Evaluation of a New Real-Time PCR Method to Detect *Salmonella* Enteritidis in Whole Shell Eggs

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BACKGROUND

Salmonella enterica serovar Enteritidis (*Salmonella* Enteritidis, or SE) and *Salmonella* Typhimurium, account for nearly half of all *Salmonella* illnesses in the US. The CDC estimates that 75% of SE outbreaks are associated with the consumption of raw or poorly cooked shell eggs. SE can infect chicken eggs before the shell is formed leading to internal contamination, unlike other fecal-associated *Salmonella* strains that are found on egg shell surfaces. The significance of monitoring shell eggs for the presence of SE has become apparent with the recent nation-wide SE outbreak linked to the consumption of eggs from Wright County Egg and Hillandale Farms. By the end of August 2010, over 2500 illnesses have been reported. The CDC reports the current SE outbreak is the largest SE outbreak since tracking began in the mid-1970's. Over a half-billion eggs, representing 32 brands, were recalled.

ABSTRACT

A Real-time PCR assay was recently constructed to detect *Salmonella* Enteritidis in shell eggs and poultry houses to support the needs of the poultry industry due to recent U.S. FDA regulations. The new regulations, affective July 9, 2010, require commercial shell egg producers perform routine environmental tests of poultry houses for presence of SE (1). If SE is detected in the environment then further tests are required on the eggs, in which 1000 eggs (50 pools of 20 eggs) must be tested every 2 weeks over an 8 week period. These new regulations are predicted to reduce the number of human SE infections resulting from egg consumption by nearly 60 percent. A complete workflow was created to include a simple, automated, sample preparation protocol for shell egg samples enriched using a rapid 24-hour enrichment method, followed by Real-time PCR detection. The complete workflow was validated against the US FDA Bacteriological Analytical Manual (BAM) reference method (2) using an unpaired study design. The Real-time PCR method was shown to be equivalent to the FDA BAM reference method using chi-square statistical anlysis.

METHODS

PCR Assay Design
Multiple PCR assays were designed and one was selected based on predicted specificity of hybridization to SE versus related exclusion strains. A 10x primer-probe mix was prepared containing 900 nM SE primers, 250 nM SE probe, 450 nM Internal Positive Control (IPC) primers, 125 nM IPC probe, and 5x IPC template.

Egg Sample Preparation and Enrichment
Whole shell eggs were disinfected with alcohol:iodine. Approximately 250 eggs were combined into a bulk sample for the uninoculated control, and a bulk lot consisting of approximately 900 eggs were inoculated with SE (ATCC 13706) at a concentration of 0.2-2 CFU per 1000 g. Egg pools consisting of approximately 20 eggs (1000 g) were prepared. Twenty inoculated and 5 uninoculated egg pools were enriched according to the FDA BAM Chapter 5 protocol for *Salmonella* n egg samples.. A second set of 20 inoculated and 5 uninoculated egg pools were combined with 100 mL of 10X TSB per pool, and then incubated at 35°C for 24 hours.

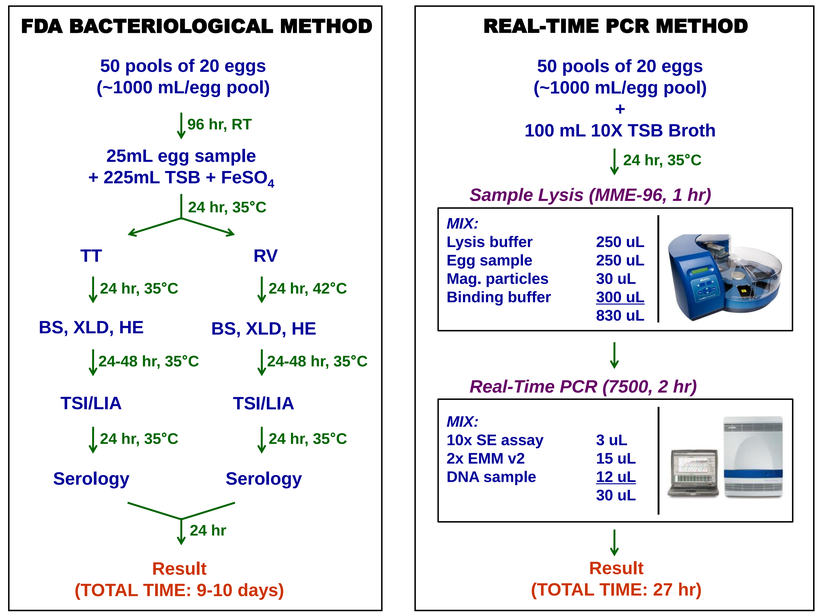
DNA Extraction
A new sample preparation method was developed based on the PrepSEQ® NA Extraction Kit that allowed for complete automation on the MagMAX™ Express-96 Magetic Particle Processor. The Sample Preparation workflow is outlined in Figure 1.

Real-Time PCR
Real-time was run on the 7500 Fast instrument using standard conditions (95 ºC for 10 min; 40 cycles at 95 ºC for 15 seconds and 60 ºC for 60 seconds). The PCR reaction setup is outlined in Figure 1.

