UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

Uso de Bacteriófagos Recombinantes para Detecção Rápida de *Escherichia coli* O157:H7 e *Salmonella* spp. em Alimentos

Nathanyelle Soraya Martins de Aquino

Porto Alegre
2021

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USO DE BACTERIÓFAGOS RECOMBINANTES PARA DETECÇÃO RÁPIDA DE *ESCHERICHIA COLI* O157:H7 E *SALMONELLA* SPP. EM ALIMENTOS

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul, como parte dos requisitos necessários à obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Eduardo Cesar Tondo

Porto Alegre

2021

CIP - Catalogação na Publicação

DE AQUINO, NATHANYELLE SORAYA MARTINS
Uso de Bacteriófagos Recombinantes para Detecção Rápida de Escherichia coli O157:H7 e Salmonella spp. em Alimentos / NATHANYELLE SORAYA MARTINS DE AQUINO. -- 2021.
137 f.
Orientador: EDUARDO CESAR TONDO.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Porto Alegre, BR-RS, 2021.

1. SALMONELLA. 2. E. COLI O157:H7. 3. BACTERIÓFAGOS. 4. MÉTODOS DE DETECÇÃO. 5. SEGURANÇA DE ALIMENTOS. I. TONDO, EDUARDO CESAR, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

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Título da tese: Uso de Bacteriófagos Recombinantes para Detecção Rápida de *Escherichia coli* O157:H7 e *Salmonella* spp. em alimentos

Submetida como parte dos requisitos para obtenção do grau de

DOUTORA EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

Aprovada em:_____ /_____ /_____

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Dedico este trabalho aos queridos Natanael Aquino (in memorian), Socorro Aquino (in memorian) e Maria Aquino (in memorian). Eu ainda sinto o amor de vocês por mim.

AGRADECIMENTOS

Agradeço a Deus, primeiramente, por estar viva e por me guiar nesta etapa da minha vida, assim como tem feito sempre.

Agradeço aos meus pais que são os meus maiores incentivadores na vida. Obrigada por acreditarem em mim, por terem me permitido sair de casa para tentar alçar voos maiores. Sem o apoio de vocês eu não teria conseguido.

Agradeço ao meu irmão por cuidar dos nossos pais. Sentir o seu apoio sempre que compartilho alguma pequena vitória é muito importante para mim. Agradeço também à minha cunhada pelo incentivo e por estar perto da minha família.

Agradeço à minha vó Assunção, pelas orações, pela preocupação e pelo apoio financeiro. Agradeço também a todos os meus tios, tias, primos e primas pelo carinho, pela torcida e pela recepção maravilhosa sempre que eu volto para casa.

Agradeço à minha orientadora de mestrado e amiga, profª Laura, por ser meu primeiro incentivo e apoio para fazer a seleção do doutorado.

Agradeço ao meu querido orientador profº Eduardo Tondo, pelo seu apoio durante os últimos 4 anos. Obrigada pelo tempo dedicado à minha orientação desde o início até a correção deste trabalho.

Agradeço aos meus colegas do laboratório de Microbiololgia e Controle de Alimentos pelas palavras de incentivo, pela ajuda intelectual e pelos inúmeros momentos divertidos que tornaram a caminhada mais leve. Em especial gostaria de agradecer à Vera Masutti pela amizade e por todo o esforço para que os trâmites legais dessa parceria pudessem ser executados. Obrigada também à Susana Elias por estar comigo do início ao fim desta pesquisa e à Fabíola pela prontidão sempre que eu precisei.

Agradeço à minha família de Porto Alegre, que foram os primeiros a me acolher e me dar suporte nessa fase que se iniciou em 2017. Jovênia, Carla, Gabi, Carol e Fábio, obrigada por abrirem as portas e me deixar passar esse tempo incrível com vocês. Jô obrigada por ser como uma mãe, obrigada pelo incentivo e pelo suporte. Carla obrigada por sua alegria contagiante, por sua motivação e também por sempre vibrar com as minhas conquistas. Obrigada Judite, pelo seu apoio desde o mestrado, obrigada pelo carinho, pelo incentivo e por cuidar da Margot todo esse tempo.

Agradeço aos meus amigos do GYPSY, Débora, Diego, Daniel, Giovana, Maria Emília, Gabriel, Maria, Ju e Camily. Muito obrigada pela parceria e por terem me ajudado a manter a sanidade durante esse tempo de isolamento social. Em especial à Maria por ter desenhado as artes deste trabalho.

Agradeço à empresa financiadora desta pesquisa, LabCorp, pelo aporte financeiro e por ter nos confiado este projeto tão importante para a empresa. Em especial gostaria de agradecer aos pesquisadores Steve Erickson e Minh Nguyen pelos treinamentos, apoio intelectual e por estarem sempre disponíveis a contribuir.

Agradeço à UFRGS, ICTA e PPGCTA pela cessão de laboratórios, pela estrutura física e pelo corpo de funcionários. À banca examinadora deste trabalho pelo tempo dedicado e pelas contribuições.

Agradeço à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos durante o curso do doutorado.

RESUMO

Escherichia coli O157:H7 e *Salmonella* spp. são dois dos mais importantes microrganismos causadores de Doenças Transmitidas por Alimentos (DTA) no mundo. Nos últimos anos, o Brasil vem liderando as exportações mundiais de carne bovina e avícola, alimentos que podem ser veículos destes patógenos. Antes da exportação, esses alimentos devem ser testados para garantir a ausência dos microrganismos citados e, quanto menor for o tempo de análise, maior é a vantagem competitiva das empresas exportadoras. Neste estudo, foram avaliados dois novos ensaios para detecção de *E. coli* O157:H7 e *Salmonella* spp. em alimentos. PhageDx *E. coli* O157:H7 Assay e PhageDx *Salmonella* Assay são métodos baseados no uso de bacteriófagos recombinantes, capazes de detectar *E. coli* O157:H7 e *Salmonella* spp*.* em tempos menores que outros métodos. Até o presente estudo, esses *kits* não haviam sido testados para a detecção de cepas brasileiras. Logo, o objetivo deste trabalho foi avaliar a especificidade, limite de detecção e sensibilidade dos *kits* PhageDx *E. coli* O157:H7 Assay e PhageDx *Salmonella* Assay em detectar *E. coli* O157:H7 e *Salmonella* spp. isoladas no Brasil. Primeiramente, realizou-se o teste de inclusividade com 55 isolados de *Salmonella* spp. e 14 isolados de *E. coli* O157:H7. Em seguida, foi determinado o limite de detecção *in vitro* dos *kits*. Para isto, três concentrações diferentes (1, 10 e 100 UFC/poço de microplaca) dos microrganismos avaliados foram submetidas a 2 h de infecção fágica a 37 ºC. A terceira etapa deste trabalho foi determinar o limite de detecção dos *kits* em alimentos. Para o *kit* PhageDx *Salmonella* Assay, inoculou-se 1 mL do coquetel de *Salmonella* spp. (concentrações de 1, 10 e 100 UFC) em 25 g de alface, peito de frango, salsicha de frango, patê de frango e *nuggets.* Em seguida, as amostras foram acrescidas de 75 mL de água peptonada tamponada e enriquecidas a 41 º C por 7 h antes de serem submetidas à 2 h de infecção fágica a 37 ºC. Utilizando a mesma metodologia mencionada acima, testou-se o *kit* PhageDx *E. coli* O157:H7 Assay. Neste teste, 1 mL do coquetel de *E. coli* O157:H7 nas mesmas concentrações, foi adicionado a 25 g de carne moída e alface, e a 25 mL de leite pasteurizado e água mineral. Posteriormente, caldo triptona de soja foi adicionado às amostras e a mistura foi incubada por 5 h a 41 º C antes de serem submetidas à 2 h de infecção fágica a 37 ºC. Além disso, o PhageDx *E. coli* O157:H7 Assay teve seu desempenho comparado a um *kit* de PCR em tempo real para pesquisa de *E. coli* O157:H7 e *Escherichia coli* enterohemorrágicas, respectivamente, em carcaças bovinas. Com relação aos resultados obtidos, verificou-se que todos os isolados testados foram identificados pelo seu respectivo *kit* e o limite de detecção *in vitro* foi de 100 UFC/poço, em apenas 2 h, ou seja, sem enriquecimento das amostras. Os *kits* também foram capazes de detectar 1 UFC dos patógenos em 25 g (ou 25 mL) de alimentos, após 5 e 7 horas de enriquecimento, para *E. coli* O157:H7 e *Salmonella* spp., respectivamente. Apenas a detecção de *Salmonella* spp. em alface apresentou um limite de detecção ligeiramente superior aos demais alimentos, isso é, 10 UFC/25 g. O *kit* PhageDx *E. coli* O157:H7 Assay não detectou a presença *E. coli* O157:H7 em nenhuma das 100 carcaças bovinas avaliadas. Em concordância com este resultado, o *kit* de PCR em tempo real não detectou a presença de *E. coli* enterohemorrágica. Os ensaios avaliados demonstraram ser satisfatórios para detecção de *E. coli* O157:H7 e *Salmonella* spp. isoladas no Brasil, apresentando sensibilidade, especificidade e rapidez ao avaliarem diferentes matrizes alimentares. A detecção de uma única célula de *Salmonella* spp. em 25 g de amostra, após aproximadamente 9 h de ensaio, representa uma redução significativa de tempo

em comparação aos métodos tradicionais e rápidos disponíveis atualmente no mercado. O mesmo foi observado para o *kit* de *Escherichia coli* O157:H7, cujo tempo de análise foi de cerca de 7 h. Além disso, os *kits* demonstraram ser fáceis de executar, tornando-os ferramentas promissoras para a detecção de *E. coli* O157:H7 e *Salmonella* spp. em diferentes alimentos.

Palavras-chave: Métodos de detecção; segurança de alimentos; patógenos alimentares; bovinocultura, avicultura.

ABSTRACT

Escherichia coli O157:H7 and *Salmonella* spp. are two of the most important microorganisms causing Foodborne Diseases (FD) in the world. In recent years, Brazil has been leading world exports of beef and poultry, foods that can be vehicles for these pathogens. Before exportation, these foods must be tested to ensure the absence of the microorganisms cited, and the shorter the time of analysis, the greater the competitive advantage of exporting companies. In this study, two new assays for detection of *E. coli* O157:H7 and *Salmonella* spp. in food were evaluated. PhageDx *E. coli* O157:H7 Assay and PhageDx *Salmonella* Assay are methods consisting of recombinant bacteriophages, capable of detecting *E. coli* O157:H7 and *Salmonella* spp. in shorter times than other methods. Until the present study, these kits had not been tested for the detection of Brazilian strains. Therefore, the objective of this study was to evaluate the specificity, limit of detection and sensitivity of PhageDx *E. coli* O157:H7 Assay and PhageDx *Salmonella* Assay in detecting *E. coli* O157:H7 and *Salmonella* spp. isolated in Brazil. First, an inclusivity test was performed with 55 isolates of *Salmonella* spp. and 14 isolates of *E. coli* O157:H7. Next, the in vitro detection limit of the kits was determined. For this, three different concentrations (1, 10 and 100 CFU/microplate well) of the evaluated microorganisms were submitted to 2 h of phage infection at 37 ºC. The third step of this work was to determine the detection limit of the kits in food. For the PhageDx *Salmonella* Assay kit, 1 mL of the *Salmonella* spp. (concentrations of 1, 10, and 100 CFU) was inoculated into 25 g of lettuce, chicken breast, chicken sausage, chicken pâté, and nuggets. The samples were then added to 75 mL of buffered peptone water and enriched at 41 °C for 7 h before being submitted to 2 h of phage infection at 37 °C. Using the same methodology as mentioned above, the PhageDx *E. coli* O157:H7 Assay kit was tested. In this test, 1 mL of the *E. coli* O157:H7 cocktail at the same concentrations was added to 25 g of ground beef and lettuce, and to 25 mL of pasteurized milk and mineral water. Subsequently, tryptone soy broth was added to the samples and the mixture was incubated for 5 h at 41 ºC before being submitted to 2 h of phage infection at 37 ºC. In addition, the PhageDx *E. coli* O157:H7 Assay performance was compared to a real-time PCR kit to screen for *E. coli* O157:H7 in bovine carcasses. Regarding the results obtained, it was verified that all isolates tested were identified by their respective kit and the in vitro detection limit was 100 CFU/well, in only 2 h, that is, without enrichment of the samples. The kits were also capable of detecting 1 CFU of the pathogens in 25 g (or 25 mL) of food, after 5 and 7 hours of enrichment, for *E. coli* O157:H7 and *Salmonella* spp., respectively. Only the detection of *Salmonella* spp. in lettuce had a slightly higher detection limit than the other foods, i.e. 10 CFU/25 g. The PhageDx *E. coli* O157:H7 Assay kit did not detect the presence of *E. coli* O157:H7 in any of the 100 bovine carcasses evaluated. In agreement with this result, the real-time PCR kit did not detect the presence of enterohemorrhagic *E. coli*. The assays evaluated proved to be satisfactory for detection of *E. coli* O157:H7 and *Salmonella* spp. isolated in Brazil, showing sensitivity, specificity and rapidity in evaluating different food matrices. The detection of a single *Salmonella* spp. cell in 25 g of sample, after approximately 9 h of assay, represents a significant reduction in time compared to traditional and rapid methods currently available on the market. The same was observed for the *Escherichia coli* O157:H7 kit, whose analysis time was about 7 h. In addition, the kits proved to be easy to perform, making them promising tools for the detection of *E. coli* O157:H7 and *Salmonella* spp. in different foods.

Keywords: Detection methods; food safety; foodborne pathogens; cattle, poultry.

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INTRODUÇÃO

O Brasil é o principal exportador de carne bovina e de frango no mundo (ABIEC, 2021; ABPA, 2021). Assegurar a qualidade e especialmente a segurança destes produtos é de fundamental importância para mantê-los dentro do competitivo mercado internacional da carne.

Escherichia coli O157:H7 é um dos mais severos patógenos alimentares. A ingestão de aproximadamente 50 células desse microrganismo pode causar diarreia hemorrágica, insuficiência renal e morte (FORSYTHE; 2013, p. 221; HESSEL; TONDO, 2019, p. 135). Os principais alimentos envolvidos na transmissão de *E. coli* O157:H7 são os produtos cárneos bovinos consumidos crus ou insuficientemente tratados termicamente, assim como leite cru. Isto pode ser explicado pelo fato dos ruminates serem um reservatório natural desta bactéria (CDC, 2015; WHO, 2018). Além disso, a contaminação fecal da água e de outros produtos, principalmente vegetais consumidos crus, pode ser fonte de *E. coli* O157:H7 (WHO, 2018).

Salmonella spp. é apontada como uma das quatro principais causas globais de doenças diarreicas (WHO, 2018). Estima-se que a cada ano, ocorram, no mundo, 153 milhões de casos de gastrenterite e 57.000 mortes causadas por *Salmonella* não tifóide (NTS) (HEALY; BRUCE, 2019). No Brasil, *Salmonella* foi o principal responsável por DTA, dos anos 2000 a 2017 (BRASIL, 2018). Os sintomas de salmonelose costumam ser febre, dor abdominal, diarréia, náusea e vômito (CDC, 2019) e os produtos de origem animal, principalmente produtos avícolas e leite cru estão entre as principais fontes de salmonelose em humanos (WHO, 2018).

O Brasil, como importante fornecedor de alimentos em nível global, necessita de métodos de detecção de *E. coli* O157:H7 e *Salmonella* spp. e, de forma ideal, esses métodos devem ser confiáveis, acessíveis e rápidos, a fim de que os resultados estejam disponíveis o mais rápido possível (HAUGE *et al*., 2017). Embora os métodos tradicionais sejam considerados o “padrão-ouro” para a detecção de patógenos alimentares, eles são demorados e laboriosos, levando a perdas econômicas significativas (STONE *et al*., 2019).

As principais etapas envolvidas na detecção de microrganismos por métodos tradicionais são o pré-enriquecimento de amostras, o enriquecimento seletivo, o cultivo em meios seletivos diferenciais, testes bioquímicos e confirmação sorológica

do microrganismo isolado (RATNAM *et al*., 1988; SCHMELCHER; LOESSNER, 2014; ZHANG *et al*., 2017). No caso de *Salmonella* e *E. coli* O157:H7, no mínino três dias são necessários para obter o resultado negativo. Havendo colônias suspeitas, as etapas de confirmação podem resultar em mais uma ou duas semanas de análise.

A análise de alimentos por métodos rápidos é uma necessidade da indústria de alimentos. Ultimamente, técnicas de detecção baseadas em Reação em Cadeia de Polimerase (PCR), técnicas imunológicas, espectrometria de massa e citometria de fluxo fazem parte da rotina laboratorial de muitas indústrias de alimentos. Estes métodos reduzem significativamente o tempo de análise e são utilizados principalmente como *screening* de um número elevado de amostras*.* Amostras negativas são liberadas rapidamente, geralmente, em até 24 h. No entanto, as amostras positivas detectadas pelos ensaios rápidos devem ser confirmadas pela metodologia tradicional. Apesar disso, o uso de métodos rápidos como *screening*, justifica-se pela possibilidade de conhecer o *status* sanitário do alimento mais rapidamente (NACMCF, 2019; SCHMELCHER; LOESSNER, 2014; WANG *et al*., 2016). A maioria dos métodos rápidos ainda necessita da etapa de pré-enriquecimento, consumindo de 12 a 24 horas. Além disso, métodos como PCR e ELISA apresentam pontos negativos como a incapacidade de distinguir células vivas das mortas dos patógenos-alvos, necessidade de equipamentos caros, manipulação excessiva da amostra e necessidade de pessoal treinado para a execução dos testes (NACMCF, 2019; SCHMELCHER; LOESSNER, 2014; WANG *et al*., 2016).

A utilização de bacteriófagos em métodos de detecção de microrganismos vem despertando o interesse de diversos pesquisadores (BROWN *et al*., 2020; MEILE *et al*., 2020; NGUYEN *et al*., 2020; ZELCBUCH *et al*., 2021). Isso pode ser justificado porque tais métodos podem apresentar vantagens frente aos métodos tradicionais e também aos métodos rápidos já desenvolvidos. Alta especificidade com o patógeno-alvo, rápida infecção dos microrganismos-alvo, distinção entre células vivas e células mortas, custo de produção razoável e normalmente não requerem equipamentos caros e nem pessoal treinado para execução, são algumas das principais vantagens dos métodos de detecção de patógenos por bacteriófagos (BAI *et al*., 2016; RIPP *et al*., 2008).

Recentemente, o *Laboratory Corporation of America Holdings* (“LabCorp”), sediado em St. Paul – USA, desenvolveu dois *kits* baseados em bacteriófagos

recombinantes para detecção de *E. coli* O157:H7 e *Salmonella* spp. Bacteriófagos selvagens foram modificados geneticamente para expressar NanoLuc (Nluc), uma luciferase originária do camarão *Oplophorus gracilirostris*. Nluc possui apenas 19 kDa, é 150 vezes mais brilhante do que outras luciferases e reage com um substrato de furimazine (HALL *et al*., 2012). Os *kits* de detecção desenvolvidos, baseiam-se na infecção das células-alvos pelos bacteriófagos recombinantes, os quais se multiplicam dentro das células infectadas. Durante a replicação dos bacteriófagos, ocorre a produção de luciferase NLuc e a adição do substrato à amostra desencadeia uma reação bioluminescente, que pode ser facilmente identificada por um luminômetro.

Em análises prévias realizadas pelo fabricante, os *kits* demonstraram ser rápidos, sensíveis e específicos na detecção de patógenos alimentares, o que poderia ser vantajoso às indústrias brasileiras, caso pudessem detectar patógenos circulantes em alimentos no Brasil.

OBJETIVOS

Objetivo Geral

Avaliar a especificidade, limite de detecção e sensibilidade dos kits PhageDx *E. coli* O157:H7 Assay e PhageDx *Salmonella* Assay em detectar *E. coli* O157:H7 e *Salmonella* spp. isoladas no Brasil.

Objetivos Específicos

1. Avaliar a capacidade dos *kits* PhageDx *E. coli* O157:H7 Assay e PhageDx *Salmonella* Assay em detectar *in vitro E. coli* O157:H7 e *Salmonella* spp, respectivamente, isoladas no Brasil;
2. Determinar o limite de detecção *in vitro* dos *kits* PhageDx *E. coli* O157:H7 Assay e PhageDx *Salmonella* Assay;
3. Analisar a sensibilidade do *kit* PhageDx *E. coli* O157:H7 Assay em diferentes matrizes alimentares contaminadas artificialmente com um *pool* de *E. coli* O157:H7 isoladas no Brasil;
4. Analisar a sensibilidade do *kit* PhageDx *Salmonella* Assay em diferentes matrizes alimentares contaminadas artificialmente com um *pool* de *Salmonella* spp. isoladas no Brasil.

CAPÍTULO 1- REVISÃO BIBLIOGRÁFICA

1.1 Agronegócio brasileiro

O agronegócio tem importância inquestionável na economia brasileira. Em 2020, o agronegócio teve participação de 26,6% no Produto Interno Bruto (PIB) do Brasil. Neste mesmo ano, as exportações derivadas deste setor representaram 54% (100.701,91 US$ milhões) de todo o valor obtido com exportações (ABIEC, 2021).

É importante ressaltar que o Brasil também destaca-se como grande contribuinte para o agronegócio global, mantendo-se entre os líderes nas exportações de café, açúcar, suco de laranja, soja, milho, etanol, carne suína, bovina e de frango, entre outros (PWC, 2013). Segundo o relatório anual da Associação Brasileira da Indústria de Alimentos, no ano de 2020, os alimentos produzidos no Brasil foram importados por 190 países (ABIA, 2021).

1.1.1 Indústria de carne bovina

O Brasil é o maior exportador mundial de carne bovina. No ano de 2020, foram produzidas 10,32 milhões de toneladas equivalente carcaça (TEC), sendo 7,63 milhões (73,93%) destinados ao consumo interno e 2,69 milhões (26,07%) destinados à exportação (ABIEC, 2021). Apesar do cenário de pandemia vivido no último ano, o Brasil registrou um aumento de 8% nas exportações de carne bovina (ABIEC, 2021).

A exportação desses produtos e de seus derivados gerou 8.478,21 milhões de dólares em 2020, representando 5% do total gerado por todos os produtos exportados pelo Brasil e 10% do total gerado pelas exportações referentes ao agronegócio. A carne *in natura*, continua sendo o principal produto exportado (85,29%) e a China destaca-se como o principal destino da carne bovina brasileira (ABIEC, 2021).

O mercado interno também tem uma parcela expressiva na economia, uma vez que cerca de 74% da produção é comercializada internamente. Além disso, o Brasil tem se mantido entre os principais consumidores de carne bovina, ocupando atualmente, o terceiro maior consumo *per capita* do mundo, com 36,39 kg/ano (ABIEC, 2021).

O consumo de carne bovina é motivado dentre vários fatores, por ser uma excelente fonte de proteína. Sua composição química é rica em aminoácidos essenciais para o organismo, minerais, como ferro, zinco, fósforo; e vitaminas, incluindo as vitaminas do complexo B (OJHA *et al*., 2016, p. 337).

As projeções apontam que o Brasil continuará se destacando entre os maiores produtores de carne bovina nos próximos anos. Estima-se que Brasil, China, União Europeia e Estados Unidos devem produzir juntos, quase 60% da produção global de carne até 2029. O Brasil destaca-se ainda como um dos países onde ocorrerá grande crescimento produtivo, influenciado pelo suprimento abundante de recursos naturais, alimentos, disponibilidade de pastagens, ganhos de produtividade e, em certa medida, à desvalorização do Real (OECD/FAO,2020, p. 172).

1.1.2 Indústria de produtos avícolas

Além de maior exportador de carne bovina, o Brasil é o país que mais exporta carne de frango no mundo. No ano de 2020, foram 4.231 mil toneladas enviadas principalmente para China, Arábia Saudita e Japão (ABPA, 2021). Economicamente isto representou U$ 6.097 milhões. O consumo interno detém 69% de toda a produção, sendo o consumo *per capita* de 45,27 kg/ano (ABPA, 2021). Houve um aumento de quase 3 kg comparados com o ano anterior. Em pesquisa realizada pela Associação Brasileira de Proteína Animal (ABPA), observou-se que 80% da população brasileira consome carne de frango no mínimo de 2 a 3 vezes por semana e não observou-se distinção do consumo deste tipo de proteína animal entre as classes sociais. Outro dado interessante demonstra que mais de 50% da população brasileira declara ter conhecimento de que o Brasil é o maior exportador de carne de frango do mundo (ABPA, 2021).

O Brasil é atualmente o terceiro maior produtor de carne de frango, atrás dos Estados Unidos e da China (ABPA, 2021). A previsão estima crescimento de 3% no ano de 2022, baseada na forte demanda externa e no aumento do consumo interno. As exportações de carne de frango continuarão se beneficiando do Real desvalorizado e o aumento no consumo interno pode estar relacionado com o consumo de carnes menos caras, como a de frango. Ambos os cenários supracitados são oriundos da pandemia (USDA, 2021).

1.2 Patógenos alimentares e Segurança dos Alimentos

Patógenos alimentares são responsáveis por grandes perdas econômicas e sociais tanto em países em desenvolvimento, quanto em países desenvolvidos. Nos países em desenvolvimento, esses microrganismos representam uma das principais causas de doença e morte, enquanto nos países desenvolvidos, todos os anos, milhões de pessoas apresentam casos de gastrenterite (D'AGOSTINO; COOK, 2015, p. 83).

Os alimentos podem estar contaminados por microrganismos ou por suas toxinas. No ambiente de processamento, mesmo carcaças provenientes de animais saudáveis, podem ser veículos de microrganismos patogênicos, contaminando equipamentos e propagando patógenos pela cadeia alimentar (DAS *et al*., 2017).

Normalmente idosos, imunodeprimidos, grávidas e crianças são os grupos da população mais afetados pelas DTAs, sendo denominados como grupo de risco (MARTINOVIĆ *et al*., 2016; REI *et al*., 2017). Segundo a Organização Mundial da Saúde, crianças menores de 5 anos de idade representam 40% da população afetada, com 125 mil óbitos por ano. Estima-se que 600 milhões de pessoas no mundo apresentam alguma enfermidade por consumir alimentos contaminados e que 420 mil morrem todos os anos (OMS, 2017). O aumento na expectativa de vida da população incrementa consideravelmente o grupo de risco, o que pode aumentar os surtos e proporcionar impactos expressivos na saúde pública (MARTINOVIĆ et al., 2016; REI et al., 2017).

Devido à globalização e o comércio internacional de alimentos, a segurança dos alimentos vem se tornando um tema cada vez mais importante. O intenso comércio de alimentos visa abastecer mercados internos e distantes, suprindo necessidades de consumidores cada vez mais exigentes. Logo, as indústrias, as comunidades científicas e os órgãos de regulamentação devem trabalhar para reduzir, controlar ou evitar que patógenos adentrem nas cadeias de fornecimento de alimentos e sejam causadores de surtos de origem alimentar (D'AGOSTINO; COOK, 2015, p. 83).

Apesar da evolução no processamento de alimentos, ainda existe a preocupação de que alimentos, principalmente os de origem animal, sejam veículos de patógenos alimentares. Alguns dos patógenos alimentares mais importantes fazem

parte da microbiota de bovinos, suínos e frangos, o que facilita a presença desses microrganismos nos produtos derivados desses animais. Apesar dos esforços das indústrias, ainda não há tecnologia capaz de eliminar totalmente *Salmonella* spp. e *E. coli* de carnes frescas, evidenciando a necessidade de conscientização da população a respeito de cuidados como cocção adequada e prevenção da contaminação cruzada nas cozinhas (TONDO; GONÇALVES, 2021). Além disso, as mudanças sociais ocorridas, como a globalização no comércio de alimentos, a busca por alimentos minimamente processados, aumento dos grupos de risco, patógenos resistentes e manipulação inadequada de alimentos são fatores que podem comprometer a segurança do consumidor (BUNCIC *et al*., 2014).

1.2.1 *Escherichia coli* O157:H7

Escherichia coli O157:H7 faz parte de um grupo de microrganismos comensais que estão presentes no trato gastrintestinal dos bovinos. De acordo com Brashears e Chaves (2017), a presença deste patógeno nos rebanhos bovinos independe da localização geográfica onde os mesmos se encontram. A Autoridade Europeia de Segurança dos Alimentos (EFSA) estima que a prevalência desta bactéria é de 0,2 a 2,3% em animais individuais, 1,5% a 13,7% em rebanhos e 5,5% a 20,2% em carcaças (EFSA, 2013b).

Por serem excretados nas fezes, a transferência deste patógeno entre os animais pode ocorrer durante o confinamento, transporte ou remoção do couro, onde a pele contaminada entra em contato com as carcaças (ARTHUER *et al*., 2007; BUNCIC *et al*., 2014; KOOHMARAIE *et al*., 2005; VISVALINGAM; HOLLEY, 2018). A contaminação dos humanos por este microrganismo pode ocorrer através do contato direto ou indireto com as fezes, contaminação cruzada de carcaças e/ou utensílios durante a produção ou o consumo de carnes e derivados sem tratamento térmico adequado (BUNCIC *et al*., 2014).

A nomenclatura *E. coli* O157:H7 é designada para expressar o 157º antígeno somático (O) e o 7º antígeno flagelar (H) do patógeno (MEAD; GRIFFIN, 1998). Esta bactéria faz parte do grupo das *Escherichia coli* Enterohemorrágicas, caracterizadas pela produção de toxinas Shiga ou também denominadas verotoxinas (PAGE; LILES, 2013). Karmali *et al*. (1983) identificaram pela primeira vez este patógeno em dois

surtos de colite hemorrágica, enfermidade caracterizada por cólicas abdominais e diarreia sanguinolenta. A complicação dessa enfermidade é a síndrome hemolítico-urêmica (SHU), marcada por anemia hemolítica, trombocitopenia e lesão renal aguda, com alto índice de mortalidade (KOOHMARAIE *et al*., 2005; MEAD; GRIFFIN, 1998; PAGE; LILES, 2013).

Devido à gravidade das enfermidades atribuídas a este patógeno e suas consequências para a saúde dos consumidores, nos Estados Unidos há a política de "tolerância zero" para a presença de *E*. *coli* O157:H7 nos alimentos. Produtos como carne moída crua ou seus derivados são considerados adulterados e não aptos à comercialização quando detectada a presença desta bactéria (BRASHEARS; CHAVES, 2017).

