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

Hans-Henno Dörries[1], Dayna M. Harhay[2], Kerry D. Brader[2], Monali Gandhi[3], Ivonne Remus-Dörries[1], Cordt Grönewald[1], Patrice Chablain[1], Rebecca L. Olsen[3], and Stacy K. Stoltenberg[3].
[1]Hygiena® Diagnostics GmbH, Hermannswerder 17, Potsdam, Germany 17743
[2]USDA-ARS Meat Animal Research Center, Clay Center, Nebraska, USA 68933
[3]Hygiena®, 941 Avenida Acaso Camarillo, California, USA 93012

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INTRODUCTION:

Salmonella remains a significant health concern causing millions of cases of foodborne illness annually. The CDC estimates *Salmonella* bacteria causes 1.35 million infections, more than 26,000 hospitalizations, and over 400 deaths in the United States each year. Food is the source of a majority of these illnesses.

While there are established performance standards set by the USDA to reduce the prevalence of *Salmonella* in poultry, there has been no significant reduction in the number of human infections from *Salmonella* in poultry products.

The USDA and the food industry work to find a balance to continue to offer wholesome, nutritious meat products while reducing the risk of illness from *Salmonella* for humans. The established performance standards measure the prevalence of *Salmonella*, but the risk of human illness is associated with the amount of *Salmonella* in a product and its virulence.

There is a need in the poultry and beef industry for a rapid method to identify the risk of *Salmonella* in food samples based on virulence or pathogenicity, instead of prevalence alone.

PURPOSE:

1. Development of a real-time PCR assay to detect *Salmonella* in relevant food samples based on genes associated with pathogenicity as researched by USDA.
2. Evaluate the performance of the assay in detecting the pathogenicity of samples previously characterized by USDA as highly pathogenic *Salmonella* (HPS).
3. Demonstrate the inclusivity and exclusivity of the assay.

REGISTERED TRADEMARKS

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METHODS:

The USDA Agriculture Research Service (USDA-ARS) conducted a study to characterize differences in virulence gene content among *Salmonella* serovars most commonly linked to outbreaks and human illness, and serovars more commonly found as commensals associated with food animals. Primers and probes for the PCR assay were selected based on the virulence genes and targets researched by USDA-ARS and shared via a CRADA. A gel-based PCR assay was developed by USDA-ARS, and a real-time lyophilized PCR assay was developed by Hygiena®.

The lyophilized PCR assay was developed to detect three HPS targets and *Salmonella* spp. In order to check the results regarding PCR inhibition, an internal control was added as a fifth target. The assay was tested on FSIS *Salmonella*-positive enrichments and isolates prepared by USDA from each of the four major commodities: chicken, turkey, pork, and beef.

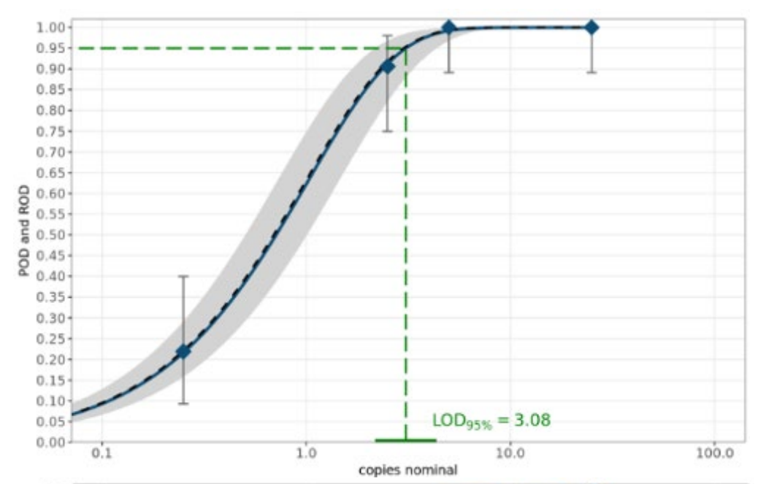
In this study, 30 turkey samples, 36 chicken samples, 33 pork samples, and 30 beef samples were evaluated using the newly developed real-time PCR assay.

