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Research Article

Phage-Based Assay for the Detection of *Salmonella* in Brazilian Poultry Products

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Abstract

Salmonella is one of the most common microorganisms responsible for foodborne diseases worldwide, and its rapid and accurate detection is necessary for food safety. Bacteriophages are a promising tool for detecting bacterial foodborne pathogens due to their safety, specificity, rapid propagation, and capacity to differentiate living and dead cells. The PhageDx *Salmonella* Assay is a new *Salmonella* detection method composed of recombinant bacteriophages encoding a luciferase reporter gene. While this method has been validated in the United States to detect *Salmonella* in ground turkey and powdered infant formula, it has not been validated in other countries, and its performance in other matrices is unknown. In this study, the performance of the PhageDx *Salmonella* Assay was evaluated using *Salmonella* strains isolated in Brazil. 55 isolates from food and food processing environments in Brazil were examined and successfully detected using the recombinant bacteriophages employed by this method. As Brazil is the number one exporter of chicken globally, this method was also validated in several chicken-based food matrices. Using a pre-

enrichment of 7 hours, it was possible to detect one CFU per 25 g on chicken meat, sausage, pâté, and chicken nuggets. The total analysis time was 9 hours, shorter than other *Salmonella* detection methods currently available. The method proved to be easy to execute, sensitive, and fast, making it a promising tool for the Brazilian poultry industry.

Keywords: Bacteriophage; Diagnostics; Food; Chicken; Salmonellosis; Bacteria

1. Introduction

Salmonella is one of the major foodborne pathogens worldwide [1]. Each year, 153 million cases of gastroenteritis and 57,000 deaths caused by nontyphoidal salmonellae (NTS) are estimated globally [2]. In addition, NTS is the foodborne bacterial zoonosis most recurrent in Brazil [3]. *Salmonella* is a significant problem for food production and public health [4]. In many countries, the limit in Food Safety Criteria for *Salmonella* is the absence of the pathogen in 10 g or 25 g of food [5-8]. Detection methods for *Salmonella* must be accurate and sensitive enough to detect a single colony-forming unit (CFU) in each sample. The time required to carry out the analysis and determine the presence of pathogen is one of the most important factors to be considered when choosing a detection method. Traditional culture methods produce a negative result after approximately three days, while a positive result may need ten or more days to identify certain *Salmonella* serovars. Rapid methods based on molecular biology or immunoenzymatic reactions need approximately 24 to 30 h to detect *Salmonella* [9,10], and positive results need to be confirmed by the traditional methods, resulting in additional time [11]. The use of bacteriophages (phages) to detect foodborne pathogens has garnered increased interest in recent years [12-14]. Several characteristics of phages make them very useful in commercial methods for food pathogen detection. Evolving alongside their hosts, the host range of each phage may vary from an entire bacterial genus (broad) to only a few specific strains within a species (narrow). The natural host range of each phage can be exploited to provide the desired specificity to a detection assay. In terms of sensitivity, bacteriophage have a short lifecycle, typically about one to two hours, facilitating rapid detection of the presence or the absence of host pathogens. Another benefit of phage is that viable bacteria are needed for their replication. This means that phage-based detection methods can differentiate between living and dead pathogens [15-17]. Finally, phages are widely considered safe and do not pose a health risk following exposure [18]. Thus, bacteriophages are a promising tool for the rapid detection of bacterial foodborne pathogens. Although phages can be used in several ways, one approach for detection utilizes genetically modified reporter phage. In this method, wild-type phages are engineered to carry a reporter gene which, after infection, is expressed and can be measured, for example, by bioluminescence or fluorescence [15]. As exogenous genes are expressed when target pathogen cells are infected, they produce an easily detectable signal for rapid identification of bacterial hosts [17]. One example of a reporter is NanoLuc®, a luciferase engineered by Promega™ from the deep-sea shrimp (*Oplophorus gracilirostris*). NanoLuc® is a 19 kDa protein that utilizes imidazopyrazinone substrate (furimazine) in an ATP-independent reaction to generate a signal that is 150 fold brighter than either firefly or Renilla luciferase [19]. The PhageDx *Salmonella* Assay is a recently published and validated phage-based method for *Salmonella* detection in food [20]. This kit contains recombinant phages that have incorporated NanoLuc® luciferase gene into their genome. This method was previously shown to broadly detect all *Salmonella* species and could accurately identify the presence of *Salmonella*. Additionally, the