O surto ocorrido entre novembro de 1992 e fevereiro de 1993 envolvendo a rede de *fast food* "Jack in the box", foi a motivação para a implantação da política de tolerância zero. No estado de Washington, onde ocorreu a maioria dos casos, foram notificados 501 casos, 151 hospitalizações (31%), 45 casos de SHU (9%) e três mortes. Durante a investigação, observou-se que a cocção dos hambúrgueres realizada de acordo com a política da empresa, era insuficiente para inativar a bactéria. O centro do alimento atingia temperaturas abaixo de 60 ºC e atribuiu-se a isto, a causa do surto (BELL *et al*., 1994). Esse acontecimento teve uma proporção tão significativa, que foi um dos pontos discutidos na primeira reunião de gabinete do presidente Bill Clinton em 1993, logo após sua posse como presidente dos Estados Unidos. A partir desta reunião, várias medidas foram realizadas nos meses seguintes, como a obrigação da implementação do sistema de Análise de Perigos e Pontos Críticos de Controle (HACCP) para todas as fábricas de carne e aves inspecionadas pelo governo federal nos Estados Unidos (MURANO; CROSS; RIGGS, 2018).

Após o episódio de 1993, os surtos envolvendo hambúrgueres de *fast food* foram controlados, isto provavelmente devido à padronização dos processos de cozimento da carne moída (JAY *et al*., 2004). No entanto, ainda são descritos surtos relacionados ao consumo de carne bovina. No período de 2006 a 2021, os Estados Unidos relataram 28 episódios de surtos causados por *E. coli* O157:H7, envolvendo tanto produtos de origem animal quanto produtos de origem vegetal (CDC, 2022). Os detalhes desses surtos podem ser observados no Quadro 1. Neste período houve 1388 pessoas infectadas, 659 hospitalizações, 139 casos de SHU e 13 mortes. Pelo

menos 1/3 dos surtos estavam ligados diretamente ao consumo de carne bovina (CDC, 2022).

No Brasil, há apenas um registro descrito onde se relacionou a ingesta de alimento contaminado com *E. coli* O157:H7 e surto de origem alimentar. Esse episódio ocorreu em Campinas, em 2001, onde dois pacientes apresentaram diarreia, sem a ocorrência de SHU. O alimento envolvido foi carne mal cozida (CVE/SES-SP, 2011).

Um surto causado por *E. coli* O157 NM (não-móvel) foi registrado em 2005. Nesta ocasião, um estudante de 13 anos foi hospitalizado por apresentar diarreia sanguinolenta e dor abdominal. As investigações levaram ao isolamento desta bactéria na salada de tomate e queijo que havia sido preparada na cantina da escola (SANTOS *et al*., 2017). No final de 2019 foram registrados os primeiros casos fatais de *Escherichia coli* O157 no sul do Brasil. Na ocasião, quatro crianças da mesma escola no munícipio de Santa Maria - RS, foram hospitalizadas por apresentarem diarreia intensa, vômitos e febre. Além disso, a mãe de uma criança foi diagnosticada com SHU. *E. coli* O157 NM foi isolada das fezes de uma criança. Duas crianças vieram a óbito, uma por insuficiência renal e outra por insuficiência respiratória. Com base nos sintomas e isolamento bacteriano, as mortes foram atribuídas a *E. coli* O157 NM associada com *Campylobacter jejuni (*BARTZ *et al*., 2022).

Uma possiblidade para a escassez de dados que relacionem doenças transmitidas por alimentos no Brasil e esta bactéria, é a não obrigatoriedade de pesquisa de *E. coli* O157:H7 em alimentos envolvidos em surtos. Somado a isto, as técnicas empregadas para identificá-la não estão implementadas na maioria dos laboratórios Centrais (LACENs), responsáveis pelos diagnósticos laboratoriais nas áreas de Vigilância Ambiental, Epidemiológica e Sanitária (DE PAULA *et al*., 2014).

Em revisão realizada por Castro *et al*. (2019), observou-se a prevalência de *Escherichia coli* produtora de toxina Shiga (STEC) no Brasil com base em artigos científicos disponíveis na literatura. Em rebanho de bovinos, as taxas de contaminação variaram de 17,5% a 71,0%, e os principais sorogrupos detectados foram O157:H7, O113:H21 e O111. Já na carne bovina, as taxas de prevalência variaram de 0 a 27,5%. Neste trabalho, 22 artigos científicos sobre a presença de contaminação de STEC em humanos foram avaliados. Em 22% dos estudos, a presença de STEC foi descrita em relatos de casos ou em amostras coletadas de

pacientes com SHU. Os sorogrupos envolvidos foram O26:H11, O103:H2, O165, O157, O157:H7 e O104:H4.

Quadro 1- Surtos de *E. coli* O157:H7 reportados nos Estados Unidos no período de 2006 a 2021.

Fonte	Número de Casos	Número de Hospitalizações*	Número de Casos SHU**	Número de mortes	Ano
Espinafre	199	102	31	3	2006
Taco Bell	71	53	8	0	2006
Hambúrguer de carne moída	40	21	2	0	2007
Pizza congelada	20	8	4	0	2007
Carne moída bovina	49	27	1	0	2007
Massa de biscoito crua pré-embalada e refrigerada	72	34	10	0	2009
Carne bovina	23	12	2	0	2009
Carne bovina e avícola	21	9	1	0	2010
Alface romana	33	12	3	0	2010
Queijo	38	15	1	0	2010
Avelã com casca	8	4	0	0	2011
Bolonha (“salame” bovino)	14	3	0	0	2011
Alface romana	56	33	3	0	2012
Espinafre orgânico	33	13	2	0	2012
Saladas prontas	33	7	2	0	2013
Carne bovina moída	12	7	0	0	2014
Salada de frango	19	5	2	0	2015
Brotos de alfafa	11	2	0	0	2016
Produtos de carne bovina	11	7	1	0	2016
Manteiga de soja	32	12	9	0	2017
Folhas verdes	25	9	2	1	2017
Alface romana	210	96	27	5	2018
Alface romana	62	25	2	0	2018
Salada picada fresca de girassol	10	4	1	0	2019
Alface romana	167	85	15	0	2019
Desconhecida	32	15	1	1	2020
Folhas verdes	40	20	4	0	2020
Desconhecida	22	11	3	1	2021
Saladas embaladas	10	4	2	1	2021
Espinafre *baby*	15	4	3	1	2021

Fonte: Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (CDC)

* Os números de hospitalizações estão relacionados aos números de informações médicas que o CDC tinha disponível

Surtos domésticos relacionados a este patógeno foram descritos por Soborg *et al*. (2013), na Dinamarca. Os autores relataram 11 casos confirmados de *E. coli* O157:H7 em 9 famílias distintas entre 18 de setembro a 28 de outubro de 2012. A faixa etária dos envolvidos variou de 3 a 68 anos. Os sintomas não ocorreram devido ao consumo de algum alimento em uma rede de *fast food*, mas a investigação sugeriu

que a fonte do surto foi o alimento preparado na casa dos envolvidos. O alimento em comum consumido pelas famílias durante o período de interesse foi carne moída e algumas famílias relataram que durante o consumo, o centro da carne estava vermelho, sugerindo cozimento insuficiente.

Esses dados referentes a surtos, tanto domésticos quanto pelo consumo de alimentos fora de casa, somado à prevalência desta bactéria nos rebanhos evidencia a necessidade de uso das Boas Práticas (BP) durante o abate, a produção e preparação de alimentos, em especial à carne bovina, para reduzir o risco de transmissão deste patógeno (NOBILI *et al*., 2017).

Além disto, é necessário salientar que a política de tolerância zero, dos EUA, assim como o controle exigido em outros países, inclusive o Brasil, faz com que os produtos sejam testados e a ausência desta bactéria seja comprovada, o que torna uma prática onerosa para as indústrias (KOOHMARAIE *et al*., 2005).

1.2.2 *Salmonella* spp.

O gênero *Salmonella* spp. é composto por bactérias Gram-negativas, anaeróbias facultativas, pertencentes à família das Enterobactereaceae. Este gênero está dividido em duas espécies, denominadas *Salmonella bongori* e *Salmonella enterica*. A espécie *S. bongori* é formada apenas pela subespécie bongori, enquanto a espécie *Salmonella enterica* é composta pelas subespécies enterica, salamae, arizone, diarizone, houteane e indica. As subespécies são ainda divididas em mais de 2500 sorovares distintos. Mais de 99 % dos sorovares pertecem à espécie *S. enterica,* e dentro deste grupo a espécie *Salmonella enterica* subespécie enterica é a maior causadora de doenças em humanos (GRIMONT; WEILL, 2007; WHO, 2018). Os principais sorovares responsáveis por casos de salmonelose humana são *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Stanley e *S.* Newport (EFSA-ECDC, 2016). Salmonelose não-tifoide, que refere-se a doenças causadas por todos os sorotipos de *Salmonella,* exceto Typhi, Paratyphi A, Paratyphi B (tartarato negativo) e Paratyphi C, está entre as principais causas de diarreia bacteriana em todo o mundo (HEALY; BRUCE, 2019).

Estas bactérias podem ser encontradas no trato gastrintestinal de animais e humanos. Os animais domésticos criados para consumo humano, como aves, suínos

e bovinos são as principais fontes de infecção humana por *Salmonella* (MAFI; ORENSTEIN, 2018, p. 386). Nos EUA, no período de 2018 a 2021, foram registrados 32 surtos provocados por *Salmonella* (Quadro 2), resultando em 4899 pessoas doentes, 1235 hospitalizações e 5 mortes (CDC, 2022). Alimentos de origem animal e vegetal estavam envolvidos nos surtos, destacando-se dois surtos ocorridos pelo consumo de cebolas, responsáveis por aproximadamente 44% dos doentes registrados com salmonelose, nos últimos 3 anos.

Quadro 2- Surtos de *Salmonella* spp. reportados nos Estados Unidos no período de 2018 a 2021.

Alimentos	Número de Casos	Número de Hospitalizações*	Número de mortes	Ano
Pasta de Gergelim	8	0	0	2018
Produtos crus de frango	129	25	1	2018
Carne moída	403	117	0	2018
Ovos	44	12	0	2018
Produtos de frango	25	11	1	2018
Produtos crus de peru	358	133	1	2018
Salada de macarrão	101	25	0	2018
Cereal	135	34	0	2018
Melão pré-cortado	77	36	0	2018
Ovos	45	11	0	2018
Coco seco	14	3	0	2018
Salada de frango	265	94	1	2018
Kratom	199	50	0	2018
Brotos crus	10	0	0	2018
Coco ralado congelado	27	6	0	2018
Frutas cortadas	165	73	0	2019
Carne moída	13	9	1	2019
Mamão	81	27	0	2019
Atum cru congelado	15	2	0	2019
Melões pré-cortados	137	38	0	2019
Carne moída de peru	7	1	0	2019
Cogumelos orelha-de-pau	55	6	0	2020
Pêssegos	101	28	0	2020
Cebolas	1127	167	0	2020
Palitos de salame	34	7	0	2021
Frutos do mar	115	20	0	2021
Cebolas	1040	260	0	2021
Carnes - *Italian Style*	40	12	0	2021
Salada verde embalada	31	4	0	2021
Camarão cozido congelado	9	3	0	2021
Produtos crus de frango recheado, empanado e congelado	36	12	0	2021
Brie de caju	20	5	0	2021
Carne moída de peru	33	4	0	2021

Fonte: Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (CDC)

No Brasil*, Salmonella* spp. é apontada com um dos principais agentes causadores de surtos alimentares (BRASIL, 2019). Ovos e produtos à base de ovos, carne de ave *in natura*, processados e miúdos estão na lista dos alimentos mais envolvidos nos surtos, no período de 2009 a 2018 (BRASIL, 2019).

Em trabalho realizado por Ritter *et al*. (2019) observou-se que uma cepa de *Salmonella* Enteritidis, denominada SE86, conhecida por causar mais de 90% dos surtos de salmonelose no Rio Grande do Sul no período de 1999 a 2013, apresentou em seu genoma inúmeros genes de resistência a antimicrobianos, resistência térmica e ácida, fatores de virulência e genes relacionados à adesão. Isso pode explicar porque este patógeno foi um dos principais causadores de surtos no Brasil. Genes de resistência foram encontrados nesse sorovar e em mais 51 isolados de *S*. Enteritidis obtidos de surtos de origem alimentar ocorridos em diferentes cidades do estado do Rio Grande do Sul (RS), de 2003 a 2015 (MASCITTI *et al*., 2021). Os autores realizaram o sequenciamento completo de genoma em 52 isolados e analisaram comparativamente com 65 genomas adicionais do Centro Nacional de Informação Biotecnológica (National Center for Biotechnology Information – NBCI). Os resultados dessa pesquisa demonstraram que todos os isolados de *S.* Enteritidis do sul do Brasil agruparam-se no clado epidêmico global disseminado mundialmente originado na década de 1980. Isso significa que o mesmo clone de *S.* Enteritidis estava causando surtos no Brasil e no mundo.

Na indústria, monitorar a presença de *Salmonella* spp. é importante, tanto com relação ao critério de segurança de alimentos, quanto devido à restrições que a presença deste patógeno pode ocasionar nas importações e exportações dos alimentos. A análise de presença ou ausência desta bactéria por métodos tradicionais pode limitar a análise de um número elevado de amostras, devido ao custo e ao consumo de tempo. Por esse motivo, técnicas de triagem eficazes e rápidas são necessárias para melhorar o processo de análise (PULIDO-LANDÍNEZ, 2019).

1.3 Controle de *Escherichia* coli O157:H7 e *Salmonella* spp. no Brasil

O Brasil apresenta potencial para fornecer 40% da demanda global adicional de alimentos esperada para 2050 (PWC, 2013). Controlar a circulação dos principais

patógenos transmitidos por alimentos é importante para garantir acesso dos alimentos brasileiros à mercados internacionais.

A Instrução Normativa n° 60, de 23 de dezembro de 2019, estabelece a lista de padrões microbiológicos para alimentos prontos para oferta ao consumidor. Essa legislação institui que *Salmonella* spp*., Escherichia coli,* microrganismos aeróbios mesófilos e Estafilococos coagulase-positiva sejam pesquisados em carnes e/ou produtos cárneos. Nessa legislação específica, não há a obrigatoriedade da pesquisa de *E. coli* O157:H7 nem nesse grupo de alimentos e em nenhum outro (BRASIL, 2019). Ao contrário de *E. coli* O157:H7, a pesquisa e, mais precisamente a ausência de *Salmonella* spp., é exigida em praticamente todos os grupos alimentares descritos nesta legislação (BRASIL, 2019).

Com relação às medidas de controle nacionais adotadas para estes patógenos, pode-se citar o Programa Nacional de Controle de Patógenos – PNCP.
O PNCP é caracterizado por um conjunto de ações que visam reduzir a prevalência de patógenos em produtos de origem animal que estão sob fiscalização do Sistema de Inspeção Federal - SIF. Além disso, este programa julga as ações de controle adotadas pelos estabelecimentos para gerenciar o risco a fim de preservar a segurança do alimento (MAPA, 2020). Dentro do programa, estabeleceu-se o plano para controle de *Escherichia coli* produtora de toxina Shiga (STEC) (O157:H7, O26, O45, O103, O111, O121 e O145) e *Salmonella* spp. em carne de bovinos, através da Norma Interna DIPOA/SDA nº1, de 17 de junho de 2015 (BRASIL, 2015). Esta norma aprova:

> os procedimentos para a coleta e análise de *Escherichia coli* verotoxigênica e *Salmonella* spp. em carne de bovino *in natura* utilizada na formulação de produtos cárneos, cominutados, prontos para serem cozidos, fritos ou assados (BRASIL, 2015).

Para a coleta das amostras, a Norma Interna DIPOA/SDA n º1, de 17 de junho de 2015 exige um plano amostral N60. Este plano, define que cada amostra, será composta por 60 pedaços pequenos e finos da superfície de aparas de desossa, da carne de cabeça, esôfago ou diafragma. A mesma amostra deve ser utilizada no laboratório para pesquisa de STEC *e Salmonella* spp*.* A coleta das amostras oficiais é realizada por servidores públicos que atuam na inspeção federal e as análises fiscais são realizadas pelos Laboratórios Federais de Defesa Agropecuária (LFDAs), próprios

do MAPA e que estão vinculados à Coordenação Geral de Laboratórios Agropecuários (CGAL) (MAPA, 2020).

A partir de junho de 2019 entrou em vigor a Instrução Normativa SDA nº 60, de 20 de dezembro de 2018. Esta instrução estabelece o controle microbiológico em carcaça de suínos e em carcaça e carne de bovinos nos abatedouros frigoríficos sob inspeção federal, incluindo análises para monitoramento de Enterobacteriaceae, *Salmonella* spp. e *E. coli* sorogrupos O157:H7, O26, O45, O103, O111, O121 e O145 (BRASIL, 2018). Esses estabelecimentos passaram a realizar amostragens para o programa de autocontrole (realizado por eles), além da amostragem oficial realizada pelo SIF já preconizada desde a Norma Interna de 2015.

Com relação à coleta de amostras para o grupo de STEC, não houve alteração em comparação com a legislação anterior. Já a amostragem para pesquisa de *Salmonella* spp. passou a ser realizada em superfícies de carcaças de suínos e bovinos, através da técnica por esfregadura.

No quadro 03 pode-se observar os resultados da verificação de STEC e *Salmonella* spp. em carne de bovinos no período de 2015 a 2020 (MAPA, 2015, 2016, 2017, 2018, 2019, 2020, 2021). A amostragem foi realizada de acordo com a legislação vigente na época. A porcentagem de *Salmonella* spp. detectada variou de 0,7 % em 2018 a 2,57 % em 2016. Com relação às STEC (exceto *E. coli* O157:H7), a máxima porcentagem foi de 0,26 % em 2019. *E. coli* O157: H7 só foi detectada nos anos de 2016, 2019 e 2020. No ano de 2020 ocorreu a maior detecção deste patógeno, 0,16% (MAPA, 2021).

Quadro 3- Resultados da verificação oficial de *E. coli* produtora de Shiga-toxina (STEC), *E. coli* O157:H7 e *Salmonella* spp. em carne de bovinos *in natura* pelo Serviço de Inspeção Federal no período de 2013 a 2020.

ANO	Patógeno	Nº de amostras analisadas	Nº de amostras com presença do patógeno	%
Agosto de 2013 - outubro de 2014	*E. coli* O157:H7	442	0	0
2015	*Salmonella* spp. / 325 g	982	17	1,7
	E. coli STEC (sorogrupos O26, O45, O103, O111, O121 e O145)		4	0,4
	E. coli O157:H7		0	0
2016	*Salmonella* spp. / 325 g	933	24	2,57
	E. coli STEC (sorogrupos O26, O45, O103, O111, O121 e O145)	937	1	0,11
	E. coli O157:H7	938	1	0,11
2017	*Salmonella* spp. / 325 g	1310	21	1,60
	E. coli STEC (sorogrupos O26, O45, O103, O111, O121 e O145)		2	0,15
	E. coli O157:H7		0	0
2018	*Salmonella* spp. / 325 g	1286	9	0,7
	E. coli STEC (sorogrupos O26, O45, O103, O111, O121 e O145)		2	0,15
	E. coli O157:H7 / 325 g		2	0,15
2019	*Salmonella* spp. / 325 g	1523	24	1,57
	E. coli STEC (sorogrupos O26, O45, O103, O111, O121 e O145)		4	0,26
	E. coli O157:H7 / 325 g		0	0
2020	*Salmonella spp.* / 325 g	1884	32	1,7
	E. coli STEC (sorogrupos O26, O45, O103, O111, O121 e O145)		1	0,05
	E. coli O157:H7 / 325 g		3	0,16

O monitoramento de *Salmonella* spp. em carcaças de frangos era regido pela Instrução Normativa nº 70, de 6 de outubro de 2003 (BRASIL, 2003). O objetivo desta norma era:

> Estabelecer o monitoramento e controle de *Salmonella* spp. nos estabelecimentos avícolas comerciais de frangos e perus de corte e nos estabelecimentos de abate dessas aves registrados no Serviço de Inspeção Federal (SIF), com objetivo de reduzir a prevalência desse agente e estabelecer um nível adequado de proteção ao consumidor (BRASIL, 2003, Art. 1).

Em 2013, o PNCP iniciou o Programa exploratório para pesquisa de *Salmonella* spp. em carcaças de frangos, através da Norma Interna SDA nº 2, de 11 de outubro de 2013. O objetivo desta Norma foi realizar uma avaliação sistemática do Programa de Redução de Patógenos disposto na Instrução Normativa nº 70, de 06 de outubro de 2003 (MAPA, 2015). Essa legislação foi atualizada e em fevereiro de 2017, entrou em vigência a Instrução Normativa nº 20, de 21 de outubro de 2016 (BRASIL, 2016). A partir de então, o controle e monitoramento de *Salmonella* spp. se estendeu à toda a cadeia de produção de frangos e perus, incluindo estabelecimentos avícolas comerciais e os estabelecimentos de abate. Foi determinado também que estes estabelecimentos instituam em seus programas de autocontrole, ações de controle e monitoramento de *Salmonella* spp., desde a matéria-prima até o produto final (BRASIL, 2016). No caso dos estabelecimentos de abate de frangos, esta Instrução estabelece o monitoramento de *Salmonella* spp. em carcaças inteiras de frangos coletadas de forma aleatória, imediatamente após o gotejamento e antes da embalagem primária (BRASIL, 2016). A prevalência máxima aceitável de *Salmonella* spp. é de 20%.

Estão dispostos no Quadro 04 os resultados das análises oficiais realizadas pelo Serviço de Inspeção Federal entre 2013 e 2021 (MAPA, 2015, 2016, 2017, 2018, 2019, 2020, 2021) .

Quadro 4 - Resultados da verificação oficial de *Salmonella* spp. em carcaças de frangos pelo Serviço de Inspeção Federal no período de 2013 a 2021.

Período	Nº de abatedouros de frango	Nº de amostras analisadas	Nº de amostras com presença do patógeno	%
Outubro de 2013 - junho de 2014	89	856	150	17,52
2016	143*	1922	330	17,17
Fevereiro de 2017 - março de 2018	132	2592	466	17,97
Março de 2018 - fevereiro de 2019	134	2791	352	12,71
Janeiro de 2019 - abril de 2020	134	2831	427	15,08
Janeiro de 2020 - fevereiro de 2021	137	2881	369	12,81

***** Valor referente ao número de abatedouros de frango e peru. Não foi possível obter estes dados separadamente.

Durante os três primeiros anos de monitoramento, a prevalência de *Salmonella* spp. foi de aproximadamente 17%. Em seguida, houve uma redução de quase 30% na ocorrência de *Salmonella* spp. em comparação ao ano anterior. Esse resultado pode estar relacionado com as medidas de controle adotadas na cadeia produtiva, podendo impactar diretamente na segurança dos consumidores da carne de frango brasileira (MAPA, 2018). No ano de 2020, houve redução nas detecções de *Salmonella* em frango em 15% quando comparado ao ano anterior (MAPA, 2021).

O controle de *Salmonella* é responsável por expressivos investimentos por parte das indústrias e agências reguladoras no Brasil. Por exemplo, ainda na Instrução Normativa nº 20/2016 (BRASIL, 2016), é destacada a necessidade de estar atento às possíveis exigências adicionais impostas por países importadores:

> As ações de controle e monitoramento de *Salmonella* spp. previstas nesta Instrução Normativa serão realizadas sem prejuízo ao cumprimento de exigência complementares em acordos bi ou multilaterais com os países importadores de produtos de origem animal (BRASIL, 2016, Art. 83).

1.4 Análise microbiológica de Alimentos

Comercializar alimentos seguros, atraentes e nutritivos é um desafio para a indústria de alimentos (BATT, 2016). Os testes microbiológicos são fundamentais para o gerenciamento e qualidade microbiológica dos alimentos (ZWIETERING; ROSS; GORRIS, 2014, p. 244). O objetivo principal destes testes é minimizar as DTAs e consequentemente garantir um alimento seguro, preservando a saúde do consumidor (MAGNÚSSON *et al*., 2012).

Durante muitos anos, os métodos baseados em culturas microbiológicas foram os principais meios de detecção de microrganismos e sua caracterização foi baseada em análises microscópicas e testes bioquímicos (BATT, 2016). Apesar de alguns métodos convencionais serem oficiais e recomendados em publicações de referência como aquelas da APHA (American Public Heath Association), ICMSF (International Commission on Microbiological Specifications for Foods) e a FDA (Food and Drug Administration), existem alguns entraves na sua utilização. O tempo de análise, por exemplo, é um dos principais pontos negativos. O tempo gasto para realização das análises, influenciará na liberação de laudos e impactará economicamente, uma vez que os produtos necessitam do *status* sanitário, antes de serem comercializados. Acrescenta-se ainda os gastos com meios de cultura, excesso de trabalho de laboratório e necessidade de grande volume de vidraria (HÚNGARO *et al*., 2014, p. 226).

A detecção de *Salmonella* spp. conforme a ISO 6579:2002 (2002) compreende as etapas de pré-enriquecimento (20 - 24 h), enriquecimento seletivo (22 - 24 h), semeadura em placas contendo meios seletivos (24 - 48 h) e confirmação por provas bioquímicas e provas sorológicas (24 - 48h). Neste método são necessários pelo menos 3 a 4 dias para liberação de resultados negativos e 7 a 10 dias para os resultados positivos, confirmados pelas provas adicionais. Já para o isolamento de *E. coli* O157:H7 segundo a ISO 16654:1998 (1998) são necessárias as etapas de enriquecimento seletivo (20 - 24h), isolamento pela técnica de separação imunomagnética (IMS), semeadura em placas contendo meios seletivos (24 h) e realização das provas bioquímicas e sorológicas (3 - 5 dias). Um resultado negativo pode ser emitido em 2 dias de análise, enquanto um resultado positivo pode demorar até 10 dias para ser confirmado.

As dificuldades observadas nos métodos tradicionais, principalmente com relação ao tempo, estimularam o desenvolvimento de métodos mais rápidos para análise microbiológica de alimentos (HÚNGARO *et al*., 2014, p. 226). Os métodos rápidos mais utilizados ultimamente são baseados em Reação em Cadeia de Polimerase (PCR), técnicas imunológicas, espectrometria de massa e citometria de fluxo. Apesar da agilidade dessas técnicas em comparação aos métodos tradicionais, algumas desvantagens podem ser apontadas, como a necessidade de etapas de pré-enriquecimento de 12 a 24 horas, equipamentos caros, dificuldade no preparo das amostras e a impossibilidade de distinguir entre células vivas e mortas dos patógenos-alvo (ADAMS, 2014, p. 32; SCHMELCHER; LOESSNER, 2014; WANG *et al*., 2016). Novos métodos estão sendo desenvolvidos com o objetivo de diminuir o tempo de análise, sem perder a sensibilidade e a especificidade na detecção dos microrganismos-alvos (BATT, 2016; GONZALES-BARRON, 2017).

1.4.1 Bacteriófagos como métodos rápido para detecção de patógenos

Bacteriófagos, ou fagos, são vírus que infectam exclusivamente bactérias (SCHMELCHER; LOESSNER, 2014). Twort e d'Herelle foram os primeiros a observarem os fagos, no início do século XIX. Sua primeira aplicação foi como agentes antimicrobianos, no entanto, a descoberta dos antibióticos fez com que seu uso fosse drasticamente reduzido (REES; SWIFT; BOTSARIS, 2014, p. 194).

Assim como qualquer outro vírus, bacteriófagos são parasitas intracelulares obrigatórios, logo a penetração bem-sucedida na célula bacteriana é fundamental para continuação do seu ciclo de vida (RAKHUBA *et al*., 2010). A adsorção, interação física entre as proteínas de ligação dos vírus e os receptores presentes nas células-alvo, é o primeiro passo para a infecção da bactéria (DOWAH; CLOKIE, 2018). As proteínas de ligação presentes nos bacteriófagos são responsáveis por identificar corretamente o hospedeiro, ligando-se aos receptores localizados na superfície celular (SILVA *et al*., 2016). Os receptores podem variar de acordo com a natureza da parede celular das bactérias. Em bactérias gram-positivas, peptidoglicano e ácidos tecóicos são os principais componentes envolvidos na adsorção dos fagos. No caso das bactérias Gram-negativas, inúmeros receptores já foram identificados, sendo eles,

principalmente, proteínas localizadas na membrana celular e lipopolissacarídeos. Além disso, flagelos, *pili* e cápsulas, estruturas que não estão localizadas na parede celular das bactérias, atuam como receptores para certos fagos de bactérias Gram-negativas (DOWAH; CLOKIE, 2018; RAKHUBA *et al*. 2010; SILVA *et al*., 2016). O grau de especifidade do fago para detectar um gênero, uma espécie ou uma cepa bacteriana está relacionado à natureza dos receptores (RAKHUBA *et al*. 2010). Além disso, em uma mesma célula bacteriana mais de um receptor pode estar envolvido no processo de adsorção (SILVA *et al*., 2016).

Quando a ligação entre o bacteriófago e a célula hospedeira é realizada com sucesso, esta etapa se torna irreversível e o bacteriófago inicia a alteração conformacional para injetar o acido nucléico na célula hospedeira (STONE *et al*., 2019). Após a inserção do material genético, inicia-se a fase de replicação em uma das duas principais rotas, a lítica ou lisogênica. Na rota lítica, a expressão dos genes dos fagos orienta o metabolismo da célula hospedeira para replicação de DNA e síntese de proteínas. As proteínas virais são montadas e o genoma é empacotado nos capsídeos. No final do ciclo, ocorre a produção das proteínas, holinas e endolisinas. As holinas são responsáveis por produzir buracos na parede interna da membrana plasmática bacteriana, resultando na interrupção do metabolismo do hospedeiro, uma vez que a síntese de ATP é interrompida. As endolisinas acessam os buracos realizados pelas holinas e atingem a parede celular das bactérias, realizando a digestão enzimática do peptidoglicano. A célula é então lisada e ocorre a liberação dos fagos descendentes (DENNEHY; ABEDO, 2021; MONTEIRO *et al*., 2019;). No ciclo lisogênico, o genoma do fago é incorporado ao genoma da bactéria, muitas vezes, permanecendo integrado por longos períodos (DOWAH; CLOKIE, 2018). É na fase da adsorção que os fagos lisogênicos (temperados) “decidem” por qual rota irão prosseguir. Ambiente e/ou células com poucos recursos e alta densidade de fagos no meio são fatores que influenciam os mesmos a optar pelo ciclo lisogênico (ŁOŚ *et al*., 2021, p. 124-125). Dentro das células, os fagos se replicam, não necessariamente como vírus, mas como elemento genético sem capsídeo, denominado de profago. Para que isso ocorra, os fagos silenciam os genes que não serão necessários para o profago, como o gene sintetizador do capsídeo. Além disso, alguns genes poderiam codificar produtos tóxicos ao hospedeiro o que comprometeria a relação benéfica existente. Os profagos, no

entanto, podem entrar na via lítica, por um processo chamado de indução de profago (ŁOŚ *et al*., 2021, p. 125). A indução é estimulada quando as bactérias hospedeiras são expostas a inúmeras condições adversas como dessecação, luz Ultra-Violeta (UV) ou radiação ionizante, agentes mutagênicos e químicos. Nessas condições, ocorre a produção de proteases que irão destruir as proteínas repressoras dos genes do fago. A expressão do genes, reverterá o processo de integração (excisão) e iniciará o ciclo lítico, culmimando na morte bacteriana e expulsão de novos fagos no ambiente (MAYER, 2015).

