Development of a Multiplex Real-Time PCR Assay for the Detection of Highly Pathogenic *Salmonella* (HPS) in Beef, Poultry and Pork

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INTRODUCTION

Salmonella continues to be a threat to human health, and most of the Salmonellosis cases are attributed to food sources, according to the CDC.[1] While there are over 2,600 different types of *Salmonella*, human illness data suggests that certain *Salmonella* (*Salmonella* of concern (SoC); Figure 1.) are better at causing disease than others.

The USDA and industry work to find a balance between continuing to offer wholesome, nutritious meat products and reducing the risk of illness from *Salmonella* for humans. The poultry, beef and pork industries would benefit from a rapid and easy-to-use test to identify the risk of *Salmonella* in food samples based on virulence or pathogenicity, instead of simply presence or absence.

The data presented demonstrate the accuracy of a novel real-time (RT) assay for detecting SoC in poultry, beef and pork enrichments. The data further highlight an important limiting principle of all RT detection assays and demonstrate how examination of the Ct value of the *Salmonella* spp. marker engineered in this assay can help identify enrichment samples containing sufficient concentrations of *Salmonella* to produce HPS results with increased accuracy.

PURPOSE

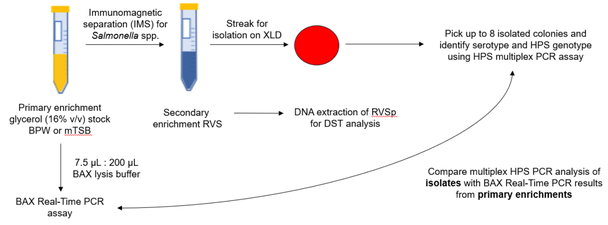
This study evaluated the performance of the multiplex BAX® RT-PCR assay for detecting *Salmonella* of increased concern for human health, in 696 primary enrichment samples from poultry, beef and pork products collected as part of the FSIS regulatory testing program (2021-2022). These enrichments had previously been characterized for serotype and HPS genotype using culture isolation methods[2] as well as with CRISPR-SeroSeq Deep Serotyping (DST) methods.[3] These well characterized enrichments provided a robust "truth-set" for assessing the accuracy of the novel RT-PCR assay.

REGISTERED TRADEMARKS

METHODS

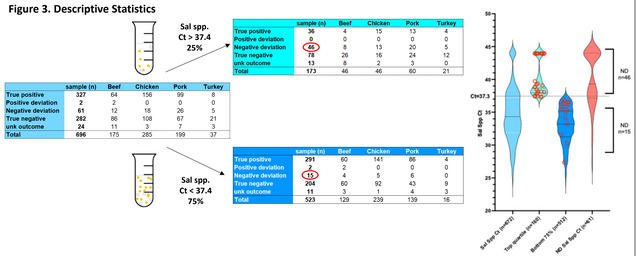
The multiplex, real-time PCR assay was designed with three HPS targets originally identified as part of a multiplex PCR assay[2] (HPS-B, HPS-A, HPS-X) associated with virulence, combined with a *Salmonella* spp. target and internal control. Enrichment samples demonstrating amplification of 2 or 3 BAX targets were classified as BAX positive (positive for the presence of HPS), while those amplifying 1 BAX marker were classified as negative for the presence of HPS. The assay was tested on 696 *Salmonella*-positive enrichment samples (beef, n=175; chicken, n=285; pork, n=199; turkey, n=37) which had been previously characterized for serotype and HPS genotype using 1) culture isolation methods, 2) the HPS multiplex PCR assay[2], and 3) CRISPR-SeroSeq[3] (Figure 2). PCR amplification curves were generated on the BAX® System Q7 and interpreted with an algorithm prototype developed specifically for the assay. The BAX RT assay results from the enrichment samples were compared to the serovar and HPS genotype profiles based on the isolates from the samples (Figure 3).

Figure 2. Culture and Molecular Methods of Characterization



RESULTS

Figure 3. Descriptive Statistics



	sample (n)	Beef	Chicken	Pork	Turkey
True positive	327	64	156	99	8
Positive deviation	3	2	0	0	0
Negative deviation	61	12	18	26	5
True negative	282	86	108	67	21
unk outcome	24	11	3	7	3
Total	696	175	285	199	37

	sample (n)	Beef	Chicken	Pork	Turkey
True positive	36	4	15	13	4
Positive deviation	0	0	0	0	0
Negative deviation	66	8	13	20	5
True negative	78	26	16	24	12
unk outcome	13	8	2	3	0
Total	173	46	46	60	21

	sample (n)	Beef	Chicken	Pork	Turkey
True positive	291	60	141	86	4
Positive deviation	2	2	0	0	0
Negative deviation	15	4	5	6	0
True negative	204	60	92	43	9
unk outcome	11	3	1	4	3
Total	523	129	239	139	16

Figure 4. Concordance assessment

Real-time (RT) assay results were categorized as:

- **True Positive (TP)** - Serotype/HPS result matches RT assay result (BAX positive called when HPS identified with culture and/or DST)
- **True Negative (TN)** - Serotype/HPS result matches RT assay (BAX negative or not Sal call matches culture and/or DST)
- **Positive Deviation (PD)** - Enrichment was positive for HPS with RT assay when culture and/or DST results were HPS negative (where isolate/DST results indicate two or more non-HPS serovars, amplifying different BAX markers were present in the enrichment)
- **Negative Deviation (ND)** - Primary enrichment was negative for HPS with RT assay, while culture and/or DST of secondary enrichment confirmed the presence of HPS serotype(s)
- **Unknown (unk)** - RT assay identified HPS present, but culture and/or DST results did not. HPS could have been present but not culturable (i.e., Dublin in Beef enrichment)

