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Isothermal Amplification and Lateral Flow Nucleic Acid Test for the Detection of Shiga Toxin-Producing Bacteria for Food Monitoring

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Abstract: Foodborne bacteria have persisted as a significant threat to public health and to the food and agriculture industry. Due to the widespread impact of these pathogens, there has been a push for the development of strategies that can rapidly detect foodborne bacteria on-site. Shiga toxin-producing *E. coli* strains (such as *E. coli* O157:H7, *E. coli* O121, and *E. coli* O26) from contaminated food have been a major concern. They carry genes *stx1* and/or *stx2* that produce two toxins, Shiga toxin 1 and Shiga toxin 2, which are virulent proteins. In this work, we demonstrate the development of a rapid test based on an isothermal recombinase polymerase amplification reaction for two Shiga toxin genes in a single reaction. Results of the amplification reaction are visualized simultaneously for both Shiga toxins on a single lateral flow paper strip. This strategy targets the DNA encoding Shiga toxin 1 and 2, allowing for broad detection of any Shiga toxin-producing bacterial species. From sample to answer, this method can achieve results in approximately 35 min with a detection limit of 10 CFU/mL. This strategy is sensitive and selective, detecting only Shiga toxin-producing bacteria. There was no interference observed from non-pathogenic or pathogenic non-Shiga toxin-producing bacteria. A detection limit of 10 CFU/mL for Shiga toxin-producing *E. coli* was also obtained in a food matrix. This strategy is advantageous as it allows for timely identification of Shiga toxin-related contamination for quick initial food contamination assessments.

Keywords: Shiga toxin; recombinase polymerase amplification; nucleic acid detection; lateral flow assay; food safety; Shiga toxin-producing *E. coli*

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1. Introduction

Food contamination has persisted as a global problem that has worsened in recent years. These contamination events have a significant impact on not just public health but also on the food and agriculture industry [1,2]. Several bacterial species are implicated in foodborne illness with a range of frequency and severity [3]. One particular subset of bacteria implicated in food contamination and foodborne illnesses is known as Shiga toxin-producing *E. coli* (STEC). These bacteria are hallmarked by their ability to produce Shiga toxin, a virulent protein [4]. STEC strains produce two different Shiga toxins, Shiga toxin 1 and/or Shiga toxin 2 [5]. These two toxins are structurally similar and have the same mechanism of pathogenicity but are antigenically separate from each other.

Shiga toxins are part of a group of AB5 protein toxins that work through the blockage of protein synthesis in eukaryotic cells. The A subunit of the toxin is responsible for the toxin's activity, while the B subunit allows for the toxin to bind to the globotriaosylceramide (Gb3) receptors located on the membranes of several types of mammalian cells [6]. Shiga toxins are not native to *E. coli*, and the DNA encoding the toxins are instead part of lambdoid prophages that had infected these *E. coli* strains previously [7]. These prophages

infect the *E. coli*, and then the phage DNA is incorporated into the bacterial chromosome. Within these prophages and in the subsequent bacterial chromosome, the *stx* sequences are downstream of a tight promoter and, as such, are not expressed under normal conditions. For toxin production to occur, the phage must be induced via the triggering of the bacterial SOS signal (stress conditions), dependent on the RecA protein of the host [8]. After this induction, the prophage DNA is excised from the host DNA and replicated separately [9]. During this replication, the phage proteins and Shiga toxins are produced by the bacterial host, and then at the end of the induction process, cells are lysed. This lytic release of Shiga toxins often leads to complications such as Hemolytic Uremic Syndrome (HUS) [10]. This release of toxins and phage by STEC can also cause the normal intestinal flora to pick up the *stx* genes and, as a result, produce additional Shiga toxins, intensifying the infection [11].

There are over a hundred STEC strains of bacteria, and these strains can possess *stx1*, *stx2*, or both *stx* genes [12]. Therefore, the simultaneous detection of both *stx* genes is a better strategy to monitor the presence of any STEC bacteria without having to perform individual-strain-specific gene detection. The majority of the literature reports are targeted at the detection of *E. coli* O157:H7 as it is the most common STEC strain implicated in food contamination in the USA [13]. Not many publications have focused on non-O157:H7 STEC strains, even though these strains pose an equal health threat as the O157:H7 STEC strain and are also frequently implicated in food outbreaks [14]. In general, current bacterial detection methods are often time-consuming, expensive, or require personnel or laboratory space to conduct the assays [15]. With these types of methods, results can take anywhere from a few days to weeks. This leaves ample opportunity for contamination to go unnoticed. Therefore, it is of importance to have rapid detection methods, especially those that can quickly provide an idea of any STEC contamination. There have been many recent advances in the on-site detection of pathogenic foodborne bacteria overall, including multiplexed assays [3,16–18]. However, challenges still remain with regards to the complexity and cost of assay without compromising the required sensitivity.

