Preliminary testing of *Escherichia coli* for use in RPA experiments

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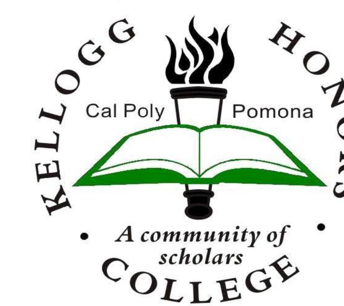


Abstract

Recombinase polymerase amplification (RPA) is a DNA amplification technique that has been utilized to detect various bacteria. Unlike PCR -- which requires strict temperature control - RPA is isothermal, allowing testing in a point of care setting with relative ease. Shiga toxin-producing *Escherichia coli* (STEC), is a prominent foodborne enteropathogen capable of causing illnesses in low concentrations. RPA has been previously utilized as a means of detecting STEC in real time, with high specificity, sensitivity, and predictive value in a laboratory setting. In the future, this technology could be applied in the field to determine contamination of crops, milk, blood, and other field samples without the need and expense of laboratory PCR testing. In this project, four types of non-disease-producing *Escherichia coli* were cultured, grown, and identified, then subsequently tested for optical density and plated for colony counting. Testing produced preliminary standard curves by which to determine the numbers of bacteria associated with different concentrations. These initial steps pave the way forward for future testing, in which known numbers of STEC bacteria will be spiked in samples (milk, blood), for determination of the feasibility of detection by RPA in a field setting.

Introduction

Escherichia coli O157:H7 is one of 100 STEC producing serotypes of *E. coli* known to cause haemolytic colitis and haemolytic uraemic syndrome in humans with relatively low numbers of organisms (as little as 10 colony forming units). Compared to other serotypes, *E. coli* O157:H7 possesses a high potential for mortality. Detecting presence of O157:H7 before it enters the supply chain is crucial to preventing illness. RPA has been implicated as a possible means of detecting O157:H7 in point of care settings - meaning, in the field, with no laboratory necessary. RPA is an isothermal process, therefore unlike other methods of DNA amplification like PCR, which require controlled temperature changes to promote DNA cleaving and amplification, RPA can proceed in ambient temperatures, proceeding between 37 and 42 degrees Celcius. Use of the Twista device (TwistDx Co) can be accomplished at point of care with relative ease, eliminating the need to send samples off to a lab, which might be a costly and timely procedure. The ability to utilize a real-time, isothermal method of DNA amplification for detection of a prominent foodborne pathogen like O157:H7 in field samples may potentially reduce the prevalence of these pathogens in the supply chain, lowering incidence of illness. RPA has been previously shown to be able to detect O157:H7 with high sensitivity and specificity - one study was also able to detect O157:H7 in milk samples, along with two other prominent foodborne pathogens. The next step in this process is to determine the validity of these procedures for use on field samples (i.e. milk, blood, feces, etc.). To do this, known numbers of bacteria will need to be spiked into samples, and tested with RPA to determine the ability of the procedure to determine presence of O157:H7 in subsequently smaller concentrations. This project undertakes the preliminary testing necessary for these future goals.

Methods

Four strains of non-disease causing *E. coli* were chosen for the preliminary testing. Initially, these strains were: (1) *E. coli* O157:H7 #TCC 43888, (2) *E. coli* O157:H4 PSU1, (3) *E. coli* O157:H7 PSU 5, and (4) *Salmonella Braenderup* ATC BAA644. (4) was later replaced with *E. coli* GFP O157. To verify the identity of the bacteria, these four strains and a sterile control were cultured in tryptic soy broth (TSB) and grown overnight in a 32°C incubator, then streaked for isolation on tryptic soy agar (TSA) plates. Following incubation overnight at 32°C, resulting isolated colonies were streaked onto microscope slides and gram stained for visualization with a microscope.

