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Evaluating Recombinant Bacteriophages for Rapid Detection of Escherichia coli O157:H7 in Foods

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Abstract

Escherichia coli O157:H7 is an important foodborne pathogen that is increasingly being isolated around the world. Since this microorganism is the causative agent of haemorrhagic colitis (HC) and fatal haemolyticuremic Syndrome by hemorrhagic colitis (HC) and fatal hemolyticuremic Syndrome (HUS), rapid and accurate detection methods must be available to contribute to food safety. This study evaluates the performance of a recombinant bacteriophage-based assay (PhageDx) for the detection of *E. coli* O157:H7 in vitro and across various food matrices. The assay was tested using *E. coli* O157:H7 strains isolated in Brazil from lettuce irrigation water, bovine carcasses, and hides. The accuracy, sensitivity, and reproducibility of PhageDx were assessed using three inoculum levels (1, 10, and 100 CFU) in ground beef, lettuce, pasteurized milk, and mineral water. PhageDx successfully detected 1 CFU/25 g or mL of *E. coli* O157:H7 within 7.5 hours, after a 5-hour enrichment step, and detected 100 CFU in just 2.5 hours without enrichment. In analysis of 100 bovine carcasses, PhageDx results were consistent with real-time PCR, with no false positives or negatives. These findings suggest that PhageDx is an effective tool for rapid detection of *E. coli* O157:H7, supporting its potential application in food safety monitoring.

Keywords: luminescence; Assay; Detection; Food; Pathogen.

Introduction

Escherichia coli O157:H7 is an enterohemorrhagic *E. coli* (EHEC) currently recognized as one of the most significant foodborne pathogens globally [1,2]. This microorganism is the etiological agent of a range of illnesses, from mild gastroenteritis to severe diseases such as hemolytic colitis (HC) and hemolytic uremic syndrome (HUS) [6]. *E. coli* O157:H7 has been responsible for numerous foodborne outbreaks [3], for example a recent multi-state outbreak occurred in the United States affecting 83 people, resulting in 27 hospitalizations, 2 cases of HUS, and 1 fatality [4]. The pathogenicity of *E. coli* O157:H7 is linked to several virulence factors, with the eae gene and Shiga toxins (Stx1 and Stx2) being of particular importance. The eae gene facilitates the attachment of the microorganism to host intestinal cells, while Stx1 and Stx2 disrupt protein synthesis, contributing to the severity of HC and HUS [5, 6]. The presence of these virulence factors is crucial for the development of diseases, with the eae gene playing an essential role in bacterial adhesion to the intestinal lining, while Stx1 and Stx2 are directly involved in the pathogenic process and aggressivity of symptoms [7]. Given its low infectious dose and concentration in foods, the detection of *E. coli* O157:H7 demands selective, sensitive, and accurate methods. Furthermore, in

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food production industries, the ability to obtain rapid results is a critical feature of detection methods, enabling quick decision-making and contributing to food safety. Currently, there are several methods available for detecting *E. coli* O157:H7 in food, but many of these are complex, costly, and time-consuming. The most commonly used protocols involve plate culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assays (ELISA), all of which require an overnight enrichment step before pathogen detection. Traditional plate culture is particularly laborious, taking between 2 to 7 days to complete, because it involves multiple steps such as pre-enrichment, selective enrichment, plating on agar media, biochemical testing, and serological confirmation. While PCR offers faster results, it can produce false positives due to the detection of genes associated with virulence factors that may not be exclusive to *E. coli* O157:H7. Moreover, PCR methods are unable to differentiate between viable and non-viable cells. ELISA assays also typically require additional enrichment or purification steps, further prolonging analysis time. Depending on the method, both PCR and ELISA can take anywhere from 5 to 25 hours to provide results, with certified methods commonly used in large food companies taking approximately 24 hours to generate results [1, 8].