Here we designed a strategy to detect the two *stx* genes in a single reaction to be able to determine the presence of any STEC bacteria. We employ simple chemical lysis of a sample followed by isothermal DNA amplification for both genes and a lateral flow assay-based visualization of the two *stx* genes, *stx1* and *stx2*. One promising isothermal amplification technique for on-site applications is recombinase polymerase amplification (RPA), which is utilized in this study. This method works at a temperature of 39 °C, and the entire amplification reaction can be performed in only 20 min. RPA products are then visualized on a lateral flow assay strip. For that, primers corresponding to two genes are labeled with biotin or digoxin that allow immobilization of the amplification product on the strip and also interact with a visual reporter (gold nanoparticles) [19,20]. These assay strips are useful for on-site detection methods as they are easy to use and easy to interpret. More specifically, these lateral flow strips are able to perform dual gene detection on a single strip. This cuts down on the number of tests used to detect two different genes. This saves time and resources, allowing for a greater number of samples to be tested without sacrificing cost or time.

The strategy described in this study aims to provide a broad initial screening tool for foodborne contamination via the detection of *stx* genes in a manner that is rapid, inexpensive, and simple to run. This is advantageous as there is no need for any significant equipment. Additionally, the inexpensive and rapid nature of this strategy allows for several tests to be run at any given time with quick results. This can help facilitate prescreening of samples for possible contamination prior to any further or more rigorous testing using culture or other laboratory methods. Furthermore, the ability to detect more than one target allows for detection of all STEC bacteria that produce Shiga toxins in a single test strip without the need for individual tests for each strain. A full depiction of this strategy can be seen in Figure 1.

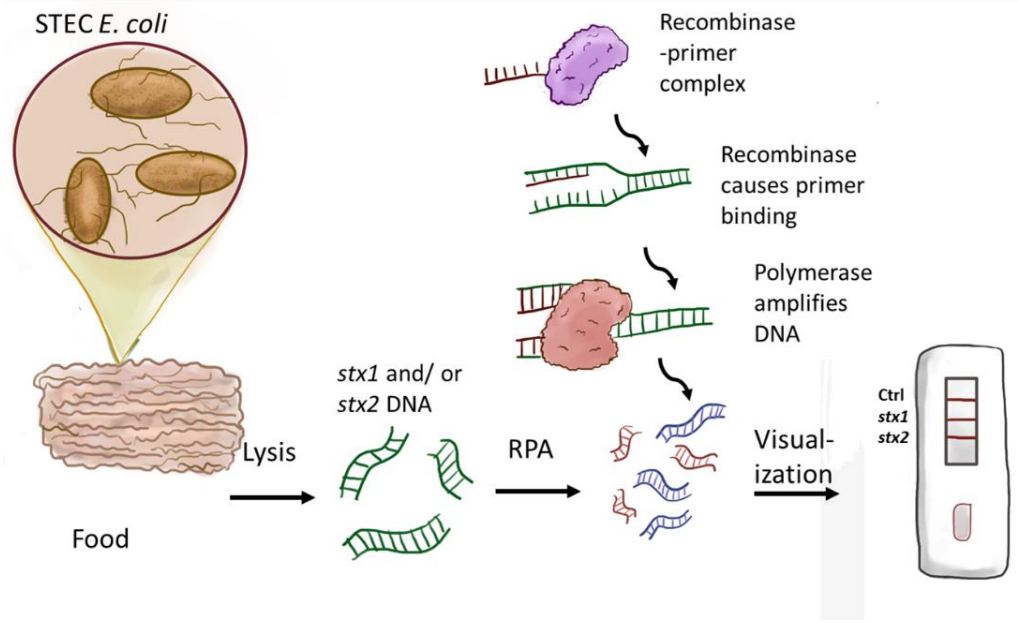


Figure 1. Schematic representation of the work performed in this study. Briefly, *E. coli* is lysed within a sample and then the *stx* DNA is amplified with RPA before being visualized on a lateral flow assay strip.

2. Materials and Methods

2.1. Materials

All bacterial strains used in this manuscript have been purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Oligos were purchased from Sigma (St. Louis, MO, USA). Milenia Hybridetect 2T Lateral Flow paper strips were purchased from Milenia Biotec (Gießen, Germany). TwistDx RPA kits were obtained from TwistDx (Cambridge, UK). Herring Sperm DNA was obtained from New England Biosciences (Ipswich, MA, USA). Triton-X 100 and RT-PCR grade water were obtained from ThermoFisher (Waltham, MA, USA). Sodium hydroxide and other buffers and salts were obtained from VWR (Radnor, VA, USA). Ground chicken was procured from a local chain supermarket.

