Optimization of Phage-Based Pathogen Detection During Sample Shipment

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Optimization of Phage-Based Pathogen Detection During Sample Shipment

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Abstract. Foodborne illnesses are diseases caused by pathogen microorganisms present in the food matrix. Various organizations regulate the laws and guidelines that must be followed to avoid contamination of food with pathogen microorganisms. The presence of pathogen microorganisms is sufficient cause to deny the entry of food shipments. That is the reason that rapid detection of pathogen microorganisms is of vital importance. In this study, a bioluminescence detection method for *Escherichia coli* O157:H7 was evaluated. For this the ΦV10 *Lux* + *Kanamycin resistance* was used. A Randomized Complete Block Design (RCB) was used to measure the effect of seven concentrations of *E. coli* O157:H7, seven concentrations of ΦV10 *Lux* + *Kanamycin resistance*, two incubation temperatures, and five enrichment times before the addition of kanamycin. The data were analyzed using a Chi-square test. The sensitivity, specificity, accuracy, positive and negative predictive values of the method were also calculated, obtaining 95, 100, 96, 100 and 36%, respectively. There was no difference in the bioluminescence detection when using bacteria concentrations from 5 Log CFU/mL to 7 Log CFU/mL; phage concentrations from 7 Log PFU/mL to 8 Log PFU/mL (P = 0.9432); 1, 2, 3, 4 or 5 hours enrichment time before adding kanamycin (P = 0.9889) or incubation temperatures of 21 or 37 ˚C (P = 0.9364).

Keywords: Bacteriophage, bioluminescence, detection, *E. coli* O157:H7.

Resumen. Las enfermedades transmitidas por los alimentos son causadas por microorganismos patógenos presentes en la matriz alimentaria. Existen varias organizaciones que regulan las leyes y pautas que deben seguirse para evitar la contaminación de los alimentos con microorganismos patógenos. La presencia de microorganismos patógenos es suficiente para negar la entrada de envíos de alimentos. Esa es la razón por la cual la detección rápida de microorganismos patógenos es de vital importancia. En este estudio, se evaluó un método de detección de bioluminiscencia para *Escherichia coli* O157:H7, para lo cual, se utilizó el ΦV10 Lux + resistencia a la kanamicina. Se usó un diseño de Bloques Completos al Azar (BCA) para medir el efecto de siete concentraciones de *E. coli* O157:H7, siete concentraciones de ΦV10 Lux + resistencia a la kanamicina, dos temperaturas de incubación, y cinco tiempos de incubación antes de la adición de kanamicina. Los datos se analizaron mediante una prueba de Chi-cuadrado. También se calcularon, la sensibilidad, especificidad, precisión, valor predictivo positivo y valor predictivo negativo del método, obteniendo 95, 100, 96, 100 y 36 respectivamente. No hubo diferencia en la detección de bioluminiscencia cuando se usan concentraciones de bacterias de 5 Log UFC/mL a 7 Log UFC/mL; concentraciones de fagos de 7 Log UFP/mL a 8 Log UFP/mL (P = 0.9432); 1, 2, 3, 4 o 5 horas como tiempo de incubación antes de agregar kanamicina (P = 0.9889) o temperaturas de incubación de 21 o 37 ˚C (P = 0.9364).

Palabras clave: Bacteriófago, bioluminiscencia, detección, *E. coli* O157:H7.

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1. INTRODUCTION

Annually, almost 9 million cases of foodborne illnesses are reported in the United States, 250,000 of those are cases caused by Shiga Toxin producing *Escherichia coli* infections (Scallan *et al.* 2011). Seventy-three thousand of these are *E. coli* O157:H7, which causes about 2100 hospitalizations and 60 deaths. Studies have shown that the main reservoir of *E. coli* O157:H7 is cattle, so it is generally associated with meat (Rangel *et al.* 2005); but it is also problematic in fruits, vegetables, and water. In general, *E. coli* lives in the intestine of mammals, and its presence in food indicates fecal contamination. However, the consumption of the O157:H7 strain can result in illness with symptoms including stomach pain, diarrhea (hemorrhage), and vomiting (Croxen & Finlay 2010, CDC 2014). The infection that it produces is usually of short duration, and the treatment is to stay hydrated; however, it can become lethal in young children. *E. coli* O157:H7 infections have been associated with hemolytic uremic syndrome, kidney failure, hemolytic anemia, and in some cases death (Mele *et al.* 2014).

In 1994, the U.S. Department of Agriculture Food Safety and Inspection Service implemented the zero-tolerance law for meat because a low infective dose of bacteria can make a person sick, approximately 10-100 cells (USDA-FSIS 2011). *E. coli* O157:H7 is a high risk to public health (Cloke *et al*. 2015; Feng *et al.* 2017). and detection of one cell is vital.