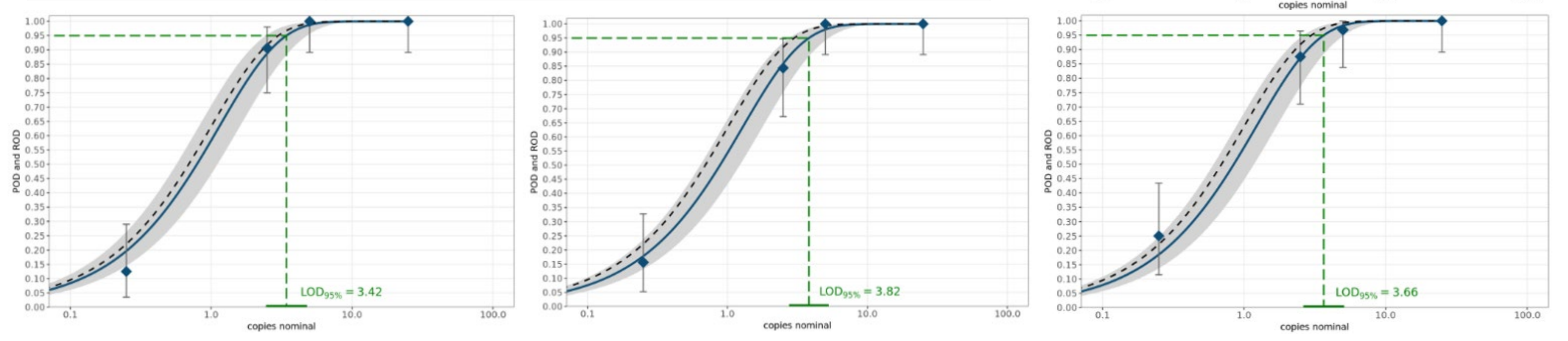
Pure, RNA-free DNA of *Salmonella* Enteritidis and *Salmonella* Dublin was quantified by droplet dPCR (dPCR; Stilla, naica® instrument) and used in a POD approach to estimate the absolute $LOD_{95\%}$. DNA concentrations of 25, 5, 2.5, 0.25, and 0.025 copies/reaction were tested in 32 replicates each.

RESULTS:

Sensitivity (Absolute Limit of Detection ($LOD_{95\%}$))

Table 1. $LOD_{95\%}$ in Copies/reaction	
HPS-B	3.08
HPS-A	3.42
HPS-X	3.82
***Salmonella* spp.**	3.66





S. Enteritidis	Number of positive vs total number of samples; DNA Concentration [copies/µL; (copies/reaction)] (CV [%])				
Target	1 (25)	0.2 (5)	0.1 (2.5)	0.01 (0.25)	0.001 (0.025)
HPS-B	16/16; 32.29 (0.41)	16/16; 34.59 (1.30)	13/16; 33.82 (1.04)	4/16; 36.43 (0.51)	0/16; -
HPS-A	16/16; 32.82 (0.23)	16/16; 34.96 (0.83)	15/16; 36.14 (1.34)	2/16; 36.96 (0.10)	0/16; -
HPS-X	16/16; 30.81 (0.27)	16/16; 32.78 (0.94)	13/16; 33.82 (1.04)	3/16; 36.12 (2.53)	0/16; -
Salm. spp.	16/16; 32.02 (0.42)	15/16; 34.76 (1.00)	15/16; 35.34 (0.78)	4/16; 36.37 (0.69)	0/16; -
IPC	16/16; 29.36 (0.34)	16/16; 29.22 (0.36)	16/16; 29.37 (0.29)	16/16; 29.51 (0.50)	16/16; 29.91 (0.23)