2.2. Bacterial Lysis and DNA Collection in Media

The protocol followed was previously established in our earlier published work [21]. Briefly, 1.0 mL of culture at dilutions ranging from 10^6–1 CFU/mL was removed and centrifuged for 5 min at 4000× *g*. Supernatant was aspirated, and the pellet was resuspended in 100 µL of lysis buffer (50 mM NaOH and 5% Triton-X 100 (TX-100)). This suspension was vortexed and then allowed to incubate for 10 min at room temperature. Samples were then either immediately used or stored at −20 °C.

2.3. Shiga Toxin Gene Amplification Using RPA

The recombinase polymerase amplification reaction components are first added to a PCR tube. These components include 29.5 µL rehydration buffer, 2.4 µL of forward primers for both *stx1* and *stx2*, 2.4 µL of reverse primers for both *stx1* and *stx2* (Table 1), and 5.4 µL of water. Primers are added to the sides of the tube to prevent interactions from reducing potential background. A 2.0 µL of sample was then transferred to the wall of the reaction tube. These tubes were then centrifuged and transferred to the reaction tubes containing the manufacturer-provided lyophilized reaction. A total of 2.55 µL of magnesium acetate (MgOAc) was added to the caps, and the tubes were then centrifuged before immediately incubating for 20 min at 39 °C. Amplification reaction products were immediately visualized on lateral flow strips before being stored at −20 °C.

Table 1. Primer sequences used in this study.

Primer Name	Primer Sequence
stx1 F	5′-FAM-TTTTATCGCTTTGCTGATTTTTCACATGTT-3′
stx1 R	5′-DIG-CAAACCGTAACATCGCTCTTGCCACAGACT-3′
stx2 F	5′-FAM-CAGAGATATCGACCCCTCTTGAACATATAT-3′
stx2 R	5′-Biotin-GTATAACTGCTGTCCGTTGTCATGGAAACC-3′

2.4. Lateral Flow Assay

Milenia Hybridetect 2T lateral flow strips were purchased from Milenia Biotek (Gießen, Germany). Lateral flow strips and buffers were first allowed to acclimate to room temperature prior to use. A total of 100 µL of manufacturer-supplied buffer was pipetted into a 96-well plate. Samples were diluted 1:50, and then 10 µL of this dilution was pipetted onto the strip, and then strips were allowed to incubate in the running buffer for 5–10 min before removing for visualization. Lateral flow assays are visualized and interpreted with the naked eye with no need for extensive equipment. This is because the gold-conjugated anti-FAM antibodies embedded in the test strips recognize the FAM label present on the forward primers and becomes incorporated in the amplification product. This is what generates the visual color change in the control and test lines. Amplification products are captured on the test lines via interactions with either the biotinylated primer or the digoxigenin-labeled primer. A positive result is represented by either two lines if only one *stx* gene is present or three lines if both *stx* genes are present. A negative result is represented by a single line at the top of the strip. This topmost line is used as a means for quality control and assay validity. In this case, *stx2* amplification product *is* represented by the bottom test band, and *stx1* amplification product is represented by the middle band.

2.5. Solid Food Sample Handling and Spiked Sample Studies

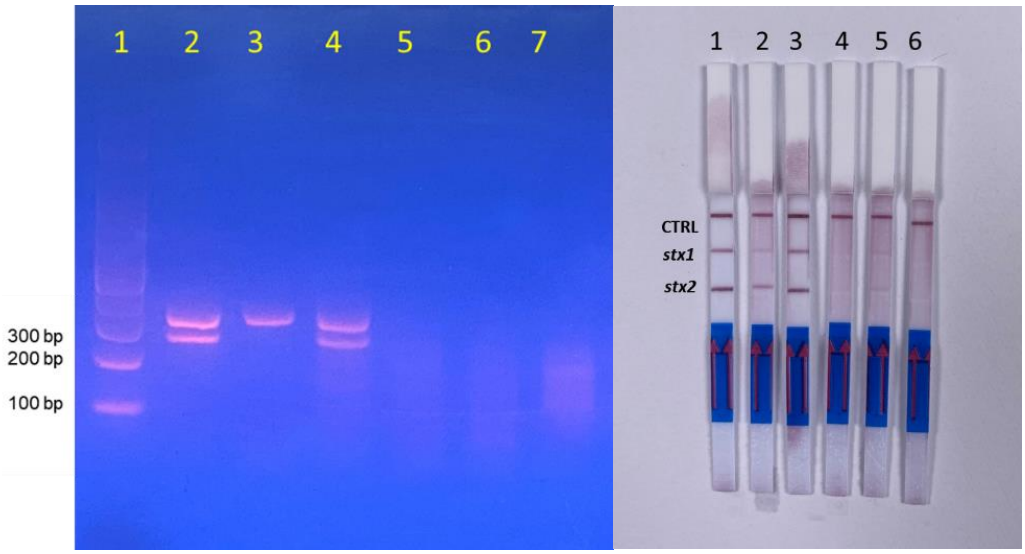
This method was established in a prior study in our laboratory [21]. Ground chicken was purchased from a local chain supermarket and homogenized into a 10 mL 10% *w*/*v* suspension using 1x PBS and a mortar and pestle. Into this suspension, 100 µL of bacterial culture per mL of liquid volume was added. Total concentrations ranged from 10^6 to 10 CFU/mL. Samples were then incubated for 10 min with 1.00 mL of lysis buffer (50 mM NaOH and 5% TX-100). Lysate was directly used for RPA. RPA samples were then visualized on a lateral flow strip as described in earlier methods.