Traditionally *E. coli* O157:H7 detection methods involve preparing culture media, enrichment, incubation, colony isolation, identification, biochemical tests, and serological tests (Bastidas 2018). Although there are reliable methods and referred to as the gold standard, they are time-consuming and often laborious (Poltronieri *et al.* 2016). Sampling, sample storage, and sample transportation are critical since the accuracy of microbiological analysis results depend on the sample management (Sánchez-Romeroa *et al.* 2017). Foodborne pathogen detection requires rapid and robust detection methods, as they can detect the presence of a pathogen in less time than traditional methods, because of the need for safe food for human consumption (Law *et al.* 2015).

Due to its perishable nature, a shipment of food cannot wait long for the results of microbiological analysis; in response to the need, rapid methods including both DNA and antibody-based assays have been created (Maurer *et al.* 1999). Immunoassays are detection methods for pathogens that are faster, more sensitive, and more convenient than traditional culture-based assays, (FDA 2001; Feng *et al.* 2017). Antibody assays rely on the high binding specificity of the antibody to the antigen to give accurate results.

There are alternative methods of pathogen detection, such as bacteriophage detection. Bacteriophages are organisms that need to reproduce within a host, in this case, bacteria, to be considered as living organisms. They are highly selective, so they can only infect a particular host organism and even show specificity at the strain level. Usually, the phages have two life cycles: lytic and lysogenic. The lytic cycle is the most common and begins bacterial cytoplasm where it replicates and creates virus proteins. These proteins are packed inside the head capsid, and then the lysis of the bacteria occurs. The lysogenic cycle differs with the lytic in that it does not lyse the cell but inserts its genetic material into the bacteria's genome. Therefore, every time the bacteria

divides, the DNA of the virus replicates and passes to the daughter cells. This can be maintained until the environmental conditions change, and the phage enters again into the lytic cycle.

The high specificity of the phages makes them ideal for the detection of pathogens in particular. Phages in which the lysogenic cycle predominates are used with reporter genes. Frequently, a reporter gene is linked to another gene of interest. In this way, it is easy to with the bacteria's infection by the phage; the phage injects its genetic material into the identify gene's presence from a simple change in cell physiology.

Bioluminescence occurs when luciferase enzymes oxidize luciferin to produce light (Hall *et al.* 2012). Bioluminescence is usually a better detection method because the light is directly related to the number of viable cells, which makes a quick method, and a more reliable method than PCR.

The genes *lux* and *LUC* have been commonly used as a bioluminescence reporter bacteriophage-based detection system (Hagens & Loessner 2007). There are previous studies with other pathogens that obtained good results. A *Salmonella* detection assay was carried out with P22 *lux,* it detected 10 CFU of *Salmonella* with 6 hours of pre-incubation (Chen & Griffiths 1996). In another phage-based application, A511 (*Listeria*-specific phage) was modified with a *Vibrio harveyi lux*AB protein to create A511:*luxAB*. A single-tube luminometer detected 10^2 cells mL^{-1} after 2 hours of incubation. With the inclusion of an enrichment step, the phage detected one *Listeria monocytogenes* cell per gram of artificially contaminated salad (Loessner *et al.* 1996).

There are numerous other based bacteriophage reporter applications. Recently a NanoLuc based reporter bacteriophage for the detection of *E. coli* O157:H7 has been constructed from ΦV10 (Zhang *et al.* 2016). They reported the detection of 5 cells in 40 mL of ground meat in nine hours. Studies with bacteriophages and reporter genes are still under investigation (Baker *et al.* 2015; Zhao *et al.* 2016). In this study, phage ΦV10 *Lux+Kanamycin resistance* will be used. It is also important to note that phage using reporter genes only detect viable cells.

The objectives of this study were:

- Determine the effect of different factors affecting E. *coli* O157:H7's growth or the bioluminescence response.

- Determine the limits of detection.

2. MATERIALS AND METHODS

Location
All of the experiments were carried out at the Biosensor Development Laboratory in the Philip E. Nelson Hall of Food Science building at Purdue University in West Lafayette, Indiana, United States of America.

***Escherichia coli* O157:H7 C7279 Test Culture Preparation**
For this study *Escherichia coli* O157:H7 C7279 was used, this strain was obtained from an apple cider outbreak. To prepare the test culture, a single colony was selected from a previously plated petri dish and inoculated into a flask with Luria broth (LB) with the help of a sterile inoculating loop. The culture was labeled and incubated at 37 ˚C on a shaker overnight.

The next morning, several Eppendorf tubes were prepared with a mix of 500 µL of the culture and 500 µL of glycerol for long term storage. Glycerol maintains the viability of *Escherichia coli* O157:H7 cultures during long time storage at low temperatures. The tubes were put in a rack and kept at -80 ˚C in a freezer.

Starting an *Escherichia coli* O157:H7 C7279 culture
An Eppendorf tube was used to inoculate 100 mL of LB, the culture had to be thawed and vortexed before addition. The flask was labeled and incubated at 37 ˚C on a shaker overnight. For all of the experiments which involve an *Escherichia coli* O157:H7 C7279 culture, the culture was started the day before.