A grande especificidade dos bacteriófagos, que fez com que seu uso fosse diminuído em terapias fágicas, é uma característica extremamente interessante em processos de identificação ou detecção de microrganismos (RICHTER *et al*., 2018; SCHMELCHER; LOESSNER, 2014).

1.4.1.1 Abordagens utilizadas em sistema de detecção de patógenos

Vários métodos têm sido estudados para a utilização de fagos como ferramentas de detecção de bactérias. Dentre eles estão métodos baseados na lise das células hospedeiras, detecção de ATP, medida de impedância ou condutividade, ensaio de amplificação de fagos, métodos utilizando fagos como moléculas de afinidade e fagos repórteres modificados geneticamente (SCHMELCHER; LOESSNER, 2014).

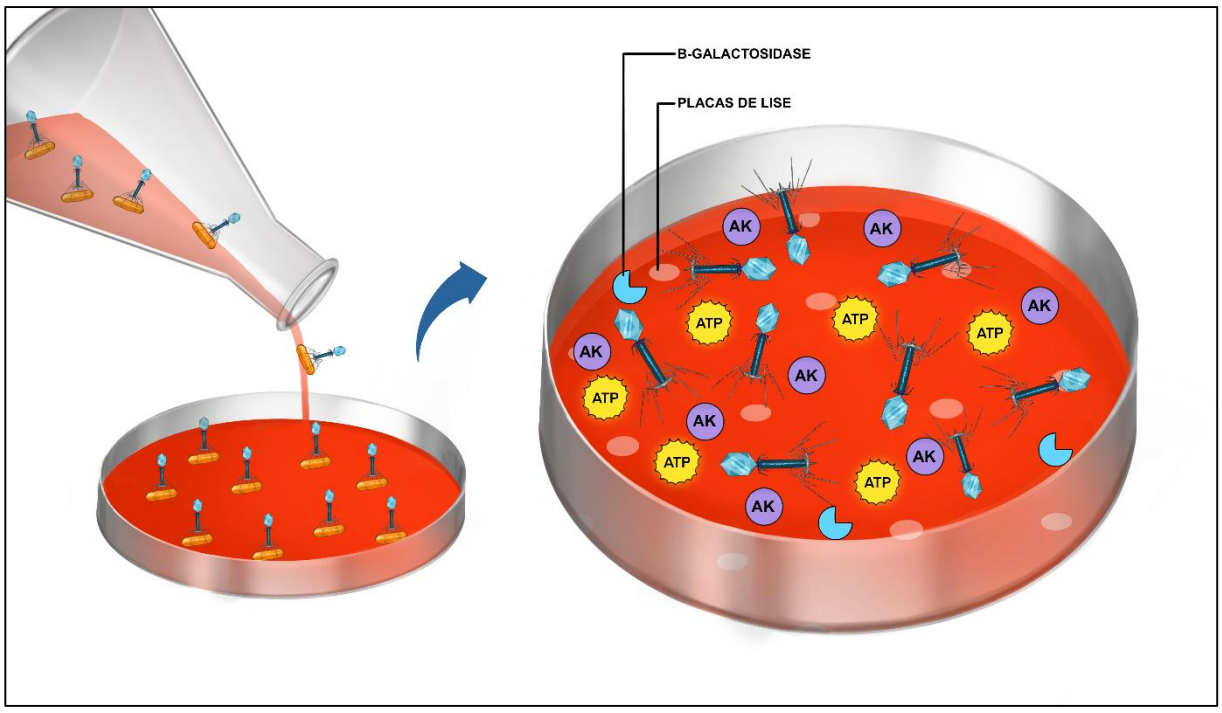
Na última etapa do ciclo lítico ocorre a lise da célula bacteriana. Esta etapa pode ser utilizada no processo de detecção de microrganismos. Na detecção com base na lise induzida por fago, as progênies podem ser enumeradas ou o conteúdo intracelular pode ser utilizado como marcadores (HUSSAIN *et al*., 2021) (Figura 1).

A enumeração é o método mais simples e consiste na contagem de placas de lise. As amostras contendo os patógenos e os fagos são misturados e inicia-se o processo de adsorção e infecção das células bacterianas. Em seguida, um viricida é adicionado para inativar os bacteriófagos que não participaram da infecção da célula. Como as células-alvo geralmente estão presentes em quantidades pequenas nas amostras, o que tornaria difícil sua visualização no plaqueamento, após a adição do viricida, adiciona-se células auxiliares às amostras. A lise dessas células auxiliares

será realizada pelos fagos que estavam presentes nas células bacterianas iniciais das amostras. Logo, o número de placas de lise das células auxiliares corresponderão diretamente ao número de células-alvo provenientes da amostra analisada (HUSSAIN *et al*., 2021; RICHTER *et al*., 2018; SCHMELCHER; LOESSNER, 2014).

Alguns marcadores intracelulares utilizados para determinar a lise bacteriana são ATP (Adenosina trifosfato), AK (Adenilato quinase) e β- *D*- galactosidase (SCHMELCHER; LOESSNER, 2014). Os métodos de detecção desses marcadores baseam-se em catálise enzimática cujos produtos da reação serão colorimétricos, luminescentes ou quimioluminescentes (RICHTER *et al*., 2018). O extravasamento do conteúdo intracelular altera a condutividade do meio e essa alteração pode ser medida por impedância ou condutividade (SCHMELCHER; LOESSNER, 2014). Durante a multiplicação dos microrganismos, moléculas maiores não carregadas, são transformadas em moléculas menores, carregadas positivamente, tornando o conteúdo intracelular liberado altamente condutor (RICHTER *et al*., 2018). Como a impedância é definida como a resistência ao fluxo de uma corrente alternada por meio de material condutor, a presença das bactérias-alvo retardará as alterações na impedância do meio (SCHMELCHER; LOESSNER, 2014).

Figura 1- Detecção de microrganismos com base na lise induzida por fago.

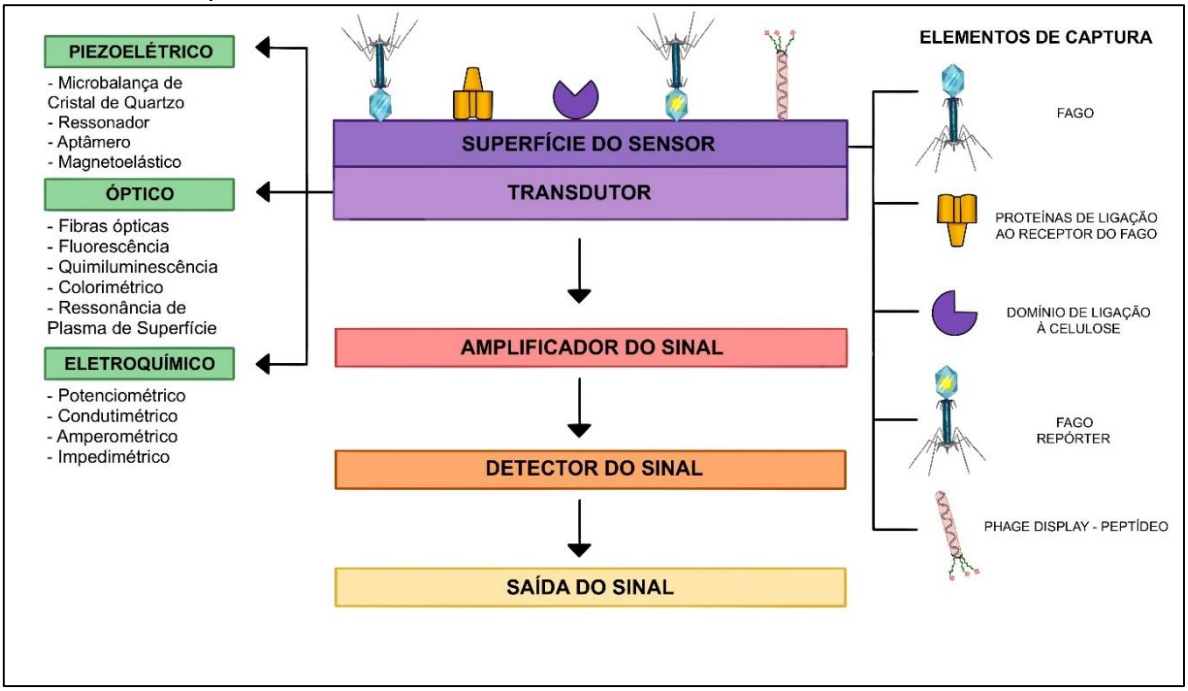


Fonte: Própria (2021)

Uma outra abordagem utilizada é o emprego de fagos ou componentes de fago como moléculas de afinidade. Nesse caso, ocorre a detecção da célula-alvo sem a necessidade do processo de infecção (SCHMELCHER; LOESSNER, 2014). As proteínas de fago responsáveis pela adsorção do fago a uma célula hospedeira específica, podem ser integradas em sistemas para a detecção de patógenos (HUSSAIN *et al*., 2021). Esta tecnologia foi desenvolvida pela BioMérieux no *kit* VIDAS® UP, e está disponível para detectação de *Salmonella*, *Listeria* e *E. coli* O157 em alimentos (http://www.biomerieux.com).

Os fagos também podem atuar como elementos de captura em um biossensor (Figura 2). De maneira geral, um biossensor é definido como um dispositivo analítico que detecta o analito, amplifica, processa e gera um sinal mensurável (HUSSAIN *et al*., 2021). Os sistemas que utilizam biossensores são compostos por um elemento de captura, uma superfície de sensor, uma plataforma de transdução, um amplificador, um detector e uma saída de sinal. A sensibilidade e especificidade do sistema geral dependem do sinal de transdução empregado e qual biosonda é usada (Stone et al., 2019).

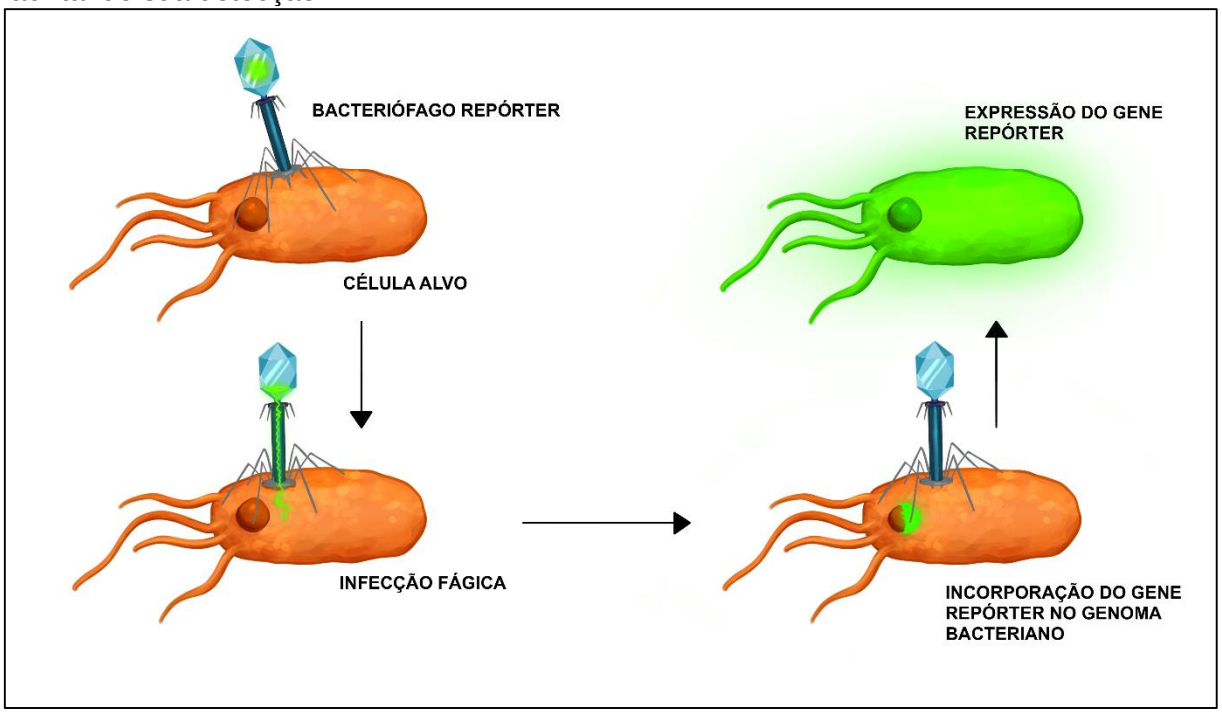
Figura 2- Os fagos e seus componentes (proteínas de ligação e domínios de ligação) como elementos de captura em um biossensor.



Fonte: Própria (2021)

Fagos modificados geneticamente, tais como fagos repórteres, também podem ser ferramentas úteis para detecção (Figura 3). Nesse método, os fagos têm seu genoma modificado através da incorporação de um gene de bioluminescência ou fluorescência, por exemplo. Esse gene não se expressa sozinho no fago, somente após a infecção de um hospedeiro viável, os genes são ativados, resultando na emissão de um sinal detectável de bioluminescência, fluorescência ou conversão enzimática de um substrato cromogênico (STONE *et al*., 2019).

Figura 3- Bacteriófagos repórter inserem seus genes para marcar a célula hospedeira facilitando sua detecção.



Fonte: Própria (2021)

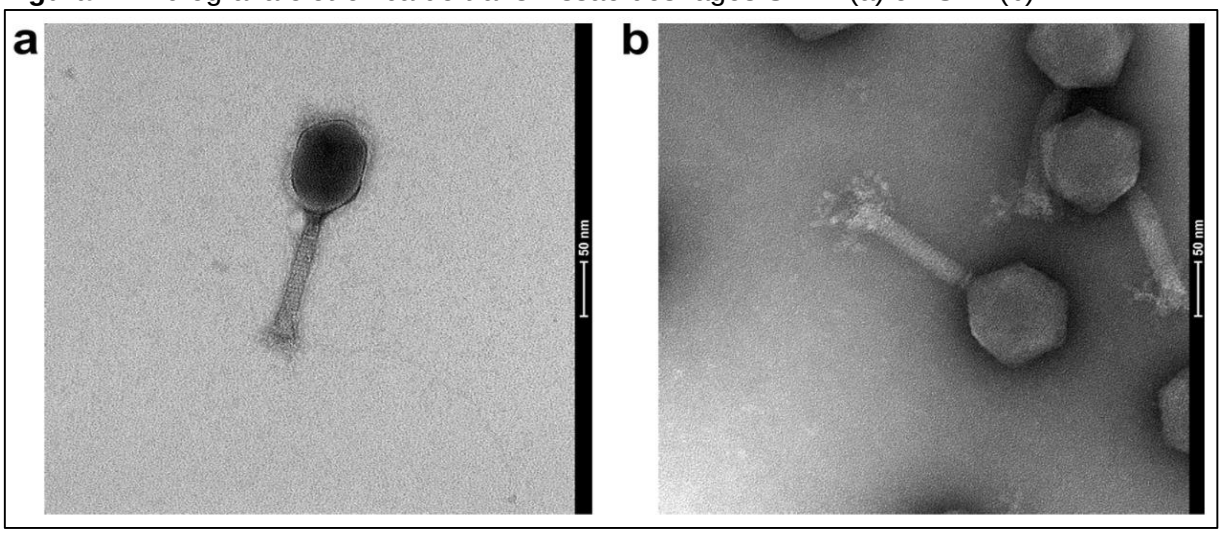
Os ensaios com fagos repórter são rápidos, sensíveis e detectam apenas células viáveis. Também são robustos e devido a alta especificidade, normalmente não necessitam de purificação da amostras (SCHMELCHER; LOESSNER, 2014).

1.4.1.1.1 PhageDx *Salmonella* Assay e PhageDx *Escherichia coli* O157:H7 Assay

Os *kits* PhageDx *Salmonella* Assay e PhageDx *E. coli* O157:H7 Assay estudados neste trabalho, foram desenvolvidos pela empresa americana *Laboratory Corporation of America Holdings* (LABCORP). Os fagos presentes nestes kits são

fagos recombinantes. Na Figura 4 podemos observar os bacteriófagos SEA1 e TSP1, que deram origem aos fagos presentes no *kit* PhageDx *Salmonella* Assay. Esses fagos foram escolhidos em uma triagem com 53 outros fagos. Após a inserção do gene NanoLuc no genoma dos bacteriófagos, eles passaram a ser denominados SEA1.NL e TSP1.NL. Ao entrar em contato com amostras contendo células de *Salmonella*, esses bacteriófagos recombinantes realizam o reconhecimento e infecção das células em aproximadamente 2 horas. Em seguida, o substrato furimazine é adicionado produzindo furamide e luminescência na presença de oxigênio. O *kit* PhageDx *Salmonella* Assay foi registrado na AOAC® sob Nº 121904.

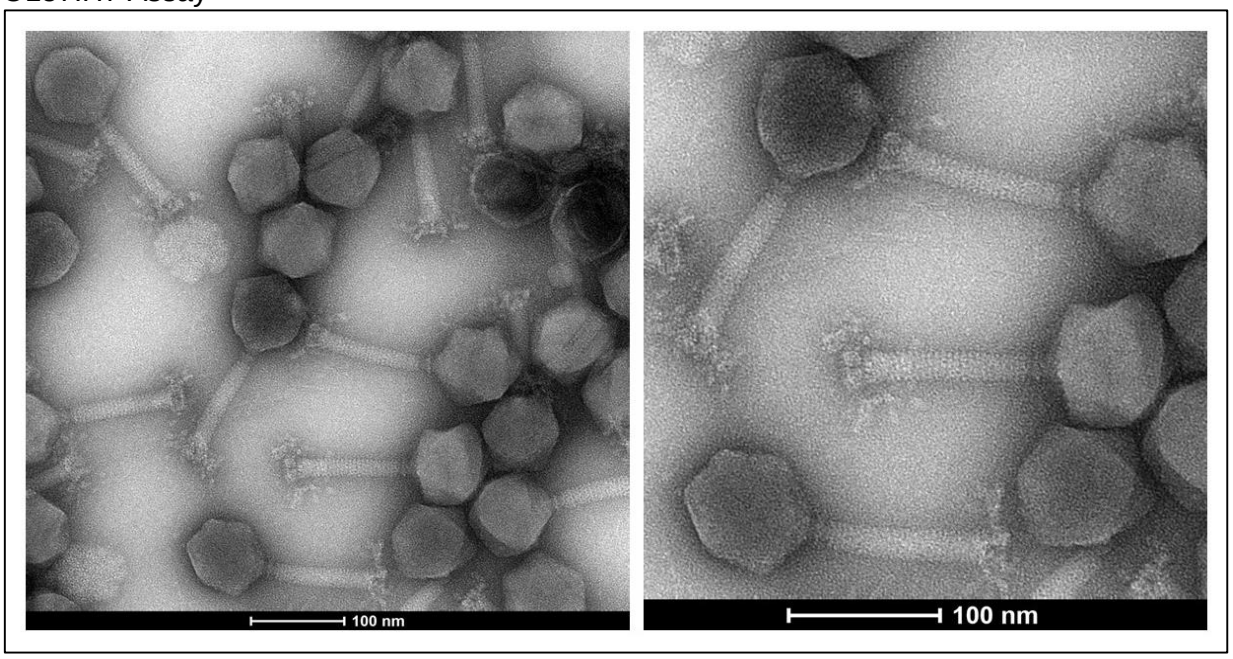
Figura 4- Micrografia eletrônica de transmissão dos fagos SEA1 (a) e TSP1 (b)



Fonte: Nguyen et al. (2020)

O *kit* PhageDx *E. coli* O157:H7 Assay é formado por um único fago (Figura 5). O método foi registrado na AOAC sob Nº 081601 e o princípio do método é o mesmo do citado acima. A presença de *E. coli* O157:H7 é determinada incubando a amostra com o fago e adicionando-se o substrato da luciferase. A detecção da luz proveniente da reação enzima-substrato é realizada em um luminômetro. Ausência de luz indica que não havia *E. coli* O157: H7 presente na amostra analisada.

Figura 5 - Micrografia eletrônica de transmissão do fago presente no *kit PhageDx E. coli* O157:H7 Assay



Fonte: Imagem gentilmente cedida pelo pesquisador Stephen Erickson (LabCorp)

O ofício circular Nº 43/2021/CGCOA/DIPOA/SDA/MAPA de 11 de agosto de 2021 autoriza laboratórios não credenciados pelo MAPA a realizar análises microbiológicas e físico-químicas em produtos de origem animal e água, utilizando métodos escolhidos pelo próprio laboratório, desde que sejam reconhecidos por órgãos de renome e gerem resultados auditáveis. Os laboratórios de autocontrole, aqueles utilizados por estabelecimentos sob o Sistema de Inspeção Federal (SIF) e que realizarão os ensaios e emitirão os resultados, podem ser escolhidos livremente pelas empresas. Os métodos utilizados para as análises devem permitir a rastreabilidade das amostras, desde a recepção até a emissão do resultado final. Quando o país ou bloco importador não exigir um método a ser utilizado e sim parâmetros para os resultados de cada ensaio, o laboratório de autocontrole deverá utilizar-se de métodos validados e reconhecidos internacionalmente, tais como AOAC, AFNOR, Métodos ISO, MicroVal, NordVal, entre outros (BRASIL, 2021).

Em vista disto, os *kits* PhageDx *Salmonella* Assay e PhageDx *E. coli* O157:H7 Assay podem ser utilizados nacionalmente para detecção de *Salmonella* spp. e *E. coli* O157:H7 em carnes de aves, bovinos e demais alimentos.

CAPÍTULO 2- Artigos

Os materiais e métodos, resultados e discussão desta tese são apresentados a seguir na forma de artigos científicos.

O artigo 1 descreve o método de elaboração do *kit* PhageDx *Salmonella* Assay, bem como sua utilização na detecção de *Salmonella* spp. em carne moída de peru e em fórmula infantil. No artigo 2 é apresentada a utilização do *kit* PhageDx *Salmonella* Assay para detecção de cepas de *Salmonella* spp. isoladas no Brasil e a detecção deste patógeno em diferentes alimentos a base de frango. O artigo 3 corresponde aos resultados obtidos na determinação do limite de detecção do *kit* PhageDx *Salmonella* Assay para cepas de *Salmonella* spp. e *pool* de *Salmonella* spp. oriundas do Brasil. Além disso, determina-se o limite de detecção de *Salmonella* spp. em alface orgânica hidropônica. No artigo 4 descreve-se o limite de detecção *in vitro* e em alimentos para o *kit* PhageDx *E. coli* O157:H7 Assay.

Ao final dos artigos há uma discussão geral sobre os trabalhos realizados nesta tese de doutorado.

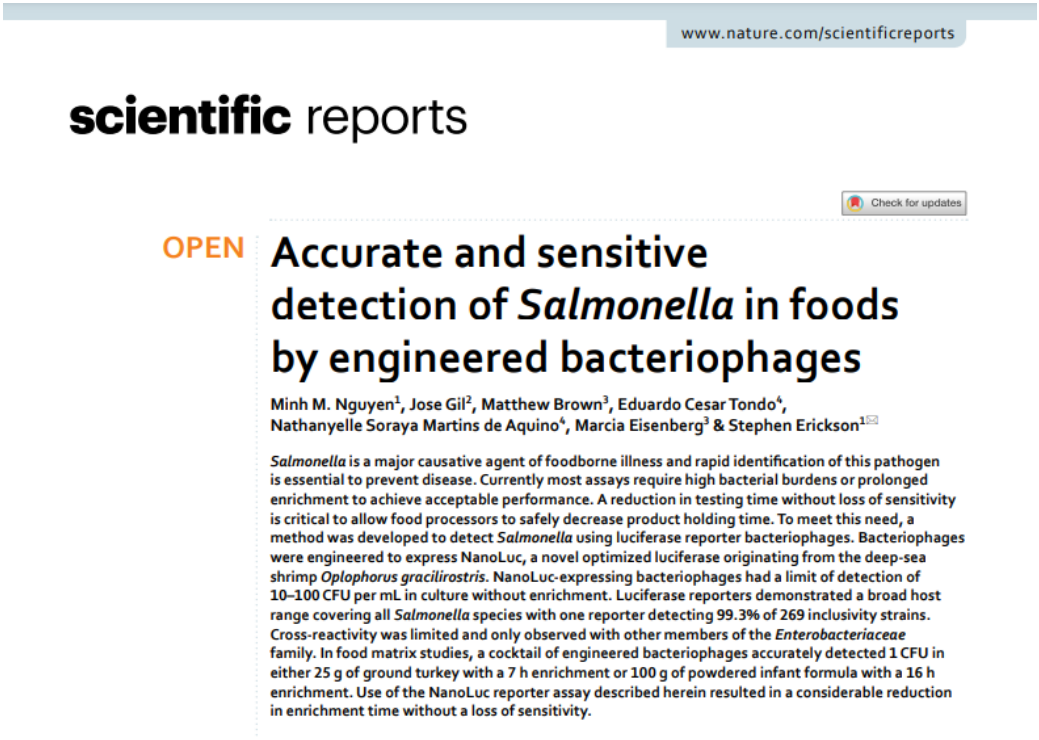
2.1 Artigo 1

Accurate and Sensitive Detection of *Salmonella* in Foods by Engineered Bacteriophages

Minh M. Nguyen, Jose Gil, Matthew Brown, Eduardo Cesar Tondo, Nathanyelle Soraya Martins de Aquino, Marcia Eisenberg and Stephen Erickson

Article published in the Scientifc Reports – Journal

The article was formatted according to the Scientifc Reports – Journal



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Accurate and sensitive detection of *Salmonella* in foods by engineered bacteriophages

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Salmonella **is a major causative agent of foodborne illness and rapid identification of this pathogen is essential to prevent disease. Currently most assays require high bacterial burdens or prolonged enrichment to achieve acceptable performance. A reduction in testing time without loss of sensitivity is critical to allow food processors to safely decrease product holding time. To meet this need, a method was developed to detect *Salmonella* using luciferase reporter bacteriophages. Bacteriophages were engineered to express NanoLuc, a novel optimized luciferase originating from the deep-sea shrimp *Oplophorus gracilirostris*. NanoLuc-expressing bacteriophages had a limit of detection of 10–100 CFU per mL in culture without enrichment. Luciferase reporters demonstrated a broad host range covering all *Salmonella* species with one reporter detecting 99.3% of 269 inclusivity strains. Cross-reactivity was limited and only observed with other members of the *Enterobacteriaceae* family. In food matrix studies, a cocktail of engineered bacteriophages accurately detected 1 CFU in either 25 g of ground turkey with a 7 h enrichment or 100 g of powdered infant formula with a 16 h enrichment. Use of the NanoLuc reporter assay described herein resulted in a considerable reduction in enrichment time without a loss of sensitivity.**

Abstract
Salmonella is a major causative agent of foodborne illness and rapid identification of this pathogen is essential to prevent disease. Currently most assays require high bacterial burdens or prolonged enrichment to achieve acceptable performance. A reduction in testing time without loss of sensitivity is critical to allow food processors to safely decrease product holding time. To meet this need, a method was developed to detect *Salmonella* using luciferase reporter bacteriophages. Bacteriophages were engineered to express NanoLuc, a novel optimized luciferase originating from the deep-sea shrimp *Oplophorus gracilirostris*. NanoLuc-expressing bacteriophages had a limit of detection of 10 to 100 CFU per mL in culture without enrichment. Luciferase reporters demonstrated a broad host range covering all *Salmonella* species with one reporter detecting 99.3% of 269 inclusivity strains. Cross-reactivity was limited and only observed with other members of the *Enterobacteriaceae* family. In food matrix studies, a cocktail of engineered bacteriophages accurately detected 1 CFU in either 25 g of ground turkey with a 7 h enrichment or 100 g of powdered infant formula with a 16 h enrichment. Use of the NanoLuc reporter assay described herein resulted in a considerable reduction in enrichment time without a loss of sensitivity.

Introduction

Salmonella is one of the most common foodborne pathogens resulting in over 93 million cases of salmonellosis and 150,000 deaths every year globally[1]. Within the US, it is estimated to cause over a million infections annually and is the leading cause of hospitalizations and deaths from foodborne illnesses[2]. These infections also represent a substantial economic burden with an annual cost of illness estimated at over $3 billion in the US alone[3].

The genus *Salmonella* consists of two species: *enterica* and *bongori*. *Salmonella enterica* is further divided into six taxonomically recognized subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*[4]. Critically, 99% of *Salmonella* isolates from human cases in the US are *Salmonella enterica* subspecies *enterica*[5]. This subspecies can be further differentiated into over 1,500 serovars[6]. The most prevalent serovars associated with foodborne disease outbreaks in the US are Enteritidis (32% of outbreaks), Typhimurium (13%), Heidelberg (8%) and Newport (7%)[7].

Accurate and timely detection of contaminated food prior to sale is essential in preventing foodborne illness. The current gold standard method for *Salmonella* detection requires at least three days, consisting of multiple sample enrichments and subsequent plating on selective agar[8,9]. An additional 24 h is also required, at minimum, to confirm any presumptive positives identified, traditionally using

biochemical analysis. Although laborious, this method can detect a single *Salmonella* colony forming unit (CFU) in a 25-gram sample.