In recent years, the use of bacteriophages for pathogen detection has gained significant attention due to their rapid, sensitive, and cost-effective nature [9 –15]. Bacteriophages, viruses that specifically infect bacteria, offer several advantages when detecting foodborne pathogens. Notably, bacteriophages are safe for humans, as they do not infect human cells. They exhibit high specificity, targeting only certain bacterial genus, species or serotypes, which enhances detection accuracy. Upon infecting their host bacteria, phages replicate rapidly, producing large quantities of new phages, which may result in bacterial cell lyses. Importantly, phages can only replicate in viable bacterial cells, providing a reliable method for identifying live pathogens and reducing the likelihood of false positives [16,17]. The Phage Dx E. coli O157:H7. Assay is an innovative detection method that uses a recombinant bacteriophage expressing luciferase to detect *E. coli* O157:H7. Upon infecting the target bacterium, the phage expresses luciferase, making it possible the emission of light when exposed to a specific substrate. The emitted light can then be detected by a luminometer, providing a rapid indication of the presence or absence of *E. coli* O157:H7 in the food samples [18]. This method has a short enrichment step (5 hours), which aroused the interest of our research group in testing the assay with *E. coli* O157:H7 isolated in Brazil. Although *E. coli* O157:H7 was not previously considered a significant risk in Brazilian food, recent studies have increasingly reported its presence in food samples from the country [19 – 22]. This raises concerns about whether the pathogen has recently entered the country [23] or whether earlier detection methods lacked sufficient sensitivity to identify circulating strains of *E. coli* O157:H7. Epidemiological data show that illnesses caused by *E. coli* O157:H7 have been linked to the consumption of undercooked ground beef, raw vegetables, milk, and contaminated water [6]. Brazilian production of all these foods is very significant and important, especially beef, as Brazil is the world's largest exporter. The objective of this study is to evaluate the performance of PhageDx recombinant bacteriophages for the detection of *E. coli* O157:H7 in vitro and in various food matrices.

Materials and Methods

PhageDx *E. coli* O157:H7 Assay

The PhageDx *E. coli* O157:H7 assay (PhageDx), developed by Laboratory Corporation of America Holdings (Labcorp), was provided as a donation to the Federal University of Rio Grande do Sul (UFRGS) after a scientific agreement was established. The assays were shipped from Saint Paul, Minnesota, USA to Brazil in refrigerated containers (<7°C). The assay utilizes a genetically engineered bacteriophage capable of specifically infecting *E. coli* O157:H7 in food samples. The procedure begins by incubating 25g or 25mL of food samples in 225mL of Tryptic Soy Broth (TSB) medium for 5 hours. Following this incubation, bacteriophages are added to the samples. If *E. coli* O157:H7 is present, the bacteriophage infects the bacterial cells and expresses the luciferase enzyme. After adding the substrate, the enzyme produces a bioluminescent signal, which is detected using a GloMax® Navigator Luminometer (Promega Corp., Madison, WI, USA). The detailed procedure is described below in section 2.3. The assay reads above 150 relative light units (RLU) to indicate the presence of *E. coli* O157:H7 in the sample [24]. Each sample was measured in duplicate with a 3-minute delay and 1-second integration period. The threshold of 150 RLU and the measurement protocol were defined by the assay manufacturer and applied as the standard in this study.

Experimental Design

PhageDx was evaluated in vitro for its ability to detect *E. coli* O157:H7 strains isolated from various sources, including lettuce irrigation water, bovine carcasses, bovine hides, and positive control strains from different laboratories in Brazil. The strains used are detailed in Table 1. Each sample was measured in duplicate, and the average RLU of these readings was recorded as results. This procedure was repeated in two independent tests for each strain, resulting in four values per strain to ensure accuracy and reproducibility. To determine the detection limit of PhageDx, *E. coli* O157:H7 samples were inoculated at three different concentrations: low (1 CFU), medium (10 CFU), and high (100 CFU), separatelly, into 10 wells of a microtiter plate. Negative controls, containing only TSB, phages, and detection reagents, were also tested.