method was confirmed to work in two matrices, ground turkey and powdered infant formula. The performance of the PhageDx *Salmonella* Assay in other matrices is unknown, and further validation is needed to facilitate the broader use of this technology. Brazil is currently the largest exporter of chicken in the world [21]. Therefore, the ability to verify the safety of chicken-based food products with a rapid and accurate method would be a great benefit to the Brazilian poultry industry. In this study, we assessed the PhageDx *Salmonella* Assay for this purpose. The assay was challenged with Brazilian isolates of *Salmonella*, and the performance of this method was examined in various chicken-based food matrices.

2. Materials and Methods

2.1 PhageDx Salmonella Assay

The PhageDx Salmonella Assay is a new method developed by the Laboratory Corporation of America (LabCorp) and registered in AOAC® (Certificate No 121904). This assay comprises two recombinant bacteriophages that have had the NanoLuc® gene inserted in their genome by homologous recombination. They were individually tested in work performed by Nguyen et al. upon contact with samples contaminated with Salmonella, the phages will express the NanoLuc® luciferase, and the pathogen can be detected in a luminometer. In previous tests [20], it was determined that readings of 750 relative light units (RLU) in the luminometer indicate the presence of Salmonella, and readings below this value indicate the absence of the pathogen. The bioluminescence test performed in this work is further detailed below in 2.4.

2.2 Inclusivity test

To evaluate the ability of the assay to detect *Salmonella* strains circulating in Brazil, various *Salmonella* serovars isolated from Brazil were used. Initially, all isolates used in this work were confirmed by a Real-time PCR developed by Souza et al. to identify *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg. Amplification conditions were as described in the above work. Samples that presented cycle threshold (Ct) values lower than 20 were confirmed as *Salmonella*. So, 55 strains isolated from foods and food-related salmonellosis outbreaks were chosen (Table 1). Inclusivity test was assessed using the same method previously described for this kit to determine if the Assay phages infected these strains [20]. *Salmonella* strains were cultured overnight in 5 ml of Tryptic Soy Broth - TSB (Kasvi, São José do Pinhais, Brasil) at 37 ºC, then diluted to an OD_{600} of 0.2 as measured in microplate reader (Loccus LMR 96, Brasil), equivalent to approximately 10^8 CFU/ml. Cell counts were confirmed by plating on Tryptic Soy Agar-TSA (Kasvi, São José do Pinhais, Brasil). Following dilution, stationary-phase cells were infected without pre-enrichment, as described in 2.4.

2.3 *Salmonella* detection in chicken and chicken-based food products

A cocktail of *Salmonella* strains was prepared for testing food matrices. We simulated a scenario for foods that could be contaminated with more than one serovar. So, six serovars were cultured overnight as described in 2.2. The serovars included in the cocktail were *S.* Minnesota (MIN_FOOD), *S.* Enteritidis (SE86), *S.* Saintpaul (SP_BOVINE), *S.* Infantis (IF 70), *S.* Heidelberg (121), and *S.* Typhimurium (17131). One ml of each culture was added to a tube, and the pooled sample was centrifuged, at 4°C, for 10 min at 2810x *g* (CIENTEC CT-5000R, Brazil). The supernatant was then discarded, and the pellet was washed three times with sterile 0.1% peptone water. After the final wash, cells were re-suspended in sterile 0.1% peptone water to a concentration of OD_{600} of 0.2 or approximately 10^8 CFU/ml. Cell counts were confirmed by plating on TSA. The *Salmonella* cocktail