A reduction in total testing time is highly desirable and can be achieved by a decrease in detection and/or enrichment time. Assays using PCR, ELISA, latex agglutination, mass spectrometry, and even meta-genomic sequencing, have been explored as rapid alternative methods of *Salmonella* detection in food matrices[10-14]. Additionally, capture of *Salmonella* by antibodies, DNA aptamers, or bacteriophages has been used to concentrate samples and reduce traditional enrichment times[15-17]. While these rapid approaches have been largely successful, most available methods still require at least 18 h of enrichment to detect 1 CFU in 25 g of product.

Bacteriophages (phages) have also been examined as a foundation for sensitive and accurate detection of foodborne pathogens[18,19]. One particularly promising phage-based approach for *Salmonella* detection involves the use of luciferase reporter phages[20-22]. This method requires an engineered phage, traditionally encoding the luciferase gene cassette *lux* from *Allivibrio fischeri*. If a sample contains viable contaminating *Salmonella*, infection with the recombinant phage will yield a detectable bioluminescent signal. A novel luciferase, NanoLuc, has been recently engineered from the deep-sea shrimp *Oplophorus gracilirostris*[23]. This luciferase is only 19 kDa, 150 times brighter than other luciferases and reacts with a novel furimazine substrate with low background noise[24]. These characteristics suggest that NanoLuc would be a superior choice as a luciferase reporter in phage-based assays. Although yet to be achieved in *Salmonella*, NanoLuc reporter phages have been recently described mediating sensitive and rapid detection of *Escherichia coli* O157:H7 or *E. coli* in ground beef and water[25-27].

The objectives of this study were: (1) to develop and characterize the first NanoLuc reporter phage assay for *Salmonella* and (2) to assess its performance to detect this pathogen in ground turkey and powdered infant formula (PIF).

Results

Characterization of *Salmonella* phages SEA1 and TSP1. Preliminary studies led to the selection of two lytic *Salmonella* bacteriophages, SEA1 and TSP1, for assay development. Using one-step growth curves, the replication cycle time was determined to be 35 to 40 min and 60 to 70 min for SEA1 and TSP1, respectively. The burst size of SEA1 was found to be approximately 30 pfu per cell, while TSP1 produced a larger

burst size of approximately 100 pfu per cell. Thus, the replication cycle time and burst size for SEA1 and TSP1 are similar to those reported for other *Salmonella* phages[28]. To facilitate plasmid design for homologous recombination, DNA from SEA1 and TSP1 was extracted and sequenced. While genome curation and annotation are beyond the scope of this study, preliminary analysis revealed a genome size of approximately 162 kbp for SEA1 and 157 kbp for TSP1. BLAST analysis of SEA1 revealed considerable homology to *Salmonella* phage vB_SenM-S16 (NC_020416). This phage was previously described as a 160 kbp Myovirus possessing a remarkably broad host range strictly within the *Salmonella* genus[29]. BLAST analysis of TSP1 revealed considerable homology to *Salmonella* phage SFP10 (NC_016073). This phage was previously described as a 158 kbp Myovirus specific for *E. coli* O157:H7 and *Salmonella* isolates[30]. The morphology of SEA1 and TSP1 was visualized by transmission electron microscopy (Fig. 1a,b). Both SEA1 and TSP1 have contractile non-flexible tails. Based upon these micrographs and supported by sequence homology, SEA1 and TSP1 are also predicted members of the *Myoviridae* family[31].

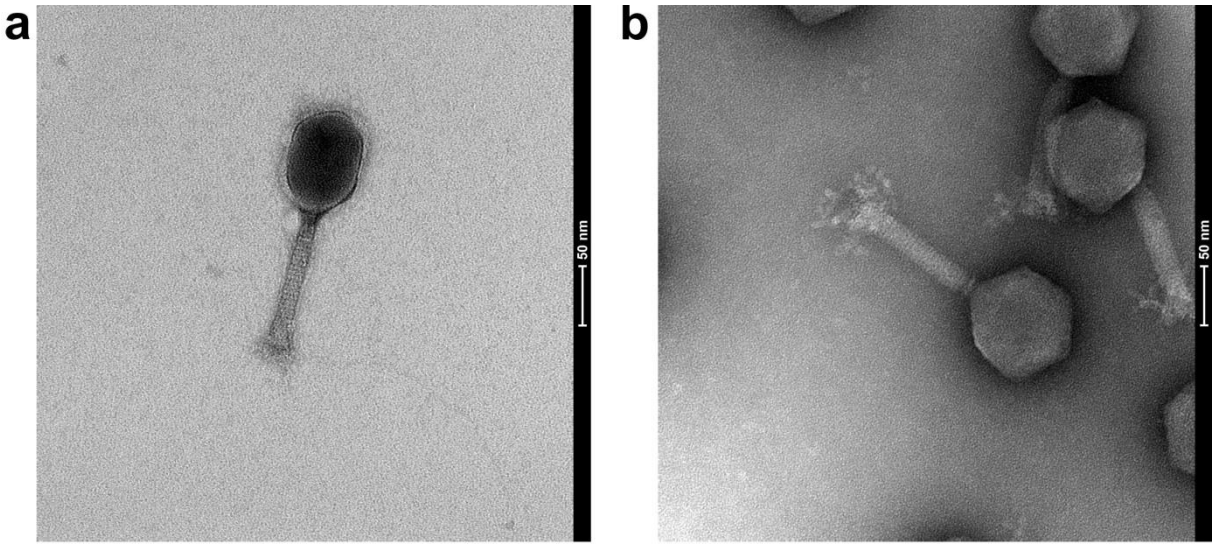

Figure 1. Transmission electron micrograph of (a) SEA1; (b) TSP1

Construction of NanoLuc-expressing recombinant bacteriophages. Generation of NanoLuc-expressing recombinant SEA1 and TSP1 was performed using homologous recombination (Fig. 2a,b). Homologous flanks were designed to direct insertion downstream of the predicted major capsid protein, a strategy previously used to generate luciferase reporter phage for *Listeria*[32]. This insertion site was not expected

to disrupt any predicted genes. Recombination donor plasmids were generated containing these regions of homology flanking a codon-optimized NanoLuc gene under a T4 late promoter. *Salmonella* transformants containing these donor plasmids were infected with SEA1 and TSP1. Recombinant NanoLuc-expressing bacteriophages (SEA1.NL and TSP1.NL) were isolated from this reaction, passaged to purity, and confirmed by DNA sequencing.

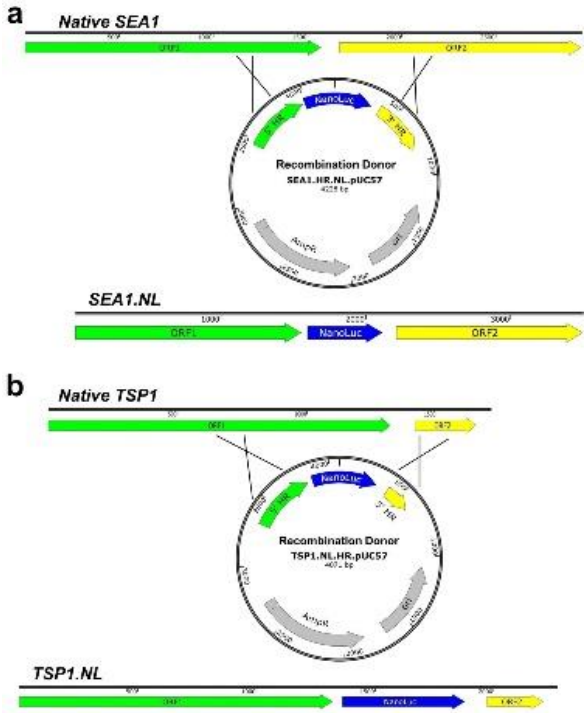

Figure 2. Generation of NanoLuc expressing bacteriophages by homologous recombination. (a) SEA1 recombination donor assembled in pUC57. ORF1 of SEA1 indicates the predicted major capsid protein while ORF2 has homology to head vertex proteins; (b) The TSP1 recombination donor assembled in pUC57. ORF1 of TSP1 indicates the predicted major capsid protein while ORF2 is a hypothetical protein of unknown function. Graphics were generated using SnapGene (GSL Biotech LLC, Chicago, IL).

Limit of detection of engineered bacteriophage reporters. Successful detection of pathogens must be capable of finding small numbers of contaminating cells. Although SEA1.NL and TSP1.NL were expected to produce considerable amounts of luciferase following infection, the exact number of required host cells to produce a detectable signal over background was unknown. To determine the limit of detection of these engineered phages, infections were performed with inoculums ranging from 1 to 10,000 CFU. After a 2 h infection, the amount of luciferase produced in each condition was determined with a luminometer following the addition of substrate (Table 1). Background from medium and reporter alone (negative control) was minimal,

averaging 117 and 51 RLU for SEA1.NL and TSP1.NL, respectively. The standard deviation (SD) of background was also low, allowing signal from luciferase production to be easily recognized. Signal above background was detected, on average, from a single cell for both SEA1.NL and TSP1.NL. Average RLU from either reporter phage infection increased proportionately with the number of *Salmonella* cells, reaching values over 100 times background with only 100 CFU. Signal from TSP1.NL was consistently higher than SEA1.NL at equivalent cell counts, possibly reflective of the higher burst size of TSP1. RLU variability, as measured by coefficient of variation (CV), was expected at low cell counts, where the probability of no cells being present in a replicate is increased. Individual replicates that received no cells by chance will yield only a background signal, which will contrast starkly with replicates containing live cells. This effect would be most pronounced at one, two, and five CFU, where the highest variation is observed. These results confirm the functionality of SEA1.NL and TSP1.NL, revealing a clear correlation between average RLU and presence of *Salmonella*. Remarkably, a detectable signal above background could be demonstrated with a single log phase CFU after only a 2 h infection. This single cell signal was greater than twice background, a standard cutoff used by others to determine positive detection from luciferase reporter phages[25].

Reporter	CFU	# of Replicates	Avg. RLU	SD	% CV	S/B
SEA1.NL	0	6	117	7	6	1.0
	1	10	287	499	174	2.4
	2	10	365	382	105	3.1
	5	10	1,285	1,172	91	11.0
	10	10	2,205	960	44	18.8
	100	10	12,453	4,685	38	106.4
	1,000	6	169,643	26,610	16	1,449.9
	10,000	6	2,313,504	223,614	10	19,773.5
TSP1.NL	0	6	51	12	24	1.0
	1	10	207	255	123	4.1
	2	10	362	497	137	7.1
	5	10	627	704	112	12.3
	10	10	2,667	2,163	81	52.1
	100	10	20,920	5,011	24	408.9
	1,000	6	241,224	19,632	8	4,714.5
	10,000	6	4,585,851	144,389	3	89,625.8

Table 1. Limit of detection of bacteriophages SEA1.NL and TSP1.NL. Abbreviations: CFU = colony forming units, RLU = relative light units, SD = standard deviation, CV = coefficient of variation, and S/B = Signal over background. *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC 19585) was used for TSP1.NL while *Salmonella enterica* subsp. *enterica* ser. Choleraesuis (ATCC 7001) was used for SEA1.NL. Strains were diluted from log phase cultures and infected with the indicated reporter phage for 2 h. Signal over background was defined as average RLU over average RLU without cells.

Inclusivity of SEA1NanoLuc and TSP1NanoLuc detection. Since most human infections are the result of *Salmonella enterica* subsp. *enterica*, a panel of 245 members of this subspecies was assembled. These strains, spanning 84 distinct serovars, were infected for 2 h with either SEA1.NL or TSP1.NL and assessed for luciferase production. A threshold of 750 RLU was used to establish positive detection for inclusivity and all further testing. This static value was selected for increased stringency during host range testing and to allow consistency across complex matrices with variable background autoluminescence. When testing stationary phase cells without enrichment, SEA1.NL and TSP1.NL produced a positive signal from 243 of 245 and 129 of 245 *Salmonella enterica* subsp. *enterica* strains, respectively (Table 2). Data for individual serovars are provided (Supplementary Table 1). Only two *Salmonella* strains were negative with both reporter phages, one strain of serovar Enteritidis (out of 27 tested) and one strain of serovar Kentucky (out of 3 tested). Although less common, *Salmonella bongori* and *Salmonella enterica* subspp. *arizonae, diarizonae, houtenae, indica* and *salamae* may facilitate human disease and cannot be disregarded. Additional 24 *Salmonella* strains were assessed, including at least one representative of every currently recognized *Salmonella enterica* subspecies and the only other *Salmonella* species, *bongori*. SEA1.NL produced a positive signal from all 24 strains, while TSP1.NL detected six of these strains (Table 2). RLU values for all inclusivity strains are provided (Supplementary Table 2). Overall, SEA1.NL yielded an impressively broad host range, successfully detecting 99.3% of *Salmonella* tested in this study. TSP1.NL, on the other hand, detected just over one half (50.2%) of strains tested, indicating a substantially narrower host range. Of interest, TSP1.NL produced substantially higher signal than SEA1.NL for the Agona serovar of *Salmonella enterica* subsp. *enterica*. With six strains of this serovar tested, the median RLU signal was 73,530 for SEA1.NL and 51,280,951 for TSP1.NL (Supplementary Table 2). While no false negatives were observed with these six strains, the lowest signal observed with SEA1.NL was a mere 2,958 RLU compared to 6,195,000 RLU with TSP1.NL. This particular serovar has been the source of several food-related outbreaks, including contaminated infant milk products.[33-35] Overall, the median signal from positively detected *Salmonella* strains was 103,257,000 RLU for SEA1.NL and 206,576,768 RLU for TSP1.NL. These values are substantially above the 750 RLU threshold used and demonstrate the robust signal generation of these reporters.

			Positives / Total	
Genus	**Species**	**Subspecies**	**SEA1.NL**	**TSP1.NL**
Salmonella	*enterica*	*enterica*	243/245	129/245
		salamae	6/6	4/6
		arizonae	6/6	1/6
		diarizonae	6/6	1/6
		houtenae	2/2	0/2
		indica	1/1	0/1
	bongori	N/A	1/1	0/1
	Non-typeable	N/A	2/2	0/2
		Summary:	267/269	135/269

Table 2. Detection of diverse *Salmonella* by SEA1.NL and TSP1.NL. Stationary phase cultures were diluted to an OD_{600} of 0.2 and infected with the indicated reporter phage for 2 h. Strains were determined to be positive when signal exceeded a detection threshold of 750 RLU. Strains were determined to be non-typeable by vendor/source.

Specificity of SEA1NanoLuc and TSP1NanoLuc detection. Methods facilitating detection of *Salmonella* contamination must possess sufficient specificity to limit cross-reactivity with the natural microbiome present in many food matrices. To determine the specificity of these NanoLuc reporter phages, an exclusivity panel of non-*Salmonella* strains was collected. Representatives of 14 species of Gram-positive and 26 species of Gram-negative bacteria were infected for 2 h with SEA1.NL or TSP1.NL and assessed for luciferase production. Unsurprisingly, no false positives were detected with Gram-positive bacteria, likely due to considerable differences in surface structures when compared to the *Salmonella* host (Table 3). False positives were observed, however, with several Gram-negative species. Upon infection of diluted overnight cultures with SEA1.NL, a total of eight false positives were identified from the panel of 90 strains. For this reporter, three strains of *Escherichia coli* (of 48 tested) and one strain each of *Citrobacter brakii*, *Citrobacter sedlakii*, *Serratia marcescens*, *Shigella flexneri*, and *Yersinia enterocolitica* produced a signal above the detection threshold. By contrast, TSP1.NL only produced a positive signal with one strain, a *Citrobacter sedlakii*, from the entire exclusivity panel. RLU values for all exclusivity strains are provided (Supplementary Table 3). Of note, five of the eight false positives encountered with SEA1.NL were below 103,000 RLU, which is 1,000-fold lower than the median RLU signal observed with *Salmonella* true positives. Only two false positive strains, one *Escherichia coli* and one *Citrobacter sedlakii* generated signal indistinguishable from most of the *Salmonella* strains. These results highlight the limited potential for false positives from related Gram-negative bacteria.

			Positive / Total	
Type	**Genus**	**Species**	**SEA1.NL**	**TSP1.NL**
Gram-negative	*Acinetobacter*	*calcoaceticus*	0/1	0/1
	Citrobacter	*braakii*	1/1	0/1
		freundii	0/1	0/1
		koseri	0/1	0/1
		sedlakii	1/1	1/1
		werkmanii	0/1	0/1
		youngae	0/1	0/1
	Cronobacter	*sakazakii*	0/1	0/1
	Edwardsiella	*tarda*	0/1	0/1
	Enterobacter	*cloacae*	0/1	0/1
		kobei	0/1	0/1
	Escherichia	*coli*	3/48	0/19
		fergusonii	0/1	0/1
		hermanni	0/1	0/1
	Hafnia	*alevi*	0/1	0/1
	Klebsiella	*aerogenes*	0/1	0/1
		oxytoca	0/1	0/1
		pneumonia	0/1	0/1
	Morganella	*morganii*	0/1	0/1
	Pluralibacter	*gergoviae*	0/1	0/1
	Proteus	*vulgaris*	0/1	0/1
	Pseudomonas	*aeruginosa*	0/1	0/1
	Serratia	*marcescens*	1/1	0/1
	Shigella	*flexneri*	1/1	0/1
		sonnei	0/1	0/1
	Yersinia	*enterocolitica*	1/1	0/1
Gram-positive	*Bacillus*	*cereus*	0/1	0/1
		subtilis	0/1	0/1
	Enterococcus	*faecalis*	0/2	0/2
		faecium	0/1	0/1
	Listeria	*grayi*	0/1	0/1
		innocua	0/1	0/1
		ivanovii	0/1	0/1
		seeligeri	0/1	0/1
		welshimeri	0/1	0/1
	Staphylococcus	*aureus*	0/3	0/3
		epidermidis	0/1	0/1
		haemolyticus	0/1	0/1
		hominis	0/1	0/1
		saprophyticus	0/1	0/1
		Summary:	8/90	1/61

Table 3. Exclusivity of SEA1.NL and TSP1.NL. Stationary phase cultures were diluted to an OD_{600} of 0.2 and infected for 2 h with the indicated reporter phage. Samples were positive when the luminescent signal exceeded a threshold of 750 RLU.

Detection of *Salmonella* contamination in food matrices. Based upon these results, SEA1.NL and TSP1.NL were combined into a single phage cocktail. TSP1.NL was expected to supplement the signal intensity of many *Salmonella* strains without compromising the specificity achieved with SEA1.NL alone. Further, an enrichment period was added to the workflow to promote recovery, growth, and detection of single

cell *Salmonella* contamination. This method of phage detection is referred to as the PhageDx method for *Salmonella*.

Although accurate detection of *Salmonella* cells was achieved in pure culture, food products are complex test matrices that may present additional challenges and complications. To determine if the PhageDx method could mediate detection in such environments, two distinct relevant food matrices were selected. As a representative of a ground meat product, raw ground turkey was chosen and has previously been associated with a nationwide outbreak in the United States[36]. For the second matrix, powdered infant formula was selected to model detection in dried food products, which itself has been linked to multiple outbreaks in infants[37]. Both matrices were pre-screened prior to use to evaluate the presence of pre-existing contamination. *Salmonella* was not detected endogenously from portions of either matrix used in this study. Although appearing free of *Salmonella*, each gram of homogenized ground turkey did yield approximately 40 CFU on non-selective media. The powdered infant formula used in this study was found to contain little endogenous flora (0 CFU per gram).

Portions (25 g) of raw ground turkey were either uninoculated, inoculated with a low-level of *Salmonella* (~1 CFU), or inoculated with a high-level of *Salmonella* (~10 CFU). Following a 7 h enrichment, samples were infected with a reporter phage cocktail for 2 h and checked for luciferase production. No false positives were detected among uninoculated test portions (Table 4). As anticipated, partial positives for the “low” inoculum and all positives for the “high” inoculum were obtained with two *Salmonella enterica* subsp. *enterica* serovars. Importantly, the PhageDx assay agreed with a culture-based confirmation method for all 30 samples. Dynabead isolation and CHROMagar *Salmonella* were utilized for this comparison as both methods have demonstrated excellent performance in a variety of food matrices[38-41]. These results indicate that the PhageDx method is capable of accurately detecting low levels of *Salmonella* contamination in raw ground turkey.

Serovar	Inoculum	RLU	PhageDx	Culture
	None	230	Negative	Negative
	None	269	Negative	Negative
	None	308	Negative	Negative
	Low	227	Negative	Negative
	Low	280	Negative	Negative
	Low	283	Negative	Negative
	Low	15,287	Positive	Positive
Newport	Low	228,509	Positive	Positive
	Low	241,967	Positive	Positive
	Low	258,108	Positive	Positive
	Low	379,592	Positive	Positive
	Low	496,149	Positive	Positive
	Low	921,481	Positive	Positive
	High	5,037,793	Positive	Positive
	High	5,060,438	Positive	Positive
	None	203	Negative	Negative
	None	203	Negative	Negative
	Low	168	Negative	Negative
	Low	207	Negative	Negative
	Low	209	Negative	Negative
	Low	219	Negative	Negative
	Low	223	Negative	Negative
Muenster	Low	244	Negative	Negative
	Low	14,893	Positive	Positive
	Low	76,050	Positive	Positive
	Low	347,209	Positive	Positive
	Low	690,403	Positive	Positive
	High	1,065,705	Positive	Positive
	High	1,834,854	Positive	Positive
	High	2,683,763	Positive	Positive

Table 4. Detection of *Salmonella enterica* in inoculated portions of ground turkey (25 g). *Salmonella enterica* subsp. *enterica* serovars were diluted from stationary phase cultures and inoculated into pre-screened portions of ground turkey. Strain 27869 (ATCC) and OCT084 (USDA) were used as serovar Newport and Muenster, respectively. Equilibrated samples were enriched for 7 h, and infected with a cocktail of SEA1.NL and TSP1.NL for 2 h before being assessed for luciferase production. Inoculums consisted of either no CFU "None", approximately 1 CFU per 25 g "Low" (1.1 CFU for Newport, 1.2 CFU for Muenster), or approximately 10 CFU per 25 g "High" (11.8 CFU for Newport, 8.9 CFU for Muenster). A detection threshold of 750 RLU was used to determine positive samples. Samples were confirmed by a culture-based method involving Dynabead isolation and plating on CHROMagar *Salmonella*.

Portions (100 g) of PIF were evaluated in a similar manner to raw ground turkey, except a 16 h enrichment was used. No false positives were detected among uninoculated test portions (Table 5). Both serovars of *Salmonella enterica* subsp. *enterica* produced the anticipated partial positives for the "low" inoculum and all positives for the "high" inoculum. As seen previously with ground turkey, the PhageDx method agreed with a culture-based confirmation method for all 30 PIF samples. These results indicate that the PhageDx method is also capable of accurately detecting low

levels of *Salmonella* contamination in PIF.

Serovar	Inoculum	RLU	PhageDx	Culture
Heidelberg	None	339	Negative	Negative
	None	354	Negative	Negative
	None	376	Negative	Negative
	Low	285	Negative	Negative
	Low	288	Negative	Negative
	Low	333	Negative	Negative
	Low	343	Negative	Negative
	Low	395	Negative	Negative
	Low	432	Negative	Negative
	Low	461	Negative	Negative
	Low	259,893,152	Positive	Positive
	Low	461,765,216	Positive	Positive
	Low	501,775,552	Positive	Positive
	High	498,655,520	Positive	Positive
	High	1,031,197,312	Positive	Positive
Reading	None	271	Negative	Negative
	None	305	Negative	Negative
	Low	302	Negative	Negative
	Low	334	Negative	Negative
	Low	2,820	Positive	Positive
	Low	10,667	Positive	Positive
	Low	16,944	Positive	Positive
	Low	43,975	Positive	Positive
	Low	162,731	Positive	Positive
	Low	167,912	Positive	Positive
	Low	458,206	Positive	Positive
	Low	481,718	Positive	Positive
	High	170,377	Positive	Positive
	High	340,074	Positive	Positive
	High	365,167	Positive	Positive

Table 5. Detection of *Salmonella enterica* in inoculated portions of powdered infant formula (100 g). *Salmonella enterica* subsp. *enterica* serovars were diluted from stationary phase cultures, dried down, and inoculated into pre-screened portions of PIF. Strain SL476 (FDA) and 52317.1 (USDA) were used as serovar Heidelberg and Reading, respectively. Equilibrated samples were enriched for 16 h, diluted ten-fold, and infected with a cocktail of SEA1.NL and TSP1.NL for 2 h before being assessed for luciferase production. Inoculum consisted of either no CFU “None”, 1 CFU per 100 g “Low”, or 5 CFU per 100 g “High”. A detection threshold of 750 RLU was used to determine positive samples. Samples were confirmed by a culture-based method involving plating on CHROMagar *Salmonella*.

Discussion

Rapid, accurate, and sensitive detection of foodborne pathogens is essential to maintain a safe and effective food supply. Despite achieving desired sensitivity and accuracy, many commercially available assays require extensive enrichment and operate under a timeframe of days, not hours. This study details the development and performance of the PhageDx rapid detection method for *Salmonella*, a prevalent and

important contaminant of food products. To our knowledge, this method represents the first development and use of NanoLuc reporter bacteriophages in *Salmonella*.

Initial screening of 53 bacteriophages led to two promising candidates, SEA1 and TSP1, being chosen for assay development. These phages were predicted based on morphology to be members of the *Myoviridae* family (Fig. 1). NanoLuc-expressing recombinants of these phages (SEA1.NL and TSP1.NL) were engineered using homologous recombination (Fig. 2). These reporters were evaluated using a 2 h infection, no enrichment, and varying amounts of log phase *Salmonella*. A clear correlation between luminescent signal over background and *Salmonella* was observed with SEA1.NL and TSP1.NL (Table 1). Critically, a single CFU of *Salmonella* produced, on average, a detectable signal above background. This result underscores the advantages of tying reporter production to lytic phage, which rapidly adsorb, infect, replicate, and lyse host cells. The limit of detection of this method is also aided by the robust and specific luminescent signal produced by NanoLuc and its substrate, furimazine[23]. The reporter phages engineered in this study thus demonstrated remarkable sensitivity, a promising trait that can be leveraged to shorten assay time.

The inclusivity of NanoLuc reporter phages was evaluated with diluted stationary phase cultures, no enrichment, and a 2 h infection. Using a detection threshold of 750 RLU, SEA1.NL was able to positively detect 99.3% of 269 *Salmonella* strains (Table 2). The extensive range of this reporter suggests that SEA1 utilizes a receptor common to almost all *Salmonella*. Of note, the *Salmonella* bacteriophage S16, which has considerable homology to SEA1, has been found to bind OmpC, an outer member protein that is well-conserved among *Salmonella*[29,42]. Regardless of the receptor used, the median signal from detected strains was over 100 million RLU, well beyond the threshold used (Supplementary Table 2). Unlike the broad coverage of SEA1.NL, TSP1.NL detected only 50.2% of these strains while retaining strong median signal over 200 million RLU among positives. The narrower host range of TSP1 may represent the utilization of a less common receptor. Possible mechanisms include various outer membrane proteins or LPS O-antigen modifications found to act as receptors in other *Salmonella* phage[43]. Overall, the broad inclusivity of SEA1.NL is well-suited for detection across the *Salmonella* genus, while TSP1.NL may provide a supportive benefit with signal intensity in particular serovars, such as Agona.

Only two strains of *Salmonella* could not be detected by either reporter, one representative each of *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Kentucky. Importantly, our results do not indicate an inability to detect these serovars entirely as 27 other Enteritidis and two other Kentucky strains were positive (Supplementary Table 1). The exact mechanism behind these two false negatives is unknown, although it is plausible these two strains lack a common *Salmonella* receptor. Alternatively, a litany of phage resistance mechanisms have been described such as those mediating restriction-modification, abortive infection, and extracellular matrix production[44]. Future studies may refine or expand upon the phage cocktail, highlighting the flexible and modular nature of this approach.

The exclusivity of each reporter phage was evaluated in the same fashion as inclusivity. Of 90 non-*Salmonella* strains examined, false positives were detected with eight strains for SEA1.NL (Table 3). These eight strains belonged to the family *Enterobacteriaceae* with representatives of five genera including *Escherichia*, *Citrobacter*, *Serratia*, *Shigella*, and *Yersinia*. It appears likely that the receptor of SEA1, while conserved among most *Salmonella*, is not restricted to this genus. Some outer membrane proteins, including OmpC, are conserved among members of this family and may participate in this cross-reactivity[45-47].

Among the eight false positives, only one strain of *Citrobacter sedlakii* and one strain of *Escherichia coli* could mimic the intensity of a typical *Salmonella* isolate (Supplementary Table 3). Although the mechanism behind this variation in signal intensity is not known, it is feasible that non-*Salmonella* may lack certain co-receptors conducive to phage infection. TSP1.NL, on the other hand, cross-reacted with only one strain from the entire exclusivity panel, the same *Citrobacter sedlakii* observed with SEA1.NL. This supports the previous notion regarding the narrower host range of this phage. TSP1.NL has considerable homology to the *Salmonella* bacteriophage SFP10, which was previously reported to recognize both *Salmonella* and *E. coli* O157:H7[30]. Despite this homology, TSP1.NL did not produce a positive result for any O157:H7 strain tested in our study, which included some of the same strains previously tested with SFP10. These data support the notion that TSP1.NL does not share this property with SFP10.

The performance of SEA1.NL and TSP1.NL in food matrices was evaluated as part of the PhageDx *Salmonella* assay. Critically, this method involves minimal processing,

no sample cleanup, and can be adapted as needed depending on the properties of the matrix. When compared to a culture-based method, the PhageDx assay correctly identified 100% of artificially contaminated raw ground turkey samples with a 7 h enrichment (Table 4). Detection of a single *Salmonella* CFU in 25 grams of product, a required sensitivity benchmark, was achieved. Importantly, no false positives were observed, suggesting that the natural flora of this matrix (40 CFU per g) did not contain problematic *Enterobacteriaceae* strains or burdens. Similar performance was achieved in PIF, where the PhageDx assay also correctly identified 100% of artificially contaminated PIF samples with a 16 h enrichment (Table 5). The lack of false positives in this matrix was unsurprising, given the lack of natural flora observed in this study (0 CFU per g). The ability of phage reporters to function in various food matrices has been previously observed and is further supported by this study[48,49]. In summary, the PhageDx method agreed 100% with the longer culture-based method in both food matrices and was capable of accurate and sensitive detection of *Salmonella* contamination.