de Aquino NSM, et al., J Biotechnol Biomed 2024
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Each well was measured twice with the luminometer, and the average reading was used as the final value per well. This process was conducted in three independent assays, yielding 30 data points per inoculum level (1, 10 and 100 CFU/well), ensuring a robust assessment of the assay's sensitivity. The sensitivity of the PhageDx assay was further evaluated with artificially contaminated food samples, including ground beef, pasteurized whole milk, organic curly lettuce, and non-carbonated mineral water. For the low (1 CFU) and medium (10 CFU) inocula, 10 wells were tested per microplate, while for the high inoculum (100 CFU), 4 wells were tested. Each well was measured twice with the luminometer, and the average RLU was recorded. Three independent tests were performed for each food matrix, resulting in 30 measurements for the low and medium inocula, and 12 for the high inoculum level, totaling 288 measurements across the food matrices.

PhageDx Ability to Detect *E. coli* O157:H7 Strains

Each *E. coli* O157:H7 strain was cultured overnight in 5 mL of TSB at 37°C. The cell concentration was standardized to 10^8 CFU/mL using a microplate reader (Loccus LMR 96, Brazil). To confirm the bacterial concentration, samples were plated on Tryptic Soy Agar (TSA) and incubated for 24 hours at 37°C. The strains were then added to a 96-well plate, with 100 µL of each sample and 10 µL of phage solution. After 2 hours of incubation at 37°C, 65 µL of a solution containing 50 µL 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. Luminance was measured using a luminometer. Experiments were performed in duplicate.

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).

Strain	Avg. RLU	Origin
L14	6.51 x 10^6	Lettuce irrigation water;
BB	3.37 x 10^6	Organic lettuce irrigation water;
CC	5.77 x 10^6	Organic lettuce irrigation water;
A1P2	7.63 x 10^6	Bovine carcass after hide removal and before evisceration;
A3P1	8.30 x 10^6	Bovine hide after bleeding;
A8P1	1.08 x 10^7	Bovine hide after bleeding;
A10P1	9.24 x 10^6	Bovine hide after bleeding;
A9	8.38 x 10^6	Bovine carcass after hide removal and before evisceration;
A10P2	7.74 x 10^6	Bovine carcass after hide removal and before evisceration;
A39	3.28 x 10^6	Bovine hide after bleeding;
AH3	3.61 x 10^6	Bovine carcass after hide removal and before evisceration;
AMA2	7.62 x 10^6	Positive control from LMCA – ICTA*
FIO	2.31 x 10^6	Positive control from FIOCRUZ**
USP	9.09 x 10^6	Positive control from USP***
E. coli ATCC 25922 (non-O157:H7)	1.07 x 10^2	American Type Culture Collection

* LMCA - ICTA: Microbiology and Food Control Laboratory (ICTA/UFRGS);

**INCQS 00171/FIOCRUZ: Fundação Oswaldo Cruz;

*** USP: Universidade de São Paulo;

PhageDx In Vitro Detection Limit

To assess the detection limit, a cocktail of five *E. coli* O157:H7 strains (USP, CC, A1P2, FIO and A8P1) was prepared. After standardization to 10^8 CFU/mL, the strains were mixed and serially diluted to concentrations of 10, 100, and 1000 CFU/mL. Each dilution (100 µL) was added to the wells of a 96-well plate, resulting in three inoculum levels: low (1 CFU/well), medium (10 CFU/well), and high (100 CFU/well). A total of 30 samples were evaluated at each inoculum level. The bacterial concentration was confirmed using the procedure described in section 2.3.