was then serially diluted with sterile 0.1% peptone water to 10^2, 10^1, and 10^0 CFU/ml. All food samples, poultry meat, poultry sausage, chicken *pâté*, and chicken nuggets, were purshed at the supermarket of Porto Alegre/Brazil. Before the test, they were previously tested to ensure the absence of *Salmonella* (ISO 6579-1:2017) [23]. To determine assay performance in each matrix, 25 g of each type of food were placed inside a Whirl-Pak® sterile filter bag (Nasco, Fort Atkinson, WI, USA) and 1 ml of 1, 10, or 100 CFU/ml dilutions of the *Salmonella* pool was added. 75 ml of pre-warmed (41±1ºC) Buffered Peptone Water - BPW (Merck, Darmstadt, Germany) was then added, and the sample was blended on a stomacher (Stomacher® 400, Seward, England) for 30s. The samples were incubated at 41±1ºC for 7 h, followed by bioluminescence assay as described in 2.4. 7 h of pre-enrichment was chosen to mirror the duration of enrichment used previously in the closest validated matrix, ground turkey.

2.4 Bioluminescence assay

Bioluminescence assay was performed using either 100 µl (for inclusivity) or 150 µl (for food matrices) of samples prepared according to sections 2.2 and 2.3, respectively. Each sample was added separately to a well of a 96-well white plate (Thermo Scientific™, Massachusetts, USA) and 10 µl of the recombinant phage cocktail from the PhageDx *Salmonella* Assay were added to each well following 2 h incubation at 37 °C. While the samples were incubating, the lysis/luciferase master mix was prepared. This reagent (Nano-Glo® Luciferase Assay System, Promega Corp., Madison, WI) is composed of 50 µl of Assay Buffer, 15 µl 5X Lysis Buffer and 1 µl Luciferase Substrate. After the 2 h incubation, 65 µl of lysis/luciferase master mix were added to each well, and the 96-well plates were read immediately in a GloMax® Navigator Luminometer (Promega, Fitchburg, USA) with the following parameters: 3 min delay, 1 s integration, and two reads. Samples were evaluated using a cut-off of 750 RLU, as recommended by the manufacturer. For the inclusivity test, negative controls consisted of TSB (Kasvi, Brazil), recombinant phage cocktail, and lysis/luciferase master mix (Nano-Glo® Luciferase Assay System, Promega Corp., Madison, WI). For the detection of chicken and chicken-based food products, negative controls were composed of the uninoculated food matrix added of BPW (Merck, Darmstadt, Germany), recombinant phages cocktail, and lysis/luciferase master mix (Nano-Glo® Luciferase Assay System, Promega Corp., Madison, WI). All assays were performed in triplicate. Each *Salmonella* strain was tested 6 times, and for the food samples, the low and medium inoculum (1 and 10 CFU/ml) were tested 30 times, and the high inoculum (100 CFU/ml) was tested 12 times. Means were calculated using Excel® version 2016 (Microsoft Co., Ltd. Redmond, WA., EUA).

3. Results

3.1 Inclusivity test

All 55 Brazilian *Salmonella* strains tested were detected by the phage cocktail, as shown in Table 1. We observed differences in signal intensity between the strains (Table 1). RLU values ranged from 750 (cut-off for a positive sample, according to the Assay producer) to 10^9. The strains of *S.* Enteritidis produced RLU numbers ranging from 10^7-10^9, except strain 4135, which produced 10^4 to 10^6 RLU. *S.* Heidelberg strains demonstrated greater variation in RLU production, ranging from 750 to 10^9. Strain *S.* Heidelberg 507 had the highest range of signals (10^7 – 10^9 RLU). Strains *S.* Heidelberg 124 and 126 generated RLU from 750 to 10^3, and the others *S.* Heidelberg strains produced 10^4 to 10^6 RLU. All strains in this group were isolated from chicken carcasses or the poultry processing environment. *S.* Hadar produced 10^4-10^6 RLU. All

strains of *S.* Typhimurium, *S.* Infantis, *S.* Minnesota, *S.* Newport, *S.* Saintpaul, and *S.* Anatum produced 10^7 to 10^9 RLU.