Previous attempts to utilize bacteriophage for *Salmonella* detection in food matrices have been met with mixed success. Utilizing a combination of bacteriophage and immunomagnetic separation, previous studies have successfully detected one to three CFU per 25 g of food [50]. This method required 20 h to complete but, as a result of the limited host range of the phage (SJ2), could only reliably detect *Salmonella enterica* subsp. *enterica* serovar Enteritidis. Bacteriophage have also been combined with real-time PCR to achieve rapid detection of low burdens in spiked chicken samples [51]. This approach was able to detect eight *Salmonella* CFU per 25 g portion within 10 h. Once again, however, the limited host range of the phage restricted reliable detection to one serovar, Enteritidis. Similar to the approach used in this study, several recombinant luciferase reporter phages have previously been assessed in food matrices. A luciferase-expressing recombinant of the temperate phage SPC32H demonstrated the ability to detect as few as 22 CFU per g (550 CFU per 25 g) in only 2 h from food matrices [49]. Although promising with sensitivity and speed, this phage was specific for the serovar Typhimurium, preventing its use in *Salmonella* species detection. P22, another temperate bacteriophage of *Salmonella*, was also assessed as a luciferase recombinant [20]. This reporter phage demonstrated excellent performance in feed and environmental samples with a 16 h assay time. As with all other described approaches,

detection of *Salmonella* by P22 recombinants was limited by the narrow host range of this phage and was serovar dependent. Thus, while previous bacteriophage-based methods have achieved excellent sensitivity and time to results, the PhageDx method uniquely affords broad detection of *Salmonella* independent of serovar in food matrices.

Ultimately, new methods support the continued goal of preventing contaminated products from reaching consumers. Shorter enrichment times are highly desirable, allowing issues to be detected early with limited product holding time. The PhageDx *Salmonella* assay leverages the sensitivity of two engineered NanoLuc-expressing bacteriophages to achieve rapid detection of single cell *Salmonella* contamination. This study demonstrates the noteworthy capabilities of bacteriophage reporter assays to facilitate accurate pathogen detection in a variety of matrices.

Methods

Bacterial strains. All bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), University of Georgia (Athens, GA), University of Iowa (Iowa City, IA), United States Department of Agriculture (USDA) (Clay Center, NE), Michigan State University STEC Center (East Lansing, MI), and the Food and Drug Administration (FDA) (College Park, MD). Unless otherwise indicated, bacterial strains were routinely cultured overnight in tryptone soy broth (TSB) (Oxoid, Hampshire, United Kingdom) at 37 °C with shaking at 225 revolutions per minute (rpm).

Wild-type phage isolation. The *Salmonella* bacteriophage SEA1 was obtained from Dr. Francisco Diez-Gonzalez's laboratory at the University of Minnesota. SEA1 is a broad-spectrum *Salmonella* phage of the *Myoviridae* family previously isolated from waste effluents[52]. *Salmonella* phage TSP1 was isolated from sewage samples obtained from the Metropolitan Waste Water Treatment Plant in St. Paul, Minnesota. Samples were clarified by centrifuging in a swinging bucket rotor at 4,700 x g for 10 min and filtering the supernatant through a 0.45 µm filter (Nalgene, Rochester, NY). A mixture containing 2 mL of this filtrate, 1 mL of 3× TSB, and 150 µl of an overnight culture of *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC 19585) was incubated at 37 °C with shaking at 225 rpm for 18 h. After this incubation, the sample

was once again centrifuged at 4,700 x g for 10 min and the supernatant filtered through a 0.45 µm filter. The presence of phage was initially evaluated by spot testing on ATCC 19585 and confirmed by plating for single plaques using the classical overlay method[53]. Individual plaques were picked, resuspended in TSB, and subsequentially plated again for single plaques. Single plaque selection was repeated five times to obtain pure, single phage cultures.

Generation of high titer stocks of wild-type and recombinant bacteriophages. High titer wild-type and recombinant phage stocks were made using broth lysates. To this end, 100 mL of logarithmic (log) phase *Salmonella* cells at an OD_{600} of 0.2 were infected at a multiplicity of infection (MOI) of 0.05. SEA1 and SEA1.NL used strain ATCC 14028 while TSP1 and TSP1.NL used strain ATCC 19585. After allowing 5 min for adsorption, infected cells were diluted into 400 mL of prewarmed TSB and incubated at 37 °C with shaking at 250 rpm until lysis was apparent. The phage lysate was clarified by centrifuging at 14,900 x g using a type 19 rotor in an Optima XE-90 ultracentrifuge (Beckman Coulter, Brea, CA) for 10 min at 4 °C. Phages were concentrated by centrifuging again at 14,900 x g for 2 h at 4 °C. The phage pellet was resuspended in TMS buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, and 300 mM NaCl) then treated with RNase and DNase I. Phages were further purified on a sucrose density gradient (10-30%) in TMS. The phage band was removed and sedimented at 107,200 x g using a SW41 Ti rotor in an Optima XE-90 ultracentrifuge for 30 min at 4 °C. Finally, the phage pellet was resuspended in SM buffer (50 mM Tris-HCl pH 7.5, 8 mM $MgSO_4 \cdot 7H_2O$, 100 mM NaCl, and 0.01% (w/v) gelatin) and the titer determined by serial dilution and plaque counting.

Bacteriophage characterization. DNA was isolated from the phages and sequenced by Laragen Inc. (Los Angeles, CA) using Illumina MiSeq whole genome sequencing followed by Contig assembly. DNA was isolated by heating 5×10^9 plaque forming units (pfu) at 90 °C for 5 min. DNA was purified from protein by three phenol/chloroform extractions. After removal of phenol/chloroform, 0.1 volume of 3 M sodium acetate and two volumes of ethanol were added to aqueous phase. DNA was precipitated at -80 °C, pelleted, then washed two times with 70% ethanol. The DNA pellet was dried and then resuspended in deionized water and used for sequencing.

The burst size and replication cycle time of phages were determined using a traditional one-step growth curve on their respective host strains[54].

Transmission electron microscopy of SEA1 and TSP1 was performed using 400 mesh grids coated with a thin carbon film. Glow discharged grids were floated on cesium chloride density gradient purified phage samples, then stained with 2% uranyl acetate. Images were captured on a Tecnai G2 Spirit BioTWIN at 30 kV.

Construction of homologous recombinant plasmids. Plasmids were designed to generate NanoLuc-expressing recombinant bacteriophages through homologous recombination (HR). Constructs containing a codon-optimized NanoLuc (Promega Corp., Madison, WI) gene under a T4 late promoter and flanked by regions of homology to the respective phage genome were designed. Codon optimization for *Salmonella* was performed using a codon optimization tool (Integrated DNA Technologies, Coralville, IA). Homologous flanks were designed to direct insertion downstream of the predicated major capsid protein. The SEA1 cassette consisted of 500 bp upstream of the desired insertion site, followed by the σ70 promoter -10 consensus sequence, a Shine-Dalgarno ribosomal entry site consensus sequence, a NanoLuc codon optimized for *Salmonella*, then 500 bp of downstream phage SEA1 sequence. The TSP1 cassette was similarly designed with the following exception. A 300 bp downstream homologous sequence was used, followed by a stop codon and transcriptional terminator. Differences in design were due to initial difficulties in the construction of the TSP1 HR plasmid which were overcome by the addition of a stop codon and transcriptional terminator. Constructed cassettes targeting TSP1 and SEA1 were assembled and inserted into the multiple cloning site of pUC57 by GeneWiz (South Plainfield, NJ). These recombinant constructs were expected to facilitate insertion of NanoLuc into a phage late gene region without disrupting any predicted genes.

Integration of the NanoLuc into the phage genome by homologous recombination. Electrocompetent *Salmonella* were generated as described previously[55,56]. *Salmonella enterica* subsp. *enterica* ser. Typhimurium strains ATCC 14028 and ATCC 19585 were selected as recombinant hosts for SEA1 and TSP1, respectively. Electrocompetent bacteria were combined with 100 ng of homologous

recombination plasmid DNA and subjected to a 1.8 kV single pulse using a MicroPulser electroporation apparatus (Bio-Rad Laboratories, Hercules, CA). Transformants were isolated following overnight growth on Luria-Bertani (LB) agar containing 100 µg/mL carbenicillin.

Resistant colonies were selected, grown in LB containing 100 µg/mL ampicillin, and infected with SEA1 or TSP1 respectively at various MOIs (0.1-10). Samples were incubated at 37 °C with 220 rpm shaking for 3 h. Following infection, cultures were centrifuged for 2 min at 6,800 x g. The supernatant was collected, filtered through a 0.45 µm filter, and washed with TMS on a 100 kDa pore protein concentrator PES column (Pierce Biotechnology, Rockford, IL). This was plated as previously described for single plaque isolation. To identify recombinants, candidate plaques were picked, mixed with a diluted overnight culture, and monitored for luciferase expression. Once a NanoLuc-producing isolate had been found for each bacteriophage, it was sequentially passaged at least four times from a single plaque to ensure the stability and purity of the recombinant. After isolation, high titer stocks of SEA1NanoLuc (SEA1.NL) and TSP1NanoLuc (TSP1.NL) were prepared as described previously. Homologous recombination was verified by genome sequencing, as described previously, and confirmed that the desired recombinants had been generated.

Limit of detection of phages SEA1NanoLuc and TSP1NanoLuc. Log phase *Salmonella* cells (OD_{600} of 0.1 to 0.5) were diluted in TSB to obtain desired CFU/mL. *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC 19585) was used for TSP1.NL while *Salmonella enterica* subsp. *enterica* ser. Choleraesuis (ATCC 7001) was used for SEA1.NL. 100 µl was then transferred to a 96-well plate and infected with 10 µl of phage reagent (1.2×10^7 pfu/mL in TSB) for 2 h at 37 °C. Luciferase detection solution was prepared as a master mix for each experiment, consisting of 50 µL of NanoGlo buffer, 15 µL Renilla lysis buffer, and 1 µL of NanoGlo substrate (Promega Corp., Madison, WI) per sample well. Following infection, 65 µl of this luciferase detection solution was added to each well and the samples read in a GloMax Navigator luminometer (Promega Corp., Madison, WI) using a 3 min wait time and 1 sec integration. Six to ten replicates of each dilution were measured and results averaged. Signal output was relative light units (RLU). Wells containing no *Salmonella* were used to determine background from media, phage, and detection reagents alone.

Inclusivity and exclusivity of phages SEA1NanoLuc and TSP1NanoLuc. Inclusivity and exclusivity assays were carried out to determine the coverage and specificity of recombinant phages. Overnight stationary phase cultures were diluted in TSB to an OD_{600} of 0.2 (approximately 1.6×10^8 CFU/mL). Aliquots of 100 µl were transferred to 96-well plates and infected with 10 µl of phage reagent. After 2 h of incubation at 37 °C, 65 µl of luciferase detection solution was added to each well. Luminescence was measured as previously described. Positive results were evaluated using a cutoff of 750 RLU.

Inoculation of raw ground turkey and powdered infant formula (PIF). Raw ground turkey (85% lean/15% fat Jennie-O, Wilmar, MN) was pre-screened using the PhageDx and culture-based confirmation method described below. Samples of ground turkey were also homogenized and plated on a non-selective agar (tryptone soy agar) to evaluate pre-existing contamination levels. Once the absence of endogenous *Salmonella* had been confirmed, the matrices were inoculated with the indicated *Salmonella enterica* serovars. Strain 27869 (ATCC) and OCT084 (USDA) were used, serovars Newport and Muenster, respectively. A liquid inoculum culture was prepared by transferring a single *Salmonella* colony from a TSB plate into TSB broth and incubating the culture for 18–24 h at 37 °C. Following incubation, the culture was diluted to the target level in buffered peptone water (BPW) (Oxoid, Hampshire, United Kingdom). Inoculums were plated to determine CFU level. Target CFUs levels were an average of 2-20 CFU/mL for low level and 20-100 CFU/mL for high level inoculums. Aliquots of 100 µl of designated inoculum were used to inoculate turkey samples. Based on averaged replicate plating, CFU inoculum per 25 g sample were 1.1 and 1.2 for low level and 11.8 and 8.9 CFU for high level of serovar Newport and Muenster, respectively. Prior to analysis samples were held for 48–72 h post-inoculation at 2-8 °C to allow for equilibration. Low level inoculated samples were expected to yield fractional positive results (25-75% positive), and a high level expected to yield all positive results. Negative control samples were uninoculated. All samples were assessed in a blinded manner as testers were unaware of the inoculum given to each test portion.

For PIF (Up & Up milk-based infant formula with iron, Target, Minneapolis, MN), *Salmonella enterica* was also grown in TSB for 18–24 h at 37 °C. Strain SL476 (FDA) and 52317.1 (USDA) were used, serovars Heidelberg and Reading, respectively. The culture was diluted in BPW, reconstituted in PIF and placed into a speed vacuum for 4–8 h until the sample was completely dried. Contaminated PIF was allowed to equilibrate for 2-4 weeks at room temperature (20-25 °C). After equilibration, an aliquot of dried inoculum was resuspended in 1 mL of BPW and plated to determine CFU level. Using this determined CFU, dried inoculum was then diluted into additional PIF matrix to achieve a low level (1 CFU/100 g) or high level (5 CFU/100 g). Low level inoculated samples were expected to yield fractional positive results (25-75% positive), and a high level expected to yield all positive results. Negative control samples were uninoculated. Samples of PIF were screened prior to inoculation to evaluate pre-existing contamination as described above for ground turkey. As with ground turkey, PIF was also assessed in a blinded manner.

PhageDx *Salmonella* detection assay for raw ground turkey and PIF. Raw ground turkey (25 g) was placed in a filter bag (Nasco WhirlPak, Fort Atkinson, WI), homogenized, and enriched with pre-warmed (41 °C) BPW in a 1:3 ratio (25 g ground turkey: 75 mL BPW) for 7 h at 41 °C. Powdered infant formula (100 g) was placed in a sample bag with pre-warmed (37 °C) BPW in a 1:3 ratio (100 g PIF: 300 mL BPW) and enriched for 16 h at 37 °C. After enrichment, a 150 µL direct sample for raw ground turkey, or a 150 µL 1:10 diluted sample for PIF, was transferred to a 96-well plate. Volumes of 10 µL of phage reagent were added and samples were incubated at 37 °C for 2 h. Then, 65 µL of luciferase detection solution was added. Luminescence was measured as previously described. Ground turkey samples and powdered infant formula samples with signals ≥750 RLUs were considered positive.

Culture-based confirmation method for *Salmonella*. For raw ground turkey, all samples were culturally confirmed by treating 1 mL of 24 h enriched samples with Dynabeads anti-*Salmonella* (Life Technologies AS, Norway) and then plating beads onto *Salmonella* selective chromogenic plates, CHROMagar *Salmonella* (DRG International, Springfield, NJ). For PIF samples, 100 µl of 24 h enriched samples were plated directly onto *Salmonella* selective chromogenic plates. Plates were incubated

for an additional 24 h at 37 °C. The presence of mauve colonies (1-3 mm) indicated a sample positive for *Salmonella*. The 24 h enrichment used in culture-based confirmations was an extended incubation of the samples previously tested with the PhageDx method, allowing for matched comparison.

Data availability

Unannotated raw genome assemblies of SEA1 and TSP1 are available upon request. All other data generated or analyzed during this study are included in this published article (and its supplementary information files). Researchers receiving resources generated in this study may be asked to sign a Materials Transfer Agreement that covers potential commercial use.

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Acknowledgements

The authors wish to express their gratitude to Dwight Anderson and Francisco Diez-Gonzalez for their insightful review and feedback regarding this manuscript.

Author contributions

Study was conceived by M.N. and S.E. Methods were developed by J.G., M.N. and S.E. Investigation was performed by J.G., M.N., and N.A. The original draft was written

by J.G, M.B., M.N., and S.E. Review and editing were performed by all authors. Figures were prepared by M.B., M.N. and S.E. Supervision was provided by E.T., M.E., M.N., and S.E. The project was conducted under the direction of S.E. All authors have read and agreed to this version of the manuscript.

Competing interests

Several authors (J.G., M.B., M.E., M.N., and S.E.) are employees of Laboratory Corporation of America Holdings (LabCorp). LabCorp provided all funding for this study. M.B. is a shareholder in LabCorp. S.E. and J.G. are inventors on issued United States patent (9,482,668 B2) relevant to the methods described in this work. N.A. and E.T. declare no potential conflict of interest.

Additional information

Supplementary information is available for this paper.

Supplementary information

	Positives / Total			Positives / Total	
Serovar	**SEA1.NL**	**TSP1.NL**	**Serovar**	**SEA1.NL**	**TSP1.NL**
Abaetetuba	1/1	0/1	Meleagridis	3/3	1/3
Abony	1/1	1/1	Menden	1/1	1/1
Adelaide	3/3	0/3	Michigan	1/1	1/1
Aequatoria	1/1	0/1	Minnesota	1/1	0/1
Agona	6/6	6/6	Mississippi	1/1	0/1
Alachua	1/1	1/1	Monophasic	2/2	2/2
Amsterdam	1/1	0/1	Montevideo	11/11	2/11
Anatum	6/6	1/6	Muenchen	6/6	2/6
Bareilly	1/1	1/1	Muenster	2/2	0/2
Benfica	1/1	0/1	Newport	17/17	4/17
Bispebjerg	1/1	1/1	Ngili	1/1	0/1
Braenderup	1/1	0/1	Ohio	1/1	0/1
Brandenburg	1/1	1/1	Oranienburg	1/1	0/1
Bredeney	1/1	0/1	Panama	4/4	4/4
Breukelen	1/1	0/1	Paratyphi A	1/1	1/1
Cerro	3/3	0/3	Paratyphi B	2/2	2/2
Champaign	1/1	0/1	Paratyphi C	2/2	2/2
Chester	1/1	1/1	Pomena	1/1	0/1
Choleraesuis	3/3	1/3	Potsdam	1/1	0/1
Derby	4/4	4/4	Pullorum	2/2	1/2
Dublin	8/8	5/8	Reading	2/2	2/2
Eko	1/1	0/1	Remo	1/1	0/1
Enteritidis	27/28	27/28	Rubislaw	1/1	0/1
Gallinarum	1/1	1/1	Saintpaul	3/3	2/3
Give	2/2	0/2	San Diego	1/1	1/1
Hadar	3/3	0/3	Schwarzengrund	1/1	1/1
Havana	2/2	0/2	Senftenberg	6/6	1/6
Heidelberg	9/9	9/9	Simsbury	1/1	0/1
Hvittingfoss	1/1	1/1	Stanley	1/1	1/1
Illinois	1/1	0/1	Taksony	1/1	0/1
Infantis	5/5	2/5	Tallahassee	1/1	0/1
Javiana	2/2	1/2	Tennessee	5/5	1/5
Kahla	1/1	0/1	Thompson	5/5	1/5
Kalamu	1/1	0/1	Typhi	1/1	1/1
Kentucky	2/3	0/3	Typhimurium	29/29	28/29
Kiambu	2/2	2/2	Uganda	1/1	0/1
Lexington	1/1	0/1	Urbana	1/1	0/1
Liverpool	1/1	0/1	Vellore	1/1	0/1
Livingstone	1/1	0/1	Virchow	1/1	0/1
London	2/2	0/2	Wagadugu	1/1	1/1
Manhattan	1/1	0/1	Weltevreden	1/1	0/1
Mbandaka	3/3	0/3	Worthington	1/1	0/1
Summary:				243/245	129/245

Supplementary Table 1: Reporter inclusivity with individual *Salmonella enterica* subsp. *enterica* serovars. Stationary phase cultures were diluted to an OD_{600} of 0.2 and infected with indicated reporter phage for 2 h. Strains were determined to be positive when signal exceeded a detection threshold of 750 relative light units (RLU).

Species	Subsp.	Serovar	Source	Strain ID	SEA1.NL	TSP1.NL
enterica	*enterica*	Abaetetuba	ATCC	35640	81,474,472	41
enterica	*enterica*	Abony	ATCC	BAA-2162	13,025,342	150,014,544
enterica	*enterica*	Adelaide	USDA	43128	2,925,056	64
enterica	*enterica*	Adelaide	USDA	SEP293	1,948,410	61
enterica	*enterica*	Adelaide	UIA	DMSO08	20,620,000	25
enterica	*enterica*	Aequatoria	USDA	1345	240,941,440	173
enterica	*enterica*	Agona	FDA	SARB1	59,249,952	418,878,912
enterica	*enterica*	Agona	UGA	SLR 141	11,465	25,062,894
enterica	*enterica*	Agona	UGA	N/A	1,552,205	77,499,008
enterica	*enterica*	Agona	USDA	42113	62,850	6,195,000
enterica	*enterica*	Agona	USDA	SEP054	2,958	7,694,021
enterica	*enterica*	Agona	UIA	DMSO09	84,210	355,100,000
enterica	*enterica*	Alachua	UIA	DMSO12	364,253,920	295,961,568
enterica	*enterica*	Amsterdam	USDA	41084	36,980,000	57
enterica	*enterica*	Anatum	FDA	SARB2	5,719,141,376	1,126
enterica	*enterica*	Anatum	UGA	SLR 377	162,681,744	192
enterica	*enterica*	Anatum	USDA	31064.1	16,800,000	43
enterica	*enterica*	Anatum	USDA	NOV091	123,844,296	42
enterica	*enterica*	Anatum	UIA	DMSO13	162,770,832	62
enterica	*enterica*	Anatum	ATCC	9270	44,377	37
enterica	*enterica*	Bareilly	UGA	73	185,602,848	1,535
enterica	*enterica*	Benfica	USDA	AUG071	455,400	56
enterica	*enterica*	Bispebjerg	ATCC	9842	159,974,128	137,063,104
enterica	*enterica*	Braenderup	USDA	52115	81,969	32
enterica	*enterica*	Brandenburg	USDA	AUG053	662,700	664,800,000
enterica	*enterica*	Bredeney	USDA	61003.2	11,740,000	41
enterica	*enterica*	Breukelen	ATCC	15782	2,778,540	35
enterica	*enterica*	Cerro	USDA	31011.1	407,600,160	29
enterica	*enterica*	Cerro	USDA	DEC021	360,353,696	370
enterica	*enterica*	Cerro	USDA	V2-577.2	292,269	114
enterica	*enterica*	Champaign	ATCC	700139	100,568,304	77
enterica	*enterica*	Chester	ATCC	11997	4,558,113,280	474,715,712
enterica	*enterica*	Choleraesuis	ATCC	12011	134,298,560	1,547
enterica	*enterica*	Choleraesuis	ATCC	10708	170,185,728	291
enterica	*enterica*	Choleraesuis	ATCC	7001	98,668,592	75
enterica	*enterica*	Derby	FDA	SARB9	12,198	1,419
enterica	*enterica*	Derby	FDA	SARB10	107,414	197,188
enterica	*enterica*	Derby	FDA	SARB11	197,045,648	71,512,520
enterica	*enterica*	Derby	USDA	41088.2	1,327,000	9,945,000
enterica	*enterica*	Dublin	FDA	SL477	91,407,056	645
enterica	*enterica*	Dublin	FDA	SARB12	5,843,639,296	178
enterica	*enterica*	Dublin	FDA	SARB13	1,522,073	130,845,176
enterica	*enterica*	Dublin	FDA	SARB14	5,853,678,080	135,589,440
enterica	*enterica*	Dublin	USDA	63205	304,334	896
enterica	*enterica*	Dublin	USDA	JUL052	45,290	47
enterica	*enterica*	Dublin	UIA	DMSO30	754,300	416,900,000
enterica	*enterica*	Dublin	ATCC	15480	1,740,486	20,416,442
enterica	*enterica*	Eko	USDA	33006.2	18,190,000	111
enterica	*enterica*	Enteritidis	FDA	SL1224	46,333,504	342,584,576
enterica	*enterica*	Enteritidis	FDA	SL1301	149,998,512	279,901,088
enterica	*enterica*	Enteritidis	FDA	SL1302	73,158,880	334,015,808
enterica	*enterica*	Enteritidis	FDA	SL1303	49,470,164	382,513,856
enterica	*enterica*	Enteritidis	FDA	SARB16	35,758,692	840,515
enterica	*enterica*	Enteritidis	FDA	SARB17	94,282,760	309,580,832
enterica	*enterica*	Enteritidis	FDA	SARB18	163,330,640	317,366,144
enterica	*enterica*	Enteritidis	FDA	SARB19	94,616,696	279,196,960
enterica	*enterica*	Enteritidis	UGA	430	135,373,664	280,150,080
enterica	*enterica*	Enteritidis	UGA	457	388,313,360	230,051,880
enterica	*enterica*	Enteritidis	UGA	1294	419,753,056	195,128,248

Species	Subsp.	Serovar	Source	Strain ID	SEA1.NL	TSP1.NL
enterica	*enterica*	Enteritidis	UGA	61697	257,343,776	175,864,400
enterica	*enterica*	Enteritidis	UGA	14027-J	126,145,192	370,417,920
enterica	*enterica*	Enteritidis	UGA	16037-L	114,888,112	206,576,768
enterica	*enterica*	Enteritidis	UGA	180-88	119,358,888	358,823,040
enterica	*enterica*	Enteritidis	UGA	565-88	153,713,504	3,818
enterica	*enterica*	Enteritidis	UGA	H2292	138,354,640	249,213,184
enterica	*enterica*	Enteritidis	UGA	H4267	193,152,624	247,790,912
enterica	*enterica*	Enteritidis	UGA	H4638	320,765,888	419,026,432
enterica	*enterica*	Enteritidis	UGA	ME-14	114,413,392	207,639,888
enterica	*enterica*	Enteritidis	UGA	MH45931	356,041,792	354,058,432
enterica	*enterica*	Enteritidis	UGA	S276	604,654	3,233,180
enterica	*enterica*	Enteritidis	UGA	S293	15,881,310	383,002,720
enterica	*enterica*	Enteritidis	UGA	S294	9,607,902	390,469,472
enterica	*enterica*	Enteritidis	UGA	S421	19,267,712	404,313,408
enterica	*enterica*	Enteritidis	UGA	S492	219	481
enterica	*enterica*	Enteritidis	UIA	4-52-41	3,841,278	18,726,718
enterica	*enterica*	Enteritidis	ATCC	13076	124,696,224	411,170,976
enterica	*enterica*	Gallinarum	UIA	4-50-39	117,686,904	354,388,800
enterica	*enterica*	Give	USDA	63213	221,097,088	225
enterica	*enterica*	Give	ATCC	9268	1,275,440	638
enterica	*enterica*	Hadar	UGA	14145-L	195,416,064	64
enterica	*enterica*	Hadar	UGA	MH44684	11,075,670	69
enterica	*enterica*	Hadar	UGA	VA07170803	208,948,640	87
enterica	*enterica*	Havana	UGA	99-109840	26,656,554	40
enterica	*enterica*	Havana	UGA	MH84665	166,512,368	748
enterica	*enterica*	Heidelberg	FDA	SL476	153,022,592	25,652,106
enterica	*enterica*	Heidelberg	FDA	SL486	896,572	145,342,928
enterica	*enterica*	Heidelberg	FDA	SARB23	1,088,842	131,853,408
enterica	*enterica*	Heidelberg	FDA	SARB24	8,853,631	406,794,480
enterica	*enterica*	Heidelberg	UGA	6316-J	207,102,224	399,253,088
enterica	*enterica*	Heidelberg	USDA	31026.1	139,058	28,912,046
enterica	*enterica*	Heidelberg	UIA	DMSO38	146,097,376	357,844,448
enterica	*enterica*	Heidelberg	ATCC	8326	226,117,904	294,694,144
enterica	*enterica*	Heidelberg	FDA	SL1225	896,115	355,300
enterica	*enterica*	Hvittingfoss	USDA	63008.2	237,217,104	998
enterica	*enterica*	Illinois	ATCC	11646	4,224,671,232	62
enterica	*enterica*	Infantis	UGA	MH95276	109,502,112	1,939
enterica	*enterica*	Infantis	USDA	31061	736,700,032	43
enterica	*enterica*	Infantis	USDA	JUL301	7,549	348
enterica	*enterica*	Infantis	UIA	DMSO43	2,865,999,872	1,129
enterica	*enterica*	Infantis	ATCC	51741	145,144,336	97
enterica	*enterica*	Javiana	UGA	17339-0	2,877,672	173
enterica	*enterica*	Javiana	ATCC	10721	124,645,424	7,005,335
enterica	*enterica*	Kahla	ATCC	17980	7,296,855,040	202
enterica	*enterica*	Kalamu	USDA	63279.2	893,600,000	54
enterica	*enterica*	Kentucky	ATCC	9263	57,531,032	46
enterica	*enterica*	Kentucky	USDA	31028	1,984,247	55
enterica	*enterica*	Kentucky	USDA	1315	113	47
enterica	*enterica*	Kiambu	USDA	51316	2,900,058	324,337,344
enterica	*enterica*	Kiambu	USDA	DEC174	5,626,906	352,658,208
enterica	*enterica*	Lexington	UGA	9492-M	28,256,152	53
enterica	*enterica*	Liverpool	USDA	AUG365	348,709,856	56
enterica	*enterica*	Livingstone	USDA	52327.2	118,300	45
enterica	*enterica*	London	USDA	43290	2,763	41
enterica	*enterica*	London	USDA	JUL218	1,378,000	48
enterica	*enterica*	Manhattan	USDA	1342	1,518,669	45
enterica	*enterica*	Mbandaka	UGA	74	196,287,272	55
enterica	*enterica*	Mbandaka	USDA	42136	3,563,000	705
enterica	*enterica*	Mbandaka	USDA	NOV145	257,196	43