PhageDx Sensitivity for Detection of *E. coli* O157:H7 in Artificially Contaminated Foods

The bacterial cocktail used for contaminating food samples was prepared as described in section 2.4, with an additional centrifugation step (2253 g for 10 min at 4°C) to pellet the bacteria, followed by three washes with 0.1% (w/v) peptone water. The cocktail was adjusted to concentrations of 1, 10 and 100 CFU/mL using peptone water. Food samples (ground beef, pasteurized whole milk, non-carbonated mineral water, and organic curly lettuce) were purchased from a local supermarket, transported in a refrigerated cooler (<7°C), and confirmed to be negative for *E. coli* O157:H7 by pre-incubation in modified Tryptic Soy Broth (mTSB) and analysis on Sorbitol MacConkey agar. The food samples (25g or 25mL) were placed in sterile Whirl-Pak® filter bags, and 75 mL of mTSB was added. Each sample was inoculated with 1 mL of bacterial cocktail at concentrations of 1, 10 and 100 CFU/mL. After 5 hours of incubation at 41°C, bioluminescence was measured as described in section 2.3.

Comparison of PhageDx and Real-Time PCR

Samples of 100 cattle carcasses (325g each) were collected from Brazilian slaughterhouses and incubated in 975 mL of STEC Enrichment Broth (SEB) at 41.5°C for 10 to 20 hours. After incubation, the samples were analyzed for the presence of Shiga-toxin *E. coli* (STEC) using real-time PCR iQ-Check STEC VirX kit (Bio-Rad). Concurrently, 1 mL of SEB broth was sent to the Microbiology and Food Control Laboratory (LMCA -ICTA/UFRGS) for analysis using the PhageDx assay. Upon arrival, the samples were thawed, homogenized,

de Aquino NSM, et al., J Biotechnol Biomed 2024
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and 150 µL of each sample was added to a 96-well plate. Phage solution (10 µL) was added to each well, followed by a 2-hour incubation at 37°C. The bioluminescence assay was performed as described in section 2.3. All experiments were conducted in duplicate.

Results

All *E. coli* O157:H7 strains tested (Table 1) were detected by the PhageDx. The strains generated RLU values between 10^6 – 10^7. The non-O157:H7 *E. coli* strain (ATCC 25922) used as a negative control had an average RLU of 1.07 X10^2, which was below the lower limit of the method (150 RLU). Table 2 shows the percentage of wells out of a total of 30 wells for each inoculum level (low, medium and high) that were positive after incubation for 2 hours following the addition of bacteriophage solution. As expected, the percentage of detection varied with the inoculum concentration, with higher percentages of positive samples observed as the inoculum level increased. The detection limit of the PhageDx in different food matrices is demonstrated in Table 3. In summary, the assay was able to detect E. coli O157:H7 in all food samples contaminated with 1, 10 and 100 CFU/25g or mL after 5 hours of enrichment. Similar to the in vitro sensitivity test results, higher numbers of RLU were observed, when the number of cells inoculated in the foods increased. The highest RLU values were observed when 100 CFU were inoculated in lettuce (8.20x10^6) and mineral water (1.88x10^6), followed by ground beef (9.15x10^5) and pasteurized milk (2.60x10^5). The lowest RLU values were obtained when 1 CFU/25g was inoculated in pasteurized milk (4.75 x10^3) and ground beef (7.68 x10^3).

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.

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

The negative controls, i.e., samples that were not inoculated, had even lower RLU values. The average RLUs found in these samples were 2.69 x 10^1 (mineral water), 6.00 x 10^1 (lettuce), 8.56 x 10^1 (milk) and 8.68 x 10^1 (ground beef). All 100 bovine carcasses tested negative for the presence of *E. coli* O157:H7 when tested by iQ-Check STEC VirX and PhageDx. None of the methods detected false-positive samples and both methods correctly identified the positive controls.