Serotype	Strain identification	RLU*	Source
Enteritidis (n= 25)	4953	+ + + +	Cake with confetti
	4955	+ + + +	Fried pastel
	4979	+ + + +	Mayonnaise salad
	2476	+ + + +	Ground beef
	4515	+ + + +	*Unknown food*
	8667	+ + + +	*Unknown food*
	9477	+ + + +	*Unknown food*
	11181	+ + + +	*Unknown food*
	427	+ + + +	Roasted beef
	540	+ + + +	Bacon
	544	+ + + +	Ham
	547	+ + + +	Tomato
	1199	+ + + +	Roasted Pork and beef frankfurter
	1581	+ + + +	Homemade mayonnaise
	8166	+ + + +	Beef
	17255	+ + + +	*Unknown food*
	SE86	+ + + +	Chicken cake
	1409	+ + + +	Cake
	1410	+ + + +	Cake
	4135	+ + +	*Unknown food*
	4787	+ + + +	Homemade mayonnaise
	6383	+ + + +	Cookie cake
	8596	+ + + +	Rice
	9667	+ + + +	*Unknown food*
	340	+ + + +	*Unknown food*
Heidelberg (n= 16)	112	+ + +	Chicken carcass
	118	+ + +	Chicken carcass
	410	+ + +	Chicken carcass
	506	+ + +	Chicken carcass
	507	+ + + +	Chicken carcass
	610	+ + +	Chicken carcass
	702	+ + +	Chicken carcass
	121	+ + +	Chicken slaughterhouse
	122	+ + +	Chicken slaughterhouse
	123	+ + +	Chicken slaughterhouse
	124	+ +	Chicken slaughterhouse
	125	+ + +	Chicken slaughterhouse
	126	+ +	Chicken slaughterhouse
	127	+ + +	Chicken slaughterhouse
	129	+ + +	Chicken slaughterhouse
	130	+ + +	Chicken slaughterhouse
Typhimurium	9667	+ + + +	*Unknown food*
	340	+ + + +	*Unknown food*
	9688	+ + + +	Blood sausage
	9692	+ + + +	Jelly roll
	17131	+ + + +	Shredded chicken
	5209	+ + + +	Salami
	11368/2	+ + + +	Refrigerated beef
	12037	+ + + +	Rice with chicken heart and sausage

Infantis (n=1)	IF 70	+ + + +	Lettuce
Hadar (n=1)	HD_LET	+ + +	Lettuce
Minnesota (n=1)	MIN_FOOD	+ + + +	*Unknown food*
Newport (n=1)	NP_BOVINE	+ + + +	Bovine hide
Saint Paul (n=1)	SP_BOVINE	+ + + +	Bovine hide
Anatum (n=1)	AT_BOVINE	+ + + +	Bovine hide

Table 1: Detection of Brazilian *Salmonella* strains isolated from food and food-related salmonellosis outbreaks at concentration 10^8 CFU/ml by the PhageDx *Salmonella* Assay.

*Number of plus signs indicates light emission in Relative Light Unit (RLU): + +, 750 – 10^3; + + +, 10^4 – 10^6; + + + +, 10^7 – 10^9. Overnight growth of *Salmonella* strains was standardized to 10^8 CFU/mL in TSB. After that, the strains were submitted to 2- hour infection with the phage cocktail, so then the reagents were added, and the reading was done in a luminometer.

3.2 Detection of *Salmonella* in chicken-based food matrices

Considering the limit of detection (LOD) as the lowest amount of a target that the Assay can detect 95% of the time, the LOD for artificially contaminated chicken products was 1 CFU/25g (Table 2), before 7 hours of enrichment. The RLU obtained by the samples ranged from 1.04 x 10^5 (1 CFU/25 g of chicken meat) to 4.11 x 10^7 (100 CFU/25 g of chicken nuggets).