Species	Subsp.	Serovar	Source	Strain ID	SEA1.NL	TSP1.NL
enterica	*enterica*	Meleagridis	UGA	92	145,308,436	50
enterica	*enterica*	Meleagridis	USDA	11008.1	156,641,760	690
enterica	*enterica*	Meleagridis	USDA	FEB095	256,379,600	1,303
enterica	*enterica*	Menden	ATCC	15992	3,560,914,176	4,494
enterica	*enterica*	Michigan	UGA	N/A	30,269,623	1,534
enterica	*enterica*	Minnesota	USDA	52329.1	22,570	45
enterica	*enterica*	Mississippi	UIA	DMSO49	3,029,000	461
enterica	*enterica*	Monophasic	UGA	103	91,616,056	284,687,536
enterica	*enterica*	Monophasic	UGA	102	140,004,336	277,650,400
enterica	*enterica*	Montevideo	FDA	SL1317	28,373	397,032,784
enterica	*enterica*	Montevideo	FDA	SARB30	8,468,552	568
enterica	*enterica*	Montevideo	FDA	SARB31	68,566,088	47
enterica	*enterica*	Montevideo	UGA	VA07123001	202,937	1,181
enterica	*enterica*	Montevideo	UGA	VA07171801	56,984	59
enterica	*enterica*	Montevideo	UGA	VA07172202	153,583,056	40
enterica	*enterica*	Montevideo	UGA	VA07172205	141,925,920	340
enterica	*enterica*	Montevideo	UGA	90	173,869,856	49
enterica	*enterica*	Montevideo	USDA	31056.1	261,277,616	402
enterica	*enterica*	Montevideo	USDA	AUG369	204,514,096	80
enterica	*enterica*	Montevideo	ATCC	8387	100,979,464	71
enterica	*enterica*	Muenchen	FDA	SL1314	2,438,382,080	15,295
enterica	*enterica*	Muenchen	FDA	SARB32	1,992,084,608	48
enterica	*enterica*	Muenchen	FDA	SARB33	4,619,074,048	542
enterica	*enterica*	Muenchen	FDA	SARB35	138,832,368	52
enterica	*enterica*	Muenchen	USDA	63299	2,368,000,000	857
enterica	*enterica*	Muenchen	USDA	OCT080	3,567,180	46
enterica	*enterica*	Muenster	USDA	31053	3,963,531	41
enterica	*enterica*	Muenster	USDA	OCT084	77,933	51
enterica	*enterica*	Newport	FDA	SL254	144,886,768	27
enterica	*enterica*	Newport	FDA	SL317	362,322,688	71
enterica	*enterica*	Newport	FDA	SARB36	2,961,632,512	52
enterica	*enterica*	Newport	FDA	SARB37	5,417,735,680	88
enterica	*enterica*	Newport	FDA	SARB38	1,479,245,952	1,179
enterica	*enterica*	Newport	UGA	11590-K	106,605,958	46
enterica	*enterica*	Newport	UGA	MH57137	273,264	245,019
enterica	*enterica*	Newport	UGA	55	190,814,768	657
enterica	*enterica*	Newport	UGA	57	142,928,016	27
enterica	*enterica*	Newport	UGA	78	134,727,168	45
enterica	*enterica*	Newport	UGA	88	154,595,584	44
enterica	*enterica*	Newport	USDA	63283	2,550,000,128	53
enterica	*enterica*	Newport	USDA	DEC069	233,181,760	269
enterica	*enterica*	Newport	UIA	DMSO55	2,304,000,000	3,323
enterica	*enterica*	Newport	ATCC	6962	4,816,355,328	41
enterica	*enterica*	Newport	ATCC	27869	85,592,176	46
enterica	*enterica*	Newport	FDA	SL1223	19,064	980
enterica	*enterica*	Ngili	ATCC	19127	3,135,790,592	25
enterica	*enterica*	Ohio	USDA	52307	393,667,680	526
enterica	*enterica*	Oranienburg	ATCC	9239	70,838,972	52
enterica	*enterica*	Panama	FDA	SARB39	400,174	303,791,744
enterica	*enterica*	Panama	FDA	SARB40	5,163,048,448	315,158,432
enterica	*enterica*	Panama	FDA	SARB41	4,836,790,272	339,360,064
enterica	*enterica*	Panama	ATCC	7378	91,537,192	11,462
enterica	*enterica*	Paratyphi A	ATCC	9150	608,260	174,519,328
enterica	*enterica*	Paratyphi B	ATCC	10719	169,020,552	67,675,424
enterica	*enterica*	Paratyphi B	USDA	SEP358	340,476,128	334,997,408
enterica	*enterica*	Paratyphi C	ATCC	BAA-1714	134,794,720	1,022,829
enterica	*enterica*	Paratyphi C	ATCC	BAA-1715	864,868	78,991
enterica	*enterica*	Pomena	UIA	DMSO63	57,010	110
enterica	*enterica*	Potsdam	ATCC	25957	4,518,902,272	27

Species	Subsp.	Serovar	Source	Strain ID	SEA1.NL	TSP1.NL
enterica	*enterica*	Pullorum	UIA	SL297	5,138,000	53
enterica	*enterica*	Pullorum	ATCC	13036	81,527,524	2,362,583
enterica	*enterica*	Reading	USDA	52317.1	84,120	18,230
enterica	*enterica*	Reading	USDA	SEP245	793,160	620,200
enterica	*enterica*	Remo	USDA	43164.2	3,339,264	153
enterica	*enterica*	Rubislaw	UIA	DMSO67	105,790,272	407
enterica	*enterica*	Saintpaul	ATCC	9712	82,576,576	415,529,792
enterica	*enterica*	Saintpaul	FDA	SARB55	1,218,853,760	81,410,360
enterica	*enterica*	Saintpaul	FDA	SARB56	26,428,884	687
enterica	*enterica*	San Diego	USDA	APR025	9,522,000	103,000,000
enterica	*enterica*	Schwarzengrund	USDA	13092.2	7,521,089	26,487,490
enterica	*enterica*	Senftenberg	ATCC	43845	2,366	118
enterica	*enterica*	Senftenberg	FDA	SL1315	333,627,104	41
enterica	*enterica*	Senftenberg	USDA	31072.1	23,980,000	36
enterica	*enterica*	Senftenberg	USDA	SEP160	261,634,272	52
enterica	*enterica*	Senftenberg	FDA	SARB59	27,823,494	1,282
enterica	*enterica*	Senftenberg	UGA	15106q	9,993,046	35
enterica	*enterica*	Simsbury	ATCC	12004	1,540,680,960	37
enterica	*enterica*	Stanley	ATCC	7308	68,169,976	16,221
enterica	*enterica*	Taksony	USDA	32133	41,560,000	359
enterica	*enterica*	Tallahassee	ATCC	12002	2,089,881	56
enterica	*enterica*	Tennessee	FDA	SL1517	157,676,160	33
enterica	*enterica*	Tennessee	FDA	SL487	8,807,423	236
enterica	*enterica*	Tennessee	FDA	SL490	107,866,232	439
enterica	*enterica*	Tennessee	FDA	SL63	4,317,481,984	40
enterica	*enterica*	Tennessee	FDA	TW880	1,870,579,072	1,004
enterica	*enterica*	Thompson	UGA	11842M	137,041,760	445
enterica	*enterica*	Thompson	UGA	15371-K	1,482,658	970
enterica	*enterica*	Thompson	USDA	32117	1,587,000,064	52
enterica	*enterica*	Thompson	USDA	DEC142	242,732,464	510
enterica	*enterica*	Thompson	UIA	DMSO76	101,072,624	69
enterica	*enterica*	Typhi	ATCC	6539	3,280,334,592	26,008
enterica	*enterica*	Typhimurium	USDA	31049.2	52,521,196	33
enterica	*enterica*	Typhimurium	USDA	AUG247	40,871,196	16,143
enterica	*enterica*	Typhimurium	UIA	SL1344	355,395,488	292,480,608
enterica	*enterica*	Typhimurium	ATCC	19585	310,010,656	365,391,456
enterica	*enterica*	Typhimurium	FDA	SL1226	379,277,792	407,384,192
enterica	*enterica*	Typhimurium	FDA	SL1279	385,405,440	342,914,944
enterica	*enterica*	Typhimurium	FDA	SL1280	132,328,016	379,735,136
enterica	*enterica*	Typhimurium	FDA	SL1281	183,160,000	381,709,760
enterica	*enterica*	Typhimurium	FDA	SL1282	108,731,616	406,282,688
enterica	*enterica*	Typhimurium	FDA	SL1283	126,172,176	398,853,888
enterica	*enterica*	Typhimurium	FDA	SL1284	2,840,402,176	362,198,336
enterica	*enterica*	Typhimurium	FDA	SL1285	80,220,416	364,551,952
enterica	*enterica*	Typhimurium	FDA	SL1286	28,294,146	351,644,144
enterica	*enterica*	Typhimurium	FDA	SL1287	24,037,144	356,556,720
enterica	*enterica*	Typhimurium	FDA	SL1288	18,726,582	400,137,712
enterica	*enterica*	Typhimurium	FDA	SL1289	51,395,736	347,941,952
enterica	*enterica*	Typhimurium	FDA	SL1290	70,877,360	352,810,112
enterica	*enterica*	Typhimurium	FDA	SL1291	203,489,200	370,731,456
enterica	*enterica*	Typhimurium/DT104	FDA	SL1292	794,836,928	151,668,624
enterica	*enterica*	Typhimurium/DT104	FDA	SL1293	1,155,311,616	409,543,680
enterica	*enterica*	Typhimurium	FDA	SARB65	2,761,012,224	416,909,792
enterica	*enterica*	Typhimurium	FDA	SARB66	1,850,801,408	312,075,760
enterica	*enterica*	Typhimurium	FDA	SARB67	4,024,307,456	244,597,312
enterica	*enterica*	Typhimurium	FDA	SARB68	2,178,136,320	112,672
enterica	*enterica*	Typhimurium	UGA	DT104	216,010,224	418,062,816
enterica	*enterica*	Typhimurium	UGA	9115199	101,437,336	302,145,952
enterica	*enterica*	Typhimurium	UGA	PTC 1	92,841,960	358,664,448

Species	Subsp.	Serovar	Source	Strain ID	SEA1.NL	TSP1.NL
enterica	*enterica*	Typhimurium	FDA	SL1294	1,158,475,904	419,573,952
enterica	*enterica*	Typhimurium/DT104b	FDA	SL1278	103,077,872	357,650,848
enterica	*enterica*	Uganda	USDA	51278.2	4,075,762	44
enterica	*enterica*	Urbana	ATCC	9261	336,161,120	34
enterica	*enterica*	Vellore	ATCC	15611	173,703,568	533
enterica	*enterica*	Virchow	ATCC	51955	3,199,765	477
enterica	*enterica*	Wagadugu	USDA	53298	3,699,000,064	992
enterica	*enterica*	Weltevreden	ATCC	BAA-2568	8,591,441,920	642
enterica	*enterica*	Worthington	ATCC	BAA-2085	12,412,392	40
enterica	*arizonae*	-	ATCC	BAA-1577	150,926,136	1,238,439
enterica	*arizonae*	-	ATCC	33952	71,166,208	63
enterica	*arizonae*	-	ATCC	29933	4,076	23
enterica	*arizonae*	-	ATCC	BAA-731	1,321,403	291
enterica	*arizonae*	-	ATCC	13314	1,552,442	41
enterica	*arizonae*	-	ATCC	12323	4,482,480,512	429
enterica	*diarizonae*	-	ATCC	BAA-216	103,257,000	2,341
enterica	*diarizonae*	-	ATCC	BAA-639	6,567,382,528	37
enterica	*diarizonae*	-	ATCC	12325	7,543,692,800	36
enterica	*diarizonae*	-	ATCC	29934	5,810,232,832	37
enterica	*diarizonae*	-	ATCC	31241	6,029,409	26
enterica	*diarizonae*	-	ATCC	BAA-1579	4,268,697,856	163
enterica	*houtenae*	-	ATCC	43974	3,913,850	163
enterica	*houtenae*	-	ATCC	BAA-1580	108,530,496	23
enterica	*indica*	-	ATCC	43976	5,238,925,824	36
enterica	*salamae*	-	ATCC	6959	101,438,248	446,117,280
enterica	*salamae*	-	ATCC	700149	159,562	61
enterica	*salamae*	-	ATCC	700151	55,493,530	101
enterica	*salamae*	-	ATCC	29931	81,193	992
enterica	*salamae*	-	ATCC	700148	3,965,946,880	4,991
enterica	*salamae*	-	ATCC	43972	4,703,879	62,647,396
bongori	-	-	ATCC	43975	51,580	60
Nontypeable	-	-	USDA	63214	63,280,000	224
Nontypeable	-	-	USDA	63393	1,550,662	37

Supplementary Table 2. RLU values for *Salmonella* inclusivity of SEA1.NL and TSP1.NL. Stationary phase cultures were diluted to an OD_{600} of 0.2 and infected for 2 h with indicated reporter phage. If provided by source, *S. enterica* subsp. *enterica* serovar is indicated. Strains were obtained from either the American Type Culture Collection (ATCC), the University of Georgia (UGA), the University of Iowa (UIA), the United States Department of Agriculture (USDA), or the Food and Drug Administration (FDA). Luciferase production was quantified as relative light units (RLU) on a luminometer after the addition of substrate. Grey boxes are used to indicate strains that could not be detected by either reporter, 750 RLU detection threshold.

Genus	Species	Serovar	ATCC ID	SEA1.NL	TSP1.NL
Acinetobacter	*calcoaceticus*	-	23055	289	86
Citrobacter	*braakii*	-	51113	17,950	62
Citrobacter	*freundii*	-	8090	40	46
Citrobacter	*koseri*	-	25408	57	53
Citrobacter	*sedlakii*	-	51493	24,005,312	1,727,327
Citrobacter	*werkmanii*	-	51114	62	37
Citrobacter	*youngae*	-	29935	606	23
Cronobacter	*sakazakii*	-	BAA-894	128	65
Edwardsiella	*tarda*	-	15947	119	52
Enterobacter	*cloacae*	-	13047	53	49
Enterobacter	*kobei*	-	BAA-260	69	48
Escherichia	*coli*	-	8739	224	56
Escherichia	*coli*	-	9637	50	52
Escherichia	*coli*	O1:K1:H7	11775	67	57
Escherichia	*coli*	O111:K58(B4):H-	33780	102,500	50
Escherichia	*coli*	O157:H7	35150	42	35
Escherichia	*coli*	-	35218	214	67
Escherichia	*coli*	O157:H7	43888	53	48
Escherichia	*coli*	O157:H7	43890	62	37
Escherichia	*coli*	-	51813	74	53
Escherichia	*coli*	O157:NM	700377	40	26
Escherichia	*coli*	O121	BAA-2190	90,554	48
Escherichia	*coli*	O111:H8	BAA-2201	50	37
Escherichia	*coli*	O145	BAA-2206	56	39
Escherichia	*coli*	O103:H2	BAA-2210	279	98
Escherichia	*coli*	O145:H25	BAA-2211	749	301
Escherichia	*coli*	O145:H34	BAA-2216	22,770,638	48
Escherichia	*coli*	O45:H10	BAA-2649	379	105
Escherichia	*coli*	O157:H-	DEC7E*	56	28
Escherichia	*coli*	O157:NM	TWO6555*	37	30
Escherichia	*coli*	-	13706	25	NT
Escherichia	*coli*	O106:H12	BAA-1431	203	NT
Escherichia	*coli*	N:26	ECOR-42*	719	NT
Escherichia	*coli*	ON:HM	ECOR-45*	89	NT
Escherichia	*coli*	OM:H18	ECOR-47*	137	NT
Escherichia	*coli*	O1:NM	ECOR-35*	100	NT
Escherichia	*coli*	O2:NM	ECOR-61*	167	NT
Escherichia	*coli*	O4:H43	ECOR-67*	143	NT
Escherichia	*coli*	O7:NM	ECOR-38*	115	NT
Escherichia	*coli*	O25:N	ECOR-51*	112	NT
Escherichia	*coli*	O55:H7	DEC5E*	173	NT
Escherichia	*coli*	O78:NM	ECOR70*	181	NT
Escherichia	*coli*	O88:NM	ECOR-34*	106	NT
Escherichia	*coli*	O103:H21	ECOR-30*	485	NT
Escherichia	*coli*	O104:H21	ECOR-26*	150	NT
Escherichia	*coli*	O106:NM	ECOR17*	30	NT
Escherichia	*coli*	O116:H21	Jan105*	114	NT
Escherichia	*coli*	O121:H-	ECOR-21*	153	NT
Escherichia	*coli*	O157:H43	DEC7C*	318	NT
Escherichia	*coli*	O157:H43	DEC7D*	296	NT
Escherichia	*coli*	O157 non H7	FSIS 222.3**	736	NT
Escherichia	*coli*	O157 non H7	FSIS 66**	113	NT
Escherichia	*coli*	-	SF2.1**	98	NT
Escherichia	*coli*	-	SF1**	404	NT
Escherichia	*coli*	-	SF2.2**	368	NT
Escherichia	*coli*	-	SF5**	158	NT
Escherichia	*coli*	-	SF4**	149	NT
Escherichia	*coli*	-	SF7**	136	NT

Genus	Species	Serovar	ATCC ID	SEA1.NL	TSP1.NL
Escherichia	*coli*	-	SF9**	390	NT
Escherichia	*fergusonii*	-	35469	70	150
Escherichia	*hermanni*	-	33650	115	299
Hafnia	*alevi*	-	13337	487	69
Klebsiella	*aerogenes*	-	13048	319	101
Klebsiella	*oxytoca*	-	43165	64	81
Klebsiella	*pneumonia*	-	4352	283	66
Morganella	*morganii*	-	25830	63	73
Pluralibacter	*gergoviae*	-	33028	58	53
Proteus	*vulgaris*	-	33420	37	19
Pseudomonas	*aeruginosa*	-	27853	109	62
Serratia	*marcescens*	-	13880	86,123	43
Shigella	*flexneri*	-	12022	199,355	117
Shigella	*sonnei*	-	9290	313	52
Yersinia	*enterocolitica*	-	23715	4,209	35
Bacillus	*cereus*	-	14579	67	81
Bacillus	*subtilis*	-	6051	104	82
Enterococcus	*faecalis*	-	19433	290	165
Enterococcus	*faecalis*	-	29212	60	61
Enterococcus	*faecium*	-	19434	74	54
Listeria	*grayi*	-	25401	64	55
Listeria	*innocua*	-	33090	504	293
Listeria	*ivanovii*	-	19119	81	60
Listeria	*seeligeri*	-	35967	66	63
Listeria	*welshimeri*	-	35897	55	61
Staphylococcus	*aureus*	-	27660	429	35
Staphylococcus	*aureus*	-	29213	85	32
Staphylococcus	*aureus*	-	BAA-1721	35	34
Staphylococcus	*epidermidis*	-	14990	87	452
Staphylococcus	*haemolyticus*	-	29970	68	89
Staphylococcus	*hominis*	-	27844	299	120
Staphylococcus	*saprophyticus*	-	15305	52	44

Supplementary Table 3. RLU values for exclusivity of SEA1.NL and TSP1.NL. Stationary phase cultures were diluted to an OD_{600} of 0.2 and infected for 2 h with the indicated reporter phage. If available from source, the *E. coli* serovar is indicated. Luciferase production was quantified as relative light units (RLU) on a luminometer after the addition of substrate, 750 RLU detection threshold. * These strains were obtained from the Michigan State University STEC Center. ** These strains were obtained from the USDA. All other strains were obtained from ATCC. Strain and phage combinations that were not tested are indicated as “NT".

2.2 Artigo 2

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

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Article published in Journal of Food Science and Nutrition Research
The article was formatted according to Journal of Food Science and Nutrition Research

J Food Sci Nutr Res 2021; 4 (3): 249-258 *DOI: 10.26502/jfsnr.2642-11000077*

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 chickenbased 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. Material 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. [20] 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. [22] 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 preenrichment, 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 preenrichment 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 and Discussion

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.

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.

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 (n=8)	9667	+ + + +	Unknown food
	340	+ + + +	Unknown food
			Continued on next page
	9688	+ + + +	Blood sausage
	9692	+ + + +	Jelly roll
	17131	+ + + +	Shredded chicken
	5209	+ + + +	Salami
	11368/2	+ + + +	Refrigerated beef
	12037	+ + + +	Rice with chicken heart and sausage
Infantis	IF 70	+ + + +	Lettuce

(n=1)			
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

** 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).

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.

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%)

** 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. [20] 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. (2021) [24] 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. [20] 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. [20]. 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. [12] 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. [30]. 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.

Acknowledgments

The authors thank the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES) for the financial support to researches.

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.

Funding Information

This study was carried out as a research activity without any funding or financial support.

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2.3 Artigo 3

Evaluation of PhageDX *Salmonella* Assay for *Salmonella* Detection in Hydroponic Curly Lettuce

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Article published in Foods - MDPI
The article was formatted according to Foods – MDPI

foods

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Evaluation of PhageDX *Salmonella* Assay for *Salmonella* Detection in Hydroponic Curly Lettuce

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Abstract: Lettuce is one of the most consumed leafy vegetables worldwide and has been involved in multiple foodborne outbreaks. *Salmonella* is one of the most prevalent etiological agents of foodborne disease (FBD) in lettuces, and its detection may take several days depending on the chosen method. This study evaluates a new rapid method that uses recombinant bacteriophages to detect *Salmonella* in hydroponic curly lettuce. First, the ability of the assay to detect six *Salmonella* serovars at three different concentrations (1, 10, and 100 CFU/well) was tested. Second, the detection of *Salmonella* was tested in lettuces using a cocktail of the same *Salmonella* serovars and concentrations after a 7 h enrichment. The results of these experiments showed that the detection limit was dependent on the serovar tested. Most serovars were detected in only 2 h when the concentration was 100 CFU/well. *Salmonella* was detected in 9 h (7 h enrichment + 2 h bioluminescence assay) in all lettuce samples with 10 CFU/25 g or more. *Salmonella* detection was not influenced by natural microbiota of lettuces. This study demonstrated that the phage assay was sensitive and faster than other detection methods, indicating that it is a better alternative for *Salmonella* detection on lettuces.

Keywords: bacteriophage; diagnostics; leafy green

Citation: de Aquino, N.S.M.; Elias, S.d.O.; Tondo, E.C. Evaluation of PhageDX *Salmonella* Assay for *Salmonella* Detection in Hydroponic Curly Lettuce. *Foods* **2021**, *10*, 1795.

Abstract: Lettuce is one of the most consumed leafy vegetables worldwide and has been involved in multiple foodborne outbreaks. *Salmonella* is one of the most prevalent etiological agents of foodborne disease (FBD) in lettuces, and its detection may take several days depending on the chosen method. This study evaluates a new rapid method that uses recombinant bacteriophages to detect *Salmonella* in hydroponic curly lettuce. First, the ability of the assay to detect six *Salmonella* serovars at three different concentrations (1, 10, and 100 CFU/well) was tested. Second, the detection of *Salmonella* was tested in lettuces using a cocktail of the same Salmonella serovars and concentrations after a 7 h enrichment. The results of these experiments showed that the detection limit was dependent on the serovar tested. Most serovars were detected in only 2 h when the concentration was 100 CFU/well. *Salmonella* was detected in 9 h (7 h enrichment + 2 h bioluminescence assay) in all lettuce samples with 10 CFU/25 g or more. *Salmonella* detection was not influenced by natural microbiota of lettuces. This study demonstrated that the phage assay was sensitive and faster than other detection methods, indicating that it is a better alternative for *Salmonella* detection on lettuces.

Keywords: bacteriophage; diagnostics; leafy green

1. Introduction

Recently, the consumption of fresh-vegetable salads has gained popularity worldwide due to the high concentration of bioactive compounds such as vitamins, minerals, and antioxidants, which are important for human health [1,2]. This rapid increase resulted in consumption growth rates of 10–20% per year [3,4]. However, reports on foodborne disease (FBD) outbreaks have multiplied, many of them caused by pathogens present on fresh vegetables and salads [5]. Therefore, the microbiological safety of fresh produce has become an important public health issue. Contamination may occur at any point along the production chain [6], and there are no thermal processing steps or sanitization procedures able to completely inactivate all possible pathogens in fresh vegetables before consumption. Lettuce is the most produced and consumed leafy vegetable crop in the world. From 1980 to 2016, it was the vegetable most implicated in FBD in developed countries with *Salmonella* being the main culprit [6,7]. This pathogen is one of the most common causes of FBD worldwide and is responsible for severe economic losses, considering bacterial pathogens [8]. The *Salmonella* genus is composed of over 2700 serovars, of which 200 are commonly associated with human salmonellosis. Of these, *S*. Typhimurium and *S*. Enteritidis are the most frequently implicated in human salmonellosis [7]. The current gold standard method for *Salmonella* detection needs at least three days for a negative result due to multiple sample enrichments and plating on selective agars. In cases where a

presumptive *Salmonella* colony is found, additional steps are required to confirm its identity [9]. Nevertheless, traditional methods can detect 1 CFU of *Salmonella* in a 25 g sample which is the current acceptable limit for several foods in diverse microbiological regulations. Other assays using ELISA, latex agglutination, PCR, mass spectrometry, and metagenomic sequencing have been developed with the aim of reducing the time needed for food pathogen detection. These approaches have been successful; however, most available methods still require at least 18 h of enrichment to detect 1 CFU/25 g [10]. In addition, many of these methods could potentially detect non-viable *Salmonella* cells, resulting in the need for further confirmation testing using traditional methods. Recently, bacteriophages have been used to detect bacterial foodborne pathogens due to their safety, specificity, rapid propagation, and ability to differentiate between live and dead cells [11]. The ability to differentiate living cells and dead cells is an advantage over many rapid methods. Rapid propagation is an advantage over traditional methodology. The high specificity of bacteriophages, eliminates the need for isolation steps of the target pathogen, as used in traditional methodologies, and this decreases the total assay time. One promising phage-based approach for *Salmonella* detection is the use of recombinant phages that carry a luciferase reporter. NanoLuc® is an engineered luciferase from a deep-sea shrimp *Oplophorus gracilirostris* that is 150 times brighter than other luciferases and reacts with a novel furimazine substrate with low background noise [12]. Based on these characteristics, NanoLuc® would be a superior choice as a luciferase reporter in phage-based assays. PhageDx *Salmonella* Assay is a new method that uses recombinant bacteriophages with NanoLuc® inserted to detect *Salmonella*. This method detected several *Salmonella* serovars in in vitro conditions, showing limits of 10–100 CFU detection per mL (without enrichment). Besides this, the assay detected 1 CFU in either 25 g of ground turkey with a 7 h enrichment or 100 g of powdered infant formula with a 16 h enrichment. However, the PhageDx *Salmonella* Assay has not been tested with lettuces [12]. The objective of this study is to assess the performance of the PhageDx *Salmonella* Assay for the detection of Brazilian *Salmonella* strains in vitro and on hydroponic curly lettuce.

2. Materials and Methods

2.1. PhageDx Salmonella Assay

The method used in this study was developed by the Laboratory Corporation of America (LabCorp) and registered in AOAC® (Certificate No. 121904). The method has been described in detail in Nguyen et al. [12]. Briefly, the assay contains two recombinant bacteriophages, SEA1.NL and TSP1.NL which have had the NanoLuc® (Promega Corp., Madison, WI, USA) gene inserted in their genome by homologous recombination. The test is based on the infection of recombinant-bacteriophages in *Salmonella* spp. cells, resulting in the production of the NanoLuc® luciferase during phage replication. After a 2 h infection, luciferase substrate is added, and the sample is read on a luminometer. Readings above a pre-established cutoff of 750 relative light units (RLU) indicate the presence of *Salmonella*, and readings ≤750 RLU indicate absence of *Salmonella*. The bioluminescence assay is further detailed in Section 2.4

2.2. In Vitro Assay for Determination of Detection Limit

Six *Salmonella* serovars were used to test the limit of detection (LOD) of the assay; *S*. Enteritidis, *S*. Typhimurium, *S*. Infantis, *S*. Minnesota, *S*. Heidelberg, and *S*. Saint Paul. All serovars were isolated from foods in Brazil and are from the Laboratory of Food Microbiology and Food Control of Institute of Food Science and Food Technology of the Federal University of Rio Grande do Sul (ICTA/UFRGS). The strains were cultivated overnight in 5 mL of Tryptic Soy Broth (TSB, Kasvi, Brazil) at 37 °C. Using a microplate reader (Loccus LMR 96, Cotia, Brazil), the OD_{630} was determined and then diluted to an OD_{630} = 0.2 (or approximately 10^8 CFU/mL). Then, 1 mL of each culture was added to a 15 mL conical tube in order to form a pooled culture of *Salmonella*. This condition was tested to simulate a food matrix contaminated by several serovars. The *Salmonella* cocktail and a culture of each individual strain were serially diluted in TSB (Kasvi, Brazil) to concentrations of 1000, 100, and 10 CFU/mL. To determine the level of detection (LOD) of the PhageDx assay, 100 µL of each diluted sample were transferred to a 96-well plate, resulting in 100, 10, and 1 CFU/well. The cell concentrations were confirmed by plating each final cell suspension on Tryptic Soy Agar (TSA, Kasvi, Brazil), incubating at 37 °C for
24 h, and counting colony formation. The bioluminescence test was performed as described in Section 2.4.

2.3. Salmonella Detection on Hydroponic Curly Lettuce

The *Salmonella* cocktail was used to inoculate lettuce samples. Before inoculation, 15 mL of the cocktail was centrifuged at 4 ◦C, for 10 min at 2810× g (CIENTEC CT-5000R, Belo Horizonte, Brazil), and the supernatant was discarded. Then, the pellet was washed three times with sterile 0.1% Peptone Water (Kasvi, Brazil). After the final wash, cells were suspended in sterile 0.1% Peptone Water (Kasvi, Brazil). A microplate reader was used to determine OD630, and cells were diluted to a concentration of approximately 10^8 CFU/mL (OD_{630} = 0.2). Next, the *Salmonella* cocktail was serially diluted with sterile 0.1% Peptone Water (Kasvi, Brazil) to final concentrations of 100, 10, and 1 CFU/mL. Cell concentrations were confirmed by plating on TSA (Kasvi, Brazil) as described above. Hydroponic curly lettuce was purchased at a hypermarket in Porto Alegre (Brazil) and transported to the Laboratory of Food Microbiology and Food Control located at the Federal University of Rio Grande do Sul (ICTA/UFRGS). The lettuce, previously tested for the absence of *Salmonella* spp., was portioned in 25 g samples and individually placed inside Whirl-Pak® sterile filter bags (Nasco, Fort Atkinson, WI, USA). Lettuce samples were artificially contaminated by inoculating onto the leaf surface with 1 mL of 100, 10, or 1 CFU/mL *Salmonella* cocktail dilutions. The *Salmonella* final concentration on lettuce were 100, 10, 1 CFU/25 g. Then, 75 mL of pre-warmed (41 ± 1 ◦C) Buffered Peptone Water (BPW, Merck, Darmstadt, Germany) were added, and the samples were homogenized using a stomacher (Stomacher® 400, Seward, England) for 30 s. Finally, all the samples were incubated at 41 ± 1 ◦C for 7 h, and the bioluminescence assay was carried out as described in Section 2.4.