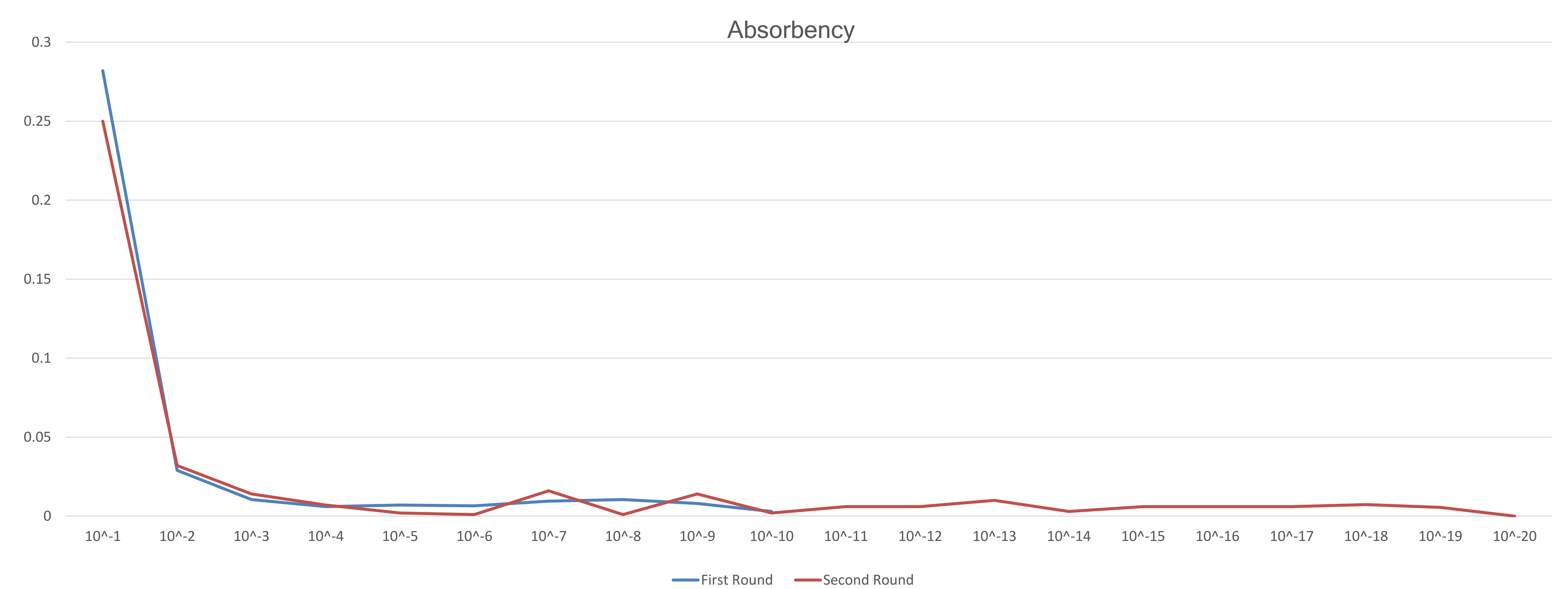
Once strains were verified, isolated colonies were transferred to TSA slants and grown overnight at 32°C to encourage growth. Following incubation, these slants were refrigerated for long-term storage. The bacteria grown on these slants were utilized in the subsequent of serial dilutions and colony counting.

E. coli O157:H7 #TCC 43888 (1) was chosen for the initial testing. A loopful of (1) was transferred from its slant into a test tube filled with 10mL TSB. After growth overnight, a 1 mL portion was transferred into a separate test tube filled with 9mL fresh broth. This process was repeated for 9 new test tubes to create a 10 fold serial dilution, with vortexing of the inoculated broths between each transfer. Samples from each dilution were transferred into cuvettes and placed into a spectrophotometer to determine absorbency. Samples (1mL) from each serial dilution were also transferred onto fresh TSA plates, spread utilizing the "hockey stick method," then incubated overnight. The isolated colonies that resulted were counted using a Colony Counter. In later weeks, serial dilutions were increased to 20 fold.

