance on the segregation of the true and false groups in the detection of *Salmonella* from cloves (1.0–2.9 log CFU/25 g) versus either *Salmonella* GeneDisc assay (Pall Corporation, Port Washington, NY) or FDA PCR method for *Salmonella* developed by FDA Pacific Regional Laboratory Southwest (PRLSW) (Tatavarthy et al., 2017).

In this study, all the five molecular methods use a closed amplification system during analysis, which minimizes the risk of the false positives due to cross-contamination, as well as provides an environment for higher reproducibility and sensitivity. Although each assay provides an easy-to-use format that requires minimal steps, the Atlas system is the easiest one. Fewer steps reduces technical errors, especially when a reaction involves multiple reagents. Nucleic acid-based tests offer rapid detection with greatly reduced enrichment time. The Atlas system utilizes the least number of steps to gain results for more than 300 samples in 8 h, or more than 500 results in 12 h (Roka Bioscience, 2018), which will be very helpful when sample numbers are large. The MDA provides for the identification of *Salmonella* positive results as quickly as 20min and negative results in 75min (new kit shortens the run time to 60min), it can execute up to 96 tests per run, while MicroSEQ kit can test 96 samples in 40min based on RapidFinder™ Express Software and 7500 Fast Real-Time PCR System, these two methods are also suitable for large sample numbers. Pro-AmpRT assay usually takes approximately 38min to test 8 samples by the Genie II/III system. The fastest assay in this study is ANSR kit, which only needs 10min to test 16 samples per run by using ANSR PDS. Both the Pro-AmpRT and ANSR methods are appropriate to detect *Salmonella* from small numbers of samples. Comparing with Atlas and MicroSEQ system, portable devices like 3M MDS, ANSR PDS, and Genie III can be carried out to the field for testing, such as in restaurants or retail shops. In addition, Genie III system and 7500 Fast Real-Time PCR are flexible open platforms for designing new methods.

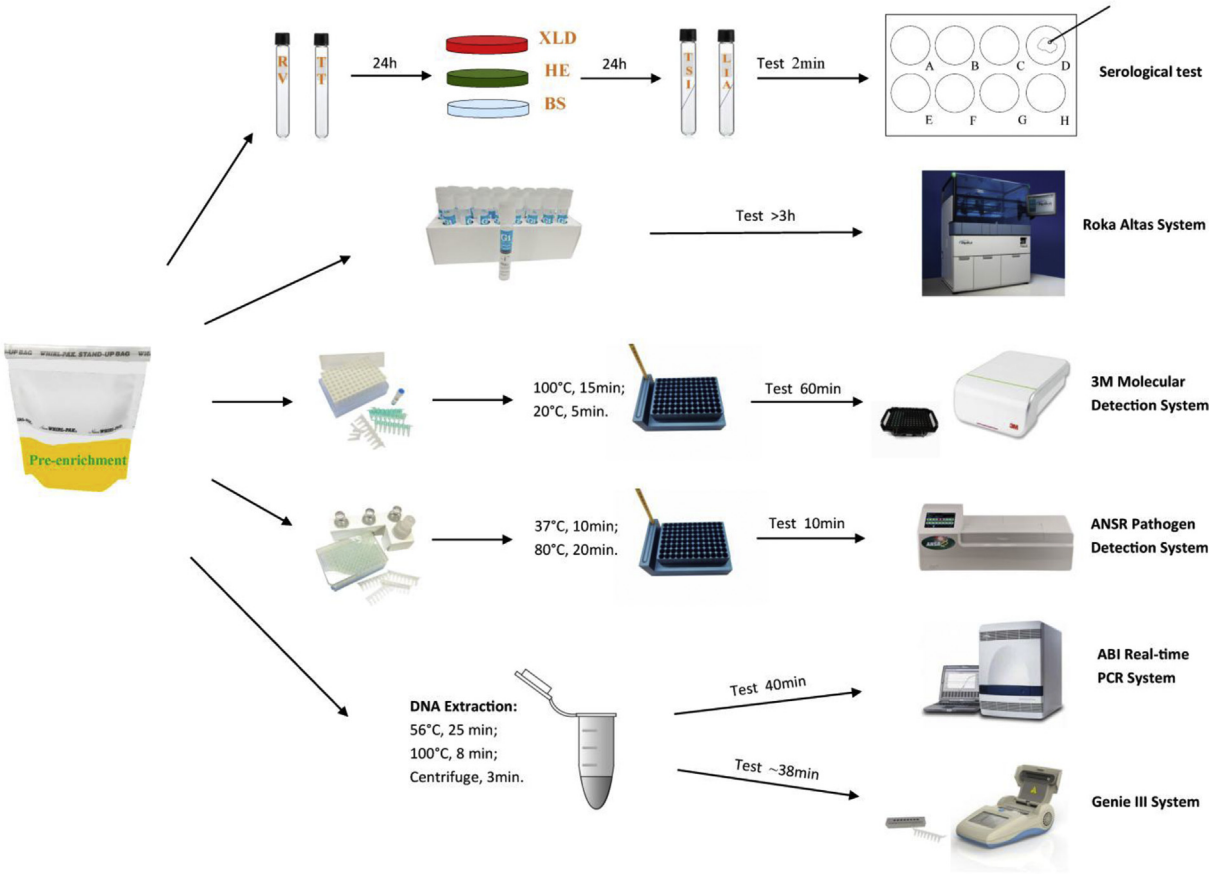

Fig. 1. Flowchart of the five molecular methods and culture method for the detection of *Salmonella*.

5. Conclusions

This study analyzed five molecular platforms for the detection of *Salmonella* in egg products. The assays allow reliable, accurate, and rapid detection of low level of *Salmonella* in food matrices in comparison with the reference culture method. There was no significance differences between each assay and the culture method result; nevertheless, the SEN assay was the most sensitive assay with the relatively better results obtained in the egg products detection and detection limit test. Based on distinctive features of these assays, like detection target, detection mechanism, test load, test time, and equipment portability, people may choose an appropriate method depending on their specific situation. Hence, with the advantage of rapid and accurate detection, these easy-to-use *Salmonella* detection assays will be applied more frequently in microbiology diagnostics to reduce *Salmonella* at any stage of food production.

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