Discussion

The advancement of rapid methods for the detection of *E. coli* O157:H7 is critical for ensuring food and water safety. Such methods must not only provide rapid results but also be easy to use, require minimal sample and reagent volumes, demand low operator skill, and incur low costs. These attributes are essential for the widespread adoption of detection methods across various settings, particularly in food safety testing [1]. Developing methods that fulfil these criteria requires a careful evaluation process, including specificity and sensitivity analyses against microorganisms found in foods from specific regions. Only after these evaluations

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

Food type	Inoculum (CFU)	Number of replicates	Avg. RLU	Detection
	0	6	8.68 x 10^1	0/6
Ground beef	1	30	7.68 x 10^3	30/30
	10	30	9.15 x 10^4	30/30
	100	12	9.15 x 10^5	12/12
	0	9	8.56 x 10^1	0/9
Pasteurized Milk	1	30	4.75 x 10^3	30/30
	10	30	4.34 x 10^4	30/30
	100	12	2.60 x 10^5	12/12
	0	6	6.00 x 10^1	0/6
Lettuce	1	30	3.41 x 10^4	30/30
	10	30	5.08 x 10^5	30/30
	100	12	8.02 x 10^6	12/12
	0	8	2.69 x 10^1	0/8
Mineral Water	1	30	1.20 x 10^4	30/30
	10	30	8.36 x 10^4	30/30
	100	12	1.88 x 10^6	12/12

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can a method be used for food safety in a given country. The specificity of a test encompasses two main aspects: inclusivity and exclusivity. Inclusivity refers to the ability of a method to detect a broad range of isolates/strains within the target microorganism, while exclusivity ensures that non-target strains are not detected [25]. The PhageDx method also demonstrated robust inclusivity, correctly identifying all 103 strains of *E. coli* O157:H7 tested in the study by Erickson et al. [18]. The method also detected all the Brazilian strains of *E. coli* O157:H7 tested by us, regardless of their origin. Erickson et al. [18] also demonstrated that PhageDx assays have good exclusivity capabilities, which was confirmed by testing 37 non-O157 *E. coli* strains, 12 big-six Shiga toxin-producing E. coli (STEC) strains and 64 non-*E. coli* strains, all of which tested negative. Corroborating these findings, our study demonstrated no false positive result with the inclusion of the non-O157 *E. coli* strain ATCC 25922 used as negative control. Sensitivity is another important characteristic for pathogen detection methods, especially when dealing with low-level contamination, which is typical for *E. coli* O157:H7 present on foods. According to our results, PhageDx was able to detect Brazilian *E. coli* O157:H7 strains without prior enrichment in approximately 80% of wells contaminated with only 10 CFU, and achieving 100% detection at 100 CFU/well. High sensitivity capacity is very important to detection methods because it is assumed that some foodborne pathogens can cause illnesses even with a single viable cell [26]. To ensure sensitivity in real-world food testing scenarios, a 5-hour enrichment step was tested to increase the concentration of target bacterial cells [27]. This enrichment step not only enhances the sensitivity of the assay but also ensures accurate detection, as bacteriophages require live cells for replication.