2.4. Bioluminescence Assay

The bioluminescence assay was performed using 100 and 150 µL of samples prepared according to Sections 2.2 and 2.3, respectively. Four or 10 replicates of each dilution were transferred to a 96-well white plate (Thermo Scientific™, Waltham, MA, USA). Samples included 4×10^2 CFU/mL, 10×10^1 and 10×10^0 CFU/mL samples. A total of 10 µL of the recombinant phage cocktail from the PhageDx *Salmonella* Assay were added to each well. The samples were incubated for 2 h at 37 ◦C. The luciferase reagent mix was prepared by combining 50 µL of NanoGlo buffer, 1 µL NanoGlo

substrate (Nano-Glo® Luciferase Assay System, Promega Corp., Madison, WI, USA), and 15 µL Renilla lysis buffer (Renilla Luciferase Assay System, Promega Corp., Madison, WI, USA). After infection, 65 µL of the luciferase reagent mix was added to each well, and the 96-well plate was read immediately in a GloMax® Navigator Luminometer (Promega, Fitchburg, MA, USA). The reading parameters used were 3 min wait time, 1 s integration, and two reads for a plate. Relative light units (RLU) were the signal output. All assays were performed in triplicate. In total, each *Salmonella* serovar and the cocktail were tested 30 times for low and medium inoculum and 12 times for the high inoculum. The results were expressed as the percentage of positive tests. The means, standard deviations, and coefficient of variation were calculated using Excel® version 2016 (Microsoft Co., Ltd. Redmond, WA, USA). In the in vitro assay, the negative controls consisted of uninoculated TSB culture medium. The negative control of the food test was uninoculated lettuce sample added to BPW (Merck, Darmstadt, Germany).

3. Results

Table 1 shows the RLUs generated by the PhageDx *Salmonella* Assay in culture tests and artificially contaminated lettuce samples. The average RLUs of the in vitro samples ranged from 182 to 26,543, while the RLUs generated by lettuces ranged from 1914 to 106,579. As expected, RLUs increased according to the size of the inoculum (Table 1) *S*. Typhimurium showed the highest RLU values even with the lowest inoculum, followed by the *Salmonella* cocktail.

Table 1. Relative Light Unit (RLU) numbers due to the detection of *Salmonella* in vitro and on curly hydroponic lettuce by PhageDx *Salmonella* Assay.

Sample	Inoculum	Number of replicates	Avg. RLU	SD	% CV
S. Minnesota	Low	30	187	41	22
	Medium	30	223	89	40
	High	12	1,475	953	65
S. Enteritidis	Low	30	182	45	25
	Medium	30	246	168	68
	High	12	724	339	47
S. Saintpaul	Low	30	198	115	58
	Medium	30	407	398	98
	High	12	3,388	2061	61
S. Infantis	Low	30	214	169	79
	Medium	30	479	415	87
	High	12	1,914	1,324	69
S. Heidelberg	Low	30	325	220	68
	Medium	30	873	426	49
	High	12	6158	1,729	28
S. Typhimurium	Low	30	1,814	1,305	72
	Medium	30	14,648	9,257	63
	High	12	16,715	15,1623	907
Salmonella cocktail	Low	30	2,323	339	15
	Medium	30	3,054	1,559	51
	High	12	26,543	9,980	38
Lettuce	Low	30	1,914	6,813	356
	Medium	30	7,347	6,207	84
	High	12	106,579	68,315	64

Strains were diluted from log phase cultures to three concentrations: low (~ 1 CFU/ mL), medium (~ 10 CFU / mL), and high (~ 100 CFU / mL). The samples were infected with the phage cocktail for 2 h at 37°C. Spiked lettuce samples were enriched for 7 hours at 41±1°C in buffered peptone water (BPW) prior to phage infection step. Luciferase substrate mix was added and RLUs were measured using a luminometer. Averages (Avg. RLU), standard deviations (SD), percent coefficient of variation (%CV) were calculated.

Table 2 contains the percentages of detection of each *Salmonella* serovar, and *Salmonella* cocktail evaluated in vitro, and the results of *Salmonella* cocktail on lettuce samples. The percentages of detection varied from 0% (when the signals emitted by samples were less than 750 RLU) to 100% (when all samples emitted signals above 750 RLU). As expected, the lowest percentages of detection were those from samples with low inoculum, while the detection percentages increased according increased the inoculum size. In in vitro tests, 100% of *S*. Saintpaul, *S*. Infantis, *S*. Heidelberg, *S*. Typhimurium, and the cocktail of *Salmonella* were detected without pre-incubation just 2 h of infection at 100 CFU/well. *S*. Minnesota and *S*. Enteritidis could also be identified at the highest inoculum concentration, but had lower detection rates, 75% and 50%, respectively. Table 2 also demonstrated that 100% of artificially contaminated lettuces presenting 10 and 100 CFU per 25 g were also detected.

Table 2. Percentage of *Salmonella* detection of *in vitro* test and curly hydroponic lettuces contaminated with the *Salmonella* cocktail.

Samples	Negative control	Low	Medium	High
S. Minnesota	0 (0/2)	0 (0/30)	0 (0/30)	75 (9/12)
S. Enteritidis	0 (0/2)	0 (0/30)	6.7 (2/30)	50 (6/12)
S. Saintpaul	0 (0/2)	3.0 (1/30)	16.7 (5/30)	100 (12/12)
S. Infantis	0 (0/2)	3.0 (1/30)	16.7 (5/30)	100 (12/12)
S. Heidelberg	0 (0/2)	10 (3/30)	60 (18/30)	100 (12/12)
S. Typhimurium	0 (0/2)	83.3 (25/30)	100 (12/12)	100 (12/12)
Salmonella cocktail	0 (0/2)	63 (19/30)	93 (28/30)	100 (12/12)
Lettuces	0 (0/2)	30 (9/30)	100 (12/12)	100 (12/12)

Log phase *Salmonella* cultures were diluted to low (~ 1 CFU/ mL), medium (~ 10 CFU/ mL), and high (~ 100 CFU/ mL) concentrations. Samples were infected with the phage cocktail for 2 h. Spiked lettuce samples were pre-incubated for 7 hours in buffered peptone water (BPW) before incubation with bacteriophages. The RLU of samples were measured using a luminometer.

4. Discussion

Since 1987, the American Public Health Association (APHA) has pointed out that rapidity and sensitivity are two critical requirements for pathogen detection methods used in food industries. Rapid detection is essential because food industries need to know as quickly as possible whether or not pathogens are present in final products. The sensitivity is important because legal requirements generally require the absence of pathogens like *Salmonella* in 25 g of food because the infective doses of these pathogens can be as low as a single cell [13]. In addition, pathogens can multiply due to time and temperature abuses during food production, increasing the risk of foodborne illnesses.

PhageDx *Salmonella* Assay was developed to meet the criteria of rapidity and sensitivity to be used in food industries worldwide. The results of this study demonstrated that assay was able to detect *Salmonella* isolated in Brazil when present on lettuces containing natural microbiota. Lettuce was chosen as a food matrix to be tested because it is one of the most consumed leafy greens worldwide and is frequently linked with foodborne salmonellosis. Besides, the PhageDx *Salmonella* Assay was not tested with this food matrix during its recent development [12]. Our results demonstrated that the PhageDx *Salmonella* Assay detected all serovars evaluated, isolated from animal sources in Brazil (from foods involved with FBD and from poultry

carcasses), generating a range of RLUs (Table 1). This was consistent with Nguyen et al. [12] findings that also demonstrated that different RLU counts were obtained from different *Salmonella* serovars. For example, 52,329.1 RLU for *S*. Minnesota USDA; 419,753,056 for *S*. Enteritidis 1294; 1,218,853,760 RLU for *S*. Saintpaul SARB55; 7549 RLU for *S*. Infantis JUL 301; 207,102,224 RLU for *S*. Heidelberg 6316-J. These RLU values were obtained after 2 h of phage infection in stationary phase *Salmonella* (concentration of approximately 10^8 CFU/mL) with the PhageDx *Salmonella* phage reagent. The same authors also found that the same *Salmonella* serovar can produce different RLU numbers. These results can be explained because the RLU numbers are dependent on the bacterial multiplication rate during the pre-enrichment period. Some strains grow more slowly than others, resulting in fewer phages and, consequently, lower RLU signals. In in vitro experiments, there was no enrichment step of the samples, which could allow the increase of *Salmonella* numbers, increasing RLUs as well.

The previously defined criteria [12] considered positive all those whose RLU signal emitted was greater than or equal to 750 RLU, which justifies the increase in the standard deviation and the variation coefficient with the increase in the number of *Salmonella* cells. This is because, in the same set of samples with the same inoculum concentration, we obtained positive samples with RLU from 831 to 4051 (data not shown). Moreover, all these samples are considered positive as they meet the criteria of ≥750 RLU.

As demonstrated in the results of Table 2, most samples contaminated with *Salmonella* serovars were 100% positive when the level of 100 CFU was used. The differences observed in the phage's ability to detect different serovars can be explained by factors other than the rate of bacterial growth. For example, *S*. Typhimurium showed a high percentage of detection even when the inoculum was as low as approximately 1 CFU/well. The adhesion stage between bacteriophages and target cells is the first step towards the success of the infection process, both in nature and in phage assays. In this stage, the bacteriophages will adhere to structures present on target cells, called receptors. These receptors can be proteins, lipopolysaccharides, teichoic acids, and capsules [14]. Due to mechanisms that have not yet been fully explained, the same phage can bind to several receptors on a target cell, facilitating the adhesion process [15]. However, changes in the structures of these receptors can totally or partially

compromise the adhesion of bacteriophages [16]. As we observed in the present study, the phage recognition of different *Salmonella* serovars was not compromised. Although all strains tested positive at the high level of 100 CFU, only *S*. Typhimurium and *S*. Heidelberg were positive at all levels, 1, 10 and 100 CFUs. The other strains tested required 100 CFU before testing positive. The detection capacity of different *Salmonella* species by the phages SEA1.NL and TSP1.NL, which compose PhageDx *Salmonella* Assay, was previously demonstrated [12]. Similar to our results, different serovars also emitted different amounts of RLUs and did not compromise the detection effectiveness of the test.

The PhageDx *Salmonella* Assay detected different *Salmonella* serovars in different concentrations in vitro. Then, we tested the *Salmonella* detection on food. Leafy vegetables eaten raw are known to be important carriers of human pathogens [17]. Lettuce has proved to be one of the most important foods for spreading FBD outbreaks in developed countries [7]. In the present study, we observed that contaminated lettuce samples incubated for 7 h before the phage infection generated higher numbers of RLUs when compared to the in vitro tests where there is no enrichment time (Table 1). These results can be explained because the number of *Salmonella* cells during the phage infection phase was higher, increasing RLU signals.

Considering the smallest inoculum (approximately 1 CFU/mL), 30% of lettuce samples were positive for *Salmonella* (Table 2). *Salmonella* counts carried out on agar plates revealed that the actual amounts of *Salmonella* inoculated on the lettuces ranged from 1.7 to 3.3 CFU/25 g in the low concentration. Considering the use of such a low number of cells as inoculum of a specific food matrix, one cannot discard the possibility that some samples were not inoculated by any pathogen cell [18], explaining the negative results obtained in some samples. Beyond that, we observed that this slight variation within the same inoculation range could vary the detection rate (data not shown). However, we do not know if it is because there were no cells to detect in the sample or because of the assay's real detection limit. When the inoculum was increased by 10 × and 100 ×, the detection percentages reached 100%.

The averages of mesophilic microorganisms and total coliform on lettuces were 6 and 4 log CFU/g, respectively (data not shown). These values are in accordance to previous studies carried out in Brazil, which demonstrated that total bacterial count ranged from 4 to 7 log CFU/g on lettuces or lettuce salads [19,20], and coliforms count

ranged from 3.11 to 4.69 log CFU/g on conventional and organic lettuce [21]. The assay detected low counts of *Salmonella* even with high amounts of mesophilic microorganisms and total coliforms on lettuce, indicating that natural microbiota did not compromise the *Salmonella* detection. One of the advantages of using bacteriophages to detect pathogens is the specificity and this was demonstrated by these results. The genetic similarity between *Salmonella*, the target microorganism, and microorganisms belonging to the coliform group did not affect the test's specificity. Furthermore, there were no false positives in the assays. All lettuce samples with their natural microbiota that were not inoculated with *Salmonella* were negative.

Ultimately, PhageDx *Salmonella* Assay, in addition to being rapid and sensitive, does not require additional technologies to detect the target microorganism. When compared to other recently published detection methods using bacteriophages, such as phage amplification combined with qPCR [22], phagomagnetic separation with enzymatic colorimetry [23], and bacteriophage amplification coupled with mass spectrometry [24], this method was simple to perform and required little equipment to carry out the assays.

In an approach similar to the one used in this work, using recombinant bacteriophages, the assay's detection limit was approximately 400 CFU/25 g of ground beef; 10 CFU/cm2 in romaine lettuce [25]. Kim et al. [25] used 5 h enrichment time and 40 min of phage infection period.

In conclusion, the method analyzed in this study demonstrated to be sensitive and specific, making it an excellent assay to be used to detect *Salmonella* on lettuces. The assay detected 100% of samples with 10 CFU/25 g, and some samples probably containing 1 CFU/25 g after 7 h of enrichment and 2 h of phage infection. This time is less than those needed by other *Salmonella* detection methods available on the market. In addition, the method was easy to perform, did not require washing or concentration steps, and was not affected by the lettuce matrix or its microbiota. These represent significant advantages over traditional and rapid methods available on the market. More tests should be performed to evaluate the PhageDx *Salmonella* Assay in other food matrices.

Author Contributions: Conceptualization, N.S.M.d.A.; S.d.O.E. and E.C.T.; formal analysis, N.S.M.d.A.; S.d.O.E.; funding acquisition, N.S.M.d.A.; investigation, N.S.M.d.A., methodology, N.S.M.d.A.; project administration, N.S.M.d.A.; supervision, E.C.T.; writing—original draft, N.S.M.d.A., S.d.O.E. and E.C.T.; writing—review and editing, N.S.M.d.A., S.d.O.E. and E.C.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors thank the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES) to research the financial support.

Conflicts of Interest: The authors declare no conflict of interest.

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2.4 Artigo 4

Viability of a Commercial Phage-based Assay for the Detection of *E. coli* O157:H7 in Brazilian Food Products

Article submetted in Viruses - Special Issue "Bacteriophages in Food Applications"
The article was formatted according to Viruses – MDPI

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Abstract: *Escherichia coli* O157:H7 is one of the most important food pathogens worldwide, being responsible for gastroenteritis to fatal hemolytic uremic syndrome. Based on the infective dose of this microorganism, usually low, detection methods used to analyze foods should be highly sensitive. Beyond that, most detection methods need a pre-enrichment step for pathogen growth before detection, which should be as short as possible to generate timely results. In this work, a bacteriophage-based method for rapidly detecting *E. coli* O157:H7 was tested in vitro and in food samples. The results demonstrated that the assay can detect 100 CFU of *E. coli* O157:H7 without the enrichment step, while after 5 h enrichment the assay was able to detect 1 CFU/25 g or mL of ground beef, milk, lettuce, and water. It was also demonstrated that there were no divergences between the results obtained through analysis by real-time PCR and by the phage-based assay in bovine carcasses. The analyzed method proved to be a rapid and sensitive alternative for the detection of *E. coli* O157:H7. In this work, it was possible to release results within approximately 7.5 h, enabling decision making on the same day as the analysis was performed.

Keywords: bacteriophage; *E. coli* O157:H7; detection; food; Shiga toxigenic *E. coli*; pathogen

1. Introduction

Escherichia coli is a fecal bacterium found in the guts of humans and other warm-blooded animals and is typically harmless to humans (WHO, 2018). However, *E. coli* O157:H7 is a pathogenic strain classified as an enterohemorrhagic *E. coli* (EHEC) and currently is one of the most important food- and water-borne pathogens in the world (Rani et al., 2021). This pathogen is responsible for illnesses that range from mild gastroenteritis to life-threatening hemorrhagic colitis and potentially fatal hemolytic uremic syndrome, particularly in infants and immunocompromised individuals. *E. coli* O157:H7 has several pathogenic factors, such as the *eae* gene and Shiga toxins, responsible for attachment and interruption of host protein synthesis, respectively (Lim et al., 2010; WHO, 2018). Due to the very low infectious dose (~ 50 CFU), a rapid, selective, sensitive, simple, accurate, and easy-to-use method for detection of pathogenic *E. coli* O157:H7 is important in environmental and food quality monitoring (Lim et al., 2010; Razmi et al., 2020).

Existing methods for *E. coli* O157:H7 detection in foods can be complex, expensive, and time-consuming, requiring trained technicians and labor-intensive work, and are prone to false positives. Most protocols require an overnight enrichment step prior to the detection method or visual identification: i.e., culture-based methods, polymerase chain reaction (PCR), enzyme-linked immunosorbent assays (ELISA). The conventional plate culture method is time consuming (2-7 days) and laborious requiring a pre-enrichment step, selective enrichment, biochemical screening, serological confirmation, and toxin testing. PCR methods have been used for *E. coli* O157:H7 identification by targeting some virulence factor-encoding genes not specific for this bacterium, and without differentiating between viable and nonviable cells. ELISA techniques often require enrichment or purification steps and pretreatments, lengthening the analysis time. Both, PCR methods and ELISA techniques, based on kit variation, could take 5–25 h for detection results (Rani et al., 2021; Razmi et al., 2020).

There is the need for affordable, rapid, sensitive, selective, and simple approaches to detect this important food pathogen (Rani et al., 2021). Recently, the use of bacteriophages (phages) has shown great promise for rapid food- and water-borne pathogen detection (de Aquino, Elias, & Tondo, 2021; de Aquino, Elias, Gomes, et al., 2021; Kim et al., 2017; Wu et al., 2021).

Phages demonstrate a high specificity for their bacterial host and are capable of rapidly replicating within their host to high numbers. Other advantages of this system are that phages are not human pathogens and only replicate in living bacterial cells, which allow the identification of viable bacteria cells, avoiding false positive results. Phages have been used to detect bacterial foodborne pathogens due to their safety, specificity, rapid propagation, and ability to differentiate between live and dead cells (Hussain et al., 2021; Schmelcher & Loessner, 2014). The “PhageDx *E. coli* O157:H7 Assay” is based on the infection of *E. coli* O157:H7 by a bacteriophage and subsequent

production of phage-encoded proteins within its specific host. The recombinant phage used in this assay expresses a luciferase reporter during infection. The presence of *E. coli* O157:H7 is determined by incubating the lysate with the appropriate luciferase substrate and detecting emitted light in a luminometer. An absence of detected light indicates that no *E. coli* O157:H7 is present in that sample (Erickson et al., 2021). The PhageDx *E. coli* O157:H7 Assay was registered by the Association of Official Analytical Collaboration (AOAC), number 081601. This protocol was followed with the exception of the prescribed centrifugation step. Therefore, the objective of this study was to assess the performance of the PhageDx *E. coli* O157:H7 Assay for the detection of *E. coli* O157:H7 strains in vitro and in ground beef, milk, lettuce, and mineral water. In addition, bovine carcasses were analyzed for *E. coli* O157:H7 and the results obtained from the PhageDx *E. coli* O157:H7 Assay and a real-time PCR method were compared.

2. Materials and Methods

2.1 PhageDx E. coli O157:H7 Assay

This is a method recently developed by the company Labcorp (Laboratory Corporation of America Holdings) that uses a genetically modified bacteriophage for detecting *E. coli* O157:H7 after a short incubation period (Erickson et al., 2021). The bacteriophage present in the PhageDx *E. coli* O157:H7 Assay kit can be seen in Figure 1. This bacteriophage will be responsible for detecting *E coli* O157:H7 in the samples. After cell infection, the addition of the substrate for the bacteriophage-encoded luciferase generates a bioluminescent signal that will be detected in a luminometer. Values above 150 relative light units (RLU) will indicate the presence of *E. coli* O157:H7 in the samples (Erickson et al., 2021). All samples were read in duplicate in a GloMax® Navigator Luminometer (Promega Corp, Madison, WI, USA) after 3 min of delay and 1 s of integration. These criteria were previously determined by the developer and used in this work as a reference.

2.2. Experimental design

The PhageDx *E. coli* O157:H7 Assay's capacity to detect *E. coli* O157:H7 isolated from food and food-processing environments was tested, using two samples of each strain. Each sample was read twice in the luminometer and the average RLU of the two readings was described as result of the test. Two independent tests were performed in this manner, totaling 4 measurements for each strain.

For the in vitro limit of detection of the PhageDx *E. coli* O157:H7 Assay, samples of each concentration were placed in 10 wells of a microplate. After that, two readings in the luminometer were averaged to represent the final value per well. Three independent tests were performed, totaling 30 measurements by concentration.

The PhageDx *E. coli* O157:H7 Assay sensitivity was tested using artificially contaminated food samples (described in 2.5). Low and medium concentration of spiked foods was tested by reading 10 wells of a microplate, and the high concentration was tested by reading 4 wells. As performed in previous tests, two readings were taken on the luminometer, and the RLU average of these readings was recorded as the value

obtained in that experiment. Three independent tests were carried out, totaling 30 measurements by concentration and food matrix for low and medium concentration and 12 measurements by concentration and food matrix for high concentration. Across all food samples, 288 phage measurements were performed.

In the in vitro analysis, the wells representing the negative controls contained TSB, phage, and detection reagents. The negative controls for food samples were composed of uninoculated food matrix with TSB, phage, and detection reagents.

Means were calculated using Excel® version 2016 (Microsoft Corp., Redmond, WA, USA).

2.3 PhageDx E. coli O157:H7 Assay capacity to detect E. coli O157:H7 isolated from food and food environments

E. coli O157:H7 strains isolated in Brazil were used to evaluate the assay detection capacity (Table 1). The strains were cultivated overnight in 5 mL of tryptic soy broth (TSB; Kasvi, São José do Pinhais, Brazil) at 37 °C, and the cell concentration was standardized in a microplate reader (Loccus LMR 96, Brazil) until the absorbance corresponded to approximately 10^8 CFU/mL. After that, the cell concentration was confirmed by plating samples on tryptic soy agar (TSA; Kasvi, São José do Pinhais, Brasil) at 37 °C for 24 h. Samples (100 µL) were added to a 96-well plate in duplicate, and 10 µL of the phage added to each well. The plate was incubated at 37 °C for 2 h. After the incubation period, 65 µL of a solution composed of 50 µL of NanoGlo buffer, 1 µL NanoGlo substrate (Nano-Glo® Luciferase Assay System, Promega Corp., Madison, WI, USA), and 15 µL Renilla lysis buffer (Renilla Luciferase Assay System, Promega Corp., Madison, WI, USA) was added to each well and the plate was read in the luminometer.

2.4 In vitro limit of detection of the PhageDx E. coli O157:H7 Assay

E. coli O157:H7 strains (USP, CC, A1P2, FIO and A8P1) were used to test the limit of detection of the assay. Overnight individual growth of each of the strains was standarized to 10^8 CFU/mL using a microplate reader (Loccus LMR 96, Brazil). After that, 1 mL of each culture was added to a falcon tube to compose a bacterial cocktail. The cocktail was decimally diluted in TSB until reach 1000, 100, and 10 CFU/mL. Finally, 100 µL was added to wells of a 96-well plate, resulting in final concentrations of 1, 10 and 100 CFU/well. Concentrations were confirmed by plating and tested for bioluminescence as described in the section 2.3.

2.5 Phage sensitivity for E. coli O157:H7 detection on foods

Ground meat products, raw milk, water, and vegetables are important sources of *E. coli* O157:H7 (WHO, 2018). Therefore, the assay's detection limit was evaluated in representatives of these food groups. The meat product was represented by knuckle ground beef. The ground beef was refrigerated and portioned onto 500 g trays in a modified atmosphere packaging. Pasteurized whole milk, non-carbonated mineral water, and organic curly lettuces were also chosen for testing. All these foods were purchased in a local supermarket of Porto Alegre city, Southern Brazil, and transported to the Laboratory of Food Microbiology and Food Control of Institute of Food Science

and Food Technology of the Federal University of Rio Grande do Sul (ICTA/UFRGS). Prior to testing, the absence of *E. coli* O157:H7 in the samples was confirmed by plating on Sorbitol McConkey agar (Neogen, Lansing, MI, USA) supplemented with cefixime tellurite (Neogen, Lansing, MI, USA) after 24 h incubation in mTSB (Modified Tryptone Soy Broth) (Thermo Scientific™ Oxoid™, UK) with novobiocin supplement (Thermo Scientific™ Oxoid™, UK).

The assay was used in the same way for all foods. First, sample portions of 25 g or 25 mL were selected and put in a Whirl-Pak® sterile filter bag (Nasco, Fort Atkinson, WI, USA). After that, artificial contamination was carried out on each sample, using 1 mL of a *E. coli* O157:H7 cocktail at concentrations 1, 10, and 100 CFU/mL. The samples were contaminated inside food bags, separately and incubated at 41°C for 5 h.

The cocktail of bacterial strains was prepared according to de Aquino, Elias, & Tondo (2021). In summary, 1 mL of the overnight growth of each strain was added to a single falcon tube, centrifuged, and washed with 5 mL 0.1% peptone water. After three more cycles of centrifugation and washing, the cocktail was serially diluted to the concentrations mentioned above.

2.6 Comparison of the PhageDx E. coli O157:H7 Assay and real-time PCR

Samples (325 g) of 100 bovine carcasses were collected in Brazilian slaughterhouses and sent to a laboratory belonging to the Unified System of Agricultural Health. Each sample was incubated for 10 to 20 h with 975 mL of STEC Enrichment Broth (SEB) medium (Bio-Rad, Hercules, California, USA) at 41.5 °C, as determined by the manufacturer of the iQ-Check STEC VirX kit. This real-time PCR assay is designed to detect virulence genes of Shiga Toxin – producing *E. coli*. After incubation, every sample was analyzed by real-time PCR and stored for analysis by PhageDx *E. coli* O157:H7 Assay. From each sample, 1 mL of the enrichment broth was collected and added to 2 mL Eppendorf tubes, frozen, and later sent to the Laboratory of Food Microbiology and Food Control of the Institute of Food Science and Food Technology for analysis by the PhageDx *E. coli* O157:H7 Assay. Upon arrival at the laboratory, the samples were thawed at room temperature for 1 h, homogenized in a shaker, and 150 µL of each sample added to a well in a 96-well plate in duplicate. After, 10 µL of the phage solution was added to each well and then incubated at 37 °C for 2 h. Thereafter, the bioluminescence test was performed as described in 2.3.

3. Results

All *E. coli* O157:H7 strains tested were identified by PhageDx *E. coli* O157:H7 Assay (Table 1). The strains obtained RLU values between 10^6 – 10^7. The non-O157:H7 *E. coli* strain (ATCC 25922), used as negative control, obtained a mean RLU of 106, wich was below the 150 RLU cut-off applied for this method.

Table 2 shows the cocktail detection percentages after 2 h of bacteriophage infection. The percentage of detection varied according to the inoculum concentration, observing an increase in the rate of detected samples with the greatest amount of target cell in the samples.

The detection limit of the PhageDx *E. coli* O157:H7 Assay in different types of food is demonstrated in Table 3. In summary, after 5 h of enrichment, the assay was able to detect *E. coli* O157:H7 in all food samples spiked with 1 CFU. Just like in vitro

sensitivity results, an increase in RLU was also observed corresponding to the increase in the number of cells present in food samples. The highest concentration of inoculum in lettuce and mineral water were the samples where the highest RLU were observed, followed by ground beef and milk. The lowest values obtained, 4.75 x 10^3 RLU and 7.68 x 10^3 RLU, were observed for the smallest inoculum added to pasteurized milk and ground beef, respectively.

To compare the PhageDx *E. coli* O157:H7 Assay to an established method, bovine carcasses where obtained and assessed for STEC contamination. All 100 samples of the bovine carcass enrichments were found to be negative for the presence of STEC using the PCR methodology according to results obtained by the partner laboratory. In agreement with these results, no sample was positive for the presence of *E. coli* O157:H7 by PhageDx *E. coli* O157:H7 Assay. Positive controls used in both methods were detected correctly.

4. Discussion

New high-throughput methods for detecting *E. coli* O157:H7 should include numerous advantages, such as: fast analysis, easy operation, small amounts of samples and reagents, low operator qualification requirements and low cost. A diagnostic method that meets these requirements becomes an excellent tool to aid in food and water safety (Rani et al., 2021). The development of methods that meet these criteria is shaped by numerous steps, from the choice of the method to be studied to specificity and sensitivity analyses before this method is tested outside the environment in which it was developed. The specificity of the method is related to two criteria, inclusivity and exclusivity. Inclusivity is the ability to detect different strains of the target microorganism and exclusivity is the ability to not identify strains that are not the target of the test.

Regarding inclusivity tests, the method developer must be aware and include in the analysis set, recurrent strains not only relevant in their country, but also relevant strains of interest to potential international end users (Wiedmann et al., 2014). The PhageDx *E. coli* O157:H7 Assay has previously proven to be inclusive by correctly detecting 100% of the 103 *E. coli* O157:H7 strains tested (Erickson et al., 2016). In this work, this method also detected 100% of tested strains, independent of the source. Regarding exclusivity, in the work of Erickson et al. (2016), 37 *E. coli* (non-O157:H7) strains, 12 big-six-STEC strains and 64 non-*E. coli* strains were analyzed and the results were negative. In this study, the standard non-O157:H7 *E. coli* strain ATCC 25922 was used as a negative control and the method did not show false positive results either.

Concerning sensitivity, the PhageDx *E. coli* O157:H7 Assay was able to detect low numbers of *E. coli* O157:H7 without a prior enrichment period. Approximately 80% of the analyzed samples containing 10 CFU/well were positive. The detection level reached 100% when each well contained approximately 100 CFU of *E. coli* O157:H7.

Sensitivity is a very important criterion when developing new methods since a single pathogen present in food can cause infection (Law et al., 2014). To improve the sensitivity of the method and knowing that food compositions are more complex than in vitro conditions, a 5 h enrichment after inoculation of the *E. coli* O157:H7 cocktail was analyzed for the food samples.

Enrichment has a double advantage, selectively increasing the concentration of target bacteria, consequently increasing the sensitivity of the assay, and ensuring that positive results are obtained from viable cells (Amagliani et al., 2018). In this case, as it is already known that bacteriophages need live cells for replication, and the aim of the enrichment is to increase the number of cells available in the samples to improve the sensitivity of the method.