ΦV10 *Lux+Kan resistance* Stock Preparation
For this study, bacteriophage ΦV10 *Lux+Kan resistance* was used. Phage ΦV10 is an *E. coli* O157:H7 specific bacteriophage. The phage was previously modified, two sets of genes were introduced into its genome: *luxCDABE*, bioluminescence reporter genes, and a gene for *Kan resistance*. This specific strain of the phage has an approximate 50% chance of starting the lytic cycle and a 50% chance of starting the lysogenic cycle.

To prepare the phage stock, a fresh culture of *E. coli* O157:H7 C7927 (24 h incubation) was inoculated with 50 µL of the phage suspension, the flask was mixed to increase the infection rate. After an hour, 100 µL of Kanamycin solution (50 µg/mL) was added and then the flask was incubated at 37 ˚C overnight on a shaker.

The next day, the culture was divided into two 50-mL-centrifuge tubes. The tubes were balanced (must weigh the same) before centrifuging at 12086.7 × *g* for 10 minutes to precipitate bacteria and cell debris into a pellet. The supernatant, containing new phageprogeny, were vacuum filtered through a 0.22 µm pore size membrane to a sterile 50 mL tube (Zhang *et al.* 2016). The stock was labeled and kept at 4 ˚C in a refrigerator.

Determination of the number of live *Escherichia coli* O157:H7 C7279 cells

Serial 1:9 dilutions were made; PBS was used to dilute the culture (1 mL of culture or previous dilution and 9 mL of PBS). Micropipettes and disposable tips were used to make the dilutions; tubes were also vortexed before continuing with the dilutions. Dilutions 10^{-5}, 10^{-6} and 10^{-7} were plated on LB agar and incubated at 37 ˚C for 24 hours. The plates were counted the next day. The number was obtained through calculations and it was reported as CFU/mL and Log CFU/mL. The plate count report was used to know the number of live cells before the enrichment.

Determination of the number of viable ΦV10 *Lux+Kan resistance*

Plaque assays were used to quantify the phage concentration. Serial 1:9 dilutions were made, sterile deionized water (SDW) was used to dilute the phage culture (1 mL of culture or previous dilution and 9 mL of SDW). Micropipettes and disposable tips were used to make the dilutions, also, tubes were vortexed before continuing with the dilutions.

Aliquots of 100 µL of phage dilutions 10^{-5}, 10^{-6} and 10^{-7} and 200 µL of *E. coli* O157:H7 C7279 culture were poured into 4 mL of fresh steamed (30 min) semisolid LB Agar and vortexed. The mixture was poured as a second layer over Petri dishes containing solid LB agar and incubated at 37 ˚C overnight. The plaques of each plate were counted the next day. The number was obtained through calculations and it was reported as PFU/mL and Log PFU/mL.

Bioluminescence based detection assay

For this experiment, all the dilutions were made the same day that they were analyzed, nothing was saved to be used in other plates. All plates of the same treatment were made and analyzed after preparation (the same day).

The bioluminescence-based detection assay consisted of a 2-dimensional matrix built on a 96-well clear-bottom black microtiter plate (Figure 1). The **X** axis (numbers) had serial dilutions of *Escherichia coli* O157:H7 C7279 (10^{-1} – 10^{-7}), the **Y** axis (letters) had serial dilutions o ΦV10 *Lux+Kan resistance* (10^{-1} – 10^{-7}) (Figure 1). An aliquot of 100 µL of both cultures was poured into the microtiter plate with the help of a multi-channel pipette. The microtiter plates were incubated for a specific time (1, 2, 3, 4, and 5 hours) at 21 and 37 ˚C. Then, 50 µL of diluted Kanamycin (50 µg/mL) were added, so the final volume in each well was 250 µL and the final concentration of the antibiotic was 10 µg/mL (Kima *et al.* 2009). Finally, the plates were incubated for 17 hours at the same temperatures.

Seven *Escherichia coli* O157:H7 C7279 concentrations, seven ΦV10 *Lux+Kan resistance* Two incubation temperatures (21 and 37 ˚C) and 5 different times between plating and adding the antibiotic (1,2, 3, 4 and 5 h) were tested. The next morning, luminescence intensity and absorbance of each well were read with a Perkin Elmer VICTOR NivoMultilabel counter and computer software.

The data were exported to Microsoft Excel. Any luminescence reading higher than 10,000 RLU (Relative Light Units) was considered positive and any absorbance reading higher than 0.1 was considered positive. All of the positive readings were reported with 1 and negative readings were

reported with a 0. A positive result consisted of a positive luminescence reading and a positive absorbance reading, the rest was taken as negative results.