Preliminary Results

	Gram Positive	Gram Negative	Observations
Plate 1	No	Yes	Rod-shaped
Plate 2	No	Yes	Rod-shaped
Plate 3	No	Yes	Rod-shaped
Plate 4	No	Yes	Rod-shaped

Table 1. Gram staining results



Graph 1. Preliminary absorbencies for *E. Coli* 0157:H7 #TCC 43888 following serial dilutions

	10^-1	10^-2	10^-3	10^-4	10^-5	10^-6	10^-7	10^-8	10^-9	10^-10
First Round	No growth	36 isolated colonies, large cluster of growth	>500, No isolated colonies	>500, Tracks of colonies, too close to accurately count	>500, Clustered growth, some isolated colonies	>500, Clustered growth, some isolated colonies	>500, Clustered growth, more isolated colonies	>500, 2 large, separate clusters; colonies more distinct	>500, Mostly isolated	>500, Mostly isolated
Second Round	No growth	No growth	>500, No isolated colonies	>500, No isolated colonies	>500, About 70 isolated colonies	>500, About 28 isolated colonies	Clustered growth, 22 well-isolated colonies	Clustered growth, 3 well-isolated colonies	No growth	No growth

	10^-11	10^-12	10^-13	10^-14	10^-15	10^-16	10^-17	10^-18	10^-19	10^-20
First Round	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Second Round	No growth	No growth	No growth	No isolated colonies	No isolated colonies	No growth	No growth	No growth	No growth	No growth

Table 2. Colony growth correlated to serial dilution

Discussion

Graph 1 above merely outlines the results of the bacterial identification tests outlined in the experimental methods. All bacteria grown were determined to be gram-negative and rod-shaped, suggestive of *Escherichia coli*. These preliminary tests were necessary before proceeding to verify the identity of the bacteria that would be tested.

All obtained results are very preliminary and further testing is still needed to establish standard curves, however these initial results do suggest a (mostly) negative correlation between number of serial dilutions and concentrations of bacteria (absorbency). A few results (specifically 10^-7 and 10^-9 in the second round of dilutions) did spike unexpectedly. No subsequent serial dilution should show higher absorbency since the process of diluting these samples inherently lowers the concentrations of bacteria with each transfer. It is possible that these results are due to human error, most likely in the handling or set up of the spectrophotometer. All results were obtained using a 600 wavelength absorbance, and each sample was tested four times to determine an average, which was then graphed above. In the future, the spectrophotometer settings will need to be reviewed for accuracy before proceeding with further testing.

Serial dilutions were increased during the second round of testing because the first round of testing yielded high numbers of colonies when plated out. In the first round of serial dilutions, no isolated colonies could be confidently counted because all colonies had grown too close together on the plate - in other words, the plate was too crowded. The optimal growth should be between 30 and 300 isolated colonies. Although the second round of testing did yield less isolated colonies, the growth was erratic at best. These results are again likely due to human error in the process of serial dilutions, plating, or in media preparation. A few of the plates used for the second round of dilutions had condensation on their lids after having been stored in the refrigerator for about two weeks - this may have hindered bacterial growth. It is also possible that in the process of sterilizing the "hockey stick" used to spread the bacteria on the plates, some of the bacteria were killed off. Sterilization involves dipping the stick into alcohol and flaming it. The "hockey stick" could have been too hot when it touched the bacteria, or residues of alcohol could have been left on it, both of which would have killed at least some of the bacteria that had been introduced onto the plate. Some of the growth followed a "bullseye" pattern that could be indicative of these mistakes - with bacterial growth on the periphery of the plate, and less or no growth in the center.

Conclusion

Although early in the process, acknowledging and remedying the issues of earlier testing will continue to improve established procedures. In time, these preliminary tests will produce solid standard curves which will be instrumental in further testing - particularly, in spiking known numbers of these strains and, eventually, *Escherichia coli* O157:H7, for detection with RPA. These tests will help to further establish the sensitivity and specificity of RPA for use in detecting STEC in field samples: an important step in helping to establish RPA as a plausible and useful technique for isothermal DNA detection in the field of this prominent and harmful foodborne pathogen.

References

All references utilized in this project are available upon request.

Acknowledgements

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