Enrichment in selective or non-selective media is commonly required to detect low levels of *E. coli* O157:H7 in food. Although this enrichment step is time-consuming, it increases the sensitivity of the assay by promoting the growth of the target cells and, in some cases, inhibiting the growth of competing microbiota. In the present study, TSB was used for enrichment without the addition of antibiotics, a notable advantage in terms of cost and ease of use. Since the PhageDx method has demonstrated specificity for *E. coli* O157:H7, the absence of antibiotics did not compromise the assay's performance, simplifying the protocol and reducing material costs. PhageDx method was certified by Association of Official Analytical Chemists (AOAC), involving five main steps - enrichment, sample concentration, phage infection, substrate addition, and reading in a luminometer. The sample concentration step, although simple, was omitted on purpose in our study, and the enrichment time was standardized to 5 hours, as recommended by the method developer. Even without sample concentration the method remained highly sensitive with short time to results, a significant advantage over traditional methods, which often require 2-5 days for pathogen detection. The PhageDx method demonstrated adequate performance in detecting *E. coli* O157:H7 at very low concentrations, with reliable results in different food matrices, including ground beef, milk, lettuce, and mineral water. Even with food samples containing as low as 1 CFU per 25 g or 25 mL, the assay detected *E. coli* O157:H7 with consistent results across different food types, underscoring the robustness and sensitivity of the method. Additionally, the bioluminescent signal generated by the NanoLuc® enzyme allowed for easy interpretation of results, providing high light emission for positive samples and low background signals for negative controls, ensuring accurate detection and reducing the likelihood of false positives [28]. When compared to previous studies using bacteriophage-based detection systems, such as those carried out by Ripp et al. [29] and Zhang et al. [30], the PhageDx method demonstrated better performance. While other studies reported longer detection times and occasional false positives, the PhageDx method achieved faster results and demonstrated greater sensitivity, detecting as little as 1 CFU of *E. coli* O157:H7 in 25 g of ground beef within only 7.5 hours. This is a significant improvement, particularly in comparison to the 9-hour detection time reported by Zhang et al. [30] or other rapid detection methods used in big food companies which require approximately 18 to 24 hours to result.

Brazil, as the largest exporter of beef, faces considerable pressure to ensure the safety of its meat products, especially in light of mandatory *Shiga*-toxin-producing *E. coli* (STEC) testing for beef intended for export [31, 32]. According to the United States Department of Agriculture (USDA), Brazil's beef production was estimated at 9.75 million tons in 2022, reflecting a growth of approximately 4.6% comparing to the previous six-month period [2]. In terms of domestic consumption, beef consumption in Brazil was estimated to be 7.14 million tons at the beginning of 2022, while the Brazilian estimative for beef exports and imports for 2023 was approximately 2.68 million tons and 70,000 tons, respectively. Although the prevalence of STEC in Brazil remains low, there is a clear need for rapid and reliable testing methods for monitoring pathogens like E. coli O157:H7 and other STECs. The PhageDx assay performed well when tested with Brazilian ground beef samples. These results indicate that PhageDx could be useful in ongoing surveillance efforts.

While the current study did not find *E. coli* O157:H7 in any of the 100 beef carcass samples tested by the iQ Check and PhageDx assay, the similarity of the results of both methods can be considered an adequate result because no false positive was found and there were no divergent results between methods. Actually, these results highlight the need for efficient and accurate testing methods reinforcing that in Brazil, the prevalence of STEC has been reported to be low, but cases of contamination do occur [20, 21]. For instance, studies have detected *E. coli* O157:H7 in various Brazilian

foods, including ground beef, milk, and vegetables [22, 33, 34]. The present study demonstrated that PhageDx is capable of detecting *E. coli* O157:H7 in various food products, indicating its utility as a rapid diagnostic tool for ensuring food safety. The PhageDx assay demonstrated to be able to detect low levels of *E. coli* O157:H7 in food and water samples in a short timeframe, which can be useful for both food safety testing and outbreak investigations. This last question is particularly important because there are already reports of foodborne outbreaks caused by *E. coli* O157 in Brazil. For example, Santos et al. [35] published the first report of human gastroenteritis caused by *E. coli* O157:NM in Brazil. In this outbreak, a 13-year-old adolescent developed bloody diarrhea and severe abdominal pain and *E. coli* O157:NM was isolated from his feces and from a tomato and cheese salad prepared in the canteen of the school where the adolescent attended. More recently, *E. coli* O157:NM was identified as the cause of death of two children in southern Brazil, but the microorganism was not found in any food analyzed [36]. These outbreaks emphasize the need to improve food safety and rapid testing methods for foodborne pathogens are essential to this goal.

Conclusion

The recombinant bacteriophages used in the PhageDx assay demonstrated a capability to detect low concentrations of viable *E. coli* O157:H7 in various food matrices. The assay successfully identified 1 cfu/25g or mL of food within 7.5 hours, highlighting its potential as a rapid pathogen detection method.

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