3. Results

3.1. RPA Optimization and Primer Selection

Primers were designed against the open reading frames for the Shiga toxin 1 gene (*stx1*) and Shiga toxin 2 gene (*stx2*). Shiga toxin gene sequences were compared across different bacterial strains to confirm their overall conservation and similarity and to check for any potential mismatch regions. For both primer sets, primers were designed for the A subunits of the toxins since its responsible for the toxin activity. Primers were designed by scanning the open reading frame and generating multiple 30 base pair sequences. Each sequence was verified as unique via Blast and compared for optimal amplification utilizing genomic DNA of *E. coli* O157:H7 (produces both Shiga toxin 1 and Shiga toxin 2), *E. coli* O121 (only produces Shiga toxin 2), and *E. coli* O26 (produces both Shiga toxin 1 and Shiga toxin 2) (ATCC). The finalized primer pairings for each *stx* gene were then used to generate two products corresponding to *stx1* and *stx2* from bacterial lysates prepared using the following strains: *E. coli* O157:H7, *E. coli* O121, and *E. coli* O26. Lysates from *P. aeruginosa* (dissimilar pathogen) and *E. coli* O6 (non-pathogenic *E. coli* strain) were used as a non-specific control. The negative control consisted of a lysis buffer sample. Amplification was performed in non-specific and negative control samples using the same primer set and conditions of RPA that were used for Shiga toxin gene amplification. RPA was chosen as our amplification technique for its relatively quick run time and its ability to work near

room temperature. This method relies on the use of a recombinase enzyme to allow for amplification to occur [22]. This recombinase creates a complex with the primers that then searches for the sequence homologous to the primers within a sample. This strand invasion allows for the DNA to be opened. The open DNA is stabilized by single-strand binding proteins, and then a DNA polymerase is able to elongate the sequence. This process is exponential and can generate a lot of the product from a small amount of target [23]. Additionally, RPA is resilient to inhibitors, capable of amplifying even in the presence of common PCR inhibitors [24]. This makes RPA a good method for performing amplification with minimal sample preparation. The product of *stx1* amplification is 220 bp in length, and the product for *stx2* amplification is 280 bp in length. The optimized primer sequences used are listed in Table 1 in the methods section described earlier. The products obtained corresponding to *stx1* and *stx2* amplification for each *E. coli* strain carrying the Shiga toxin gene were verified using DNA sequencing. An agarose gel depiction of these products can be seen in Figure 2. We expected bands for both *stx1* and *stx2* from strain *E. coli* O157: H7 (Lane 2) and *E. coli* O26 (Lane 4), only one product corresponding to *stx2* from *E. coli* O121 (Lane 3), and no bands from *P. aeruginosa*, non-pathogenic *E. coli* O6, and negative buffer control Lanes 5, 6, 7, respectively.



(**a**) (**b**)

Figure 2. (**a**) Agarose gel visualization of amplified DNA, Lane 1: 100 bp ladder, Lane 2: *E. coli* O157: H7, Lane 3: *E. coli* O121, Lane 4: *E. coli* O26, Lane 5: *P. aeruginosa*, Lane 6: *E. coli* O6, Lane 7: Negative control (**b**) Lateral flow assay visualization of the same RPA products at concentrations of 10^6 CFU/mL. 1: *E. coli* O157:H7, 2: *E. coli* O121, 3: *E. coli* O26, 4: *P. aeruginosa*, 5: *E. coli* O6, 6: Negative control.