S. Dublin	Number of positive vs total number of samples; DNA Concentration [copies/µL; (copies/reaction)] (CV [%])				
Target	1 (25)	0.2 (5)	0.1 (2.5)	0.01 (0.25)	0.001 (0.025)
HPS-B	16/16; 30.93 (0.47)	16/16; 32.85 (0.71)	16/16; 33.86 (0.89)	3/16; 35.77 (0.62)	0/16; -
HPS-A	16/16; 31.72 (0.30)	16/16; 33.45 (0.58)	14/16; 34.44 (0.67)	2/16; 34.93 (0.24)	0/16; -
HPS-X	16/16; 29.80 (0.69)	16/16; 32.16 (0.60)	14/16; 32.87 (0.65)	2/16; 32.77 (0.61)	0/16; -
Salm. spp.	16/16; 31.33 (0.62)	16/16; 33.70 (0.77)	13/16; 34.98 (0.86)	4/16; 35.95 (0.82)	0/16; -
IPC	16/16; 30.20 (0.83)	16/16; 30.09 (0.62)	16/16; 30.17 (0.75)	16/16; 30.11 (1.12)	16/16; 30.20 (0.77)

COMPARISON OF USDA ARS GEL-BASED PCR WITH REAL-TIME ASSAY FOR ISOLATES:

Table 2. Comparison of Turkey Samples (n = 30)				
	HPS-B	**HPS-A**	**HPS-X**	***Salmonella* spp.**
Agreement	30	30	28	30
Deviation	0	0	2	0
Accuracy [%]	100.0	100.0	93.3	100.0

Table 3. Comparison of Beef Samples (n = 30)				
	HPS-B	**HPS-A**	**HPS-X**	***Salmonella* spp.**
Agreement	30	29	30	30
Deviation	0	1	0	0
Accuracy [%]	100.0	96.7	100.0	100.0

Table 4. Comparison of Chicken Samples (n = 36)				
	HPS-B	**HPS-A**	**HPS-X**	***Salmonella* spp.**
Agreement	35	36	36	36
Deviation	1	0	0	0
Accuracy [%]	97.2	100.0	100.0	100.0

Table 4. Comparison of Pork Samples (n = 33)				
	HPS-B	**HPS-A**	**HPS-X**	***Salmonella* spp.**
Agreement	33	33	33	33
Deviation	0	0	0	0
Accuracy [%]	100.0	100.0	100.0	100.0

RESULTS:

INCLUSIVITY & EXCLUSIVITY

Specificity studies yielded 100% inclusivity on 204 *Salmonella* serovars (Table 5) and 100% exclusivity on 53 closely related species of *Enterobacteriaceae*.
The data from the specificity study with serovars from the in-house culture collection also show 100% agreement with the USDA-ARS gel-based PCR.

Table 5: Comparison of gel-based PCR vs. real-time PCR assay for specificity study

		Original primer sets [% positive]			HPS real-time PCR kit [% positive]			
Serotype	Number tested	HPS-B	HPS-A	HPS-X	HPS-B	HPS-A	HPS-X	S. spp.
4,[5],12:i:-	2	100	100	100	100	100	100	100
Abaetetuba	1	0	0	0	0	0	0	100
Aberdeen	1	0	100	100	0	100	100	100
Abony	2	0	100	100	0	100	100	100
Agona	1	0	100	0	0	100	0	100
Anatum	3	0	0	67	0	0	67	100
Bardo	1	100	100	100	100	100	100	100
Bareilly	2	0	0	100	0	0	100	100
Blockley	4	100	100	100	100	100	100	100
Bovismorbificans	2	0	100	100	0	100	100	100
Brandenburg	3	0	0	0	0	0	0	100
Brenderup	1	100	100	100	100	100	100	100
Cerro	2	0	0	0	0	0	0	100
Choleraesius	1	100	100	100	100	100	100	100
Concord	1	0	100	100	0	100	100	100
Cottbus	1	0	0	100	0	0	100	100
Crossness	1	0	100	100	0	100	100	100
Cubana	1	0	100	0	0	100	0	100
Derby	3	0	33	0	0	33	0	100
Dublin	8	88	100	88	88	100	88	100
Enteritidis	3	100	100	100	100	100	100	100
Gallinarum	3	100	100	100	100	100	100	100
Gallinarum-Pullorum	3	100	100	100	100	100	100	100
Hadar	5	60	100	100	60	100	100	100
Havana	1	100	100	0	100	100	0	100
Heidelberg	6	0	100	100	0	100	100	100
Hvittingfoos	1	0	100	100	0	100	100	100
Infantis	11	0	100	100	0	100	100	100
Johannisburg	1	100	0	0	100	0	0	100
Kentucky	1	0	100	0	0	100	0	100
Krefeld	1	0	100	0	0	100	0	100
Lille	1	0	100	0	0	100	0	100
Litchfield	1	100	100	100	100	100	100	100
Livingstone	4	0	50	25	0	50	25	100
London	1	0	100	100	0	100	100	100