As the level of STEC contamination in food is low, enrichment in selective and non-selective media is essential for its detection. Enrichment is often the most time-consuming step during the pathogen detection protocols. At this stage, culture media that promote the growth of target cells are normally used, and some of them also have the additional function of inhibiting the development of the accompanying microbiota. In the case of STEC, recommended culture media, BPW (ISO / TS 13136:2012), mBPWp (FDA / BAM protocol), mTSB (ISO / TS 13136: 2012) are added with antibiotics to inhibit Gram-positive bacteria and aid in the growth of Gram-negative cells such as STEC. The addition of antibiotics makes the analysis more expensive, in addition to potentially reduced sensitivity leading to false negative results (Amagliani et al., 2018).

In this work, the culture medium used for enrichment was TSB without the addition of any antibiotic, as recommended by the method developer. As the bacteriophage of the PhageDx *E. coli* O157:H7 Assay has already been shown to be specific for the microorganism in question, there was no need to include antibiotics to reduce the accompanying microbiota. The use of common culture media, such as TSB, and the potential for not using antibiotics are advantages that reduce material cost and simplify the preparation and performance of the test.

The PhageDx *E. coli* O157:H7 Assay was registered on AOAC and has five main steps: enrichment, sample concentration, phage infection, substrate addition and readout. Despite being a simple step, the concentration step performed by centrifuging 1 mL of the sample and then resuspending the pellet in TSB before incubation with the phage would be labor intensive for food industries that analyze hundreds of samples. So, this step was eliminated from the protocol and the incubation time of the samples standardized to 5 h (developer suggests 5-7 h). The objective, in addition to simplifying the protocol, was to assess whether the shorter enrichment step with the suggested culture medium and chosen time would be able to maintain the sensitivity of the method.

As shown in Table 3, in all food matrices tested, it was possible to detect burdens as low as 1 CFU/25 g or mL. The difference in the composition of the food samples did not interfere with the test sensitivity, although differences obtained in the negative controls were observed. Non-inoculated foods, i.e., negative controls, had an average RLU of 27 (mineral water), 60 (lettuce), 86 (milk) and 87 (ground beef). This large difference between the negative control and samples with a lower concentration of pathogens is important to aid in the interpretation of results in routine tests. While bright luminescence is generally desirable, low background signal is important to yield efficient test methods with high sensitivity (Hall et al., 2012). The high light emission in positive samples and the low background obtained by the PhageDx *E. coli* O157:H7 Assay is due to the enzyme NanoLuc® (Promega) present in the modified bacteriophage. Bioluminescent assays improved significantly with the discovery of this enzyme. Nanoluc is a small luciferase subunit (19 kDa) from the deep-sea shrimp *Oplophorus gracilirostris*. A half-life signal greater than 2 h with a specific activity approximately 150-fold greater than either firefly (*Photinus pyralis*) or Renilla (*Renilla reniformis*) luciferases are characteristics that make it a great tool for building bioluminescent assays.

The PhageDx *E. coli* O157:H7 Assay demonstrated good functioning compared to other bacteriophage reporter assays in the literature.

Ripp et al. (2008) used a reporter bacteriophage containing the *luxI* gene to detect *E. coli* O157:H7 in artificially contaminated apple juice, tap water and ground beef. Unlike this current work, detection in ground beef was not successful. This was due to the fact that the phage reporter used a quorum sensing type of signaling (*luxI*/*luxR*) and these samples contained natural autoinducers generating false positive results. With respect to apple juice and tap water, the sensitivity was 1 CFU/mL. However, the detection times observed were higher than in this current work. In the case of apple juice, detection took place after 6 h of enrichment and a total analysis time of 16 h. In tap water, it was detected after 6.5 h, with 6 h of enrichment.

Kim et al. (2017) were able to detect *E. coli* O157:H7 using a phage containing the lux operon without noticing interference from the food matrix. The phiV10lux phage was able to detect 10 CFU/cm^2 in romaine lettuce, 13 CFU/mL in apple juice and 17 CFU/g in ground beef. Their assay enrichment time was 5 h, followed by 40 min of phage infection.

As mentioned before, the insertion of the NanoLuc gene in bioluminescent assays has provided improved stability, higher sensitivity, and lower background. This has been proven in assays to detect different food and clinical pathogens (Brown et al., 2020; Meile et al., 2020; Nguyen et al., 2020; Zelcbuch et al., 2021; Zhang et al., 2016).

Therefore, the results obtained in this work may be compared with another work that also used Nanoluc bacteriophages to detect *E. coli* O157:H7. Zhang et al. (2016) built a NanoLuc-based phage reporter to detect *E. coli* O157:H7. The phage was able to detect 4.68 CFU in 40 mL of ground beef enrichment after approximately 9 h, demonstrating good sensitivity and rapidity in testing. For the same food, the *PhageDx E.* coli O157:H7 Assay was able to detect 1 CFU /25 g after a total of 7.5 h (5 h of enrichment, 2 h of phage infection and approximately 0.5 h for reagent addition and luminometer reading.

Brazil is the largest exporter of beef and beef cattle farming represents 5% of the revenue acquired with all exported products (ABIEC, 2021). Although STEC analysis is not mandatory for bovine products in the country, importing countries tend to demand criteria from exporters similar to those followed by their domestic products. This has caused beef exporting countries, including Brazil, to adopt mandatory standards for STEC in their export establishments, regardless of their internal regulations (FAO & WHO, 2018).

Added to this, in Brazil there is the National Program for the Control of Pathogens (PNCP), in which the official monitoring of Shiga toxin-producing *E. coli* (STEC) in beef is included. In this case, the collection of official samples is carried out by public servants who work in the federal inspection and the investigations are carried out by the Federal Agricultural Defense Laboratories (LFDAs) (MAPA, 2020). In partnership with one of these laboratories, 100 samples of carcasses were analyzed in this study. Just as the official analysis did not identify the presence of STEC, none of the samples were positive for *E. coli* O157:H7 in this work either. The results obtained are in accordance with what has been shown, in the year 2019, as STEC serogroup O157:H7 was not identified in the 1,523 samples officially analyzed (MAPA, 2020).

Despite the low prevalence of STEC observed in Brazil, the severity of these pathogens justifies their constant monitoring. In recent years, STEC have been isolated from dairy cattle feces (Moreira et al., 2003), irrigation and wash waters from organic lettuce (de Quadros Rodrigues et al., 2014) and bovine carcasses (Loiko et al., 2016). The latest data published by the official collection showed that in 2020, 3

samples were positive for *E. coli* O157:H7 and one sample was positive for STEC serogroup O26 in 1,884 bovine carcasses analyzed (MAPA, 2021). Regarding outbreaks, in 2017, one case was assigned to *E. coli* O157 due to consumption of a tomato and cheese salad prepared at the school canteen (Santos et al., 2017). In December 2019, the first deaths assigned to *E. coli* O157 NM were registered in Brazil. In this case, four children of the same school were hospitalized due to severe diarrhea, vomiting, and fever. The mother of one child was diagnosed with HUS. Unfortunately, one child died of renal failure and another due to respiratory failure (Bartz et al., 2022).

Although naturally contaminated samples were not available, the PhageDx *E.* coli O157:H7 Assay agreed with the reference results and there were no false positives. In addition, it is important to note that rapid methods are screening methods and positive samples need to be confirmed by traditional methodology. Thus, the faster the screening methods, the greater agility in decision making, whether to release the food batch or to proceed with the confirmation of positivity. In this work, it was possible to release the results in approximately 7.5 h, enabling decision making on the same day the analysis was performed.

5. CONCLUSION

The need for rapid and sensitive methods for detecting microorganisms in food and beverages encourages the development of new methods to aid in food safety. The PhageDx *E. coli* O157:H7 Assay is a rapid method that uses bacteriophage to detect *E. coli* O157:H7. The assay proved to be sensitive both in in vitro samples and in food. Food inoculated with only one *E. coli* O157:H7 cell could be detected after 5 h of incubation. Beverages, meat and vegetable products were analyzed, and the method showed its ability to be used. 7.5 h was the total analysis time, demonstrating a significant reduction in working time when compared to existing methods. Given this study, this tool can contribute to the distribution of safe food.

CRediT authorship contribution statement

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. **Eduardo Cesar Tondo**: Methodology, supervision, writing—original draft and writing—review and editing. **Carolina Fraga Alves**: Formal analysis and investigation. **Camila Camargo Drummond**: Formal analysis and investigation and **Jhuli Murineli Baia**: Formal analysis and investigation. All authors have read and agreed to the published version of the manuscript.

Funding

Financial support for this project was provided by Laboratory Corporation of America Holdings (Labcorp). Labcorp did not have a role in study design and was not involved in the decision to publish these results.

Declaration of Competing Interest

Funding in the form of financial support and reagents provided by the Laboratory Corporation of American Holdings (Labcorp). Labcorp is the manufacturer of the PhageDx *E. coli* O157:H7 Assay and holds relevant patents. Study design, data analysis, and decision to publish were carried out without Labcorp involvement. The authors Camila Camargo Drummond and Jhuli Murineli Baia are employees of Laboratório de Análises de Alimentos (Lanali) and provided bovine carcass samples for this work. Lanali has no conflict of interest.

Acknowledgments

The authors would like to thank the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) for scholarship granted to the post-graduate student.

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Figure 1. Electron Micrograph of the *E. coli* O157:H7 phage

Table 1. *E. coli* O157:H7 strains isolated from food and food production environments tested at concentration 10^8 CFU/mL by the PhageDx *E. coli* O157:H7 Assay. Results are expressed as Relative Light Units (RLU).

Table 2. Evaluation of the detection limit of the PhageDx *E. coli* O157:H7 Assay for cocktail composed for *E. coli* O157:H7 strains. The percentage indicates the percent positive samples at each concentration

Table 3. PhageDx *E. coli* O157:H7 Assay sensitivity for *E. coli* O157:H7 detection on food after 5 h of incubation.

Figure 1.

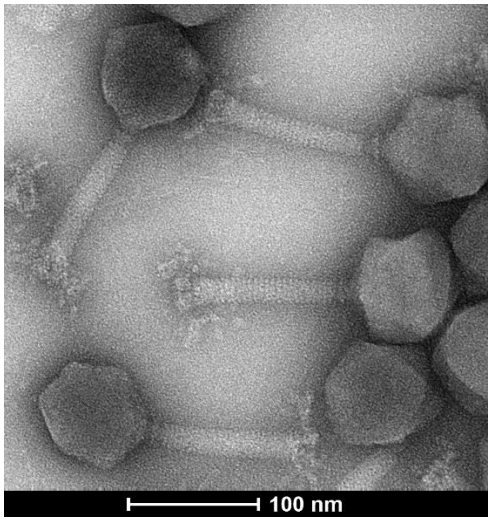

Image kindly provided by Stephen Erickson (Labcorp)

Table 1.

Strain	Average RLU	SOURCE
14	6.51×10^6	Lettuce irrigation water;
BB	3.37×10^6	Organic lettuce irrigation water;
CC	5.77×10^6	Organic lettuce irrigation water;
A1P2	7.63×10^6	Bovine carcass after hide removal and before evisceration;
A3P1	8.30×10^6	Bovine hide after bleeding;
A8P1	1.08×10^7	Bovine hide after bleeding;
A10P1	9.24×10^6	Bovine hide after bleeding;
A9	8.38×10^6	Bovine carcass after hide removal and before evisceration;
A10P2	7.74×10^6	Bovine carcass after hide removal and before evisceration;
AMA2	7.62×10^6	LMCA – ICTA *
A39	3.28×10^6	LMCA – ICTA
AH3	3.61×10^6	LMCA – ICTA
FIO	2.31×10^6	Standard FIOCRUZ**
USP	9.09×10^6	Standard USP***
ATCC 25922 (non-O157:H7)	106	American Type Culture Collection

* LMCA - ICTA: Laboratório de Microbiologia e Controle de Alimentos – Instituto de Ciência e Tecnologia de Alimentos
**FIOCRUZ: Fundação Oswaldo Cruz
*** USP: Universidade de São Paulo

Table 2.

Sample	Concentration Levels		
	1 CFU/well	10 CFU/well	100 CFU/well
Cocktail	6/30 (20 %)	25/30 (83 %)	30/30 (100%)

Table 3.

Food type	Inoculum	Number of replicates	Avg. RLU	Detection
Ground beef	Low	30	7.68×10^3	30/30
	Medium	30	9.15×10^4	30/30
	High	30	9.15×10^5	12/12
Pasteurized Milk	Low	30	4.75×10^3	30/30
	Medium	30	4.34×10^4	30/30
	High	30	2.60×10^5	12/12
Lettuce	Low	30	3.41×10^4	30/30
	Medium	30	5.08×10^5	30/30
	High	30	8.02×10^6	12/12
Mineral Water	Low	30	1.20×10^4	30/30
	Medium	30	8.36×10^4	30/30
	High	30	1.88×10^6	12/12

CAPÍTULO 3- DISCUSSÃO GERAL

O processo de globalização impactou consideravelmente o comércio de alimentos. Dentro desse contexto, o Brasil destaca-se uma vez que os alimentos produzidos nacionalmente alcançaram quase 200 países no último ano. Essas atividades representam expressiva parte na economia nacional e também o reconhecimento internacional dos produtos brasileiros. Para que o Brasil mantenha-se exportando alimentos, além de ser necessário disponibilizar produtos de qualidade e com preço competitivo, é necessário obedecer às exigências microbiológicas dos países importadores e os programas de autocontrole exigidos pela legislação brasileira.

O art. 74 do Decreto nº 9.013/2017 do MAPA exige que os estabelecimentos de produtos de origem animal possuam Programas de Autocontrole (PAC) implementados. A referida legislação determina ainda que os PAC sejam monitorados e verificados pelos estabelecimentos e os registros sejam auditáveis. O objetivo é assegurar a inocuidade, a identidade, a qualidade e a integridade dos seus produtos, desde a obtenção e a recepção da matéria-prima, dos ingredientes e dos insumos, até a expedição destes. O art. 10, inc. XVII, do mencionado decreto, dispõe que os PAC devem incluir, mas não se limitar, aos programas de pré-requisitos, Boas Práticas de Fabricação (BPF), Procedimento Padrão de Higiene Operacional (PPHO) e Análise de Pontos Críticos de Controle (APPCC) ou a programas equivalentes reconhecidos pelo MAPA (BRASIL, 2017). As BPF para estabelecimentos elaboradores e/ou industrializadores de alimentos são regidas pela Portaria nº 368/1997 do MAPA e a obrigatoriedade da implantação de Sistema de APCC nos estabelecimentos com SIF é exigida pela Portaria nº 46/1998 do MAPA (BRASIL 1997; 1998).

Métodos de detecção de microrganismos são utilizados nas indústrias de alimentos tanto para controle interno quanto para atender a critérios legais, nacionais ou internacionais. A avaliação microbiológica do produto final auxilia na verificação do *status* sanitário dos produtos antes da comercialização, além de fornecer à indústria dados que podem auxiliar em melhorias na limpeza e desinfecção das instalações, equipamentos e utensílios e também no controle dos processos.

Existem inúmeros métodos disponíveis no mercado para detecção de microrganismos. A escolha do método a ser utilizado nas indústrias pode sofrer

influência de algumas características destes métodos. Dentre os fatores mais importantes, pode-se destacar o tempo necessário para execução das análises. O setor avícola tem investido expressivamente em tecnologias e melhoramento genético para que os alimentos sejam disponibilizados com maior rapidez ao mercado. Por exemplo, a idade de abate de frangos foi reduzida significativamente. Enquanto, em 1930, as aves eram abatidas após 105 dias com 1,5 kg, hoje o abate ocorre em cerca de 42 dias e as aves possuem por volta de 2,3 kg (JESUS JUNIOR *et al*., 2007). Do mesmo modo, os métodos de avaliação microbiológica dos produtos finais devem contribuir com a agilidade buscada pelas indústrias, sendo capazes de liberar os resultados o mais rápido possível.

Os métodos microbiológicos tradicionais são baseados em cultura dos microrganismos e embora sejam mais baratos, costumam ser demorados e trabalhosos. Por isso mesmo, técnicas avançadas de detecção vêm ganhando espaço na indústria de alimentos e uma das suas principais vantagens sobre os métodos tradicionais é a redução do tempo necessário de análise. Como exemplo de técnicas avançadas pode-se citar aquelas baseadas na Reação em Cadeia da Polimerase (PCR), PCR multiplex ou PCR em tempo real, ensaios imunoenzimáticos e técnicas de espectrometria de massa, como o MALDI-TOF-MS. Embora esses métodos tenham muitas vantagens, a desvantagem em comum é o custo geralmente mais alto oriundo da necessidade de equipamentos e insumos sofisticados, bem como a exigência de operadores treinados (PACZESNY; RICHTER; HOŁYST, 2020). Os mencionados ônus, muitas vezes, são aceitos pelas indústrias em virtude destes métodos proporcionarem maior agilidade na liberação dos resultados dos alimentos a serem expedidos. Os métodos rápidos têm sido utilizados pelas indústrias para a realização de triagem de amostras e a eventual presença do microrganismo-alvo é confirmada por métodos tradicionais. Os autocontroles e as BPFs contribuem para que o número de amostras positivas seja consideravelmente inferior ao número de amostras negativas, tornando vantajosa a utilização de métodos rápidos.

Baseado no destaque do Brasil como país produtor e exportador de alimentos, e conhecendo a necessidade das indústrias por métodos rápidos para detecção de microrganismos, no presente estudo, testou-se dois novos ensaios rápidos para a detecção de *E. coli* O157:H7 e *Salmonella* spp., desenvolvidos pela Empresa americana LabCorp. A Empresa LabCorp inicialmente desenvolveu o *kit* PhageDx *E.*

coli O157:H7 Assay para a detecção rápida de um dos patógenos de maior interesse nos Estados Unidos, *E. coli* O157:H7. Por ser o maior exportador de carne bovina do mundo, e a relação já mencionada entre este alimento e o microrganismo em questão, o Brasil foi convidado a testar o *kit*. O Laboratório de Microbiologia e Controle de Alimentos - ICTA/UFRGS ingressaria no presente estudo para investigar a sensibilidade e especificidade do método utilizando cepas de *E. coli* O157:H7 oriundas do Brasil.

Apesar dos resultados interessantes apresentados pelos desenvolvedores do PhageDx *E. coli* O157:H7 Assay, as informações obtidas junto às indústrias nacionais indicavam baixa prevalência de *E. coli* O157:H7 nas carnes brasileiras, o que demonstrava que a pesquisa e análise deste patógeno, era importante, mas não urgente. Cenário completamente diferente se apresentava para *Salmonella*, um dos microrganismos de maior interesse para as indústrias brasileiras, que são responsáveis pela maior exportação de produtos avícolas do mundo. Por esse motivo, a Empresa Labcorp rapidamente desenvolveu um novo *kit,* agora para detecção rápida de *Salmonella* spp, o qual passou a ser o foco das atividades da presente Tese. Por isso, apesar do *kit* para detecção de *E. coli* O157:H7 ter sido desenvolvido primeiro, no presente estudo, os primeiros artigos publicados foram sobre o *kit* de detecção de *Salmonella*, desenvolvido em grande parte para atender as necessidades do mercado brasileiro.

Na presente Tese, o artigo 1 traz as informações sobre o desenvolvimento desse *kit*, as características dos fagos que o compõe, os resultados preliminares de exclusividade e inclusividade e os primeiros testes em alimentos. Depois de desenvolvido e testado pelo fabricante, o objetivo inicial foi avaliar a capacidade do PhageDx *Salmonella* Assay *kit* em detectar cepas de *Salmonella* spp. isoladas no Brasil. No artigo 2 pôde-se verificar que, além de detectar 100% das cepas brasileiras testadas, o limite de detecção foi de 1 CFU/25 g em todos os alimentos pesquisados. No artigo 3, foi analisada a capacidade do *kit* ser empregado também em alimentos de origem vegetal. Neste artigo, determinou-se o limite *in vitro* do método para cepas brasileiras e o limite de detecção em alface hidropônica. Para este alimento, o limite de detecção ficou levemente superior aos alimentos cárneos, 10 CFU/ 25 g.

O principal diferencial do *kit* PhageDx *Salmonella* Assay é o menor tempo necessário para a obtenção dos resultados. Nesse *kit*, as amostras são enriquecidas

por apenas 7 horas e isso reduz consideravelmente o tempo total de processamento, permitindo que os resultados sejam emitidos em apenas um dia de trabalho. Há inúmeros métodos rápidos para detecção de *Salmonella* spp. Métodos rápidos comercialmente disponíveis para detecção deste patógeno podem variar desde novos meios seletivos e técnicas convencionais modificados ou adaptados à ensaios baseados em imunologia e na investigação de DNA (LEE *et al*., 2015). Lee *et al*. (2015) listaram inúmeros métodos comerciais disponíveis para detecção de *Salmonella* spp. Os métodos descritos se encaixavam nas categorias de meios de cultura seletivos, ELISA, imunocromatográficos, biossensores, de aglutinação, de imunodifusão, dispositivos de fluxo lateral, PCR, hibridização de DNA e testes miniaturizados. Todos esses ensaios foram mais rápidos que os métodos tradicionais, mas nenhum foi tão rápido e com sensibilidade igual ao PhageDx *Salmonella* Assay.

Atualmente, já são comercializados alguns métodos rápidos capazes de fornecer resultados de análises de *Salmonella* spp. em alimentos no dia seguinte ao início da análise (LEE *et al*., 2015). Ressalta-se que o kit PhageDx *Salmonella* Assay emitiu resultados no mesmo dia e sem prejuízo da sensibilidade, o que certamente é de interesse para as indústrias de alimentos.

Assim como em qualquer técnica, o tempo total de análise pode variar com o número de amostras a serem analisadas e a destreza dos operadores. O *kit* PhageDx *Salmonella* Assay necessitou de apenas 7 h de enriquecimento e 2 h de infecção entre o fago e o microrganismo-alvo, para que a técnica detectasse *Salmonella spp.* em uma concentração de 1 UCF/ 25 g de alimento. Além disso, o *kit* necessita apenas de um luminômetro e a sua operação não é complicada.

Após finalizar as análises do *kit* de *Salmonella*, foi possível dar início aos ensaios referentes ao *kit* PhageDx *E. coli* O157:H7 Assay. Os testes de inclusividade e exclusividade realizados pelo desenvolvedor do mesmo, demonstraram que o *kit* era específico para a detecção de *E. coli* O157:H7. Nas análises de sensibilidade pode-se concluir que assim como o PhageDx *Salmonella* Assay, os resultados poderiam ser emitidos no mesmo dia do teste. Com esses achados preliminares, no presente estudo, investigou-se a eficiência do *kit* para detectar cepas brasileiras de *E. coli* O157:H7. Como observado no artigo 4 todas as cepas isoladas no Brasil foram identificadas pelo PhageDx *E. coli* O157:H7 Assay. Após finalizada esta fase, o objetivo foi investigar a redução das etapas do método. Apesar da metodologia

desenvolvida pela LabCorp já apresentar uma diminuição de tempo considerável quando comparada com os métodos disponíveis no mercado, reduzir as etapas no processamento das amostras sem perder a sensibilidade, tornaria o *kit* mais atrativo e competitivo.

Segundo Erickson, Gil e Nguyen (2021) a detecção de *E. coli* O157:H7 em amostras de carne pelo *kit* PhageDx *E. coli* O157:H7 Assay inclui o enriquecimento da amostra a 42 ºC de 6 a 7 horas (25 g) ou 9 a 10 h (375 g), transferência de 1 mL da amostra para um microtubo, centrifugação e descarte do sobrenadante, ressuspensão do *pellet* em TSB, adição do fago repórter e incubação a 37 ºC por 2 h. Em seguida, as amostras são centrifugadas novamente e transfere-se 150 µL do sobrenadante à um poço de uma microplaca onde adiciona-se os reagentes e, por fim, realiza-se a leitura das placas em um luminômetro. Nesta metodologia, cada amostra é manipulada três vezes, primeiramente são colocadas em um saco de amostra, em seguida em um microtubo para centrífuga e por último em um poço de microplaca.

Considerando a quantidade de amostras analisadas pelas empresas, estas etapas podem incrementar o tempo total da análise. Por exemplo, o tempo gasto na identificação de tubos, transferência de amostras e etapas de centrifugação, será maior quanto maior for o número de amostras. Pensando no diferencial que este método tem a oferecer frente aos métodos rápidos já disponíveis, o presente estudo avaliou a retirada da etapa de centrifugação.

No artigo 4 foi demonstrado que mesmo sem a etapa de centrifugação e padronizando-se o tempo de enriquecimento em 5 horas, o *kit* PhageDx *E. coli* O157:H7 Assay pôde detectar 1CFU de *E. coli* O157:H7 em 25 g de carne moída e alface e 25 mL de água mineral e leite pasteurizado. Como o esperado, a redução das etapas de processamento das amostras, reduziu o tempo total do método. É importante que as técnicas de detecção, além de rápidas, sejam de fácil execução. Etapas de concentração, como filtração e centrifugação, podem melhorar a sensibilidade do método, porque concentram o microrganismo de interesse, no entanto essas etapas podem ser inviáveis em laboratórios que processam um grande número de amostras.

O enriquecimento é uma etapa crucial no protocolo de detecção de patógenos, pois é responsável pela recuperação e multiplicação dos mesmos até

alcançar concentrações detectáveis (DE PAULA *et al*., 2014). Esta é geralmente a etapa mais prolongada dos métodos. Rani *et al*. (2021) revisaram tendências para diagnóstico de *E*. *coli* O157:H7 em água e alimentos. Os autores listaram inúmeros *kits* comerciais, bem como seus tempos de enriquecimento, tempos totais de análise e aplicações. Todos os *kits* necessitaram de menos tempo para execução, quando comparados à metodologia tradicional. Quando comparados com o PhageDx *E. coli* O157:H7 Assay, as 5h de enriquecimento necessárias no *kit* a base de bacteriófagos, foi expressivamente menor.

No artigo 4, 100 carcaças bovinas provenientes da amostragem oficial realizada para o PNCP foram analisadas pelo *kit* PhageDx *E. coli* O157:H7 Assay para pesquisa de *E. coli* O157:H7 e por um *kit* de PCR para a pesquisa de STEC. Tanto o *kit* investigado neste estudo quanto as análises por PCR realizada pelo laboratório credenciado, não detectaram a presença de *E. coli* O157:H7 ou STEC. Isso demonstra que o *kit* não apresentou falso positivo e também corrobora com os achados publicados pelo MAPA no decorrer do PNCP. A baixa prevalência de *E. coli* O157:H7 também reforça a vantagem do *kit* PhageDx *E. coli* O157:H7 Assay ser utilizado como método de triagem. Os resultados podem ser liberados no mesmo dia da análise, sem a necessidade de confirmação.

De maneira geral, os *kits* PhageDx *E. coli* O157:H7 Assay e PhageDx *Salmonella* Assay apresentaram benefícios frente aos métodos disponíveis. Seja pelo ponto principal que foi o tempo de análise, seja pela facilidade de execução. A utilização de microplacas nos métodos rápidos pode oferecer a vantagem de reduzir o tempo de análise e aumentar a capacidade dos métodos no processamento de um grande número de amostras (LEE *et al*., 2015). Nos métodos estudados neste trabalho, até 96 amostras, número de poços de uma microplaca, puderam ser analisadas simultaneamente. Além disso, o método demonstrou ser robusto, uma vez que pequenas variações no procedimento, como a adição duplicada de reagentes ou fagos em um mesmo poço, as quais podem ocorrer na rotina de laboratório, não afetou os resultados. É importante destacar que os *kits* exigem baixa aquisição de equipamentos, além dos que já são utilizados na rotina de laboratórios de microbiologia. O luminômetro é o único aparelho mais complexo necessário, ressalta-se, no entanto, que este aparelho não é exclusivo para o *kit* e pode ser utilizado em outras análises laboratoriais. A interpretação dos resultados é outro ponto

fundamental para evitar julgamentos errôneos. Nos artigos publicados, pode-se observar que as amostras positivas foram facilmente distinguíveis das amostras negativas e dos controles negativos. Os altos valores de RLU obtidos em amostras positivas, são uma vantagem em relação aos testes de qPCR, por exemplo, onde muitas vezes os valores obtidos perto do limiar estabelecido causam dúvida ao operador.

As vantagens já citadas aliadas ao disposto no Ofício Circular 43/2021 do MAPA (MAPA, 2021), podem permitir que os *kits* sejam implementados pelas indústrias alimentícias brasileiras. Isto forneceria ao mercado uma ferramenta rápida e precisa de detecção de patógenos e pode contribuir para que o Brasil continue destacando-se como produtor e exportador de alimentos seguros, atendendo aos critérios microbiológicos exigidos.

CAPÍTULO 4- CONCLUSÃO E CONSIDERAÇÕES FINAIS

Os *kits* de detecção PhageDx *Salmonella* Assay e PhageDx *E. coli* O157:H7 Assay demonstraram ser capazes de detectar *Salmonella* spp. e *E. coli* O157:H7 isoladas no Brasil, em matrizes alimentares produzidas nacionalmente.

Os *kits* detectaram todas cepas testadas, isoladas de alimentos envolvidos em surtos ou de ambientes de processamento.

Nos testes *in vitro,* os *kits* detectaram *Salmonella* spp. e *E. coli* O157:H7 na concentração mínima de 100 UFC/ poço, após 2 horas de ensaio.

Em 25 g de produtos avícolas, 1 célula de *Salmonella* spp. pôde ser detectada após aproximadamente 9 horas de ensaio. No caso da alface, 10 células deste patógeno puderam ser detectadas no período supracitado. Uma única célula de *E. coli* O157:H7 em 25 g de carne moída e alface ou 25 mL de leite pasteurizado ou água mineral, pôde ser detectada após aproximadamente 7 horas de análise.

Tanto o *kit* PhageDx *Salmonella* Assay quanto o *kit* PhageDx *E. coli* O157:H7 Assay forneceram resultados em menos de um dia de trabalho, o que é vantajoso para laboratórios de indústrias de alimentos.

Os resultados obtidos neste trabalho corroboraram com os resultados obtidos durante o desenvolvimento e testes preliminares realizados pelo fabricante.

As cepas brasileiras puderam sem identificadas sem perda de sensibilidade do método.

Os *kits* foram mais rápidos que outros ensaios disponíveis atualmente no mercado e demonstaram ser sensíveis e específicos para os microrganismos de interesse.

Como perspectivas futuras, planeja-se testar os *kits* em outros nichos da produção avícola, de carnes e demais produtos alimentícios e seus ambientes de produção.

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