3.2. Specificity

It is imperative in DNA detection to ensure specificity and that the method only detects the intended target gene. To assess the specificity of our detection strategy, we evaluated the specificity of the primers designed first in silico against common foodborne pathogens (Figure 3). Blast results indicated no significant similarity against the major foodborne pathogens. This type of in silico screening is an accepted method by the FDA for large pathogen screening, especially when it is not feasible to perform screening against all bacterial strains in a laboratory setting [25,26]. Additionally, we performed a Blast search for each primer set against the other Shiga toxin, meaning *stx1* primers against *stx2* and vice versa (Figure 4). Both primer pairs did not show significant similarity to the other Shiga toxin gene, indicating no significant cross-reactivity between the primers and the other Shiga toxin. Then, experimentally, we assessed our primers' specificity with a dissimilar pathogen (Pseudomonas aeruginosa) and a non-pathogenic *E. coli* variant (*E. coli* O6). Since food could be contaminated with naturally occurring non-pathogenic strains, we

needed to make sure that we would not detect those strains. For this purpose, samples of 10^6 CFU/mL of either *E. coli* O121, *E. coli* O157:H7, *E. coli* O26, *P. aeruginosa*, and *E. coli* O6 were lysed, and then RPA was performed. Results were visualized in the lateral flow assay as well as 2% agarose gel. As seen in Figure 2, our assay did not show any amplification product bands on the gel and paper strip corresponding to non-pathogenic *E. coli* or a dissimilar pathogen. Only the Shiga toxin-producing *E. coli* species produced positive results on agarose gel and paper strips. Our in silico and experimental results together indicate that there is no cross-reactivity and that our method is specific.

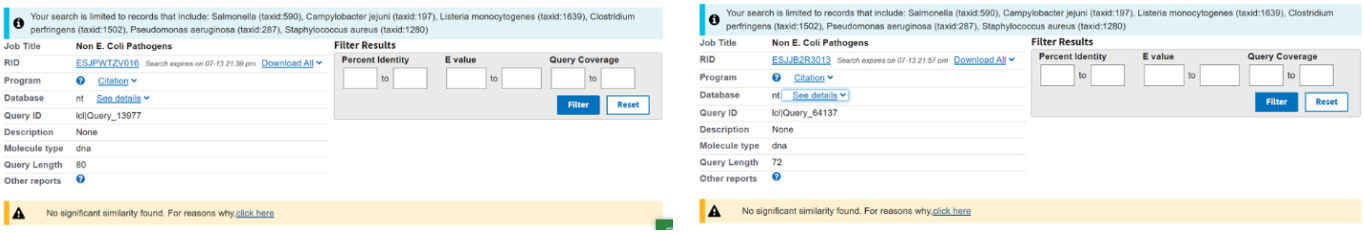


Figure 3. Blast search results of our primers for (**a**) *stx1* and (**b**) *stx2* against the following organisms: Salmonella enterica, Listeria monocytogenes, Campylobacter jejuni, Clostridium perfringens, Staphylococcus aureus. These pathogens were selected based on their implication in food outbreaks. Results searched for highly similar sequences (megablast). These results were obtained from https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 14 July 2021).

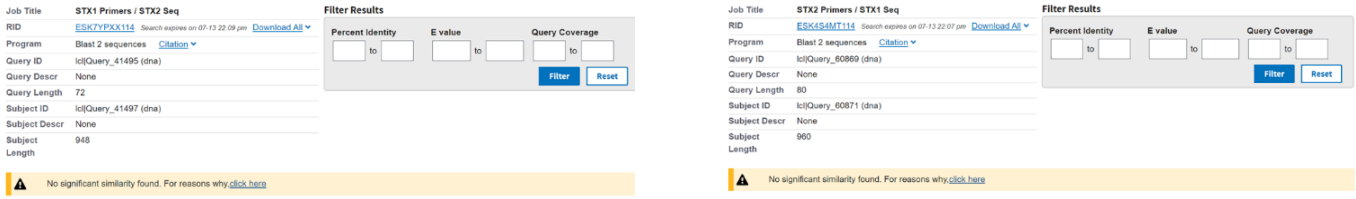


Figure 4. Primer sets were compared to their opposing toxin for cross-reactivity. (**a**) Shiga toxin 1 primers blasted against the sequence for Shiga toxin 2. (**b**) Shiga toxin 2 primers blasted against the sequence for Shiga toxin 1. These results were obtained by searching for highly similar sequences (megablast.) Results were obtained from https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 14 July 2021).

3.3. Evaluation of Sensitivity in Media

The infectious dose for STEC *E. coli* is as low as 10 cells depending upon the strain [27]. When considering this, the high sensitivity of the assay is important to prevent the potential for false negative results, which can be rather problematic in food safety monitoring. False negatives are still a challenge, with a percentage of culture analysis results yielding false negative results [28,29]. In order to test the working range of our assay in a standard condition, we lysed bacterial samples of *E. coli* O157:H7 at concentrations ranging from 10^6 to 1 CFU/mL. DNA within the lysate was amplified using RPA as described, and results were visualized on lateral flow strips shown in Figure 5. As seen in the figure, our detection limit was approximately 10 CFU/mL, which is relevant considering the infectious dose specifically for *E. coli* O157:H7 ranges from 10 to 100 cells [30]. This detection limit is also significant as it was obtained without any need for enrichment post-sampling, which drastically reduces the run time of the assay. Moreover, it only took approximately 35 min from the sample to the result, which is reasonable for on-site monitoring applications. Bands were additionally compared for the quantitative difference between the sample test lines and the control strip test lines. As expected, there was a quantitative difference between sample test lines and control test lines, as seen in Figure S2. Experimental results

were replicated in three separate experiments (from separate bacterial cultures on different days) for reproducibility. Replicate data can be seen in Figure S1.