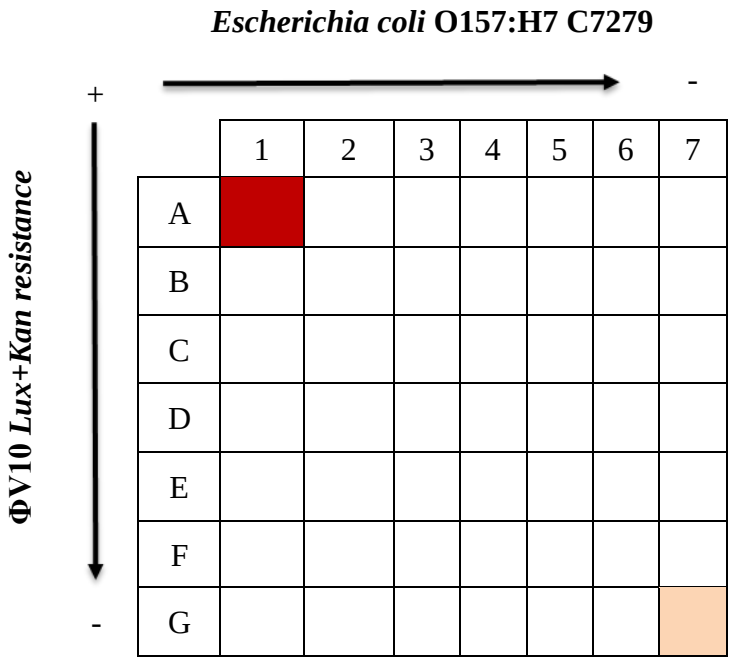

Figure 1. Microtiter plate bidimensional matrix showing the well with the highest concentration of cells and phage (red) and the least concentration of cells and phage (pink).

Calculation of parameters of sensibility, specificity, accuracy, positive predictive value, and negative predictive value

Method parameters were calculated according to the Method Validation Policy of the Honduran accreditation agency. Formulas 1, 2, 3, 4 and 5 were used:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}} \quad [1]$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{False positives} + \text{True negatives}} \quad [2]$$

$$\text{Accuracy} = \frac{\text{True positives} + \text{True negatives}}{n} \quad [3]$$

$$\text{Positive predictive value} = \frac{\text{True positives}}{\text{True positives} + \text{False positives}} \quad [4]$$

$$\text{Negative predictive value} = \frac{\text{False negatives}}{\text{False negatives} + \text{True negatives}} \quad [5]$$

Experimental design and statistical analysis

Seven concentrations of *E. coli* O157:H7, seven concentrations of ΦV10 *Lux* + *Kanamycin resistance*, two incubation temperatures, and five enrichment times before the addition of kanamycin were evaluated, resulting in 490 treatments. Each treatment had three experimental units, resulting in 1470 experimental units in total.

A Randomize Complete Block Design was used to compare the interaction between the factors such as Infection × Growth, *E. coli* O157:H7 concentration × Phage concentration, *E. coli* O157:H7 concentration × Enrichment time and *E. coli* O157:H7 concentration × Incubation temperature. The Statistical Analysis System® (SAS) version 9.4 program was used. A Chi-square test was performed. A degree of significance of 95% was used ($P < 0.05$). Parameters of sensibility, specificity, and accuracy were compared to other detection methods parameters.

3. RESULTS AND DISCUSSION

LB agar plate counts showed that the test culture had an average of 8 Log CFU/mL of *E. coli* O157:H7 C7279 approximately (Table 1) before enrichment. Plaque assay counts showed that there was an approximate average of 9 Log PFU/mL lysogens of *ΦV10 Lux+Kan resistance* (Table 2).

Table 1. *Escherichia coli* O157:H7 C7279 concentrations.

***Escherichia coli* O157:H7 C7279**					
Sample	**Dilutions**			**CFU/mL**	**Log CFU/mL**
	-6	**-7**	**-8**		
1	134	22	3	1.3×10^8	8.113943
2	96	15	1	9.6×10^7	7.982271
3	113	20	1	1.1×10^8	8.041393
4	92	19	2	9.2×10^7	7.963788
5	85	11	<1	8.5×10^7	7.929419
Average					8.006163

CFU = Colony Forming Unit, Log = Logarithms, mL = Milliliters.

Table 2. ΦV10 Lux+Kan resistance count report (number of lysogens)

ΦV10 *Lux+Kan resistance*					
Sample	**Dilutions**			**PFU/mL**	**Log PFU/mL**
	-6	**-7**	**-8**		
1	TMTC	118	10	1.2×10^9	9.079181
2	355	41	3	4.1×10^8	8.612784
3	TMTC	88	12	8.8×10^8	8.944483
4	TMTC	107	14	1.1×10^9	9.041393
5	TMTC	87	9	8.7×10^8	8.939519
Average					8.923472

PFU = Plaque Forming Unit. Log = Logarithms, mL = Milliliters, TMTC = Too many to count (>325 PFU per plate).

Data in Table 3 summarizes luminescence positive samples according to phage concentration. Data shows that the detection limit is met when *E. coli* O157:H7 C7279 concentration is $\geq 10^5$ CFU/mL and phage concentration is $\geq 10^7$ PFU/mL under *in vitro* conditions. According to the Honduran Accreditation Body, the detection limit is a "value, obtained by a given measurement procedure, with a probability β of erroneously declaring the constituent's absence in a material, given a probability α of erroneously declaring its presence". Nothing was detected below 10^3 CFU/mL of cells and 10^5 PFU/mL of phage.