		Original primer sets [% positive]			HPS real-time PCR kit [% positive]			
Serotype	Number tested	HPS-B	HPS-A	HPS-X	HPS-B	HPS-A	HPS-X	S. spp.
Manhattan	3	33	100	100	33	100	100	100
Mbanaka	1	0	100	0	0	100	0	100
Meleagridis	1	0	100	0	0	100	0	100
Mississippi	1	100	100	100	100	100	100	100
Montevideo	7	0	14	0	0	14	0	100
Muenchen	1	0	0	100	0	0	100	100
Münster	2	0	0	0	0	0	0	100
Napoli	1	0	0	100	0	0	100	100
Newport	6	0	100	100	0	100	100	100
Oranienburg	1	0	0	0	0	0	0	100
Paratyphi	4	0	0	0	0	0	0	100
Paratyphi B	9	11	22	33	11	22	33	100
Paratyphi C	1	100	100	0	100	100	0	100
Reading	3	0	33	33	0	33	33	100
Rissen	1	0	100	0	0	100	0	100
S. bongori	2	50	100	0	50	100	0	100
S. enterica subsp. *arizonae*	10	0	0	0	0	0	0	100
S. enterica subsp. *diarizonae*	8	13	0	0	13	0	0	100
S. enterica subsp. *enterica*	6	33	67	33	33	67	33	100
S. enterica subsp. *houtenae*	7	0	0	100	0	0	100	100
S. enterica subsp. *indica*	2	50	0	0	50	0	0	100
S. enterica subsp. *salamae*	8	13	13	0	13	13	0	100
Saintpaul	3	0	100	100	0	100	100	100
Senftenberg	14	0	100	0	0	100	0	100
Stanley	1	0	100	0	0	100	0	100
Stanleyville	1	0	100	0	0	100	0	100
Tennessee	1	0	100	0	0	100	0	100
Thompson	2	100	100	100	100	100	100	100
Typhimurium	5	100	100	100	100	100	100	100
Uganda	2	0	0	100	0	0	100	100
Virchow	2	0	100	100	0	100	100	100
Waycross	1	0	0	0	0	0	0	100
Weltevreden	2	100	0	100	100	0	100	100
Westhampton	1	0	100	0	0	100	0	100
	204							

SIGNIFICANCE:

The results demonstrate that highly pathogenic *Salmonella* can be quickly detected in meat and poultry using this next-generation method for *Salmonella* detection. This approach to *Salmonella* testing can enable the industry to make risk-based decisions about their product by offering an indication of when product has more infectious potential. It would help achieve the objective of simultaneously reducing 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
2. Response to Questions Posted by the Food Safety and Inspection Service: Enhancing *Salmonella* Control in Poultry Products, Journal of Food Protection, 87, 2024 https://www.fsis.usda.gov/sites/default/files/media_file/documents/NACMCF_Salmonella_2023.pdf

The research presented was conducted by scientists with the USDA-ARS US Meat Animal Research Center (USMARC). A Cooperative Research and Development Agreement (CRADA No. 58-3040-1-009) is established between USDA and Hygiena® LLC for the development of the HPS markers into a real-time assay intended for the BAX® System.