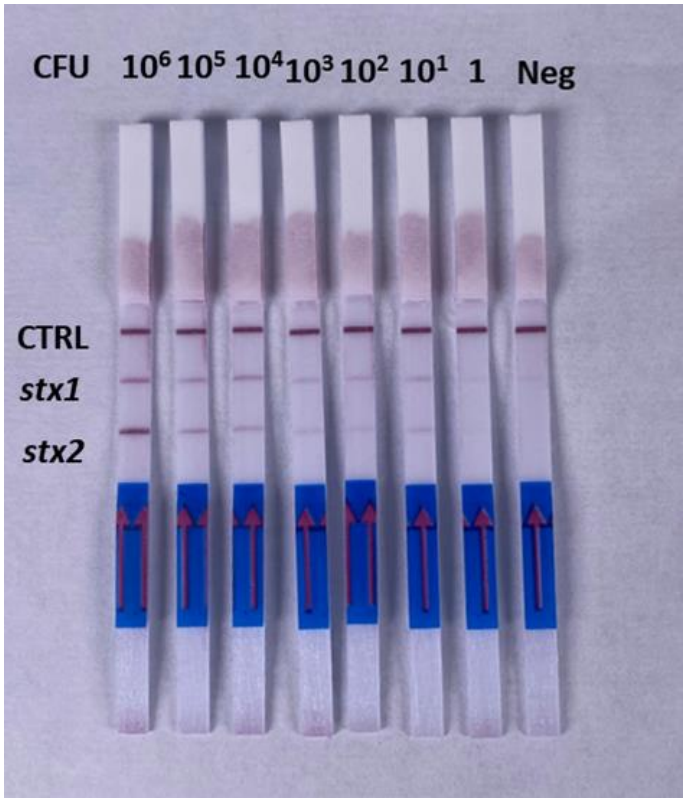


Figure 5. LOD determination in media. Genomic DNA of *E. coli* O157:H7 was harvested from varying concentrations of bacteria ranging from 10^6 to 1 CFU/mL with a negative control as lysis buffer. Results were repeated in at least three separate experiments with a representative set being shown.

3.4. Evaluation of Sensitivity in Spiked Food Samples

To demonstrate applicability in a real-world setting, we first aimed to demonstrate the detection of the *stx1* and *stx2* genes in a food matrix that is commonly implicated in STEC contamination. Many foods have been implicated in these outbreaks ranging from leafy vegetables, meat products, unpasteurized dairy, and even flour [31]. Out of these, we decided to use ground chicken as a representative food matrix. This decision was based on the knowledge that STEC *E. coli* has been identified in chicken samples and that poultry has been known to harbor STEC bacteria [32–34]. Since our media experiments yielded an LOD of 10 CFU/mL, we decided to test within the range from 10^6 to 10 CFU/mL. *E. coli* O157:H7 was spiked into a 10% *w*/*v* solution of ground chicken in sterile water. This solution was prepared using crude homogenization with a mortar and pestle. This method is simplistic and good for on-site purposes since it is inexpensive and easy to perform. These spiked samples are then lysed, and the lysate is directly used for RPA before being visualized on a lateral flow strip. Our data indicate that we are able to detect as low as 10 CFU/mL in ground chicken samples, as seen in Figure 6. Bands were compared for the quantitative difference between the sample test lines and the control strip test lines. As expected, there was a quantitative difference between sample test lines and control test lines, as seen in Figure S4. Experimental results were replicated in three separate experiments (from separate bacterial cultures on different days) for reproducibility. Replicate data can be seen in Figure S3.