The results contradict the study carried out by Zhang *et al.* in 2016. That study could detect 5.4 cells with 9.23×10^3 PFU/mL of phage. It should be noted that even though in both studies ΦV10 is used, the bioluminescence reporter genes are different, and the equipment used for the bioluminescence reading was different as well. Several studies agree that the Nanoluc gene produces luciferase with a brighter light signal than that produced by the *luxCDABE* gene (Loh & Proft, 2013; Sun *et al*. 2016).

The Log *E. coli* × Log phage interaction results show that these variables are not associated because there is not a significant difference (P = 0.9432). It means that above the detection limit (*E. coli* O157:H7 C7279 $\geq 10^5$ CFU/mL and phage $\geq 10^7$ PFU/mL), any combination of concentrations has, statistically, the same probability of being detected. The only downside is that no sample of food will ever have such a microbiological load. The detection method still needs more study and development, in order to detect lower concentrations of *E. coli* O157:H7. A longer incubation along with a longer enrichment time under optimum conditions is recommended for better results with low microbiological loads.

Table 3. Percentage of positive samples according to phage concentration.

***Escherichia coli* O157:H7 conc. Log CFU/mL**	**Phage conc. Log PFU/mL (n = 3 × 5 × 2 = 30)**							
	8	**7**	**6**	**5**	**4**	**3**	**2**	**Total**
7	100%	80%	17%	3%	0%	0%	0%	29%
6	100%	90%	27%	3%	0%	0%	0%	31%
5	87%	70%	7%	0%	0%	0%	0%	23%
4	30%	33%	0%	0%	0%	0%	0%	9%
3	7%	17%	0%	0%	0%	0%	0%	3%
2	0%	0%	0%	3%	0%	0%	0%	0%
1	0%	0%	0%	0%	0%	0%	0%	0%
Total	46%	41%	7%	1%	0%	0%	0%	14%

CFU = Colony Forming Unit, PFU = Plaque Forming Unit, Log = Logarithms, Conc. = Concentration, mL = Milliliters

Table 4 summarizes the results according to incubation temperature for treatments with more than 50% luminescence positive samples. Data shows that phage could detect *Escherichia coli* O157:H7 equally at both temperatures (P = 0.9364). According to the BAM, *Escherichia coli* is a mesophilic microorganism that lives in the intestines of warm-blooded animals, including mammals and humans (37 ˚C) (Feng *et al.* 2018). The explanation for *E. coli* O157:H7 similar detection at both temperatures could be its growth temperature range; according to the World Health Organization (WHO, 2018), *E. coli* O157:H7 can grow at temperatures from 7 to 50 ˚C, with an optimum temperature of 37 ˚C and can be destroyed at 70 ˚C. On the other hand, luminescent reaction is thermosensitive (Rienzo 2015; Oliveira & Viviani 2019), which means that high temperatures can affect the protein's ability to catalyze the reactions. Promega (2015) and Winson *et al.* (1998), agree that luciferin optimal temperature range is from 20 to 25 ˚C and can work up to a temperature of 45 ˚C.

The results for Log *E. coli* × Incubation temperature interaction show that these variables are not associated because there is not a significant difference (P = 0.9364). It means that above the detection limit (*E. coli* O157:H7 C7279 $\geq 10^5$ CFU/mL and phage $\geq 10^7$ PFU/mL), the detection method can detect *E. coli* O157:H7 at 21 or 37 ˚C obtaining similar results. It is still unknown if the method works at different temperatures. Knowing the growth range of *E. coli* O157:H7 and the working range of luciferin, it is expected to have better results between the optimum temperature of both (between 21 and 37 ˚C), so the detection method still needs more study.

Table 4. Percentage of positive samples according to incubation temperature.

***Escherichia coli* O157:H7 conc. Log CFU/mL**	**Incubation temperature (˚C) (n = 30)**		
	37	**21**	**Total**
7	93%	87%	90%
6	100%	90%	95%
5	87%	70%	78%
Total	93%	82%	88%

CFU = Colony Forming Unit, Log = Logarithms, Conc. = Concentration, mL = Milliliters, ˚C = Degrees Celsius

Table 5 summarizes the results according to enrichment time between plating and adding the antibiotic for treatments with more than 50% luminescence positive samples. Data shows that phage could detect *E. coli* O157:H7 equally at any enrichment time (P = 0.9889).

According to the BAM, *Escherichia coli* O157:H7 cultures incubate at 35 $\pm$ 0.5 ˚C for 24 hours, so it was expected that longer times would have better results than shorter times. The reason for these results may be that *E. coli* O157:H7 generation time is 20 minutes (Gibson *et al.* 2018), so even in the shortest of the times between plating and adding the antibiotic, the bacteria could duplicate three times and make sure that, with such high microbial load, the culture survived the kanamycin and could be detected.