		All Results		Sal Spp Ct <37.4		Sal Spp Ct >37.4	
		Number	%	Number	%	Number	%
Overall	Agreement	609	90.6	495	96.7	114	71.3
	Unconfirmed	63	9.4	17	3.3	46	28.8
	Total	672		512		160	
Beef	Agreement	150	91.5	120	95.2	30	78.9
	Unconfirmed	14	8.5	6	4.8	8	21.1
	Total	164		126		38	
Chicken	Agreement	264	93.6	233	97.9	31	70.5
	Unconfirmed	18	6.4	5	2.1	13	29.5
	Total	282		238		44	
Pork	Agreement	166	86.5	129	95.6	37	64.9
	Unconfirmed	26	13.5	6	4.4	20	35.1
	Total	192		135		57	
Turkey	Agreement	29	85.3	13	100.0	16	76.2
	Unconfirmed	5	14.7	0	0.0	5	23.8
	Total	34		13		21	

DISCUSSION

- Real-time PCR assays designed to detect multiple targets have the benefit of speed and reduced time to detection, but the accuracy of the results depends on a minimum level of target organisms present in the enrichment samples being tested. In this study, a threshold Ct ≤37.4 for the *Salmonella* spp. marker was a strong indicator that the *Salmonella* concentration in the enrichment was sufficient to provide accurate HPS results for >97% of samples tested, as opposed to 71% for samples with a Ct >37.4 (Figure 4). This level of target organism was achieved in 75% of the enrichment samples (n=512), with the highest observed for chicken enrichments (84%).
- The complete HPS index of the samples tested was determined by IMS, secondary enrichment in RVS, and isolate characterization. A relatively higher Ct value for the *Salmonella* spp. target was a good indicator of when the concentration is near the LOD of the assay and further enrichment or concentration may be required to properly detect the HPS markers.
- An important difference that could contribute to the occurrence of samples categorized as 'Negative Deviation (ND)' is that the sample type evaluated for the RT assay is primary enrichment, while culture and DST results are obtained from RVS secondary enrichments. As such, serovars present in low abundance in primary enrichments may be below the limit of detection (LOD) of the RT assay, but after IMS and secondary enrichment in RVS, are detectable by culture and DST methods.

Figure 1.

Summary of the correlation between the leading Serotypes of Concern (SoC) and HPS index (*HPSi ≥ 5 are highly correlated with SoC[3]) and identified as BAX(+) with RT Assay.

	SoC rank	Avg % human illness/yr	% FSIS isolates (n=1303)*	Serovar	HPS Index*	BAX result
Serotypes of Concern (SoC) - High burden of illness	1	15.36	8.37	Enteritidis	8	+
	2	12.36	0.92	Newport	7	+
	3	8.68	7.52	Typhimurium	8	+
	4	6.46	**0.00**	**Javiana**	3	-
	5	5.64	5.22	I 4,[5],12:i:-	8	+
	6	4.26	20.34	Infantis	5	+
	7	2.96	0.23	Saintpaul	6	+
	8	2.74	**0.00**	**Oranienburg**	1	-
	9	2.72	0.23	Braenderup	6	+
	10	2.4	2.76	Montevideo	2	-
	11	2.4	1.53	Muenchen	6	+
	12	2.18	0.54	Thompson	5	+
	13	2.14	0.08	Mississippi *	3	-
	14	1.22	**0.00**	Bareilly	6	+
	15	1.18	**0.00**	**Sandiego**	1	-
	16	1.1	0.08	Poona	2	-
	17	1.06	0.08	Paratyphi B	4	-
	18	1	0.54	Hadar	6	+

DISCUSSION CONTINUED

- It is important to note that the BAX RT HPS assay was previously tested on isolates from poultry, beef and pork samples with >97% accuracy. Further, it is highly specific for *Salmonella* based on previous inclusivity and exclusivity studies.
- Using archived enrichment samples that have been deeply characterized for *Salmonella* serotype[2] provides a robust "truth-set" for assessing the accuracy of novel RT detection assays and the ability to pinpoint key parameters that can be leveraged to enhance assay performance.

SIGNIFICANCE

- The Real-Time assay was able to correctly identify 91% of all enrichment samples tested, and 97% of samples that had a *Salmonella* spp. Ct ≤ 37.4.
- The results of this study further demonstrate the utility and ability of the multiplex real-time assay to detect virulence or pathogenicity markers from primary enrichment samples. Having a virulence-based approach to *Salmonella* testing can enable the industry to make risk-based decisions about their products to reduce the number of illnesses caused by *Salmonella* while continuing to provide consumers with nutritious and affordable meat.

REFERENCES

1. https://www.cdc.gov/salmonella/index.html
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3. Shariat N, Dudley EG. CRISPRs: molecular signatures used for pathogen subtyping. Appl Environ Microbiol. 2014 Jan;80(2):430-9. doi: 10.1128/AEM.02790-13. Epub 2013 Oct 25. PMID: 24162568; PMCID: PMC3911090.

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