Food	**CFU/ 25g**					
	10^0		**10^1**		**10^2**	
	RLU	P / N*	RLU	P / N	RLU	P / N
Chicken meat	1,04E+05	30/30 (100%)	1,02E+07	30/30 (100%)	2,91E+07	12/12 (100%)
Chicken Sausage	1,90E+06	29/30 (96,6%)	1,79E+05	29/30 (96,6%)	5,26E+06	12/12 (100%)
Chicken *pâté*	1,21E+06	30/30 (100%)	2,23E+07	30/30 (100%)	4,10E+07	12/12 (100%)
Chicken Nuggets	3,88E+06	30/30 (100%)	2,89E+07	30/30 (100%)	4,11E+07	12/12 (100%)

Table 2: Evaluation of the detection limit of the PhageDx *Salmonella* Assay with 25 g of chicken-based food matrices spiked with the cocktail composed of *S*. Minnesota, *S*. Enteritidis, *S*. Saint Paul, *S*. Infantis, *S*. Heidelberg and *S*. Typhimurium serovars, after 7 h of enrichment.

*P and N represent the sum of all positive samples detected and the sum of all samples analyzed in triplicates, respectively. RLU (Relative Light Unit) were calculated from the means obtained from the 30 readings for the low and medium inoculum and 12 readings for the highest inoculum. 25 g of each food type were contaminated with 1, 10 or 100 CFU of the *Salmonella* cocktail. After 7-hour incubation, 150 µl of the samples were incubated for 2 hours with the PhageDx *Salmonella* Assay kit phages. After this period, the reagents were added and the RLU was read in the luminometer.

4. Discussion

The PhageDx *Salmonella* Assay features a cocktail of two recombinant bacteriophages, each with different specificity and sensitivity. In previous studies carried out by Nguyen et al. these phages, SEA1.NL and TSP1.NL, were able to identify 267 (99%) and 135 (50%) of 269 strains of *Salmonella*, respectively. Importantly, *Salmonella* strains tested in that study were primarily from stock collections or isolates from the United States. The ability of this phage cocktail to detect Brazilian strains was thus unknown. Therefore, we evaluated the phage cocktail featured in this kit to detect diverse *Salmonella* strains isolated from food samples and suspected food-related outbreaks in Southern Brazil. Sources included cake with confetti, fried savory pastry (Brazilian pastel), mayonnaise salad, ground beef, roasted beef, bacon, ham, tomato, roasted pork, and beef frankfurter, homemade mayonnaise, beef, chicken cake, cake, cookie cake, rice, chicken carcass, blood sausage, jelly roll, shredded chicken, salami, refrigerated raw beef, rice with chicken heart and sausage, lettuce, and bovine's hide. Additionally, strains were also obtained from a chicken slaughterhouse to represent microorganisms isolated from a Brazilian poultry processing environment. Our results indicate that the phage cocktail of SEA1.NL and TSP1.NL presents in the PhageDx *Salmonella* Assay provides coverage over *Salmonella* strains circulating in Brazil. Furthermore, in a recent work carried out by Mascitti et al. it was found that all *S*. Enteritidis used in our work are part of the same monophyletic group (descended from a single ancestor), as another global epidemic lineage from around the world strains. In addition, all the strains had antimicrobial resistance genes (ARGs), such as: *aac(6')-Iaa*, *mdfA*, and *tet(34)*. These findings are important to demonstrate that the kit is able to detect important strains of *Salmonella* involved in public health cases at a global level. In our work, and in the work of Nguyen et al. it was observed that the RLU emitted during the tests may vary both within strains of the same serovar, as well as within strains of different serovars. Numerous factors may influence the success of the bacteriophage infection process and may influence the ability to detect the target pathogen, and the RLU produced. Absorption between phage-binding proteins and receptors on the bacterial surface is the first step of infection and represents the phage's ability to recognize its host and its specificity concerning the scope of target detection (strains, species or genus) [25]. This step can be compromised if the bacterial cells lose the receptor that would act as a phage-host binding site. Even if absorption does occurs, other obstacles may be present, such as degradation of genetic material inserted by the phage or mutations in the cells that prevent phage replication [26]. Additionally, to have sufficient luminescence in the sample to be distinguished from the background, phages must infect the target microorganism and produce the phage-encoded reporter (NanoLuc®). Production of phage proteins is also likely to be dependent on numerous factors, such as the growth rate of the bacteria, further contributing to signal variation between strains. Despite the observed variation in signal, it is important to highlight that all strains in this study could be detected with this phage cocktail. Furthermore, 38 of the 55 strains analyzed obtained RLU in the highest range observed, 10^7 - 10^9 RLU. The backgrounds of the assays were low and easy to be recognized. These high RLU values observed in positive samples and the low background values observed in negative controls are important during interpretations of results by operators. Meile et al. tested four luciferases, *luxAB (Vibrio harveyi), gluc (Gaussia princeps), rluc (Renilla reniformis),* and *nluc (Oplophorus gracilirostris)* (Promega, Fitchburg, USA) for reporter phage construction for *Listeria* detection. As in other studies described previously [27,19], NLuc was a highly stable