The results for Log *E. coli* × Enrichment time interaction show that these variables are not associated because there is not a significant difference (P = 0.9889). This means that there is no statistical difference above the detection limit (*E. coli* O157:H7 C7279 $\geq 10^5$ CFU/mL and phage $\geq 10^7$ PFU/mL), in when to add the kanamycin to the samples. It will help to reduce the enrichment time to the minimum.

Table 5. Percentage of positive samples related to enrichment time.

***Escherichia coli* O157:H7 conc. Log CFU/mL**	**Enrichment time (h) between plating and adding the antibiotic (n = 12)**					
	5	**4**	**3**	**2**	**1**	**Total**
7	92%	67%	92%	100%	100%	90%
6	100%	75%	100%	100%	100%	95%
5	100%	33%	75%	92%	92%	78%
Total	97%	58%	89%	97%	97%	88%

CFU = Colony Forming Unit, Log = Logarithms, Conc. = Concentration, mL = Milliliters, h = Hours

Table 6 presents the total of True Positives (TP), False positives (FP), False Negatives (FN), and True Negatives. True Positives were considered all results consisting of a positive luminescence reading and a positive absorbance reading, resulting in 158 true positive readings. True Negatives were considered all results that included a negative luminescence reading and a negative absorbance reading, resulting in 14 true negative readings. False Negatives were considered all results consisting of a negative luminescence reading and a positive absorbance reading, resulting in eight false positive readings. There are no False Positives due to the absence of results consisting of positive luminescence and a negative absorbance. Only treatments with more than 50% (detection limit) of positive luminescence readings were used for this table.

The statistical results for infection × growth interaction show that these variables are associated with a significant difference ($P < 0.0001$). This means that the infection rate depends on *E. coli* O157:H7 growth; in other words, if there is no *E. coli* O157:H7 viable cells, the bioluminescence detection method will not work. These results agree with the parameter of specificity obtained (100%), this occurs because of the use of the specific bacteriophage ΦV10 (Perry *et al*. 2009).

Table 6. Contingency table for calculating false positive and false negative results.

Pathogen infection	**Pathogen growth**	**Results**	**Values**
Yes	Yes	True positive	158
Yes	No	False positive	0
No	Yes	False negative	8
No	No	True negative	14
Total			180

Parameters obtained by the bioluminescence method.

Equation 1 is the calculation of the method's sensitivity. According to Ochoa & Orejas (1999), the sensitivity of a method detection is the probability of obtaining a positive result (bioluminescence) if the condition of interest (presence of *E. coli* O157:H7) is present. The higher the sensitivity is the method will get less false positive results.

$$\text{Sensitivity} = \frac{158}{158 + 8} = 0.951807229 \times 100 = 95\% \quad [1]$$

Equation 2 is the calculation of the method's specificity. The specificity of a method of detection is the probability of obtaining a negative result (no bioluminescence) if the condition of interest (presence of *E. coli* O157:H7) is not present. The higher the specificity is; the positive results are more reliable (Bravo-Grau & Cruz 2015).

$$\text{Specificity} = \frac{14}{0 + 14} = 1 \times 100 = 100\% \quad [2]$$

Equation 3 is the calculation of the method's accuracy. A method detection's accuracy is the degree of closeness of a measured value to the true value (Hospodsky *et al.* 2010). Accuracy is also defined as the probability of the detection method to predict the presence or absence of the condition of interest correctly.

$$\text{Accuracy} = \frac{158 + 14}{180} = 0.955555556 \times 100 = 96\% \quad [3]$$

Equation 4 is the calculation of the method's positive predictive value. According to Segura-Egea (2002), the positive predictive value of a method of detection is the probability of presenting the condition of interest (presence of *E. coli* O157:H7) because of getting a positive result in the detection test. The higher the sensitivity and the PPV are, the results of the detection test are more trustworthy.

$$\text{Positive predictive value} = \frac{158}{158 + 0} = 1 \times 100 = 100\% \quad [4]$$

Equation 5 is the calculation of the method's negative predictive value. The negative predictive value of a method of detection is the probability of not presenting the condition of interest (presence of *E. coli* O157:H7) because of getting a negative result in the detection test.

$$\text{Negative predictive value} = \frac{8}{8 + 14} = 0.363636364 \times 100 = 36\% \quad [5]$$

Table 7 shows a comparison between the Bioluminescence method and other rapid methods. According to Vizcaino-Salazar (2017), a parameter with a percentage $\leq$ 50% is considered as unacceptable, a parameter with a percentage between 50 and 79% is considered as regular, a parameter with a percentage between 80 and 94% is considered as good and a parameter with a percentage $\geq$ 95 is considered as excellent. Four out of five of the parameters obtained with the bioluminescence method qualify to be in the category of excellent, only the parameter of NPV got qualified as bad.

Table 7. Comparison between Bioluminescence method and other methods.