Statistical Analysis
Results obtained from the TaqMan® method were compared to those from the FDA BAM reference method using the Mantel-Haenzel chi-square analysis for unmatched test portions. Relative sensitivity was determined by dividing the number of confirmed positive samples from the TaqMan® method by the number of positive samples from the FDA BAM method. The false negative rate was determined by dividing the number of confirmed negative samples by the number of presumed positive samples, and the false positive rate was determined by dividing the number of confirmed positive samples by the number of presumed positive samples.

RESULTS

Figure 1. Sample Processing: FDA Method vs. Real-Time PCR



The U.S. FDA Bacteriological Analytical Manual (BAM) requires a minimum of 9 days for positive confirmation of *Salmonella* Enteritidis in shell eggs. The TaqMan® Salmonella Enteritidis Real-time PCR Method provides results in approximately 27 hours after start of enrichment.

Table 1. Summary Data Table

Inoculation Level	Inoculating Organism	U.S. FDA BAM	TaqMan® Salmonella Enteritidis Method		χ^2	Relative Sensitivity	False Negative Rate	False Positive Rate
			Presumed	Confirmed				
Experiment 1								
Control	N/A	0/5	0/5	0/5	-	-	0%	0%
Spike	*S. enterica* ser. Enteritidis ATCC 13076	16/20	16/20	16/20	0	100%	0%	0%
Experiment 2								
Control	N/A	0/5	0/5	0/5	-	-	0%	0%
Spike	*S. enterica* ser. Enteritidis ATCC 13076	11/20	13/20	13/20	0.41	118%	0%	0%

Methods comparison showed that the TaqMan® Real-time PCR method was equivalent to the FDA BAM method for detection of *Salmonella* Enteritidis in whole shell eggs. The results from chi-square analysis on two independent experiments indicated no difference between the two methods (χ^2 = 0 and 0.41 for Experiment 1 and Experiment 2, respectively). No false positive or false negative results were observed.

Figure 2. Methods Comparison: FDA BAM vs. Real-Time PCR

Experiment 1			
		Lysis	
Sample Name	FDA BAM confirmed	FAM (SE)	VIC (IPC)
1	-	ND	30.0
2	-	ND	29.9
3	+	20.3	29.0
4	+	22.9	28.7
5	-	ND	29.9
6	+	21.1	29.2
7	+	22.0	29.1
8	-	ND	34.7
9	+	19.1	30.1
10	+	18.4	30.8
11	+	19.2	29.4
12	+	20.2	29.3
13	+	18.6	29.7
14	+	18.5	29.5
15	-	ND	30.6
16	+	21.3	30.2
17	-	ND	30.1
18	-	ND	30.1
19	-	ND	30.3
20	-	ND	30.1
21	+	17.2	32.2
22	+	19.6	29.1
23	+	18.1	29.9
24	+	23.5	32.6
25	+	21.0	28.9
TOTAL +	16	16	N/A

Experiment 2			
		Lysis	
Sample Name	FDA BAM confirmed	FAM (SE)	VIC (IPC)
1	-	ND	31.0
2	+	26.2	29.9
3	-	ND	31.1
4	+	27.0	29.6
5	-	ND	32.0
6	+	27.9	30.8
7	+	25.1	29.8
8	-	ND	31.7
9	-	ND	31.6
10	+	26.2	30.2
11	+	25.1	30.0
12	-	ND	31.5
13	+	28.5	30.9
14	+	23.2	29.4
15	-	ND	32.1
16	+	25.7	30.0
17	-	ND	31.7
18	+	24.8	29.8
19	-	ND	31.7
20	+	23.6	29.3
21	-	ND	30.7
22	+	27.1	30.0
23	-	ND	31.1
24	-	ND	31.3
25	+	22.5	29.3
TOTAL +	13	13	N/A

The TaqMan® SE Real-time PCR method shows excellent correlation with the FDA BAM method for detection of SE in shell egg samples. The Ct values in the low to mid 20's is an indication of excellent enrichment. The bulk egg sample was spiked with SE at a target concentration of 0.2 to 2 cfu/1000 g in order to achieve fractional positive samples.

SUMMARY

A new method for the detection of SE in shell egg pools by Real-time PCR was developed. The workflow was designed to be simple, rapid, and more cost effective than the standard FDA BAM culture method. The method detected all SE strains tested (N=37), and demonstrated no detection of non-SE strains (N=62) (results not shown). The Real-time PCR method was found to be equivalent to the FDA BAM reference method based on chi-square statistical analysis.

CONCLUSIONS

- New FDA regulations require commercial egg producers to screen for presence of *Salmonella* Enteritidis.
- A TaqMan® Real-time PCR assay was developed to be specific for *Salmonella* Enteritidis.
- A simple and automated sample preparation protocol was created for preparing shell eggs.
- The TaqMan® Real-time PCR workflow shows equivalence in performance to the FDA BAM protocol for detection of SE in shell eggs.
- The TaqMan® Real-time PCR method provides next-day results.

REFERENCES

1. Prevention of *Salmonella* Enteritidis in Shell Eggs During Production, Storage, and Transportation; Final Rule (July 9, 2009). *Federal Register*, Vol 74 (130): 33030-33101.
2. US FDA Bacteriological Analytical Manual *Online*, Chapter 5, *Salmonella*, February 2011 Version, http://www.cfsan.fda.gov/~ebam/bam-5.html. Accessed on May 2, 2011.

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