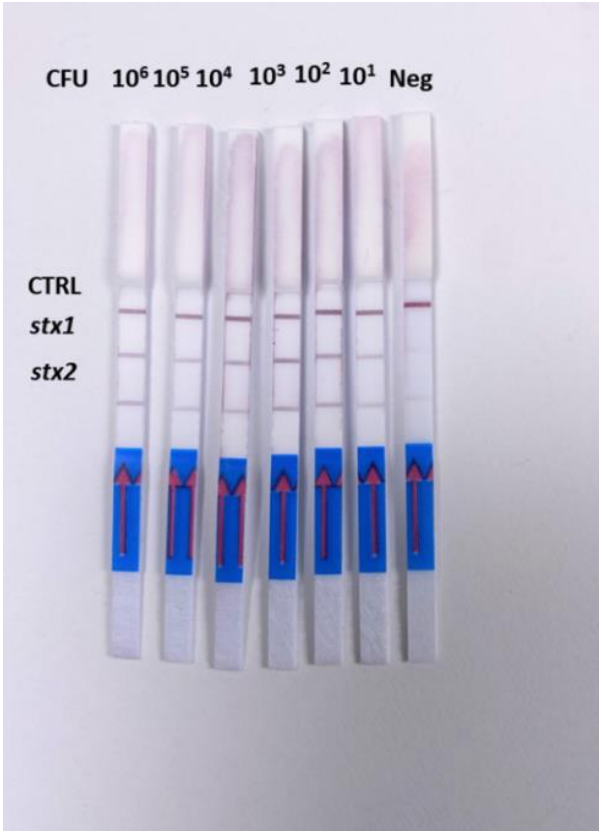
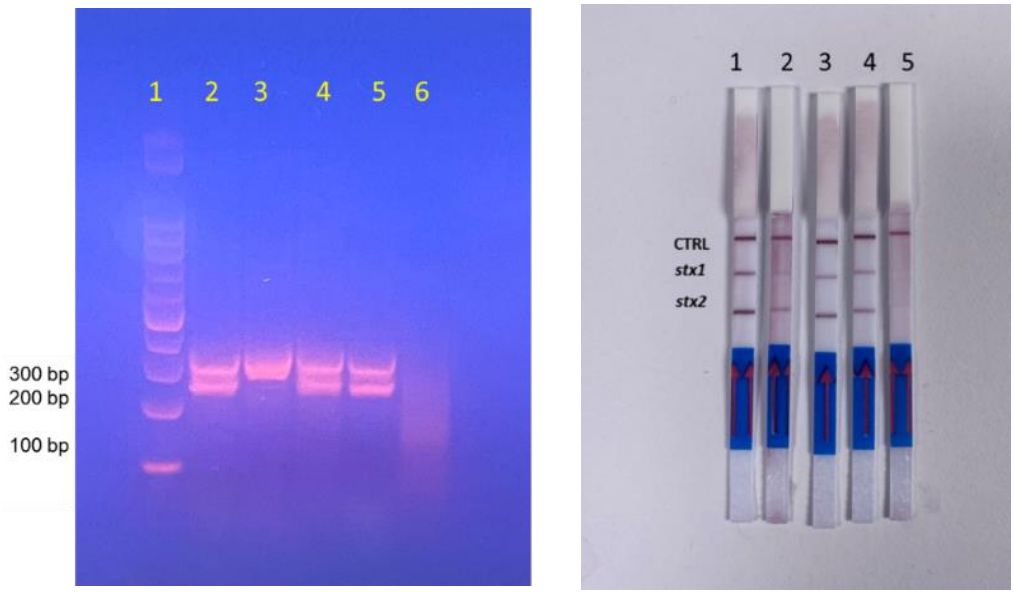


Figure 6. LOD determination in spiked chicken samples. Strips 1 to 7 show varying spiked concentrations ranging from 10^6 to 10 *E. coli* O157:H7 with a negative control in strip 7. Results were repeated in three separate experiments with one representative experiment being highlighted.

3.5. Evaluation of a Mixed Sample

While it would be rare, it is possible that there can be multiple Shiga toxin-producing bacteria present within a single sample. Additionally, it is also important to ensure that Shiga toxin can be detected from several different bacterial strains. To test and confirm that we can detect both Shiga toxins from a mix of bacterial species, we combined *E. coli* O157:H7, *E. coli* O121, and *E. coli* O26 at concentrations of 10^2/mL of each strain. We chose 10^2 CFU/mL as our final mixed sample concentration as it is a concentration that is within the range of CFU levels frequently seen in contaminated samples from various food outbreaks [35]. *E. coli* O157:H7 and *E. coli* O26 carry both *stx1* and *stx2* genes, whereas *E. coli* O121 only carries the *stx2* gene. As seen in Figure 7, we are able to detect both toxin genes separately in three different bacterial strains and also in a sample containing all three strains. This indicates that we can detect Shiga toxin from multiple bacterial strains both separately and when presented together.



(**a**) (**b**)

Figure 7. (**a**) 2% agarose gel visualization of the following: 1. DNA marker, 2: 10^6 *E. coli* O157:H7, 3: 10^6 *E. coli* O26, 4: 10^6 *E. coli* O121, 5: all three strains mixed at a concentration of 10^4 CFU/mL, 6: negative control.; (**b**) Lateral flow assay visualization of the same RPA products. 1: *E. coli* O157:H7, 2: *E. coli* O121, 3: *E. coli* O26, 4: Mixed sample, 5: Negative control. These experiments were repeated at minimum of 3 separate times on different days to ensure reproducibility. A representative image is shown.