Parameter	Bioluminescence method	PCR method[1]	VIP GOLD-EHEC Test[2]	White blood cell method[3]	Cefotaxime susceptibility method[4]
Sensitivity	95%	83%	>98%	89%	98%
Specificity	100%	98%	>99%	57%	97%
PPV	100%	99%	-	45%	78%
NPV	36%	43%	-	93%	100%
Accuracy	96%	86%	-	-	-

PPV = Positive predictive value
PPN = Negative predictive value
Source : ([2]Fahey *et al*. 2006 ; [1]Law *et al*. 2015 ; [4]Jiménez-Guerra *et al*. 2016 and [3]La Torre 2016)

Study limitations

The study had limitations because it was carried out under laboratory conditions. According to Hartung & Daston (2009), *in vitro* approaches gained importance and contributed mainly to studies, but its results cannot be attributed to shipping conditions (fluctuating temperature).

That is the reason to conduct further studies *in vivo*. Ghallab & Bolt (2014) mentioned that *in vivo* simulations are rare, but their results are more relevant and closer to reality. According to Geo *et al*. (2018), *in vivo* conditions – food matrices are complex of physical and chemical interaction of nutrients and non-nutrients. Some conditions like low pH, high temperatures and oxygen absence could make the detection impossible with this method.

Other *in vivo* conditions that could limit the detection is the presence of accompanying bacterial flora, and fluctuating temperature. In a study, Llanos *et al*. (2002) mentioned that some bacteria with aggressive growth behavior can compete and repress the growth of others. The fluctuating temperature could affect *E. coli* O157:H7 growth because when the temperature is not optimum, generation time gets longer, and as a result, there are fewer cells. If there are not enough cells, the phage cannot infect as many, therefore the bioluminescence light intensity would be lower, so this method could not detect lower concentrations of *E. coli* O157:H7.

4. CONCLUSIONS

- Under optimum pH and Aw, there was not difference in detection when using bacteria concentrations 5 to 7 Log CFU/mL; phage concentrations 7 to 8 Log PFU/mL; 1, 2, 3, 4 or 5 hours as enrichment time before adding kanamycin or incubation temperatures of 21 or 37 ˚C.

- The detection limit is met when *E. coli* O157:H7 C7279 concentration is $\geq 10^5$ CFU/mL which is higher than other detection methods, but longer enrichment time can lower the detection limit.

5. RECOMMENDATIONS

- Evaluate the effect reading the 96-well plates with a more sensitive equipment to bioluminescence.
- Evaluate the effect of different media pH in the bioluminescence result.
- Run the detection method with a food matrix to compare the bioluminescence results.
- Run the detection method including *E. coli* O157:H7 and other food safety related pathogenic microorganisms.
- Carry out the experiments with fluctuating temperatures – simulating shipping conditions.

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7. APPENDICES

Appendix 1. Media and Reagents preparation

Luria broth (LB). Luria broth (LB) was prepared by adding 10 g of Tryptone Powder, 10 g of Sodium Chloride (NaCl), and 5 g of Yeast Extract to 1 L of Deionized Water. Tryptone Powder, Sodium Chloride, and Yeast Extract were weighted with a plastic cup and a laboratory analytical balance; Deionized Water was measured with a graduated cylinder (test tube).

All was mixed in a 2-L-flask with a stir bar. The media was pH adjusted to 7.5 ± 0.2 using a pH probe and a 10 M solution of Sodium Hydroxide (NaOH). Then, 100 mL of LB were poured into 250-mL-flasks making it 10 flasks per liter. Each flask was labeled and covered with aluminum foil, autoclave tape was put on the foil to guarantee sterility. After that, the media was autoclaved at liquid cycle 40 and was put in a cabinet until use.

Luria Agar (LB Agar). Luria Agar (LB Agar) was prepared by adding 10 g of Tryptone Powder, 10 g of Sodium Chloride (NaCl), 5 g of Yeast Extract, and 17 g of Agar Powder to 1 L of Deionized Water. Tryptone Powder, Sodium Chloride, Yeast Extract, and Agar Powder were weighted with a plastic cup and a laboratory analytical balance; Deionized Water was measured with a graduated cylinder (test tube).

All was mixed in a 2-L-flask with a stir bar. The media was pH adjusted to 7.5 ± 0.2 using a pH probe and a 10 M solution of Sodium Hydroxide (NaOH). The flasks were labeled and covered with aluminum foil, autoclave tape was put on the foil to guarantee sterility. After that, the media was autoclaved at liquid cycle 40. LB Agar was cooled down to 45 ˚C so it was cool enough to touch the flasks but hot enough so the media was liquid.

Finally, LB Agar was poured into sterile empty Petri dishes, 20 mL approximately. LB Agar was let to solidify before bagging the dishes and putting them in a refrigerator at 4 ˚C until use.