enzyme that produced strong bioluminescence. Brazil is the largest exporter of chicken meat in the world [21] and poultry products are a major source of *Salmonella* contamination [28]. Rapid and accurate detection of *Salmonella* in these matrices is thus of significant importance to facilitate the timely and safe release of Brazilian poultry products into the domestic and international markets. This study has chosen four chicken products to test the sensitivity of the PhageDx *Salmonella* Assay, meat, sausage, pâté, and nuggets. The detection limit of the Assay was assessed by artificially contaminating these matrices with a pool of Brazilian *Salmonella* strains at three inoculum concentrations (10^0, 10^1, and 10^2 CFU/25 g). The LOD found in our work demonstrates that the Assay was able to detect one CFU of Salmonella spp. per 25 g on chicken products at the same day, after 7 h of enrichment and 2 h of phage infection. This detection level follows the zero-tolerance policy requirement, that is, it detects one CFU in 25 g of spiked food. This result is also important since traditional methods require at least 72 h and the rapid methods at least 24 h for *Salmonella* analysis [29]. These results are in agreement with the results found by Nguyen et al. In their study, the LOD of *Salmonella* was 1 CFU in 25 g of ground turkey with a 7 h enrichment and 100 g of powdered infant formula with a 16 h enrichment. Meile et al. developed engineered NLuc-based reporter phages for the detection of *Listeria*. The phage A511::nlucCPS detected 1 CFU of *L. monocytogenes* in 25 g of artificially contaminated milk, cold cuts, and lettuce within less than 24 h. The sensitivity of nluc-reporter phages was also evaluated by Zelcbuch et al. (2021). The LOD in their work was 10^3 cells of *Klebsiella pneumoniae* per 1 g of fecal matter. It is also important to comment on variations in food compositions, although we observed that the matrix influenced the number of RLUs emitted, this was not enough to interfere in the Assay background (data not shown). The means obtained from unspiked foods (negative control) were 267 (chicken meat), 71 (sausage), 338 (pâté), and 198 (nuggets). In other works, it can also be observed that samples of different non-inoculated foods had results below the background, although they varied among themselves. Furthermore, in the data of Table 2, it can be noted that the RLU values, even at the lowest inoculum concentrations, are easily distinguishable from the negative controls.

5. Conclusion

The recombinant bacteriophage method (PhageDx *Salmonella* Assay) evaluated in our study was able to detect all tested *Salmonella* strains. These strains were isolated from food-related industries in Brazil. Additionally, the Assay could detect 1 CFU/25g in only 9h of assay in chicken products. The total time analysis demonstrated in the present study represents a significant reduction in time of analysis compared to other technologies currently available. Furthermore, the fact that this Assay can produce positive results in the same day represents a significant advantage for routine analysis of *Salmonella*. Critically, our study extends upon previous work and validates the performance of this phage-based Assay with Brazilian *Salmonella* isolates and in different chicken-based food matrices.

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Authors' Contributions

Nathanyelle Soraya Martins de Aquino: conceptualization, investigation, methodology, project administration, writing-original draft and writing-review and editing. Susana de Oliveira Elias: Methodology, writing-original and writing-review and editing. Leonardo Vaz Alves Gomes: Formal analysis and

investigation. Eduardo Cesar Tondo: Methodology, supervision, writing-original draft and writing-review and editing. All authors have read and agreed to the published version of the manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

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