4. Discussion

In this study, we present a dual DNA detection strategy for broad detection of Shiga toxin-producing bacteria. This is beneficial since there are hundreds of strains that produce Shiga toxins encoded by the genes *stx1* and *stx2*. The detection of both toxins allows for contamination to be identified without the need for several strain-specific tests. The method developed by us utilizes simple chemical lysis followed by recombinase polymerase amplification with visual colorimetric detection on a lateral flow assay platform. This simplistic method and rapid amplification are good for on-site use due to the lack of overall complexity. On-site use is crucial in food monitoring to catch contamination ideally before the product reaches the hands of the consumer. Additionally, the inexpensive and rapid nature of this strategy would allow the user to perform multiple tests across several food samplings. This is significant because representative and proper sampling is imperative to the reliability of food-safety analysis [36].

Our detection strategy was able to distinguish between the two Shiga toxins and detect the toxin genes in several Shiga toxin-producing bacteria. Furthermore, we were able to detect as low as 10 CFU/mL of bacteria in media and 10 CFU/mL in spiked food samples. These limits of detection are within the range of the infectious dose of STEC bacteria. Additionally, this is within the range of many existing methods that target the Shiga toxin genes. (See Table 2). Our method is quicker than many of these highlighted existing methods. Furthermore, many of these methods were not designed for on-site applications, while ours is able to be readily applied for on-site analysis with considerably less equipment. Not all on-site applications reported provide the required low detection limit as demonstrated by our method since reducing the complexity of the method can compromise the detection sensitivity. Additionally, several commercial methods currently exist for the detection of STEC, ranging from nucleic acid-based techniques to immunoassays [37–39]. Our described strategy is similarly able to detect both toxins in a manner that is specific. While the commercial kits certainly have good sensitivities, our strategy offers some benefits. For example, our strategy did not require an incubation or enrichment

period. Additionally, many commercial kits are not all feasible to be used extensively in resource-poor areas. Similarly, several commercial assays are not designed with on-site use in mind. The simplistic lysis method and one-step isothermal amplification using the RPA technique are useful for on-site detection. Similarly, our strategy from sample to answer can be completed in approximately 35 min, which is reasonable for on-site applications. This strategy requires minimal equipment that can be readily available in food and agriculture sites. There was no need for sample enrichment or any intensive extraction technique, which allowed us to accomplish a fairly short sample to answer timeframe Additionally, the inexpensiveness of paper-based detection with visual detection further enhances the potential use as a broad screening tool for a wide range of STEC pathogenic bacteria.

Table 2. Literature examples of different detection strategies for STEC bacteria, focused predominantly on the detection of either the direct Shiga toxins or the *stx1* and *stx2* genes.

Methodology	Assay Run Time	Limit of Detection	Reference
Immuno-PCR	Not stated	10 pg/mL purified toxin	[40]
Real-time PCR	~1 h	5×10^3 CFU/mL	[41]
Real-time RPA	5–10 min	~5–50 CFU/mL	[42]
qPCR	Not stated	<2.7–3.7 log copies g^{-1} feces	[43]
Multiplex Real-Time PCR	Not stated	6 CFU/mL	[44]
Asymmetric PCR and LFA	Not stated	Not stated but 10 × PCR	[45]
Multiplex melting curve PCR	>6 h (because of enrichment step)	1 CFU/mL with enrichment of 6 h	[46]
Droplet digital PCR	Not stated	Not stated	[47]
Culture-Based	1–3 days	1 cell	[48]
LFA-immunoassay	<10 min	0.1 ng/mL toxin	[49]
LFA-immunoassay	~3 h	10^5	[50]
Immunoassay	<30 min	3.3×10^1 CFU/g to 1.3×10^3 CFU/g when induced with antibiotic	[51]

Ideally, this strategy would be used for a quick early determination of contamination if there is a suspicion of food contamination. We envision that this type of testing could potentially find usage in the food distribution pathway from the farms to the factories or even grocery stores. This method can be utilized to detect other pathogens in food by switching the primers to detect different targets. The ability to detect more than one pathogen simultaneously allows us to have the capability to screen more pathogenic targets in an on-site manner, which is crucial for contamination monitoring.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors10060210/s1, Figure S1: Replicate images for Figure 5. These results were obtained from different bacterial lysates on different days, Figure S2: Statistical analysis for Figure 5. Test line measurements were obtained for A: *stx1* test lane and B: *stx2* test lane using image J and analyzed for significance against the control bands using Prism software. Each intensity measurement was taken 3 times for each concentration, Figure 3: Replicate images for Figure 6. These results were obtained from different bacterial lysates on different days, Figure S4: Statistical analysis for Figure 6. Test line measurements were obtained for A: *stx1* test lane and B: *stx2* test lane using imageJ and analyzed for significance against the control bands using Prism software. Each intensity measurement was taken 3 times for each concentration.

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