Top Agar. Luria Agar (LB Agar) was prepared by adding 10 g of Tryptone Powder, 10 g of Sodium Chloride (NaCl), 5 g of Yeast Extract, and 6 g of Agar Powder to 1 L of Deionized Water. Tryptone Powder, Sodium Chloride, Yeast Extract, and Agar Powder were weighted with a plastic cup and a laboratory analytical balance; Deionized Water was measured with a graduated cylinder (test tube). All was mixed in a 2-L-flask with a stir bar. The media was pH adjusted to 7.5 ± 0.2 using a pH probe and a 10 M solution of Sodium Hydroxide (NaOH).

The flasks were labeled, covered with aluminum foil and steamed for 30 minutes. Then, the media will be cooled down to 45 ˚C, and pipetted into round-bottom tubes, 4 mL per tube approximately. The tubes were capped loosely so the tubes don't explode for the pressure, then they were put on racks; autoclave tape was put on the caps to guarantee sterility. Finally, the tubes were autoclaved at the liquid 40 cycle. Top Agar was kept in a refrigerator at 4 ˚C until use.

Continuation appendix 1

Phosphate Buffer Saline (PBS). Phosphate Buffer Saline (PBS) was prepared by adding 1.2 g of Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4), 0.8 g of Sodium Phosphate Monobasic Anhydrous (NaH_2PO_4) and 8.5 g of Sodium Chloride (NaCl) to 1 L of Deionized Water. Sodium Phosphate Dibasic Anhydrous, Sodium Phosphate Monobasic Anhydrous, and Sodium Chloride were weighted with a plastic cup and a laboratory analytical balance; Deionized Water was measured with a graduated cylinder.

PBS was mixed in a glass bottle with a stir bar and pH adjusted to 7.6 using, a pH probe and 10 M solution of Sodium hydroxide (NaOH). The bottles were capped loosely so they do not break from the pressure variations. The bottles were autoclaved at the liquid 20 cycles and after that, they were kept on the bench at room temperature (21^{o}C) until use.

Sterile Deionized Water. Sterile Deionized Water (SDW) was prepared by pouring 1 L of Deionized Water into a bottle. Deionized Water was measured with a graduated cylinder (test tube). The bottles were capped loosely so they don't explode for the pressure. The bottles were autoclaved at the liquid 20 cycle and after that, they were kept on the bench at room temperature (21^{o}C) until use.

Kanamycin Sock Solution (Kan). Kanamycin Stock Solution (Kan) was prepared by adding 2.5 g of Kanamycin Sulfate Powder to 50mL of SWD (concentration: 50 mg/mL). Kanamycin Sulfate Powder was weighted with a plastic cup and a laboratory analytical balance; Deionized Water was measured with a graduated cylinder (test tube).
Kan was vortexed in a 50-mL-Conical centrifuge tube after it was dissolved, it was filtered using a 0.2 µm filter and a disposable sterile syringe. The new tube was labeled and kept in a refrigerator at 4 ˚C until use.

Diluted Kanamycin Solution (Kan'). Diluted Kanamycin Solution (Kan') was prepared by adding 10 µL of Kan to 10 mL of LB (final concentration: 50 µg/mL). Kan was measured and transferred with a micropipette, LB was measured and transferred with a disposable serological pipette. Kan was vortexed in a 15-mL-Conical centrifuge tube, the tube was labeled and kept in a refrigerator at 4 ˚C until use.

Appendix 2. Statistical Analysis table

Interaction analyzed	Value	Probability
Infection × Growth	109.0252	< 0.0001
Log *E. coli* × Log phage	0.1169	0.9432
Log *E. coli* × Enrichment time	1.6966	0.9889
Log *E. coli* × Incubation temperature	0.1315	0.9364

E. coli = *Escherichia coli* O157:H7.
Log = Logarithm.

Appendix 3. Contingency table for Log *E. coli* × Log phage interaction

		Conc. Phage Log PFU/mL		Total
		8	**7**	
Conc. *Escherichia coli* O157:H7 Log CFU/mL	**7**	30	24	54
	6	30	27	57
	5	26	21	47
Total		86	72	158

Log = Logarithm.
CFU = Colony Forming Unit.
Conc. = Concentration.
mL = Milliliters.

Appendix 4. Contingency table for Log *E. coli* × Enrichment time interaction

		Enrichment time before adding the kanamycin (h)					Total
		5	**4**	**3**	**2**	**1**	
Conc. *Escherichia coli* O157:H7 Log CFU/mL	**7**	11	8	11	12	12	54
	6	12	9	12	12	12	57
	5	12	4	9	11	11	47
Total		35	21	32	35	35	158

Log = Logarithm.
CFU = Colony Forming Unit.
Conc. = Concentration.
mL = Milliliters.
h = Hours.

Appendix 5. Contingency table for Log E. coli × Incubation temperature interaction

		Incubation temperature (˚C)		Total
		37	**21**	
Conc. *Escherichia coli* O157:H7 Log CFU/mL	**7**	28	26	54
	6	30	27	57
	5	26	21	47
Total		84	74	158

Log = Logarithm.
CFU = Colony Forming Unit.
Conc. = Concentration.
mL = Milliliters.
˚C = Degrees Celsius